

Cell-cell Interaction Underlies Formation of Fluid in the Male Reproductive Tract of the Rat

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ABSTRACT The epithelia lining the epididymides of many species consists of several cell types. We have provided evidence that the basal cells are essential to the integrated functions of the epithelium. Basal cells, but not principal cells, and other cells in the epididymis express TRPC3 and COX-1. We have isolated basal cells from intact rat epididymis using antibody-coated Dynabeads and subjected them to whole-cell patch-clamp measurement of nonselective cation channel activity, a feature of TRPC3 protein, and Fluo-3 fluorescence measurement of intracellular Ca²⁺ concentration. The results show that a nonselective cation current blockable by La³⁺ (0.1 mM), Gd³⁺ (0.1 mM), or SKF96365 (20 μM) could be activated by lysylbradykinin (200 nM). In cells loaded with Fluo-3, addition of lysylbradykinin (100 nM) caused a sustained increase of intracellular Ca²⁺. This effect was blocked by Gd³⁺ (0.1 mM) or SKF96365 (20 μM) and was not observed in Fluo-3-loaded principal cells. Stimulation of basal cell/principal cell cocultures with lysylbradykinin (200 nM) evoked in principal cells a current with CFTR-Cl⁻ channel characteristics. Isolated principal cells in the absence of basal cells did not respond to lysylbradykinin but responded to PGE₂ (100 nM) with activation of a CFTR-like current. Basal cells, but not principal cells, released prostaglandin E₂ when stimulated with lysylbradykinin (100 nM). The release was blocked by SKF96365 (20 μM) and BAPTA-AM (0.05 or 0.1 mM). Confluent cell monolayers harvested from a mixture of disaggregated principal cells and basal cells responded to lysylbradykinin (100 nM) and PGE₂ (500 nM) with an increase in electrogenic anion secretion. The former response was dependent on prostaglandin synthesis as piroxicam blocked the response. However, cell cultures obtained from principal cells alone responded to PGE₂ but not to bradykinin. These results support the notion that basal cells regulate principal cells through a Ca²⁺ and COX signaling pathway.

KEY WORDS: transient receptor potential protein • calcium • chloride secretion • basal cells • epididymis

INTRODUCTION

The epithelial cells lining the epididymis play an active role in the formation of fluid in the epididymis where testicular spermatozoa undergo maturational changes before they acquire their fertilizing capacity and forward motility (Orgebin-Crist, 1967; Wong et al., 2001). In common with other secretory epithelia, fluid secretion into the epididymal lumen is driven by secondary active transport of chloride. The cAMP-activated chloride channels CFTR (cystic fibrosis transmembrane conductance regulator) (Huang et al., 1992, 1993; Leung and Wong, 1994a; Leung et al., 1996, 2001a) and aquaporin-9 (Pastor-Soler et al., 2001; Cheung et al., 2003) present in the principal cells are known to be responsible.

It is previously known that fluid secretion by the epithelial cells of the epididymis is under complex neurohumoral control. A number of neurohumoral factors such as serotonin (Leung et al., 1999), bradykinin (Cuthbert and Wong, 1986), angiotensin (Wong et al., 1990), vasopressin (Lai et al., 1994), and endothelin (Wong et al., 1989) stimulate secretion via lo-

cal production of prostaglandins. There are, however, peptide hormones viz CGRP (calcitonin gene-related peptide) (Leung et al., 1992) and secretin (Chow et al., 2004) that stimulate secretion without involving prostaglandins. The prostaglandin synthetase responsible for the actions of the former peptides is found to be cyclooxygenase-1 (COX-1) but not the isomeric COX-2 (Wong et al., 1999). It is further known that COX-1 is present in the basal cells but not in the principal cells and other cells of the epithelium (Wong et al., 1999; Leung et al., 2004). The COX products, largely prostaglandin E₂ (PGE₂), released to the extracellular matrix, act on the G_s protein-coupled EP2/4 prostaglandin receptors on the principal cells to increase intracellular cAMP, which then activates an apically placed CFTR to increase secretion of anions, and secondarily, water (Wong et al., 1999). In this way, via the formation of local mediators, the basal cells act as regulators of epithelial functions in much the same way

Abbreviations used in this paper: CFTR, cystic fibrosis transmembrane conductance regulator; COX, cyclooxygenase; LBK, lysylbradykinin; PD, potential difference; PGE₂, prostaglandin E₂; PSS, physiological salt solution; TRP, transient receptor potential.

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as the endothelial cells regulate vascular smooth muscle tone.

