Bradykinin-stimulated cyclooxygenase activity stimulates porcine and human vas deferens epithelial anion secretion in vitro

Fernando Pierucci-Alves, Bruce D. Schultz

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Title: Bradykinin-stimulated cyclooxygenase activity stimulates porcine and human vas deferens epithelial anion secretion in vitro

Short title: Regulation of vas deferens anion secretion

Summary sentence:
Bradykinins acutely modulate the luminal environment to which sperm is exposed in the vas deferens by enhancing epithelial cyclooxygenase activity and production of prostaglandins

Authors: Fernando Pierucci-Alves[1], Bruce D. Schultz
Department of Anatomy & Physiology
Kansas State University
Manhattan, KS 66506

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[1] Correspondence and reprint requests:
Fernando Pierucci-Alves
Department of Anatomy & Physiology
Kansas State University
1600 Denison Ave, Coles Hall 228
Manhattan, KS 66506
Phone: (785) 532-4840
Fax: (785) 532-4557
E-mail: falves@vet.ksu.edu

Abstract

Epithelia lining the male reproductive duct modulate fertility by altering the luminal environment to which sperm are exposed. Although vas deferens epithelial cells reportedly express high levels of cyclooxygenases (*Ptgs*), and activation of bradykinin (BK) receptors can lead to upregulation of PTGS activity in epididymal epithelia, it remains unknown whether BKs and/or PTGSs have any role in modulating epithelial ion transport across vas deferens epithelia. Porcine and human vas deferens epithelial cell primary cultures and the PVD9902 cell line responded to lysylbradykinin with an increase in short circuit current (I_{sc}; indicating net anion secretion), an effect that was 60-93% reduced by indomethacin. The BK effect was inhibited by the B2 receptor subtype (BDKRB2) antagonist HOE140, while the B1 receptor subtype agonist des-Arg^9-BK had no effect. BDKRB2 immunoreactivity was documented in most epithelial cells composing the native epithelium and on Western blots derived from cultured cells. Gene expression analysis revealed that the *PTGS2* transcript is 20 times more abundant than its *PTGS1* counterpart in cultured porcine vas deferens epithelia and that *BDKRB2* mRNA is likewise highly expressed. Subsequent experiments revealed that prostaglandin E2, 1-OH prostaglandin E1 (prostaglandin E receptor 4 [PTGER4] agonist) and butaprost (PTGER2 agonist) increase I_{sc} in a concentration-dependent manner, whereas sulprostone (mixed PTGER1 and PTGER3 agonist) produced no change in I_{sc}. These results demonstrate that autacoids can affect epithelial cells to acutely modulate the luminal environment to which sperm are exposed in the vas deferens by enhancing PTGS activity leading to the production of prostaglandins that act at PTGER4 and/or PTGER2 to induce or enhance anion secretion.

Introduction

Autacoids modulate cellular processes, including ion transport across several epithelia, and data supporting such functional roles have been accumulating since these compounds were first described for their potent effect on vascular endothelia [1]. Bradykinin (BK) can be synthesized in plasma by plasma kallikrein and its analog lysylbradykinin (LBK) can be synthesized on the surface of target cells by tissue kallikrein (tK) [2]. Receptors for bradykinins, specifically the B2 receptor subtype (BDKRB2), are present in tracheal and alveolar epithelia [3, 4] and in renal [5-8], corneal [9] and colonic epithelia [10, 11], where they reportedly stimulate electrogenic ion transport. In colonic epithelia, BDKRB2-stimulated ion transport is dependent on the cystic fibrosis transmembrane conductance regulator (CFTR) [11] channel that conducts both chloride and bicarbonate. *BDKRB2* transcripts are present in Sertoli cells, epididymal epithelia and prostatic epithelial cells of the rat [12, 13], and pharmacological data reveal BDKRB2 modulation of cytoplasmic calcium levels in canine prostatic epithelial cells [14]. In cultured rat epididymal epithelial cells, LBK activates prostaglandin-endoperoxide synthases (PTGS [also known as cyclooxygenases 1 and 2]) and increases local prostaglandin (PG) synthesis [15, 16]. A primary culture system in which rat epididymal epithelial cells were sorted into two populations, basal and principal cells, revealed unique response profiles to LBK. A short circuit current (I_{SC}) increase was observed across basal cells but not across principal cells, unless these were recombined with basal cells. This report indicated that PTGS1 was expressed in basal cells but not in principal cells [17]. Of greater interest to the current study are data revealing that *Ptgs* expression is highest in vas deferens epithelia [18]. Semi-quantitative protein and gene expression analysis revealed that vas deferens epithelia express both PTGS isoforms at the highest levels, when compared to other segments of the mouse reproductive tract. Likewise, intense PTGS2 immunoreactivity has been demonstrated in epithelial cells lining the human distal vas deferens and prostate [19]. To date, it remains unknown whether BKs and/or PTGSs have a role in modulating epithelial ion transport across vas deferens epithelia.

The goal of this study was to determine whether BKs modulate ion transport across vas deferens epithelia. Outcomes demonstrate BK-induced ion transport and suggest that the effects are mediated by stimulation of PTGS activity with subsequent PG synthesis. Molecular identity, localization and quantitative levels of molecular constituents of this pathway are described.

Material and Methods

Vas Deferens Tissue Acquisition and Epithelial Cell Isolation. Porcine vas deferens were surgically excised immediately postmortem from sexually mature boars at a local swine production facility, placed in ice-cold Hank's buffered salt solution (mM composition: 137 NaCl, 5.4 KCl, 0.4 KH_2PO_4, 0.6 Na_2HPO_4, 5.5 glucose) and transported to the laboratory where isolation of porcine vas deferens epithelial cells for primary culture (1^oPVD) was performed as described previously [20]. Distal human vas deferens (2 to 5 cm long) were obtained from a local hospital through procedures approved by both the University and hospital Institutional Review Boards. Isolation of human vas deferens epithelial cells for primary culture (1^oHVD) has been described previously [21].

