Neurotransmitter-stimulated ion transport by cultured porcine vas deferens epithelium

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Sedlacek, Roger L., Ryan W. Carlin, Ashvani K. Singh, and Bruce D. Schultz. Neurotransmitter-stimulated ion transport by cultured porcine vas deferens epithelium. Am J Physiol Renal Physiol 281:F557–F570, 2001.—A collagenase-based dissociation technique has been developed to routinely establish monolayer cultures of freshly isolated porcine vas deferens epithelium. Cells isolated from each tissue are transferred to 25-cm² tissue culture flasks and grown in a standard cell culture medium. Flasks reach confluency in 3–4 days, and cells are subsequently seeded onto permeable supports. Cultured cells display a monolayer cobblestone appearance and are immunoreactive to anti-ZO-1 and anti-cytokeratin antibodies. Electron microscopy is employed to demonstrate the presence of junctional complexes and microvilli. When evaluated in modified Ussing chambers, cultured monolayers exhibit a basal lumen negative potential difference, high electrical resistance (>1,000 Ω·cm²), and respond to norepinephrine, vasopressin, ATP, adenosine, and histamine, with changes in short-circuit current indicative of anion secretion. Responses are significantly attenuated in Cl⁻- and/or HCO₃⁻-free solutions. Attempts to further optimize culture conditions have shown that chronic exposure to insulin increases proliferation rates. Thus the culture method described will reliably produce viable neurotransmitter-responsive cell monolayers that will allow for the characterization of vas deferens epithelial function and associated control mechanisms.

model system; epithelia; anion transport; pH regulation; cystic fibrosis; congenital bilateral absence of the vas deferens
the disease; infertility is their only complaint, and genotyping is required to determine their CF status (3, 11, 15, 20). Furthermore, men seeking intervention for infertility have a higher incidence of “mild” mutations in the CF gene than the general population (26, 51). These observations suggest that the vas deferens is more sensitive to the loss of an anion conductance (CFTR) than any other epithelial tissue.

An aim of the present study was to develop a suitable and readily available model for vas deferens epithelium for in vitro ion transport studies. Human male genital ducts are difficult to obtain for such purposes. Mouse tissues are readily available in most laboratory settings and, because of genetically modified strains, readily manipulated at the molecular level. However, it is particularly important to note that, unlike humans who lack expression of CFTR and suffer CBVD, CF mice have a complete and patent ductal system. Thus pathological manifestations of epithelial changes are fundamentally different in humans and mice. The adult human and porcine vas deferens are similar in that they are 30–40 cm in length with distinct scrotal and abdominal components, and it has been suggested that the porcine reproductive system is an adequate model for inferences to humans (29, 30).

In this report we detail a method to routinely obtain and grow cultures of epithelial cells from porcine vas deferens that can be used as a model for ductal ion transport. Included are examples of ion transport by these epithelial cells in response to physiological hormones and neurotransmitters as well as forskolin, a direct stimulator of adenylyl cyclase activity. This system provides a reliable means by which researchers may examine the role of ion transport mechanisms and regulatory cascades that contribute to the luminal environment to which sperm are exposed.

MATERIALS AND METHODS

Tissue isolation. Mature porcine reproductive tracts including scrotum, testes, epididymides, and vas deferens (up to and including a portion of the abdominal ducts) were obtained from a local slaughterhouse ~40 min post mortem. Immediately on removal from the animal, whole reproductive tracts were immersed in ice-cold Ringer solution of the following composition (in mM): 120 NaCl, 25 NaHCO3, 3.3 KH2PO4, 0.83 K2HPO4, 1.2 CaCl2, and 1.2 MgCl2, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 40 µg/ml gentamicin, and 4 µg/ml amphotericin B. Tracts were maintained in this solution until further processing was initiated at the laboratory (2–3 h). Because the anatomic demarcation of the vas deferens from the caudal region of the epididymis is open to interpretation, we chose to focus on regions of the caudal region of the epididymis or the transitional portion of the vas deferens. Hence, regions selected for cell isolation comprised only the “straight” regions of the vas deferens (Fig. 1). Ducts collected for epithelial cell isolation ranged in length from 10 to 35 cm. Once removed, the lumen of each duct was flushed with 3 ml of fresh ice-cold Hanks’ buffered saline solution (HBSS) of the following composition (in mM): 137 NaCl, 5.4 KCl, 0.4 KH2PO4, 0.34 NaH2PO4, 5.5 glucose, pH 7.0, supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, 40 µg/ml gentamicin, and 10 µg/ml amphotericin B, and placed into ice beakers containing the antibiotic-supplemented HBSS (HBSS+A).

Epithelial cell isolation. Before dissociation of the luminal epithelial cells, tissues were first carefully cleaned of connective tissue and blood vessels and then flushed with 5 ml of HBSS+A followed by 5 ml of HBSS to remove the antibiotics from the lumen. To effect dissociation of the epithelial layer, a small volume (typically <0.5 ml) of phosphate-buffered saline for cell culture (PBScc) of the following composition (in mM), i.e., 140 NaCl, 2 KCl, 1.5 KH2PO4, 15 NaHPO4, containing 300 U/ml collagenase, 0.25% (vol/vol) trypsin, and 2.65 mM disodium EDTA, was perfused through the lumen. After the initial perfusion, the distal end of the tissue was occluded with a mosquito hemostat, and the duct was filled with dissociation solution until destended. The proximal end was then occluded with a second hemostat. Filled ducts were placed into 50-ml tubes containing chilled HBSS, immersed into a 37°C water bath, and incubated for 20–60 min, with swirling at 10-min intervals. Incubation periods of <30 or >45 min resulted in a lower proportion of isolations yielding viable epithelial cell cultures. Therefore, incubation periods of 30–45 min were employed for the majority of this work.