Calcium is a universal second messenger mediating many cellular functions. In nonexcitable cells, Ca^{2+} entry is provided by voltage-independent cationic channels, the transient receptor potential (TRP) channel family, initially found in *Drosophila* photoreceptors and later identified in various mammalian tissues by homologue screening (Clapham et al., 2001). This superfamily comprises six major subgroups of TRP proteins: TRPCs (canonical), TRPVs (vanilloid), TRPMs (melastatin), TRPA1 (AnkTM1), TRPPs (polycystins), and TRPMLs (mucolipins) (Clapham, 2003). Of these channels, the TRPC proteins are receptor-operated channels that are Ca^{2+} -permeable nonselective cation channels linked to G protein-coupled receptors (GPCRs). In many cellular systems, including epithelia, activation of TRPC channels by GPCR agonists allows cations (mostly Na^+ and Ca^{2+}) to flow into the cytoplasm, causing a rise in intracellular Ca^{2+} concentration and membrane depolarization (Large, 2002; Sydorenko et al., 2003; Gurney and Ng, 2004). In this work, we have studied the interaction between basal cells and principal cells. Emphasis is placed on the role of TRPC proteins and intracellular calcium in the basal cells.

MATERIALS AND METHODS

Cell Culture

All experiments were performed according to the guidelines of the Laboratory Animal Services Centre of the Chinese University of Hong Kong. The procedures of cell culture for the rat cauda epididymal epithelial cells were performed as previously described (Cuthbert and Wong, 1986). After enzymatic digestions, the disaggregated cells were used to form monolayer cultures for short-circuit current (*I_{sc}*) measurement or to isolate basal cells and principal cells for patch clamp and calcium influx measurement.

Short-circuit Current (*I_{sc}*) Measurement

Confluent epididymal monolayers were clamped between two halves of an Ussing chamber (World Precision Instruments) with a 0.6-cm² window. The tissue was short circuited with a voltage-clamp amplifier (DVC 1000; World Precision Instruments) as previously described (Cuthbert and Wong, 1986; Wong, 1988a).

Treatment with Antisense

In some experiments before measurement of short-circuit current, six confluent epididymal monolayers were treated with 10–25 μM TRPC3 antisense (5'-GCCACGCCTTTAGTGATAAGCAT-3') and the same number with sense (5'-ATGCTTATCAC-TAAAGGCGTGGC-3') oligonucleotides, and in other experiments six monolayers with 10–25 μM TRPC2 antisense (5'-TTGGGCGAAAGGGGATCCAT-5') and the same number with sense (5'-ATGGATCCCCCTTCGCCCAA-3'). The antisense oligonucleotides were transfected into the cells using oligofectamine reagent (Invitrogen) according to the manual described. Transfection was performed for 4 h in serum-free MEM medium and reaction was terminated by refreshing the cultures with MEM medium containing 10% FBS. Cultures were then incu-

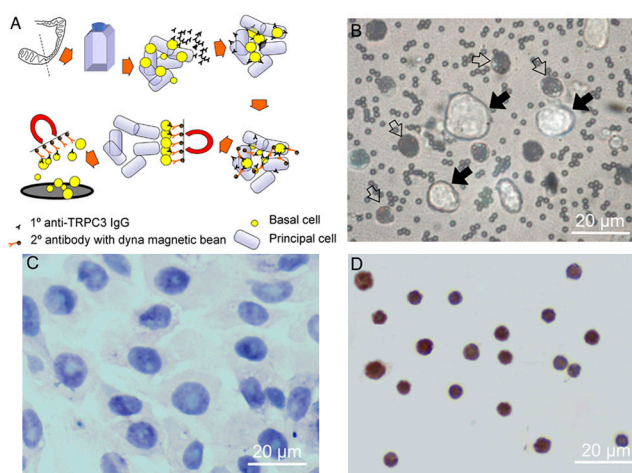


FIGURE 1. Isolation of basal cells from rat epididymis using Dynabeads. Schematic diagram showing isolation of basal cells from the rat epididymis (A). After enzymatic separation, cells were incubated with TRPC3 antibody-tagged Dynabeads, which captured (bind) basal cells (open arrows) but not principal cells (closed arrows) (B). After separation of the basal cell/Dynabeads complex with a magnet, principal cells were plated down on Petri dish and stained with hematoxylin (C). Basal cells were freed from the Dynabeads and stained with anti-COX-1 antibody (D).

bated for another 24 h before they were mounted in the Ussing chamber for short-circuit current measurement. Both oligonucleotides encompass the first 23 base pairs of the TRPC3 and TRPC2 transcripts beginning at the translation initiation AUG. The inhibition of gene expression and protein translation of target genes were confirmed by Western blot analysis.