Cell Culture. 1^oPVD, 1^oHVD and PVD9902 (an immortalized epithelial cell line derived from porcine vas deferens [22]) were seeded on 25-cm^2 tissue culture flasks or 24-well tissue culture plates and grown in Dubelcco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and 1% penicillin and streptomycin (Invitrogen) with media changes every other day. Upon reaching confluency, cells were lifted with phosphate buffer saline (PBS) containing trypsin and ethylenediaminetetraacetic acid (Invitrogen), suspended, and seeded on 1.13 or 0.33 cm^2 Transwell permeable supports (Corning-Costar, Cambridge, MA) to form polarized epithelial cell monolayers. Cells were cultured with media changes every other day until assayed in modified Ussing chambers and/or used for other experimental protocols, typically 11-14 days.

Electrophysiology. Epithelial cell monolayers were mounted in modified Ussing flux chambers (model DCV9; Navicyte, San Diego, CA), bathed symmetrically in Ringer solution (composition in mM: 120 NaCl, 25 $NaHCO_3$, 3.3 KH_2PO_4, 0.83 K_2HPO_4, 1.2 $CaCl_2$, 1.2 $MgCl_2$), maintained at 39^oC for 1^oPVD and PVD9902 or 37^oC for 1^oHVD and bubbled with 5% CO_2-95% O_2 to provide mixing and pH stability. Monolayers were clamped to 0 mV and I_{SC} was measured continuously with a voltage-clamp apparatus (model 558C; University of Iowa, Dept. of Bioengineering, Iowa City, IA). Data were acquired digitally at 1 Hz with an Intel-based computer using an MP100A-CE interface and AcqKnowledge software (version 3.7.3; BIOPAC Systems, Santa Barbara, CA). Once recordings began, this system automatically generated a 5 mV bipolar pulse every 100 secs. The resulting change in I_{SC} was used to calculate transepithelial electrical resistance (R_{TE}) according to Ohm's law. To test for various experimental hypotheses,

monolayers were exposed to different compounds: LBK, BK, des-Arg^{9}-BK, HOE140 (Bachem, King of Prussia, PA); des-Arg^{10}-HOE140 (Anaspec, San Jose, CA); PGE2, butaprost, indomethacin, amiloride, bumetanide (Sigma-Aldrich, Saint Louis, MO); 1-OH prostaglandin E1 (1-OH PGE1) and sulprostone (Cayman Chemical, Ann Arbor, MI).

Western Blot Analysis. 1^{o}PVD monolayers cultured on permeable supports were lysed and solubilized in buffer containing protease inhibitors (Calbiochem, San Diego, CA). After centrifugation at 14000 rpm and 4^{o}C for 15 min, the supernatant protein isolates were transferred to a fresh tube and quantified by a ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). Protein sample aliquots were prepared for electrophoresis by addition of loading buffer (Boston Bioproducts, Worcester, MA), ß-mercaptoethanol (Sigma-Aldrich) and heated to 95^{o}C for 5 min. Sodium dodecyl sulfate polyacrylamide gel electrophoresis was conducted with 4-20% polyacrylamide gradient pre-cast gels (Bio-rad Laboratories, Hercules, CA) at 150 V for approximately 1.5 h or until optimal separation of pre-stained protein standards (Bio-rad Laboratories) was obtained. Electro-transfer onto PVDF membranes (Millipore, Billerica, MA) was carried out in methanol-containing transfer buffer (20% v/v) at 30V for 3 h. Blots were blocked by blotting grade non-fat dry milk (Bio-rad Laboratories, 5% w/v) and exposed to 0.08 μg/ml of anti-BDKRB2 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) or anti-BDKRB2 antibody pre-adsorbed by its immunizing peptide. A suitable horseradish peroxidase (HRP) conjugated secondary antibody was employed to develop chemiluminescence signals that were imaged by a FluorChem HD2 (Alpha Innotech Corporation, San Leandro, CA).

Immunocytochemistry. Segments (~1 cm) of both proximal and distal porcine vas deferens were snap-frozen in liquid N_2 immediately after ducts were obtained and were transported to the laboratory where they were kept frozen while being embedded in tissue freezing medium (Richard-Allan Scientific, Kalamazoo, MI) for cryosectioning. Sections (18 μm) were collected onto poly-L-lysine-coated slides, fixed in 4% paraformaldehyde in PBS for 1 hour, PBS-washed five times and subjected to conventional immunocytochemistry labeling. In short, tissues were blocked with bovine serum albumin (Sigma-Aldrich) in PBS and exposed to 2 μg/ml of anti-BDKRB2 antibody, anti-BDKRB2 antibody pre-adsorbed by its immunizing peptide, or vehicle only, overnight, in a humidified chamber. Tissues were PBS-washed five times and subsequently exposed to a suitable fluorophore-conjugated secondary antibody (Alexa Fluor 488, Invitrogen) and to a fluorescent chromatin dye (TO-PRO-3, Invitrogen) for 1 hour.

Following 5 PBS-washes, cover slips were mounted with Fluoromount-G (SouthernBiotech, Birmingham, AL) and confocal microscopy was performed (LSM 510 META; Carl Zeiss Microimaging Inc, Thornwood, NY).