Freshly dissociated cells were collected from the vas deferens by blotting the duct with tissue paper to remove excess fluid and then draining the luminal contents into a 15-ml tube. The duct was flushed with 1.0 ml of growth medium DMEM (GIBCO-BRL, Grand Island, NY), supplemented with 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 100 U/ml penicillin, and 100 µg/ml streptomycin, massaged by rolling it between a gloved thumb and index finger, and again flushed with 1.0 ml of supplemented growth medium into the same tube. The resulting cell suspension was further diluted with 3 ml of growth medium and pipetted several times to facilitate the disruption of epithelial sheets that detached from the lumen. After this treatment, numerous small aggregates of cells remained. These multicell aggregates precluded any quantitative determination of cell viability. The entire volume (nominally 5 ml) of suspension was then placed into a vented, 25-cm2 tissue culture flask (Corning, Corning, NY) and incubated at 37°C in a humidified environment containing 5% CO2.

Initial cell culture and routine passage. After 18–24 h of culture, the growth media and any nonadherent cells were removed by aspiration, and cells were fed with fresh growth media. Adherent cells exhibited an epithelioid-like morphol-
ology and were monitored for growth and contamination for an additional 48 h. Bacterial or fungal contamination was observed in <10% of primary epithelial cell isolations. At the end of the monitoring period, more than one-half of the flasks contained confluent or near-confluent monolayers. Routine cell passage of confluent flasks was performed by aspirating spent media from the flask, rinsing with PBS, and then incubating the cells in 1.5 ml of dissociation media (PBS containing 5% trypsin and 2.65 mM EDTA) for 2 min, removing the dissociation solution, and allowing the cells to incubate at 37°C for an additional 4–5 min. Once the cells had detached from the flask, 5.0 ml of growth medium was added and the suspension was pipetted 5–10 times to facilitate disruption of the cell sheets. One milliliter of the suspension was added to a 25-cm² flask containing 4 ml of growth media (passage 1). Subcultures in flasks grew to confluency within 5–7 days. At the time of passage, permeable supports (1.13 cm², Snapwell, Costar, Cambridge, MA) were seeded with three to four drops of the initial suspension. The basalateral side of the support was bathed in 2.0 ml of growth medium. Subsequent passages were carried out as detailed above. Cells in culture flasks and on permeable supports were fed every other day by removing the spent media and replacing it with an equal volume of fresh growth media. Some isolations have retained the ability to form high-resistance monolayers and continue to be responsive to forskolin through >20 passages and after being frozen and thawed. However, the principal focus of this report is to describe the morphology and electrophysiology of freshly isolated and cultured vas deferens epithelial cells. Results from the first and second passage were not significantly different from one another. Therefore, data from these initial cultures have been pooled for presentation.

Electrophysiology. Porcine vas deferens epithelial cells cultured on permeable supports were evaluated for transepithelial potential difference (PD), transepithelial electrical resistance (Rₑ), basolateral short-circuit current (Iₑ), and changes in Iₑ stimulated by selected physiological and pharmacological agents using a modified Ussing chamber (model DCV9, Navicyte, San Diego, CA). For anion substitution studies, symmetrical solutions of the following composition (in mM): Cl⁻, NaHCO₃, 3.3 KH₂PO₄, 0.83 K₂HPO₄, 1.2 CaCl₂, and 1.2 MgCl₂; were designed to test the effect of specific anions on transepithelial ion transport, and changes in Cl⁻ were monitored by 10.220.33.3 on June 22, 2017 http://ajprenal.physiology.org/ Downloaded from http://ajprenal.physiology.org/ by 122.20:33.3 on June 22, 2017

Tissue preparation for histochemistry. Vas deferens were acquired and stripped of connective tissue as described above. Tissues to be used for predigestion observations were fixed overnight in 10% buffered neutral formalin (BNF; Fisher Scientific, Pittsburgh, PA) at 4°C and rinsed in PBS for histochemistry (PBSb; in mM): 5 KH₂PO₄, 15 K₂HPO₄, and 150 NaCl, pH 7.2–7.4. Small segments of each specimen (0.5–1.0 cm) were cut and placed in cryoprotectant (Sorenson’s Phosphate Buffer (in mM: 8 KH₂PO₄, 42 NaH₂PO₄ containing 30% wt/vol sucrose + 0.02% wt/vol sodium azide for 1–2 h, then mounted and frozen onto cryostat mounting stages using Tris-buffered saline Tissue Freezing Medium (Triangle Biomedical Sciences, Durham, NC). The tissue was cut into 6- or 8-μm thick sections (Leica Kryostat 1720, Wetzlar, Germany) and picked up on subbed slides (0.5% wt/vol gelatin + 0.05% wt/vol chromium potassium sulfate, single coated). The slides were allowed to air dry on a heated stage (45°C) and were stained with hematoxylin and eosin Y (Fisher Scientific) by standard techniques, and coverslips were applied with Permount (Fisher Scientific). Tissue sections were observed and digital images were acquired with light microscopy (Leica DM RX, Wetzlar).