Isolation of Basal Cells and Principal Cells

Isolation of basal cells from intact rat cauda epididymides was achieved using antibody-tagged Dynabeads (DynaL Biotech) according to the product manual. Anti-TRPC3 antibody was raised against the peptide FTYARDKWLPSPDQ, region 410–423 of the rat TRPC3 protein (Ohki et al., 2000) and purified using affinity chromatography. This peptide region has been shown to reside within the extracellular domain of TRPC3 (Entrez Protein Database GI: 14548278). After enzymatic digestions, disaggregated epididymal cells were incubated with anti-TRPC3 polyclonal antibody (1:500). The anti-TRPC3-labeled cells (mainly basal cells) were captured by Dynabeads tagged with secondary antibody. The Dynabeads captured basal cells were separated from the rest of cell populations by using a magnetic stand (Promega) and then released from the Dynabeads by 0.25% trypsin (Fig. 1). The purity of the basal cells isolated by this method was determined by immunodetection of COX-1. Cells released from the Dynabeads were incubated in MEM. They were seeded on glass coverslips and were left overnight in an incubator gassed with 5% CO_2 and maintained at 32°C. Cells were fixed with 3.7% formaldehyde and stained for COX-1 protein by rabbit anti-COX-1 antibody (1:100; Cayman Chemical). Positively stained cells were recognized as basal cells and nonstaining cells as nonbasal cells (principal cells, clear cells, myocytes, fibroblasts, etc.). Five random selected fields from each slide were counted for the number of basal cells and nonbasal cells. A total of five different batches of culture were examined. This separation technique yielded basal cells to ~86% purity. Normally, 5×10^5 cells can be obtained from 14 rat cauda epididymides. The isolated basal cells

T A B L E I

Primer Pairs Used for the Study of the Expression of TRPC and COX-1

Gene	Accession no.	Primers	Corresponding nucleotides	Expected size
TRPC1	AF061266	5'-ATGGGACAGATGTTACAAGATTTTGGG-3' (sense)	1561–1587	402 bp
		5'-AGCAAACCTCCATTCTTTATCCTCATG-3' (antisense)	1936–1962	
TRPC2	AF136401	5'-GGAGAAAGGTCCGAAAAGTAGACAC-3' (sense)	409–432	496 bp
		5'-CTCAGTCTCACTATCCTCGCCAG-3' (antisense)	881–904	
TRPC3	AB02231	5'-CCTGAGCGAAGTCACACTCCAC-3' (sense)	1269–1291	519 bp
		5'-CCACTCTACATCACTGTCCATCC-3' (antisense)	1776–1797	
TRPC4	AF288407	5'-GAAGAAACGAAGGGGTTAAGTGC-3' (sense)	1643–1666	423 bp
		5'-CATTTTTTTCTTACATAAGTGTGTCCA-3' (antisense)	2039–2065	
Trp5	AF060107	5'-CCACAGTGAAGAAGTTGACCCACAGA-3' (sense)	1224–1249	831 bp
		5'-TTAGGTTTCATCAATAGCTCTGGTCTC-3' (antisense)	2029–2054	
Trp6	NM_053559	5'-CACCGCGCGCAGACAGTCTTCG-3' (sense)	193–215	415 bp
		5'-CTTGCTGGAGTTCAGACTGGCTGGG-3' (antisense)	583–607	
COX-1	S67721	5'-TGGAGAAGTGCCAGCCCACTCCC-3' (sense)	1584–1608	206 bp
		5'-GGGGCAGGTCTTGGTGTTGAGGCA-3' (antisense)	1763–1789	
S16	X17665	5'-TCCGCTGCACTCCGTTCAAGTCTT-3' (sense)	67–90	379 bp
		5'-GCCAACTTCTTCTTGATTTCGAGCG-3' (antisense)	428–445	

were seeded onto glass slides (10^5 basal cells per glass slide) for patch clamp or $[Ca^{2+}]_i$ measurement. The unlabeled cell mixture that escaped capture by the Dynabeads contained mainly principal cells that can readily be identified by rectangular shape and microvilli upon plating on Petri dishes (Fig. 1) and functional expression of CFTR-Cl⁻ channels (Fig. 8). Both principal cells and basal cells were prepared for patch-clamp measurement and intracellular calcium measurement using Fluo-3. They were also studied for PGE₂ release.