RNA Isolation and Semi-Quantitative RT-PCR. Total RNA was isolated from epithelial cell monolayers following Ussing chamber protocols, using an RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. RNA quality was assessed with a 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). RNA concentration within isolates was determined using a ND-1000 spectrophotometer and all samples were diluted to 100 ng/μl. One-step RT-PCR was performed using a One-step RT-PCR kit (Qiagen) according to manufacturer's recommendations, with 100 ng of RNA per reaction. Single-target reactions were conducted using primer sets designed upon previously published cDNA sequences when available. Porcine PTGER1, PTGER2 and PTGER4 coding sequences had not been published. Thus, sequence homology searches were performed by querying the porcine gene index from The Institute for Genomic Research (TIGR) with the respective human sequences. Such searches yielded at least one hit with relevant homology to the *PTGER2* and *PTGER4* cDNAs and these candidate sequences were used in the design of primer sets. No porcine candidate-sequences were identified for the *PTGER1* and the human *PTGER1* sequence was used to design a primer set for the porcine target. Primer sequences, annealing temperatures, expected amplicon length and sequence accession numbers are presented in Table 1. SYBR Green I (Molecular Probes, Eugene, OR) fluorescence signals emitted at each amplification cycle were detected and quantified by a SmartCycler (Cepheid, Sunnyvale, CA). Transcript abundance measurements in any given RNA sample were performed by reactions carried out in duplicates. Melting analysis was performed at the end of every amplification phase and reaction products were subjected to agarose gel electrophoresis, sequencing and computer-based identity analysis. Acquired threshold cycle (C_t) values for target genes were normalized to the reference porcine gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) according to the amplification efficiency method [23].

Complementary DNA Sequencing. RT-PCR products resolved on agarose gels were excised and purified with a QIAquick gel extraction kit (Qiagen) and the resulting cDNA templates were sequenced with a CEQ8000 (Beckman Coulter, Fullerton, CA) according to manufacturer's recommendations.

Statistical Analysis. Paired and unpaired *t*-tests and analysis of variance (ANOVA) were performed as appropriate. These tests and the calculation of means and standard error of the mean (SEM) were performed with Microsoft Office Excel 2003 (version 11.8211.8202; Microsoft Corporation, Redmond, WA). A modified Michaelis-Menten (M-M) equation: $y = [ax]/[c + x]$, where y is the measured ΔI_{SC}, a is the maximum ΔI_{SC} ($\Delta I_{SC\ -MAX}$), x is the concentration of agonist and c is the concentration required for a half-maximal response (i.e., the apparent K_D or k_{app}) was fitted to data collected from concentration-dependency experiments. All graphs and curve fittings were made with SigmaPlot (version 6.0; Systat Software Inc., Point Richmond, CA).

Results

Bradykinins Induce Anion Secretion Across Vas Deferens Epithelia

To investigate whether BKs modulate ion transport across vas deferens epithelia, cells derived from human and porcine vas deferens, as well as the vas deferens epithelial cell line PVD9902 were used. 1^{o}PVD monolayers (n = 7 animals) revealed basal I_{SC} and R_{TE} of 0.7 ± 0.2 μA*cm^{-2} and 4400 ± 610 Ω*cm^2, respectively. LBK (500 nM), symmetrically added to the bathing media, caused a rapid bimodal increase in I_{SC} that formed a first peak before it decreased briefly and generated a second peak (Fig. 1A). $\Delta I_{SC\text{-}MAX}$ was 5.1 ± 1.0 μA*cm^{-2}. I_{SC} then receded to a nadir and rose again to a plateau that lasted several minutes. Sustained I_{SC} was partially sensitive to basolateral bumetanide (20 μM; data not shown) indicating that a portion of the I_{SC} is attributable to Cl^- secretion, as has been shown for other agonists affecting vas deferens epithelial cells [20, 24]. R_{TE} rapidly but transiently declined upon exposure to LBK. The lowest value was consistently observed at the first voltage pulse following LBK exposure and then R_{TE} recovered towards pretreatment values. This is depicted in the deflections that are of maximal magnitude at the onset of responses and their subsequent and progressive narrowing. Maximal decrease in R_{TE} ($\Delta R_{TE\text{-}MAX}$) was 3160 ± 500 Ω*cm^2. Experiments carried out with 1 nM LBK, the lowest concentration tested, revealed $\Delta I_{SC\text{-}MAX}$ of 4.5 ± 1.2 μA*cm^{-2} (n = 5 animals), which was not different from the results obtained with 500 nM. However, I_{SC} tracings produced by 1 nM formed a single transient peak that relaxed to basal levels and then rose to a plateau (Fig. 1B). To assess whether LBK produced a sustained effect, ΔI_{SC} measured between 900 and 1000 secs after the onset of responses were determined: ΔI_{SC} was 1.1 ± 0.1 μA*cm^{-2} and 0.9 ± 0.3 μA*cm^{-2} for

responses acquired with 500 nM and 1 nM LBK, respectively. At 1 nM, $\Delta R_{TE\text{-}MAX}$ was 2170 ± 670 Ω*cm^2 and, similar to that observed with 500 nM, R_{TE} was reduced to the lowest value with the onset of each response and then recovered gradually towards pretreatment values. Still in the context of the first experiments, PVD9902 monolayers (n = 6) were assessed with 500 nM LBK. From basal I_{SC} and R_{TE} at 0.4 ± 0.1 μA*cm^{-2} and 6900 ± 1400 Ω*cm^2, respectively, these monolayers responded with $\Delta I_{SC\text{-}MAX}$ of 5.6 ± 1.0 μA*cm^{-2} and $\Delta R_{TE\text{-}MAX}$ of 2720 ± 650 Ω*cm^2 (Fig. 1C). I_{SC} tracings from PVD9902 revealed a sharp and rapid rise in I_{SC} to form a single peak that declines toward baseline in about 300 s. The magnitude of responses acquired from 1^{o}PVD and PVD9902 at the tested concentrations were indistinguishable ($P < 0.05$). Subsequent experiments tested for bradykinin modulation of ion transport across 1^{o}HVD monolayers. A first 1^{o}HVD monolayer was tested with 1 nM LBK, which produced a small $\Delta I_{SC\text{-}MAX}$ (~0.8 μA*cm^{-2}). After 400 s, a second and cumulative LBK addition brought the concentration to 10 nM and the recorded $\Delta I_{SC\text{-}MAX}$ was 6.4 μA*cm^{-2} for this monolayer. Based on this single observation all subsequent experiments with 1^{o}HVD were conducted with 10 nM LBK. 1^{o}HVD epithelial monolayers (n = 6 individuals) were, at first, exposed to 10 μM amiloride, to which they consistently responded with decrease in I_{SC} (data not shown, [21]). These monolayers were then allowed sufficient time to establish a new baseline, at which point they exhibited basal I_{SC} of 3.9 ± 1.2 μA*cm^{-2} and basal R_{TE} of 550 ± 180 Ω*cm^2. Upon LBK exposure (10 nM), 1^{o}HVD monolayers responded with a rapid increase in I_{SC} that formed one single peak (Fig. 1D), in a pattern similar to 1^{o}PVD responses acquired following exposure to 1 nM LBK. 1^{o}HVD responses produced $\Delta I_{SC\text{-}MAX}$ of 9.4 ± 2.2 μA*cm^{-2} and $\Delta R_{TE\text{-}MAX}$ of 205 ± 75 Ω*cm^2. Taken together, the results presented in Fig. 1 demonstrate similar ion transport responses to LBK for vas deferens epithelial cells from 3 sources, an immortalized cell line and primary cultures of cells isolated from both porcine and human tissues.