Trichrome staining and electron microscopy of cultured cells. Cells were grown on permeable supports for 24–48 h and then fixed in 10% BNF overnight at 4°C, followed by three 15-min rinses in PBSb, and stored in PBSb + 0.02% sodium azide. Epithelial cell monolayers were dehydrated in increasing concentrations of ethanol to absolute, followed by 100% glass-distilled acetone (Electron Microscopy Sciences, Fort Washington, PA), and embedded in Epon/Araldite (Electron Microscopy Sciences). For light microscopy observations, embedded tissues were cut into 0.6- to 0.8-μm semithin sections (Ultracut E ultramicrotome, Reichert-Jung) using a diamond knife (Diatome, Fort Washington, PA). Sections were heat-fixed on glass slides and stained as previously described (33). For electron microscopy, embedded tissues were cut into 90- to 95-nm ultrathin sections and picked up on Formvar-coated copper slot grids (Electron Microscopy Sciences). The sections were then stained with Reynold’s lead citrate (44) and uranyl acetate (5% wt/vol in acidic 70% ethanol) and viewed with an electron microscope at 80 kV (Philips model EM400, Natick, MA).

Immunohistochemistry. Isolated vas deferens epithelial cells were grown on permeable supports for 24–48 h and then fixed in 10% BNF for 1–2 h at room temperature. For immunofluorescence staining, the cells were treated with 5% normal goat serum (GIBCO BRL) in PBSb + 0.2% vol/vol Triton X-100 (PBSb-Tx) to block nonspecific staining. Primary antibody (anti-Pan Cytokeratin Clone C-11, mouse monoclonal; Sigma, St. Louis, MO; anti-ZO-1, rat monoclonal, Chemicon, Temecula, CA) was diluted 1:200 in PBSh-Tx and applied for 1 h in a humidified chamber at room temperature. The cells were then rinsed three times for 15 min in PBSb-Tx, and the secondary antibody (anti-cytokeratin, Texas red-conjugated goat anti-mouse IgG, Jackson ImmunoResearch Laboratories, West Grove, PA; anti-ZO-1, FITC-conjugated goat anti-rat IgG, Chemicon), diluted 1:100 in PBSb-Tx, was applied for 3 h at room temperature. Cells were rinsed in PBSb-Tx before and then rinsed in distilled water. After drying, coverslips were applied in antifading solution (0.6% N-propyl gallate, 70% glycerin in 30 mM Tris buffer). Cells were observed and digital images captured with light microscopy (Leica DM RX microscope using 596-nm polarized light). Digital images of treated and control cells were acquired with identical numerical settings and prepared for publication in parallel using CorelDRAW (version 8.2, Corel, Ottawa, Ontario).
Cell proliferation in supplemented media. Proliferation rates of passage 1 cells were observed for each of several growth conditions. Freshly isolated cells (passage 0) were grown to confluency as described above. Cells were dissociated and dispersed, and 24-well plates were seeded at a concentration of $1.0 \times 10^5$ cells per well. Growth conditions were as follows: 1) standard growth media, or growth media supplemented with 2) 1 mM sodium pyruvate, 3) pyruvate plus 0.2 U/ml insulin, 4) pyruvate plus 0.1 µg/ml hydrocortisone, or 5) pyruvate plus insulin plus hydrocortisone. At 24-h intervals beginning at 24-h postseeding, cells were dissociated, suspended in 100 µl of media, and loaded into a hemocytometer for determination of cell density. Cells from three wells were counted at each time point. A sigmoidal equation

$$y = y_0 \frac{a}{1 + e^{-\frac{t - x_0}{b}}}$$

in which $y_0$ was the minimal number of cells (constrained to 100,000), $a$ is the maximal change in cell number, $x_0$ was the time in hours to reach half maximal response, and $1/b$ represents the rate of proliferation at $x_0$. The fit was compared to the data (SigmaPlot version 6.00; SPSS, Chicago, IL).

Chemical sources. 4,4’-Diaminostilbene-2,2’-disulfonic acid (DNDS) was purchased from Acros Organics (Fairlawn, NJ). ATP was purchased from Boehringer-Mannheim (Indianapolis, IN). Forskolin (Coleus forskohilli) was purchased from Calbiochem (La Jolla, CA). Glycerin, penicillin, streptomycin, Tris, and Triton X-100 were purchased from Fisher Scientific. Gentamicin, collagenase, and trypsin plus EDTA were purchased from Gibco BRL. Amiloride, amphotericin B, bis-Tris propane (BTP), bumetanide, carbachol, charybdoxin, clotrimalazole, histamine, hydrocortisone, insulin, isoproterenol, norepinephrine, ouabain, N-propyl gallate, pyruvate, serotonin, sodium azide, sucrose, vasopressin, and vasoactive intestinal polypeptide (VIP) were purchased from Sigma. Uranyl acetate was purchased from Tousimis Research (Rockville, MD). N-[4-methylphenylsulfonyl]-N’-[4-trifluoromethylphenyl]urea (DASU-02) was synthesized de novo in the laboratory. Other chemicals were of reagent grade or better and purchased from reputable sources.

Stock solutions of modulators for Ussing chamber experiments. Solutions were prepared as follows: forskolin, 10 mM in ethanol; carbachol and histamine, 100 mM in H2O; charybdoxin, 100 µM in H2O; isoproterenol and norepinephrine, 10 mM in 1 mM ascorbic acid; serotonin, 50 mM in 1 mM ascorbic acid; VIP and vasopressin, 1 mM in H2O; amiloride and ouabain, 10 mM in H2O; bumetanide, 20 mM in ethanol; DASU-02, 100 mM in dimethyl sulfoxide (DMSO); clotrimalazole, 300 mM in DMSO; DNDS, 5 mM in Ringer solution; ATP, 10 mM in 10 mM BTP. Forskolin, bumetanide, and VIP were stored at $-20^\circ$C. Amiloride and vasopressin were stored at 4°C. All other modulators were freshly dissolved on the day of the experiment.