RT-PCR Detection of Transient Receptor Potential (TRPC) Proteins and COX-1 mRNA

Total RNA was isolated from the epididymal epithelial cells using TRIzol reagent (Invitrogen). First-strand cDNA was obtained using Oligo(dT)₁₈ primer and Superscript II RNase H⁻ reverse transcriptase (Invitrogen). The resulting first-strand cDNA was directly used for the PCR of TRPC and COX-1 using specific primer pairs (summarized in Table I). The S16 was used as an internal standard. In general, the PCR mixtures were subjected to 30 cycles at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min.

Western Blot Analysis

Western blot analysis was performed as previously described (Leung et al., 2001b). Approximately 50–80 µg of cell protein extract was used for analysis. Anti-TRPC 3 (1:1000), polyclonal rabbit anti-TRPC1, or anti-TRPC6 (1:1000; Alomone Labs), or anti-COX-1 antibody (1:1000; Cayman Chemical) was used to detect TRPC and COX-1 proteins, respectively. Visualization of TRPC and COX-1 proteins in rat epididymal protein extracts was achieved by enhanced chemiluminescence (Western Blot Chemiluminescence Reagent Plus; NEN Life Science Products) according to manufacturer's manual.

Immunohistochemical Localization of TRPC Proteins and COX-1 in Rat Epididymis

Paraffin sections (3 µm) of rat epididymis were stained by the standard immunofluorescence method. Consecutive sections were incubated with rabbit polyclonal anti-TRPC1, anti-TRPC3 or anti-TRPC6, or anti-COX-1 antibodies diluted 1:100 overnight followed

by fluorescein (green-yellow) or Texas Red (deep red)-labeled antibody (Fluorescent Anti-Rabbit IgG Kit; Vector Labs). Slides were then mounted using VECTASHIELD mounting media (Vector Labs) for observation under a fluorescence microscope (Leica DM R system; Leica Microsystems). The images were captured by Spot-RT CCD camera (Diagnostic Instruments, Inc.). Negative controls were obtained by incubation with antigen preabsorbed antibodies.

Intracellular Calcium Concentration $[Ca^{2+}]_i$ Measurement

Cells were loaded with fluorescent dye Fluo3-acetoxymethyl ester (Fluo-3-AM; Molecular Probes) as previously described (Kwan et al., 2000). An excitation wavelength of 488 nm was provided by an MRC-1000 Laser Scanning Confocal Imaging System (Bio-Rad Laboratories), and fluorescence signals were collected using a 515-nm-long pass emission filter. Data analyses were performed with MetaFluor. The change of fluorescence intensity after drug treatments was normalized with the initial intensity.

Whole-cell Patch Clamp Recordings of Nonselective Cation Current in Basal Cells and CFTR-Cl⁻ Current in Principal Cells

Isolated basal cells, principal cells, or cocultures of principal cells with basal cells on coverslips were transferred to a 1-ml experimental chamber mounted on the stage of an inverted microscope, maintained at room temperature and either superfused at ~2 ml/min with physiological salt solution (PSS) (basal cells) or bathed unperfused in the same solution (principal cells or principal cell/basal cell cocultures) to prevent washing out of the chemical mediators. The whole-cell configuration of the patch clamp technique was used to measure macroscopic current under voltage clamp. Axopatch-200B amplifier and pClamp8 software were used for protocol generation and data acquisition (Axon Instruments). Patch pipettes were pulled from borosilicate glass (Sutter Instrument) and had resistance of 5–10 MΩ. Seals of >5 GΩ were produced on the cell surface. Current data were filtered at 2 kHz and digitized at 2–5 kHz (steps or ramps; in 5-s interval unless otherwise stated) or at 200 Hz (continuous holding current), respectively. Basal cells were held at -60 mV and principal cells at -70 mV. The input resistance was measured from the step change in current induced by a 10-mV hyperpolarizing step applied from holding potential immediately after whole-cell establishment. Cell

capacitance and series resistance were also estimated from the raised transient current. Basal cells had an input resistance of $\sim 15\ \text{G}\Omega$ and capacitance of $\sim 6.5\ \text{pF}$, whereas the corresponding values for the principal cells were $\sim 0.8\ \text{G}\Omega$ and $\sim 25\ \text{pF}$. Series resistance was not compensated and no leak current was subtracted.