Bradykinin-Induced Ion Transport across Vas Deferens Epithelia is Stimulated by Activation of BDKRB2

A subset of four assays was performed to determine which BK receptor subtype is present in and linked to ion transport across vas deferens epithelia by exposing paired 1^{o}PVD monolayers to selective agonists and/or antagonists of bradykinin 1 receptor subtype (BDKRB1) and BDKRB2. Each assay employed cells derived from one boar that gave rise to six monolayers. LBK and BK are BDKRB2-selective agonists whereas des-Arg^9-BK is a BDKRB1-

selective agonist. Results presented in Fig. 2 demonstrate that BDKRB2, but not BDKRB1 agonists stimulated I_{SC}. Furthermore, concurrent exposure to a BDKRB2 antagonist, HOE140, precluded an effect of BK on paired monolayers. The BDKRB1-antagonist, des-Arg^{10}-HOE140, despite being used at a super maximal concentration (5 μM), caused only a modest inhibition of the BK-induced change in $I_{SC,}$ which did not achieve statistical significance (n = 4). These results provide compelling evidence that BDKRB2s mediate the effect of BK and/or LBK on I_{SC} across vas deferens epithelial cells.

BDKRB2 Immunoreactivity is Present in Both Cultured Vas Deferens Epithelial Cells and in Vivo

Western blot analysis targeted expression of BDKRB2 in protein samples isolated from 1^{o}PVD monolayers. Outcomes reveal a most prominent signal with mobility consistent to the theoretical molecular weight (41 kDa), calculated for the human BDKRB2 (Fig. 3A). Four other bands of reduced intensity were present. All bands had reduced intensity in the lane that had less protein loaded. The identity of these bands is unknown although all signals were absent when the primary antibody was pre-adsorbed by the immunizing peptide regardless of the amount of protein loaded (Fig. 3B).

Immunocytochemistry and confocal microscopy analysis were carried out to investigate expression and localization of BDKRB2s in transverse sections of freshly excised porcine vas deferens. BDKRB2-immunoreactitivy was localized to cells lining the duct. Receptor immunoreactivity was present throughout the epithelium while non-epithelial cells were devoid of labeling (Fig. 3C and E). BDKRB2-immunoreactitivy was present in the same labeling pattern on sections of both proximal and distal vas deferens epithelia (not shown). Tissue sections made in a serial manner and exposed to pre-adsorbed primary antibody exhibited only very modest labeling (Fig. 3D), and sections probed with the secondary antibody only exhibited no labeling (not shown). BDKRB2-immunoreactitivy was also present at the endothelium of capillaries present in the vicinity of the epithelium and at the endothelium of veins located outside the vas deferens muscular layer (not shown).

Messenger RNA Coding for BDKRB2 But Not BDKRB1 is Present in Cultured Vas Deferens Epithelial Cells

Semi-quantitative RT-PCR analysis of RNA isolated from 1^{o}PVD monolayers derived from 3 animals revealed that the *BDKRB2* mRNA is expressed at a level equivalent to 16.45 ±

0.45 % of the total number of transcripts expressed from the housekeeping gene *GAPDH* (n = 6). The *BDKRB1* transcript was detected only once in a series of eight reactions (two reactions per RNA sample; each sample isolated from monolayers derived from one of 4 animals). C_t values for the porcine *BDKRB2* and porcine *GAPDH* mRNAs were 20.59 ± 0.24 and 17.90 ± 0.09, respectively. The *BDKRB1* positive reaction had a C_t of 38.70, which shows that even when detected, *BDKRB1* mRNA is $>10^5$ less abundant than the *BDKRB2* mRNA. Sequencing of the amplicons derived from these reactions was performed and all products proved to be of expected identity.

Bradykinins Effect on I_{SC} and R_{TE} across Cultured Vas Deferens Epithelial Cells is Indomethacin-Sensitive

To determine whether the BDKRB2-induced ΔI_{SC} includes an increase in PTGS activity, monolayers were exposed to indomethacin or vehicle control for 10 to 15 minutes prior to LBK exposure. Indomethacin was without effect on I_{SC} or R_{TE}. Subsequent exposure to LBK (500 nM) caused a $\Delta I_{SC\text{-}MAX}$ and $\Delta R_{TE\text{-}MAX}$ of 0.9 ± 0.5 μA*cm^{-2} and 1630 ± 390 Ω*cm^2 respectively. These ΔI_{SC} responses in the presence of indomethacin are less than 20% when compared to vehicle-treated controls (Fig. 4). The possibility remained that, although the $\Delta I_{SC\text{-}MAX}$ was reduced by indomethacin, net flux, measured as area under the I_{SC} curve might not have changed. Thus, the magnitude of responses were also defined over the course of 300 s and revealed an indomethacin suppressive effect (Fig. 4C). Both $\Delta I_{SC\text{-}MAX}$ and the net change in flux over 300 s were affected to a similar extent by indomethacin. In addition, the effect of LBK on R_{TE} was approximately 50% less in the presence of indomethacin (Fig. 4D).