Statistical analysis. Numerical data from Ussing chamber experiments are presented as the arithmetic mean and SE of the mean using the culture well as the experimental unit. Where appropriate, Student’s t-test was employed to assess the likelihood of population differences. A probability of a type I error of $<5\%$ was considered statistically significant.

RESULTS

Morphological and histochemical characterization of cells isolated from deferent ducts. Deferent ducts were obtained before and after cell isolation and prepared for histochemical analysis to identify the origin of the cells that were isolated for in vitro cell culture. Sections from intact ducts revealed a lumen that was lined by a pseudostratified columnar epithelium composed predominantly of principal cells (Fig. 2A). Basal cells were observed near the basement membrane. After cell isolation, tissue sections exhibited no columnar epithelium, although a layer of what appeared to be basal cells remained in contact with the basement membrane (Fig. 2B). Thus the cell isolation protocol effectively removed the columnar epithelial cells throughout the deferent duct. The vast majority of these cells are reported to be principal cells, although a small proportion of the isolated cells likely includes other types [e.g., clear cells, dark (pencil) cells, narrow cells, etc.; 2, 43].

Epithelioid cells were routinely observed in the culture flasks after cell isolation. Photomicrographs of a typical epithelial cell culture at 24 and 48 h postisolation are presented in Fig. 3. Epithelial cell isolations contained predominantly small clusters of cells, although individual cells were also observed. Cell clusters attached to the culture substrate and spread out to reach confluency in the 25-cm² flask within 72–96 h after isolation. The cultured vas deferens cells exhibited a cobblestone pattern typical of epithelial cells.

Fig. 2. Photomicrographs of porcine vas deferens tissues before (A) and after (B) the cell isolation protocol. A pseudostratified columnar epithelium composed primarily of principal cells is present before enzyme exposure. After cell isolation, the remaining cells lining the lumen are presumed to be basal cells (arrows). Six-micrometer-thick sections, paraffin embedded, hematoxylin and eosin stained, are shown. Scale bar = 50 µm.
Additional morphological studies were completed to determine whether cultured vas deferens cells exhibited characteristics commonly associated with epithelia. Cells grown in culture on permeable supports exhibited a cuboidal epithelial morphology when assessed by both light and electron microscopy. A trichrome-stained section of cultured cells is shown in Fig. 4A. The cells grew as a continuous cuboidal pseudostratified layer on the permeable support. Portions of at least five cells are displayed with nuclei at varying heights. With transmission electron microscopy, cellular and subcellular components are observed (Fig. 4B). Cultured cells exhibited microvilli and tight junctions apically, and desmosomes dispersed along the lateral membranes. Additionally, smooth endoplasmic reticulum and many mitochondria are scattered throughout the cytoplasm. In separate experiments, cells were cultured on permeable supports and probed for anti-ZO-1 and anti-cytokeratin immunoreactivity (Fig. 4, C and E). Cultured cells exhibited epitopes that were recognized to be each of these antibodies.

In summary, the results show that pseudostratified columnar epithelial cells (primarily principal cells) are removed from the vas deferens by the isolation protocol. These cells attach, disperse, and proliferate in tissue culture flasks to rapidly reach confluency. Subsequently, cells that are passaged onto permeable supports develop into a cuboidal pseudostratified epithelial monolayer in which the cells exhibit morphological and cytochemical markers commonly associated with epithelium.

Electrophysiology. Electrophysiological techniques provide a stringent test of cellular differentiation and epithelial function. Experiments were conducted 5–22 days postseeding in modified Ussing chambers to test for the development of PD_{te}, R_{te}, and ion transport activity of the epithelial cells. Cultured cell monolayers exhibited a lumen negative PD_{te} at all time points although the magnitude was significantly greater (P < 0.001) after 11 days in culture (Fig. 5A). After recording PD_{te}, epithelial cell monolayers were clamped to 0 mV, and I_{sc} was continuously recorded. Basal I_{sc} was near zero and unaffected (P > 0.5) by the number of days in culture (Fig. 5B). Transepithelial resistance was determined by assessing the effects of a 5-mV bipolar pulse on I_{sc} and calculating the resistance using Ohm’s law. Epithelial cell monolayers displayed a high R_{te} (2,400 ± 300 Ω·cm²) after 5–8 days in culture that increased (P < 0.001) and reached a plateau.
Forskolin was employed as a stimulant of the cAMP second messenger cascade to assess the responsiveness of the cell monolayers. The addition of forskolin (2 μM) to the bathing solution caused a prototypical response that was characterized by a rapid transient peak in $I_{sc}$, followed by a sustained plateau (e.g., see Fig. 8). The increase in $I_{sc}$ was consistently accompanied by a reduction in $R_{te}$ suggesting that a conductive pathway had been activated. Forskolin-stimulated increases in $I_{sc}$ were routinely observed for periods in excess of 1 h (not shown). As shown in Fig. 5, $B$ and $C$, the responsiveness of epithelial monolayers to forskolin was fully developed at the earliest time point tested. Monolayers universally responded to forskolin-dependent stimulation of adenyl cyclase with an increase in $I_{sc}$ that was paralleled by a reduction in $R_{te}$. Results of subsequent experiments strongly suggest that the increase in $I_{sc}$ is indicative of Cl⁻ and/or HCO₃⁻ secretion.