Basal cells were superfused with normal PSS, which consisted of (in mM) NaCl 140, CsCl 4.7, MgCl_2 1.2, NaH_2PO_4 1.2, CaCl_2 2.5, HEPES 10, glucose 10, pH 7.4 with NaOH. The K^+ -based pipette solution contained (in mM) KCl 35, K-gluconate 100, MgCl_2 2, MgATP 3, EGTA 0.1, HEPES 10, pH 7.2 with KOH. To further characterize and magnify the nonselective cation current, a feature of TRPC protein, a low- Ca^{2+} -PSS was used as previously described (Jung et al., 2002), which was composed of (in mM) NaCl 140, CsCl 5, CaCl_2 0.2, MgCl_2 1, glucose 10, HEPES 10, pH 7.4 with NaOH. Nimidipine ($10\ \mu\text{M}$) and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB, $50\ \mu\text{M}$) were added to block the voltage-dependent calcium currents and chloride current, respectively. The pipette solution, which was set to contain nominally $100\ \text{nM}$ $[\text{Ca}^{2+}]_i$, had (in mM) Cs-methanesulfonate 110, CsCl 25, MgCl_2 2, CaCl_2 3.62, EGTA 10, HEPES 30, and pH 7.2 with CsOH.

To isolate the CFTR Cl^- current in principal cells, the pipette and bath solutions contained Cl^- as the main permeant ion. $5\ \text{mM}$ EGTA was included in pipette solution to suppress the Ca^{2+} -activated Cl^- current. The pipette solution contained (in mM) 135 *N*-methyl-D-glucamine $^+$ - Cl^- (NMDG-Cl), 2 MgCl_2 , 3 MgATP, 5 EGTA, 10 HEPES, pH 7.2 with Tris. The NMDG-Cl external solution contained (in mM) NMDG-Cl 135, CsCl 4.7, CaCl_2 2.5, MgCl_2 1.2, NaH_2PO_4 1.2, glucose 10, HEPES 10 (pH 7.4, Tris). Cells were first bathed in normal PSS before switching to NMDG-Cl external solution. Agonists were added directly to the bath.

Assay of Basal and LBK-induced Prostaglandin E_2 Release

Lysylbradykinin (LBK)-induced PGE_2 release was measured in isolated basal cells or principal cells grown on 96-well culture plates. Cells were incubated with LBK for 30 min. The culture media were measured for PGE_2 using QSR Immunoassay Kit (Assay Designs, Inc.) according to the manufacturer's instruction. Release of PGE_2 was expressed as picogram PGE_2 release per milligram protein (Cheuk et al., 2002).

Figure Preparation

Figures were prepared using Adobe Photoshop 7.0 software.

Statistical Analysis

Data are presented as means \pm SEM. In short-circuit current measurements, unpaired two-tailed Student's *t* test was used for comparison between groups. In patch-clamp experiments, paired *t* test was used. Multiple comparison was made using one-way ANOVA with Bonferroni post-hoc test. *P* values < 0.05 were accepted as significant.

RESULTS

Effects of Chelation of Intracellular Calcium on the *I*_{sc} Responses to Lysylbradykinin and Secretin

LBK (Cuthbert and Wong, 1986) and secretin (Chow et al., 2004) have been shown to stimulate chloride and bicarbonate secretion (measured as short-circuit current, *I*_{sc}) in cultured rat epididymal epithelia. The effect of LBK but not that of secretin is mediated by an increase in prostaglandin synthesis as COX inhibitors abolish the response to the former but not to the latter.

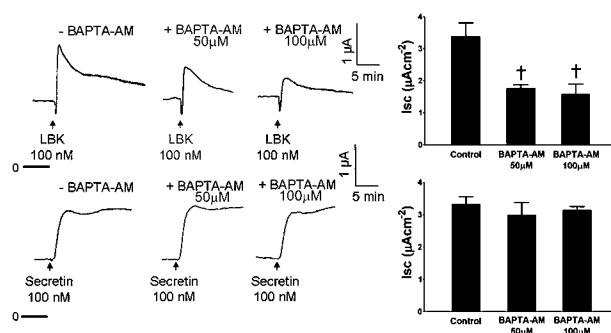


FIGURE 2. Effect of BAPTA-AM. Cultured rat epididymal epithelia (area $0.4\ \text{cm}^2$) stimulated with LBK ($100\ \text{nM}$, top), or with secretin ($100\ \text{nM}$, bottom). In each panel, the epithelium on the left shows the control response to LBK or secretin. The other two epithelia were pretreated with BAPTA-AM (0.05 or $0.1\ \text{mM}$, apical) for 1 h before addition of the hormone. Histogram shows a summary of the results. Each column represents the mean \pm SEM of six experiments. †, $P < 0.01$, compared with control.