Monolayers derived from the PVD9902 cell line and subjected to the same protocol (n = 6) displayed no effect of exposure to indomethacin (500 nM). Once exposed to LBK (500 nM), these monolayers responded with $\Delta I_{SC\text{-}MAX}$ and $\Delta R_{TE\text{-}MAX}$ of 0.39 ± 0.06 μA*cm^{-2} and 440 ± 200 Ω*cm^2 respectively, which is less than 7% of the $\Delta I_{SC\text{-}MAX}$ and less than 17 % of the $\Delta R_{TE\text{-}MAX}$ exhibited by paired monolayers not treated with indomethacin (Fig. 5).

1^{o}HVD cultures were assessed for an effect of LBK (10 nM) in the absence or presence of indomethacin (2 μM). Monolayers of 1^{o}HVD were assessed twice for effects of LBK in the absence and presence of indomethacin: either first with LBK only, then replaced in culture for 3-5 days and then evaluated a second time in the presence of indomethacin, or in the reverse order. Baseline values for indomethacin treated 1^{o}HVD monolayers (n = 6 monolayers derived from 6

donors) were 2.4 ± 1.5 μA*cm^{-2} and 721 ± 382 Ω*cm^2. Exposure to indomethacin produced no measurable changes in I_{SC} or R_{TE}. Responses to LBK within the indomethacin-treated group constituted $\Delta I_{SC\text{-}MAX}$ and $\Delta R_{TE\text{-}MAX}$ of 3.7 ± 0.5 μA*cm^{-2} and 196 ± 108 Ω*cm^2 respectively. This $\Delta I_{SC\text{-}MAX}$ is significantly less than the untreated group (Fig. 5C), but 1^{o}HVD exhibited no difference in terms of $\Delta R_{TE\text{-}MAX}$ (Fig. 5D). Taken together, these results support the inference that, in vas deferens epithelial cells, BK modulates ion transport via activation of cyclooxygenases that likely contribute to PG synthesis.

PTGS2 mRNA is Highly Expressed in Cultured Porcine Vas Deferens Epithelia

Indomethacin inhibits both PTGS1 and PTGS2. Thus, experiments were conducted to determine the relative expression of mRNA coding for the PTGS isoforms in 1^{o}PVD cells. Semi-quantitative RT-PCR was carried out with RNA isolated from monolayers derived from 5 animals. C_t values were: 18.72 ± 0.60 for *GAPDH*; 21.25 ± 0.35 for *PTGS2* and 25.68 ± 0.22 for *PTGS1*. Normalization of targeted *PTGS* mRNA to *GAPDH* reveals that the *PTGS2* transcript abundance was more than 20 times greater than the *PTGS1* transcript and as much as 20% of *GAPDH* (Fig. 6). Resulting amplicons had expected mobility on agarose gels and subsequent cDNA sequencing proved expected identity (data not shown).

PTGER4 and PTGER2 Contribute to Anion Secretion across Vas Deferens Epithelia

Changes in I_{SC} exhibited by 1^{o}PVD were recorded by a modified Ussing chamber as monolayers were exposed to cumulative concentrations of subtype selective prostaglandin E receptor agonists: sulprostone (PTGER1 and PTGER3), butaprost (PTGER2) and 1-OH PGE1 (PTGER4). PGE2, the primary product of PTGS activity, although a non-selective PTGER agonist, was also used in this concentration response study. These assays were carried out in a paired manner so that cells from each animal had at least one monolayer exposed to each compound. Results are summarized in Fig. 7. Concentration-dependent stimulation of I_{SC} was observed with PGE2, 1-OH PGE1 and butaprost, while sulprostone was without a remarkable effect, even at the greatest concentration tested. Effects at the highest concentration tested (1 μM), were not different for PGE2, 1-OH PGE1 or butaprost. However, quantitative analysis of the concentration dependence revealed some differences. Responses to 1-OH PGE1 and butaprost were well-fitted by a modified M-M function with derived macroscopic dissociation constants (k_{app}) of 20.2 ± 6.5 nM and 37.7 ± 18.4 nM, respectively. Visual inspection revealed that concentration-dependent responses to PGE2 were not fitted well by the M-M function.

Nonetheless, at every concentration tested, the response to PGE2 was greater than the response to any of the receptor-selective agonists. Thus, the rank order of potency was PGE2 > 1-OH PGE1 > butaprost >>> sulprostone, suggesting that both PTGER4 and PTGER2 can contribute to the response.

Semi-quantitative RT-PCR revealed a direct correlation between the pharmacological data described above and transcript abundance for PTGER subtypes measured in RNA isolated from 1^{o}PVD monolayers. A comprehensive read-out of *PTGER*s expression levels is summarized in Fig. 8. *PTGER4* mRNA was approximately 4 times more abundant than *PTGER2* mRNA and more than 60 times more abundant than *PTGER3* mRNA. Reactions for *PTGER1* failed to detect this transcript in 6 attempts. Single amplicons of expected mobility derived from reactions targeting *PTGER2*, *PTGER3* and *PTGER4* mRNAs were sequenced and revealed expected identity (data not shown). Thus, both pharmacological and molecular data support the conclusion that PGs acting at PTGER4 and PTGER2 contribute to ion transport that is stimulated by LBK.