To determine whether vas deferens epithelial cell monolayers could respond to physiological stimuli, various neurotransmitters, paracrine agents, and pharmaceuticals were employed as stimulants. As shown in Fig. 6, vasopressin, norepinephrine, and isoproterenol caused increases in $I_{sc}$ (as well as decreases in trans-epithelial resistance) that were qualitatively indistinguishable from a forskolin-stimulated response. Responses to adenosine were also indistinguishable from that of forskolin (not shown). Responses to the subsequent addition of forskolin were reduced in every case. VIP, ATP, and histamine were associated with smaller, yet significant ($P < 0.04$) increases in $I_{sc}$ whereas serotonin and carbachol were without effect ($P > 0.13$) on basal current. Results are summarized in Fig. 7A and show that cultured vas deferens epithelial cell monolayers consistently respond to only selected neurotransmitters. The receptor subtypes and second messenger cascades that modulate these responses remain to be determined.

Experiments were completed to pharmacologically test for the presence of selected ion transport proteins. As shown in Fig. 8A, basolateral addition of ouabain reversed forskolin-stimulated ion transport, indicating that Na⁺⁻K⁺-ATPase plays a crucial role in the response of the monolayer. Bumetanide reduced, but did not abolish, both forskolin- and norepinephrine-stimulated ion transport (Fig. 8B and $E$), suggesting that a component of the response requires the activity of the Na⁺⁻K⁺⁻2Cl⁻ cotransporter. Importantly, the inhibitory effect of bumetanide was manifested only from the basolateral side of the monolayer (Fig. 8B), strengthening the conclusion that at least a portion of the $I_{sc}$ reflects Cl⁻ secretion. Further support of this conclusion is presented in Fig. 8C. DASU-02, an inhibitor of the CFTR anion channel (38, 46–48), reduced forskolin-stimulated ion transport whereas DNDS (0.5 mM), an inhibitor of Ca²⁺-activated Cl⁻ channels, was without effect. DNDS has also been reported to inhibit Na⁺/HCO₃⁻ cotransporter (NBC) at greater concentrations (18). Basolateral exposure to DNDS resulted in a biphasic response that included both inhibition of forskolin-stimulated $I_{sc}$ and in a modest stimulation of $I_{sc}$ (Fig. 8D). This result suggests that NBC participates in the response to forskolin. The stimulatory component could reflect stimulation of other conductances (see DISCUSSION). Amiloride, an inhibitor of epithelial Na⁺ channels, was without effect on basal $I_{sc}$ (Fig. 8C).
and had no effect on either ATP-stimulated or forskolin-stimulated \( I_{sc} \) (data not shown). Amiloride's lack of effect appears to directly contradict an earlier report employing ovine vas deferens (4). The basis of this difference will be addressed in the DISCUSSION.

Basolateral \( K^+ \) conductance(s) are responsible for maintaining membrane potential during ongoing ion transport. \( Ba^{2+} \), a nonselective \( K^+ \) channel blocker, rapidly and significantly inhibited forskolin-stimulated \( I_{sc} \); inhibition was concentration dependent, and

Fig. 6. Cultured vas deferens epithelial cell monolayers selectively responded to neurotransmitters, paracrine agents, and pharmaceuticals with changes in ion transport, as indicated by changes in \( I_{sc} \). All epithelial monolayers exhibited basal currents of \(< 1 \mu A/cm^2 \) (the dotted line represents the 0-current level). Periodic deflections in the \( I_{sc} \) trace result from a 5-mV bipolar pulse \( (R_{sc} \geq 2,000 \, \Omega \cdot \text{cm}^2) \). Norepinephrine (10 \( \mu M \)), vasopressin (1 \( \mu M \)), isoproterenol (10 \( \mu M \)), and forskolin (For; 2 \( \mu M \)) caused an increase in \( I_{sc} \) (and concomitant decrease in monolayer resistance) that was characterized by a transient peak of 5–10 \( \mu A/cm^2 \) followed by a plateau phase of 3 to 6 \( \mu A/cm^2 \). Other compounds were used at the following concentrations: vasoactive intestinal polypeptide (VIP), 1 \( \mu M \); ATP, 10 \( \mu M \); serotonin, carbachol, and histamine, 100 \( \mu M \). In these experiments, all compounds were simultaneously added to both the apical and basolateral compartments. Results are typical of 4–21 observations for each agonist.

Fig. 7. A: peak and sustained changes in \( I_{sc} \) (measured as a change from baseline) to various agonists. Adenosine, isoproterenol, norepinephrine, vasopressin, ATP, and histamine were associated with significant transient \( (P < 0.003) \) and sustained \( (P < 0.04) \) increases in \( I_{sc} \). VIP, carbachol, and serotonin produced no consistent effect on \( I_{sc} \). Results from 4–21 experiments for each condition are summarized. B: inhibitors of various ion transport proteins were evaluated. Amiloride (\( \text{ENaC} \) inhibitor) had no effect on basal \( I_{sc} \) \( (P > 0.8) \). When introduced after cAMP-dependent stimulation with forskolin, \( N^\prime-(4\text{-methylphenylsulfonyl})-N^\prime(4\text{-trifluoromethylphenyl})\text{rea} \) (DASU-02; CFTR inhibitor) and bumetanide (Bum; \( \text{Na}^+ - \text{K}^+ - 2\text{Cl}^- \) cotransporter inhibitor) significantly reduced \( I_{sc} \) \( (P < 0.001; \Delta I_{sc} \approx 3 \mu A/cm^2) \). Results from 20–26 experiments for each condition are summarized.