There is evidence that COX enzymes are dependent on calcium signaling pathway, therefore we investigated the role of intracellular calcium in the secretory response to LBK and secretin by using a pharmacological agent that perturbs the Ca^{2+} signaling pathway. The results show that 1,2-bis (2-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid tetrakis acetoxyethyl ester (BAPTA-AM, 0.05 or $0.1\ \text{mM}$), a Ca^{2+} chelator that prevents an increase in intracellular free Ca^{2+} , attenuated the anion secretion response of cultured epididymal epithelia to LBK but not to secretin (Fig. 2).

RT-PCR and Western Blot Analysis of COX-1 and TRPC

Transient receptor potential proteins (TRPC) are Ca^{2+} -permeable cation channels activated by G protein-coupled receptors (Clapham et al., 2001; Clapham, 2003). To study the role of TRPC in COX-1-mediated anion secretion induced by LBK, we have studied the expression of COX-1 and TRPC in the rat epididymis. RT-PCR and Western blot analysis detected the expression of COX-1 mRNA and protein in the rat epididymis (Fig. 3 A) (Wong et al., 1999). Using a similar approach, TRPC mRNA and proteins were also detected with the predominant isoforms being TRPC 1, 3, and 6 (Fig. 3 A). TRPC 2, 4, and 5 isoforms, however, were not detected (unpublished data). Expression for TRPC 1, 3, and 6 mRNA increases from the initial segment to the cauda epididymidis. The protein profiles mirror the respective TRPC mRNA in different regions along the epididymis (Fig. 3 A).

Immunohistochemistry revealed the presence of COX-1 in the basal cells, but not in the principal cells and other cells (Fig. 3 C) (Wong et al., 1999; Leung et al., 2004). Using specific antibodies against the TRPC proteins, TRPC3 was localized only in the basal cells but not in the other cells of the epithelium and interstitial