Discussion

Results presented here demonstrate for the first time that BKs with preferential affinity to the BDKRB2 elicit an increase in I_{SC} that is indicative of anion secretion by cells lining the vas deferens. Molecular and functional data derived from both porcine and human epithelial cell models are reported that describe a role for BK and the BDKRB2 in vas deferens epithelial physiology. 1^{o}PVD cells respond to BK in a HOE140-sensitive manner and, likewise, PVD9902 respond to BKs. Native porcine vas deferens tissues exhibit epithelial BDKRB2-immunoreactivity. LBK also induces a ΔI_{SC} that is indicative of anion secretion in 1^{o}HVD. Data derived from these 3 epithelial models, along with BDKRB2-immunoreactivity in native tissue, support the inference that activation of BDKRB2 upregulates PTGS activity, mostly PTGS2, to synthesize PGs that ultimately bind to PTGER4 and/or PTGER2 to initiate pathways leading to anion secretion across the vas deferens epithelium. It is expected that such cellular events change the vas deferens luminal fluid: its volume, ion composition and pH.

The rapid BK-induced increase in I_{SC} was accompanied by a similarly rapid and drastic reduction in R_{TE}, which is suggestive of the opening of ion channels, likely CFTR. Functional hallmarks of this ion channel have been reported previously for these cell-based systems [20-22,

25]. Such observations are in keeping with the bradykinin effects observed in epithelia elsewhere in the male reproductive tract, most notably in epididymal epithelia [15-17, 26]. Epithelial systems where the qualitative aspect of BK-induced anion secretion have been at least partially elucidated, as in the kidney [5-8] and colon [10, 11], provide further support for the proposed link between BDKRB2 activation and an anion secretory effector such as CFTR in vas deferens epithelia. Both porcine and human vas deferens epithelial cells respond to submicromolar concentrations of BK with substantial anion secretion, although the concentrations employed are approximately 10 to 100 times greater than the circulating plasma levels of BK [27]. Within this context, it should be noted that epithelia lining the male reproductive tract, specifically epididymal epithelia, have been shown to express tK immunoreactivity [12], which suggests that BK synthesis can occur at the apical membrane of epithelium lining other portions of the male excurrent duct.

Results suggest that the BDKRB2 is solely responsible for the ion transport observed upon BK exposure. Stimulation was fully blocked by the BDKRB2 antagonist HOE140. Furthermore, BK, which is a BDKRB2 selective agonist, stimulated I_{SC} whereas the BDKRB1 selective agonist des-Arg^{9}-BK caused no appreciable change in I_{SC}. The BDKRB1 antagonist des-Arg^{10}-HOE140 was associated with a reduction in BK-stimulated I_{SC}, although this difference did not achieve statistical significance. This observation is consistent with reports that BDKRB1 antagonists developed from the des-Arg-fragment of HOE140 exert potent antagonistic effect on the BDKRB1 while exhibiting weak antagonistic effects on the BDKRB2 [2].

The 1^{o}PVD system expresses the BDKRB2 at the protein level as indicated by Western blot analysis. The porcine coding sequence is not fully cloned to date but the theoretical mobility of the human BDKRB2 is 41 kDa. In these assays, the most prominent band had mobility between the protein standards 37 and 50 kDa. In addition, other signals of lesser intensity were present. Two different signals were present at and below the 37 kDa marker and two other signals were present below and above the 50 kDa marker. While it becomes difficult to account for the origin of these signals, it should be noted that a signal with mobility greater than the theoretical value has been reported for the human BDKRB2 [28] and that all signals were substantially reduced or eliminated by pre-adsorption of anti-BDKRB2 with the immunizing peptide. BDKRB2 immunoreactivity was observed in the majority of cells composing the intact

porcine vas deferens epithelium. Principal, basal, clear, narrow and dark (pencil) cells are reported as cell types within this epithelium although principal cells account for the vast majority of the cells that line the duct [29, 30]. Micrographs obtained following BDKRB2 immunolabeling indicated that this receptor is present throughout most cells composing the vas deferens epithelium. The labeling pattern allows, with a reasonable degree of certainty, for the inference that principal cells express the BDKRB2 in vivo. At the transcriptional level, *BDKRB2* is highly expressed in 1^{o}PVD monolayers, which seems to place this cell model apart from others in this regard. G-protein coupled receptors - BDKRB2 included – are generally expressed at low levels in mammalian cells despite their wide participation in cellular signal transduction in several tissues. This has made necessary the development of cellular expression systems, largely based on insect cells, in order to obtain reasonable yields of BDKRB2 for various applications [31-33]. The *BDKRB1* transcript is, however, virtually absent from the transcriptome of 1^{o}PVD monolayers. As described in the results, this transcript was amplified only once out of 8 attempts using 1^{o}PVD RNA and that amplification generated a 38.7 C_t value, which indicated at least 100,000-fold fewer copies than *BDKRB2* mRNA. These data provide further support for an anion secretion pathway that is stimulated by the BDKRB2 and not the BDKRB1 in vas deferens epithelia. The BDKRB2 is present in several tissues and is constitutively expressed under physiological conditions, while the BDKRB1 is an inducible form of the bradykinin receptor that is expressed in the context of inflammation and/or trauma [2, 34]. The possibility for BDKRB1 modulation, however, does remain. BDKRB1 was transcribed, translated, and expressed at the basolateral membrane of colonic epithelia within 3 hours after being mounted in Ussing chambers [35]. The 1^{o}PVD monolayer from which that RT-PCR amplification of *BDKRB1* mRNA was derived remained in the Ussing chamber for approximately 45 minutes. That single observation where the *BDKRB1* transcript was amplified could reflect rapid induction, although this possibility remains to be tested.