\[ \Delta I_{sc} \approx 3 \mu A/cm^2 \]
60% inhibition was observed at the highest concentration tested (3 mM, Fig. 9, A and E). Clotrimazole and charybdotoxin were employed as selective inhibitors of an intermediate-conductance K⁺ channel, IK. Clotrimazole significantly reduced forskolin-stimulated Isc at the highest concentration tested (30 μM, Fig. 9, B and E) but was without effect at 3 μM (data not shown), a concentration reported to inhibit IK in other secretory epithelia (17, 18). A more potent and selective inhibitor of IK, charybdotoxin, was employed to further test for the presence of this conductance and was found to be without effect (Fig. 9, C and E). Amiloride, a blocker of the cAMP-dependent, small-K⁺ conductance (IsK), was employed to test for a contribution to forskolin-stimulated ion transport from this conductance. As shown in Fig. 9, D and E, 293B was without effect. Taken together, these results indicate that a basolateral K⁺ conductance is involved in ongoing ion transport, but that it is neither IK nor IsK. Further experiments are required to determine the identity of this Ba²⁺- and clotrimazole-sensitive K⁺ conductance(s).

Anion substitution experiments were conducted to further characterize the ionic basis of the Isc. As shown in Fig. 10, a maximal response to ATP and bumetanide requires the presence of Cl⁻ in the bathing media. Similarly, the forskolin-stimulated transient increase in ion transport required Cl⁻ to be present. The absence of HCO₃⁻ did not adversely affect these responses. Alternatively, the prolonged effect of forskolin was dependent on HCO₃⁻ in the bathing media, and, in the absence of Cl⁻, was bumetanide insensitive. In the absence of Cl⁻ and HCO₃⁻, no sustained effects of ATP or forskolin are observed. The transient peak effects of ATP and forskolin on Isc, however, are significantly different from zero and might reflect the loss of anions (i.e., trapped Cl⁻ and HCO₃⁻) from epithelial cell cytoplasm into the apical media. The sustained effect of forskolin (0.51 ± 0.23 μA) is not significantly different from zero in these conditions. These results suggest
that at least two sustained ion transport pathways are stimulated by forskolin. The first requires the presence of \( \text{Cl}^- \) and is bumetanide sensitive, and the second requires \( \text{HCO}_3^- \) and is bumetanide insensitive.

Taken together, these results demonstrate that cultured epithelial cell monolayers develop an electrically tight barrier, actively transport ions to develop a luminal-negative potential in basal conditions, and behave as functional syncitium that acutely and selectively respond to neurotransmitters and peptide hormones, with changes in ion transport that would be expected to change the composition and/or volume of luminal fluid.

**Optimization of culture conditions.** A relatively simple medium has proven itself extremely effective for the initial culture of vas deferens epithelial cells. However, we sought to determine whether commonly employed supplements would affect cell proliferation. Thus cells isolated from individual vas deferens were seeded onto 24-well plates and cultured in selected supplements, and, at specified intervals, cells were dissociated and counted (Fig. 11). The fit of a sigmoidal function to the data suggests that insulin supplementation significantly decreases the doubling time during log growth (control, \( 43.9 \pm 9.5 \text{ h/generation} \) vs. pyruvate + insulin, \( 20.0 \pm 3.3 \text{ h/generation} \)) and increases the number of cells present at growth climax (control, \( 1.5 \pm 0.3 \times 10^6 \text{ cells/well} \) vs. pyruvate + insulin, \( 5.1 \pm 0.3 \times 10^6 \text{ cells/well} \)). Pyruvate and pyruvate + hydrocortisone differed little from control. Doubling time and maximal number of cells in the presence of pyruvate + insulin + hydrocortisone was similar to that observed in the presence of pyruvate and insulin. Lag time was not affected by media supplementation. Additional studies are required to determine whether the proliferative effects of insulin would be duplicated with cells cultured on permeable supports and to determine whether ion transport by epithelial monolayers would be affected.

**DISCUSSION**

In this report, we define techniques to routinely isolate and culture vas deferens epithelial cells in sufficient quantities to conduct numerous tightly paired experiments to better understand the role of vas deferens epithelium in fertility. Results show that a population of columnar epithelial cells, predominantly principal cells, are isolated from the duct. These isolated
cells attach to standard tissue culture flasks and rapidly form a monolayer with epithelioid morphology. When passed to permeable supports, the cells express both ZO-1 and cytokeratin, biochemical markers of epithelial cells, and exhibit epithelial morphology when assessed by either light or electron microscopy. Results from electrophysiological experiments demonstrate that cultured cells exhibit functional characteristics of epithelial tissues, including a basal transepithelial potential difference, a relatively high transepithelial resistance, and the ability to actively transport ions. Most importantly, the results demonstrate that cultured porcine vas deferens epithelial cell monolayers provide differential responses to physiological and pharmacological stimuli. Thus a system has been developed to identify and study physiological mechanisms that regulate the luminal environment of the distal duct through which sperm must pass.

The results provide a framework from which to propose a basic cellular model of ion transport (Fig. 12). Included in this model are components for which highly selective inhibitors are available: the ouabain-inhibitable Na\(^+-\)K\(^+-\)ATPase and the bumetanide-inhibitable Na\(^+-\)K\(^+-\)2Cl\(^-\) cotransporter. DASU-02 has been shown to inhibit CFTR-mediated anion transport in other systems (38, 46–48), and there is good reason to expect CFTR to be functional in this epithelium because the vas deferens is severely affected in virtually all CF patients. Thus the presence and functional activity of these three components in vas deferens epithelia are reasonably assured.