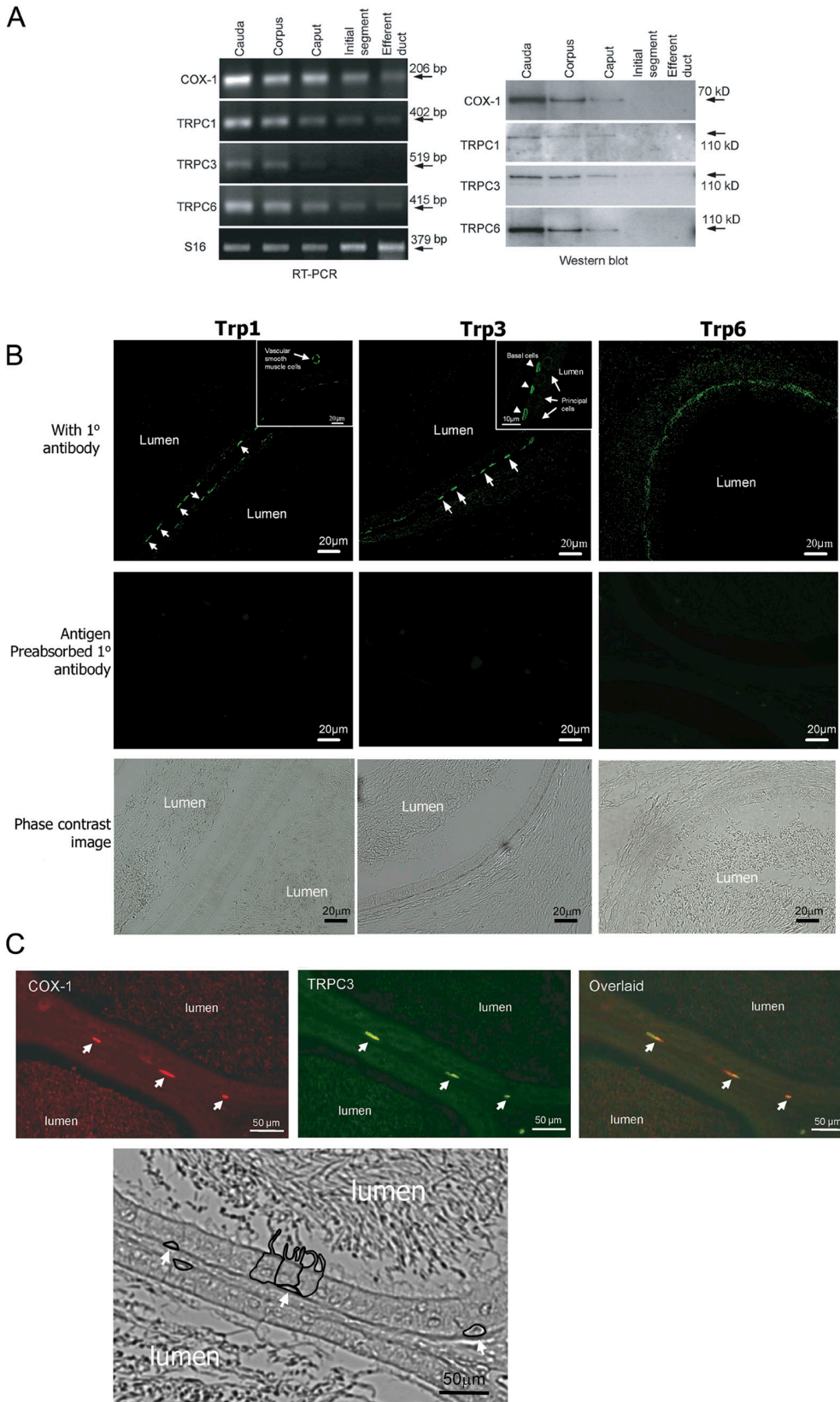


FIGURE 3. Identification and localization of TRPC and COX-1 in the rat epididymis. (A) RT-PCR and Western blot analysis of TRPC and COX-1 transcripts and proteins. RT-PCR revealed only TRPC1, 3, and 6 transcripts but not TRPC2, 4, and 5 transcripts (not depicted). The PCR products for TRPC1, 3, and 6 were more intense in the caudal than in the proximal region of the epididymis, whereas that of COX-1 was more uniformly distributed along the epididymis. The PCR product for S16 serves as the internal standard. In parallel with the RNA expression, Western blots also show the expression of TRPC1, 3, and 6 proteins in the rat epididymis. (B) Immunohistochemical localization of TRPC1, TRPC3, and TRPC6 proteins in the rat epididymis using polyclonal rabbit anti-TRPC1, anti-TRPC3, and anti-TRPC6 antibodies (1:100 dilution). Higher magnifications are shown in insets. Negative controls were obtained by incubation with antigen-preabsorbed antibodies. Phase contrast images of the corresponding stained sections are shown below. (C) Consecutive sections of the rat cauda epididymidis showing positive immunoreactivity (fluorescence) for COX-1 and TRPC3, respectively. Both proteins were restricted to basal cells in the epithelium but not to the other cell types. The two sections were overlaid to demonstrate coexistence of TRPC3 and COX-1 in the basal cells. Phase contrast image is shown to reveal the relationship of the cells. Basal cells are indicated by arrows.

tissues (Fig. 3 B). TRPC1 was in the basal cells as well as the vascular smooth muscle cells, and TRPC6 was weakly seen in the principal cells (Fig. 3 B). Labeling with anti-

COX-1 and anti-TRPC3 antibodies in consecutive sections of the rat cauda epididymidis revealed colocalization of COX-1 and TRPC3 in the basal cells (Fig. 3 C).

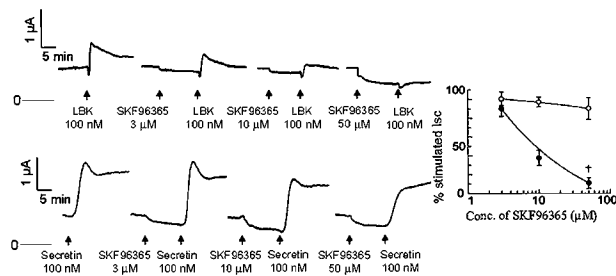


FIGURE 4. Effect of SKf96365 *Isc* responses to (A) LBK (100 nM) or (B) secretin (100 nM) in cultured rat epididymal epithelia (area 0.4 cm²) in the presence of varying concentrations of SKf96365. Inset shows the concentration–inhibition curves of SKf96365 on LBK (closed circles) and secretin (open circles). Each point shows the mean \pm SEM of six experiments. †, $P < 0.01$, compared with control.