Exposure to the non-selective PTGS inhibitor indomethacin significantly reduced LBK responses in all three vas deferens epithelial cell models tested. This observation leads to the conclusion that, similar to what has been demonstrated in epididymal epithelia [15-17], BKs activate PTGS and upregulate PG synthesis in vas deferens epithelia. PGs, in turn, bind PTGERs to initiate a pathway (or pathways) leading to anion secretion. In 1^{o}PVD, *PTGS2* expression is nearly 20 times greater than *PTGS1* expression. This observation is consistent with a previous

report that defined relative levels of these transcripts in murine vas deferens [18]. PTGSs, and most prominently PTGS2, have been subject of substantial pharmaceutical development. Recently, selective inhibitors of PTGS2 (rofecoxib, celecoxib, valdecoxib) have been actively marketed as non-steroidal anti-inflammatory drugs although rofecoxib was withdrawn. Since then, attention has turned to side-effects occurring in the kidney [36] where PTGS2 is constitutively expressed. The high levels of PTGS2 expression in the vas deferens as well as its role in modulating ion transport make it reasonable to believe that therapeutic use of these compounds may impact fertility at the level of epithelia lining the male excurrent duct.

Concentration-dependent responses to PGs are exhibited by 1^{o}PVD epithelial cell monolayers. The outcomes suggest that the PTGER4 and/or PTGER2 account for the response to PGE2 in the vas deferens. Butaprost is the most selective PTGER2 agonist available among synthetic prostaglandins and possesses a reported k_{app} of 110 nM, while 1-OH PGE1 has PTGER4 selectivity with a reported k_{app} of 190 nM [37]. 1^{o}PVD responded to both of these selective agonists at concentrations at or slightly below their reported k_{app}. PGE2 is a powerful non-selective agonist of all PTGER subtypes and its concentration-dependent changes in I_{SC} were not fitted well by the M-M function. This is consistent with the notion that two PTGER subtypes can modulate I_{SC} across vas deferens epithelia. Nonetheless, butaprost, 1-OH PGE1 and PGE2 produced virtually the same ΔI_{SC} at the greatest concentration tested, which suggests the existence of a response limiting factor, most likely the ion transporting proteins carrying out their function at a maximal rate when the greatest agonist concentration was effectively in the system. Both PTGER4 and PTGER2 are G_S-protein coupled receptors, suggesting substantial adenylyl cyclase activation. Previously reported data from this laboratory demonstrated cAMP synthesis in 1^{o}PVD upon exposure to agonists other than PG [38]. PTGER2 and PTGER4 reportedly modulate ion transport induced by PGE2 elsewhere in the male reproductive tract, specifically in epididymal epithelia [15]. The relative abundances of mRNA for prostaglandin E receptor subtypes described here for vas deferens epithelia seems to set the male excurrent duct apart from other urogenital epithelia. Gene expression analysis performed on 2 mice strains reveals that all EP receptor transcripts are expressed in the kidney of both strains but that *PTGER4* mRNA is least abundant while *PTGER1* mRNA is the most abundant [39]. In the second mouse strain, kidney levels of *PTGER4* expression are comparable to that of *PTGER1* and *PTGER3*, and well below the levels of medullary *PTGER2* mRNA. Further evidence is available that

supports high expression of the *PTGER1* and *PTGER3* transcripts in the kidney but modest expression of the *PTGER4* and *PTGER2* transcripts [40]. These *PTGER* expression profiles place the kidney as an organ system where the expected effect of PGE2 is via inositol thriphosphate and reduction in cellular cAMP levels, as opposed to signaling through phosphatidylinositol 3 (PI3)-kinase and increase in cAMP levels that, based upon our observations, is expected in the vas deferens.

PGs modulate acid and base transport. In the duodenum, PGs are required for the modulation of net bicarbonate secretion and cause alkalinization of the duodenal lumen. In this segment of the gastrointestinal tract, bicarbonate secretion is upregulated through activation of PTGER3 and PTGER4 [41-43]; cAMP-, calcium- and PI3-kinase-dependent pathways [44]; and by modulating activity of apically expressed bicarbonate exchangers such as SLC26A6 [45]. Although further investigation is needed to elucidate the qualitative aspects of anion secretion induced by BKs and PGs in vas deferens epithelium, this laboratory demonstrated that anion secretion in this tissue is bicarbonate-dependent [24]. Using a variety of ion substitution conditions and selective permeabilization of either the apical or basolateral membranes of 1^{o}PVDs, bicarbonate was necessary for the maintenance of sustained changes in I_{SC} upon forskolin stimulation and required for changes in I_{SC} to occur at the isolated basolateral membrane. Additionally, mRNA coding for a collection of bicarbonate co-transporters and exchangers is expressed in porcine vas deferens epithelia, including *SLC4A4, SLC26A3*, *SLC26A4* and *SLC26A6* [22, 24]. Sperm bicarbonate uptake, while still in the male reproductive tract, is required for adequate sperm capacitation and the development of fertilizing capacity [46]. Taken together, these data make it reasonable to conclude that the vas deferens epithelium might be a substantial, if not the main, cellular contributor of bicarbonate secretion onto the environment to which sperm is exposed.

In conclusion, BKs act on the BDKRB2 to upregulate PTGS activity and the synthesis of PGs that then act on PTGER4 and/or PTGER2 to ultimately induce anion secretion across vas deferens epithelia. Anion secretion most likely modifies the male duct luminal environment to which sperm are exposed and thus may modulate sperm function and ultimately, male fertility.

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460

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Figure Legends

Figure 1. Lysylbradykinin (LBK) induces anion secretion across vas deferens epithelial monolayers. **A**. When exposed to 500 nM, porcine vas deferens epithelial cell monolayers derived from primary cultures (1^{o}PVD) exhibit two short term peaks of short circuit current (I_{SC}) before a later and sustained rise in I_{SC} takes place. **B**. 1^{o}PVD monolayers respond to 1 nM LBK and consistently exhibit one single peak and a late rise in I_{SC}. PVD9902 cell monolayers (**C**) and primary cultures of human vas deferens epithelial cells (**D**) also respond to LBK with a transient increase in I_{SC}. Results presented are typical of 5-7 observations for each condition.