A basolateral K\(^+\) conductance is included in Fig. 12. Such a pathway is required to maintain the membrane potential in the presence of other ion transport mechanisms. Thus K\(^+\) entering the cell via the bumetanide-sensitive cotransporter can be recycled to the basolateral compartment and can allow for electrogenic anion secretion at the apical membrane. The identity of the conductance remains to be determined. Blocker pharmacology is consistent with neither IK nor IsK because of the lack of inhibition by charybdotoxin and 293B, respectively. However, inhibition by high concentrations of clotrimazole suggests some similarities with IK, and stimulation of \(I_{\text{sc}}\) by DNDS (Fig. 8D) suggests that some similarities to IsK may also be present (1, 49). Additional experiments are required to determine whether IK, IsK, and/or other K\(^+\) channels contribute to vas deferens ion transport.

Fig. 10. Agonist-induced changes in \(I_{\text{sc}}\) are dependent on the anion composition of the bathing solution. Typical responses to ATP (10 \(\mu\)M, apical only), forskolin (2 \(\mu\)M, symmetrical), and Bum (20 \(\mu\)M, basolateral only) in Ringer solutions of the indicated composition are shown in A–D. Data from this and 6 similar experiments are summarized in E. Responses to ATP, Bum, and the peak response to forskolin are significantly reduced \((P < 0.007)\) by the absence of Cl\(^-\). The sustained \((\text{Sust})\) effect of forskolin is significantly reduced in the absence of HCO\(_3\)\(^-\) \((P < 0.001)\). All epithelial monolayers exhibited basal currents of <1 \(\mu\)A/cm\(^2\) (the dotted line represents the 0-current level). Periodic deflections in the \(I_{\text{sc}}\) trace result from a 5-mV bipolar pulse \((\text{Basal } R_{\text{te}} = 3,500 \Omega \text{cm}^2)\).
Anion substitution studies indicate that both Cl\(^{-}\) and HCO\(_3\)^{-}-dependent ion transport processes are stimulated by forskolin. Activation of CFTR could account for this response, although other mechanisms must also be invoked. Two components of the response, transient and sustained, exhibited differential sensitivity to the absence of Cl\(^{-}\). Transient effects require the presence of Cl\(^{-}\) whereas sustained effects require that both anions are present. To partially account for these results, we propose that Cl\(^{-}\) enters the cell at the basolateral membrane through a bumetanide-sensitive cotransporter and exits cells across the apical membrane through CFTR. An additional ion transport mechanism is required to account for the HCO\(_3\)^{-}-dependent \(I_{sc}\) observed in the absence of Cl\(^{-}\). HCO\(_3\)^{-} may enter the cell at the basolateral membrane via NBC (inhibited by DNDS) and leave the cell through CFTR or perhaps another pathway as indicated. Alternatively, the forskolin-stimulated current in the absence of Cl\(^{-}\) could be cation absorption. However, the lack of an amiloride effect on these cells suggests that Na\(^{+}\) is not being absorbed. There are other possible interpretations of these data, including the presence of an anion exchanger or the placement of an alternative NBC (i.e., NBC3) in the apical membrane (42). However, definitive experiments to separate these possibilities and elucidate the identity and location of the transport proteins are beyond the scope of the present publication. Regardless, the sustained current in normal Ringer solution appears to be a combination of Cl\(^{-}\) and HCO\(_3\)^{-} secretion. This conclusion would also account for the rather modest effect of bumetanide in control conditions, but its virtually complete inhibition in the absence of HCO\(_3\)^{-}.

Forskolin, a compound known to activate adenylyl cyclase, consistently increases \(I_{sc}\), indicating that transport mechanisms (likely including CFTR) are modulated by this second-messenger cascade. Ion substitution studies indicated that forskolin stimulated more than one ion transport pathway. Numerous neurotransmitters were also shown to stimulate epithelial ion transport, with their receptors being discretely localized to either the apical or basolateral membrane. Depending on the neurotransmitter, both transient and sustained responses are observed, suggesting that unique pathways could be independently modulated by the various ligands. These results are particularly intriguing in light of the fact that vas deferens muscle contraction is particularly sensitive to sympathetic stimulation (i.e., norepinephrine) and that numerous transmitters (especially ATP) are coreleased with norepinephrine (52). We have demonstrated that these and other neurotransmitters acutely modulate epithelial function in this portion of the deferent duct, as well. More recent studies indicate that adenosine stimulates DASU-02-sensitive anion secretion across freshly excised vas deferens (45), providing evidence that the cultured cell system mimics the native system in this regard.
Ion channels present in vas deferens epithelial membranes remain to be fully determined. Inhibition by DASU-02 and the association of CBAVD with CF strongly suggest that CFTR participates in the response. However, we previously reported that DASU-02 inhibits CFTR with an IC50 of ~10 μM (47). Thus the basis of the $I_{sc}$ remaining in the presence of 100 μM DASU-02 is not likely mediated by CFTR. Neither Cd2$^{2+}$ (300 μM; an inhibitor of Cl$^-$/Ca2$^{2+}$ channels; data not shown) nor DNDS (500 μM; an inhibitor of Ca$^{2+}$-activated Cl$^-$ channels and outwardly rectifying Cl$^-$ channels) had any effect on forskolin-stimulated $I_{sc}$, suggesting that these conductances cannot account for the forskolin-stimulated ion transport. Given that a major component of the $I_{sc}$ is Cl$^-$ independent, these results suggest that a novel HCO3$^-$/Cl$^-$ conductive pathway might be present. Certainly, additional experiments are required to test this hypothesis.

Stimulation of ion transport was consistently accompanied by a reduction in $R_{sh}$. The simplest interpretation of these data is that a conductive pathway(s) is activated by stimulation. All measurements of ion flux were made in short-circuited conditions such that changes in $I_{sc}$ reflect only active transcellular transport that includes the activation of conductive pathways in the cell membranes (e.g., CFTR). Inhibition by DASU-02 was accompanied by an increase in $R_{sh}$ that further supports the presence of a conductive pathway in the activated cell monolayers. However, stimulants of ion transport have also been reported to modulate the resistance or ion selectivity of the paracellular pathway in leaky epithelia (5, 32). Similar effects of forskolin on the paracellular pathway of the relatively high-resistance porcine vas deferens remain a possibility but are not directly addressed by the present data.

The procedure that we describe for the culture of adult porcine vas deferens epithelial cells is less demanding than methods reported for the primary culture of either fetal human (22) or neonatal ovine (4) vas deferens epithelial cells. The collagenase technique for cell isolation provides a starting population of cells that is highly enriched for epithelial cells and includes extremely few fibroblasts. The growth media employed for porcine cells was simpler than media employed for either the ovine or human cells. Harris et al. (22), reported that insulin, cholera toxin, and hydrocortisone were “essential” for maintenance of cell morphology in these systems. These supplements were not essential for the culture of porcine cells in the present study, although insulin was shown to dramatically enhance proliferation rate. As previously stated, additional studies are required to identify the effects of these supplements on ion transport. Additionally, we have observed that chronic treatment with hydrocortisone induces the expression of an amiloride-sensitive current in basal conditions (i.e., ENaC expression; Carlin RW and Schultz BD, personal observations). Thus different culture conditions clearly account for some of the differences in results between this study and previously published reports. Differences in species and the stage of sexual maturation likely contribute to distinct outcomes as well.

The present results support and extend earlier observations in which membrane patch-voltage clamp techniques were used to identify ion conductances in vas deferens epithelial cells. Two Cl$^-$ channels were described in human vas deferens epithelial cells that might contribute to electrolyte and fluid secretion (41). The smaller of the two channels was activated by forskolin and exhibited a conductance that might be expected for CFTR. Additionally, an apical Ca$^{2+}$-activated K$^+$ channel was observed (50). These observations show that mechanisms are present that might support epithelial anion or cation transport. The present results suggest that forskolin stimulates anion secretion across this epithelia via apical Cl$^-$ channels (CFTR) that are electrically coupled to K$^+$ conductances in the basolateral membrane. A HCO3$^-$-dependent component was also observed. This could indicate that HCO3$^-$ secretion accounts for a portion of the $I_{sc}$.

The excurrent duct of the rat is probably the most widely studied of any species and thus the best characterized. Breton, Brown, and co-workers (6, 7, 10) have proposed that the epididymis and, to a much lesser extent, the vas deferens is an acid-secreting and/or bicarbonate-absorbing epithelium. In stark contrast, Wong and co-workers (34, 53) propose a mechanism for cAMP-stimulated Cl$^-$ and HCO3$^-$ secretion in rat epididymis. Our results from the porcine vas deferens support and extend observations reported by Wong et al. (54), in that we observe inhibition of cAMP-stimulated ion transport by DASU-02, ouabain, and bumetanide. Similarly, we observed that ATP (apical) and norepinephrine (basolateral) stimulated anion secretion. The apparently conflicting results can be reconciled by noting that in one case [Breton et. al. (6, 7, 10)] basal ion transport in freshly excised tissues was assessed, whereas in the other cases [Wong et. al. (34, 54) and the present study] stimulated ion transport across cultured epithelial cell monolayers was evaluated. Previous reports suggest that, in the absence of stimulation, the epididymis and the proximal vas deferens establishes an acid environment for the storage of quiescent sperm. Our results suggest that sympathetic stimulation of the vas deferens contributes to an alkalization of the lumen that could occur during the period of arousal before ejaculation. If one assumes a duct diameter of 2 mm, a length of 30 cm, and a stimulated current of 5 μA with bicarbonate secretion accounting for 80% of the current, one then calculates that during 15 min of preejaculatory stimulation, the duct might secrete as much as $0.7 \times 10^{-6}$ base equivalents into a volume of <1 ml. This magnitude of base secretion could raise the luminal pH substantially and contribute to the timely activation of sperm (12). Congenital bilateral absence of the vas deferens (CBAVD) accounts for a significant proportion of male

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1) Inhibition by higher concentrations of DNDS at the basolateral membrane might indicate that a Cl$^-$ conductance is present at the basolateral membrane. However, such a Cl$^-$ conductance would not be expected to contribute to the apical secretion of anions.
infertility (19, 20, 39). Men affected by CF, a disease of epithelial ion transport and the most common lethal recessive genetic disease of Caucasians, are almost universally (>97%) sterile due to CBAVD (3, 14, 20). Denning et al. (16) first documented azoospermia in a few CF patients who survived to adulthood some 30 years ago. Improved treatment for CF patients has expanded and will continue to expand the population affected by CBAVD in the coming years (23). However, the causal relationship between CF and CBAVD remains to be determined. The vas deferens epithelial cell model that we have developed will be informative in understanding the exquisite susceptibility of the vas deferens to mutations in CFTR and the associated changes in epithelial ion transport that contribute to duct attenuation and male infertility.

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