Figure 2. Bradykinin 2 receptor subtype (BDKRB2) mediates bradykinin-induced ion transport across 1^{o}PVD. A selective BDKRB2 agonist (bradykinin, BK) elicits responses that are absent when paired monolayers are pre-treated with the selective BDKRB2 antagonist HOE140 but not when exposed to des-Arg^{10}-HOE140, a potent bradykinin 1 receptor subtype (BDKRB1) antagonist with weak antagonist effect at BDKRB2. Paired monolayers exposed to the selective BDKRB1 agonist des-Arg^{9}-BK failed to exhibit changes in I_{SC} (n = 4). * indicates significant difference from paired monolayers exposed to BK only ($P < 0.05$).

Figure 3. BDKRB2-immunoreactivity is present in 1^{o}PVD and native porcine vas deferens epithelia. **A.** Chemiluminescence signals derived from an immunoblot probed with anti-BDKRB2. The most prominent band (arrow) reveals mobility that is consistent to the theoretical mobility calculated for human BDKRB2 (41 kDa). Signal strength is proportional to the amount

615 of protein loaded (20 and 10 μg in the left and right lane, respectively). **B.** No signal is observed when primary antibody is pre-adsorbed with immunizing peptide. Data are paired to panel A. **C-F.** Transverse sections of native vas deferens exposed to anti-BDKRB2 antibody exhibit labeling (green) localized to most cells composing the epithelium (**C** and **E**). Image **F** reveals the corresponding bright field and nuclear staining (blue) to image **E**. Sections probed with pre-
620 adsorbed primary antibody showed little immunolabeling (**D**). Scale bar is representative of 50 μm in **C** and **D** and 20 μm in **E** and **F**. Results are representative of several observations with tissues derived from 3 animals.

Figure 4. Indomethacin inhibits the effect of LBK on 1^{o}PVD ion transport. **A**. Typical responses
625 for paired monolayers exposed to LBK in the absence (left) and presence (right) of indomethacin. Summarized results (n = 7) show that LBK-induced maximal change in I_{SC} (**B**), average change in I_{SC} for 300 s (**C**) and change in R_{TE} (**D**) are 50-80% less in monolayers concurrently exposed to indomethacin. * indicates significant difference from vehicle treated control ($P < 0.05$).

630

Figure 5. Indomethacin inhibits the effect of LBK in the PVD9902 cell line and primary human epithelial cell monolayers (1^{o}HVD). PVD9902 exhibit pronounced change in I_{SC} and R_{TE} upon exposure to LBK (500 nM) that is largely precluded by indomethacin (500 nM) as indicated in panels **A** and **B** respectively (n = 6 each). 1^{o}HVD I_{SC}-responses to LBK (10 nM) are also
635 sensitive to indomethacin (2 μM) (**C**), while changes in R_{TE} are not affected (**D**) (n = 6 each). * indicates significant difference from vehicle treated control ($P < 0.05$).

Figure 6. *PTGS2* mRNA abundance far exceeds that of *PTGS1* in 1^{o}PVD. Results expressed relative to *GAPDH* mRNA. Primer sequences and protocol parameters are presented in Table 1. (n = 10 reactions for each target, RNA derived from 5 animals).

Figure 7. PTGER4 and PTGER2 mediate prostaglandin (PG) induced ion transport across 1^{o}PVD. Concentration response curves derived from cumulative concentrations of PTGER agonists applied to monolayers assayed in modified Ussing chambers are presented. Selective agonists of the PTGER4 and PTGER2 (1-OH PGE1 and butaprost, respectively) stimulate I_{SC} as does PGE2, a non-selective agonist. Sulprostone, an agonist of PTGER1 and PTGER3, failed to cause changes in I_{SC}. Sigmoidal curves represent the best fit of a modified Michaelis-Menten function to each data set. Parameters of the fits are reported in the text. Each point represents 10-12 observations.

Figure 8. *PTGER4* mRNA abundance is approximately 4 times greater than that of *PTGER2* and approximately 60 times greater than that of *PTGER3* in 1^{o}PVD. Results are expressed relative to *GAPDH* mRNA. Primer sequences and protocol parameters are presented in Table 1. (n = 6 reactions for each target, RNA derived from 3 animals).

Tables

Targeted Transcript	Sense Primer	Anti-Sense Primer	Annealing Temp. (^{o}C)	Expected Product Length (bp)	NCBI Accession
BDKRB1	GTCTGAACGCGGCGGAAATCTACC	ACTTGGACGGAGCGCAGCAGGAAC	64.3	340	AB051422
BDKRB2	CAACGTCACCGCCTGCATCATCAA	AAGGGCAGCCAGCAGACGACGAAC	61.9	240	AB051422
PTGS1	TTTGCCCAACACTTCACCCACCAG	AGCCCAGGAAGCAGCCCAAACA	60.8	293	AF207823
PTGS2	CTGGCTGCGGGAACATAATAGAG	TGGAAGGCGTCAGGCAGAAG	56.3	276	AF207824
PTGER1	GGTGGGCCAGCTTGTCGGTATCAT	CTCCCAGGCGCTCGGTGTTAGG	64.1	277	NM000955
PTGER2	GGCGCGTGTGCACCTACTTTG	AGGCGAGCACTGCGATGATGAG	60.8	324	EU241329
PTGER3	TGGGGCCGGATCACGACTG	CACGGGCTTCTCCTGGCTCTGT	58.6	349	U27083
PTGER4	AGTCCCCTTTCCCAATGA	GGCCGATCTCCCCACTAT	52.7	304	EU241330
GAPDH	CCAGCAAGAGCACGCGAGGAGGAG	CGGGGGTCTGGGATGGAAACTGGA	61.2	117	X94251

Table 1: Information associated with the performed gene expression analysis.

Bradykinin-stimulated cyclooxygenase activity stimulates vas deferens epithelial anion secretion in vitro

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