Effects of Inhibitors of TRPC Proteins on *Isc* Responses to Lysylbradykinin and Secretin

To delineate a role for Ca²⁺ influx through TRPC channels, a TRPC channel inhibitor, 1-(β -[3-(4-methoxyphenyl)propoxyl]-4-methoxyphenethyl)-1H-imidazole hydrochloride (SKf96365) (Wang and van Breemen, 1997; Halaszovich et al., 2000), was used to study LBK-induced anion secretion in cultured rat epididymal epithelium. Fig. 4 shows that pretreatment of the epithelium with the inhibitor dose dependently blocked the responses of the epithelium to LBK without affecting the responses to secretin. Furthermore, antisense to TRPC3 reduced the LBK response without affecting the secretin response (Fig. 5). In contrast, antisense to TRPC2 did not affect the response to LBK (ΔI_{sc} after TRPC2 antisense treatment, $4.47 \pm 0.38 \mu\text{A cm}^{-2}$; TRPC2 sense, $4.82 \pm 0.25 \mu\text{A cm}^{-2}$; results not significantly different). Western blot analysis has shown that TRPC3 antisense oligonucleotides reduced the expression of TRPC3 protein but not the TRPC1, TRPC6, and COX-1 protein in the rat epididymis (Fig. 5, inset). These experiments support the notion that TRPC channels have a role to play in mediating the secretory response of the epididymal epithelium to bradykinin and possibly other hormones.

TRP-like Current in Basal Cells

Isolated basal cells were subject to patch-clamp electrophysiological study to elucidate the functional role of TRPC proteins under quasi-physiological conditions. When basal cells were voltage clamped at -60 mV, dialysed with K⁺-based internal solution and bathed in normal PSS, superfusion of LBK (200 nM) evoked an inward current composed of a transient and a sustained phase (Fig. 6 A). The peak amplitude of the transient current was -20.0 ± 4.4 pA ($n = 7$). A shift of reversal potential toward positive potential was also observed (unpublished data); this may have reflected an influx of cations through TRPC channels (nonselective cation

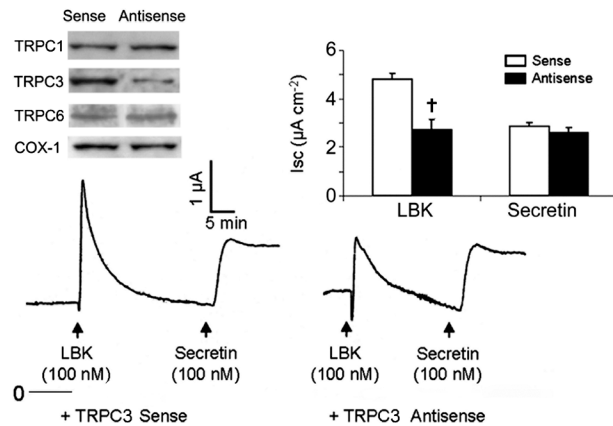


FIGURE 5. Effect of antisense oligonucleotide to TRPC3. Two matched cultured rat epididymal epithelia (area 0.4 cm²) from the same batch of cells stimulated with LBK (100 nM) followed by secretin (100 nM). The epithelium on the left was pretreated with sense and on the right antisense to TRPC3. Histogram shows a summary of the results. Each column shows the mean \pm SEM of six different experiments. †, $P < 0.01$, compared with control. The down-regulation of TRPC3 protein after antisense treatment is revealed by Western blot analysis (inset). TRPC1, TRPC6, and COX-1 expressions are not affected by TRPC3 antisense oligonucleotide.

channels), thereby causing cell membrane depolarization. No inward current was observed in the principal cells isolated from the same batch of epididymal tissues upon exposure to LBK (Fig. 6 B). The known nonselective cation channel blockers SKf96365 (10–20 μM), Gd³⁺ (0.1 mM), or La³⁺ (0.1 mM) reversed the LBK current to the baseline (unpublished data). Under the same conditions, pretreatment of basal cells with SKf96365 (20 μM) or Gd³⁺ (0.1 mM) prevented the LBK-activated current (Fig. 6, C and D). This current was presumably a nonselective cation current, probably a member of the TRP channel superfamily. To characterize this current further, we performed experiments in low extracellular Ca²⁺ concentration (i.e., 0.2 mM), as physiological level of Ca²⁺ attenuated the current (Helliwell and Large, 1996; Kamouchi et al., 1999; Jung et al., 2002). A Cs⁺-based internal solution with Ca²⁺ buffering was used to eliminate contamination of K⁺ currents and other currents activated by store depletion. To prevent chloride channel and voltage-activated Ca²⁺ channel activities, their respective blockers, NPPB (50 μM) and nomidipine (10 μM), were also included in the external solution. Under these conditions, addition of LBK still evoked an inward current in the basal cells at negative potentials, with larger current magnitude compared with that recorded in normal PSS (Fig. 6 F, inset). The current profile also consists of a transient peak (~ 400 pA) followed by a plateau phase. Addition of Gd³⁺ (0.1 mM) caused a reduction of the inward current. As demonstrated in Fig. 6 F, the current exhibits a nonlinear current–voltage (*I*–*V*) relationship

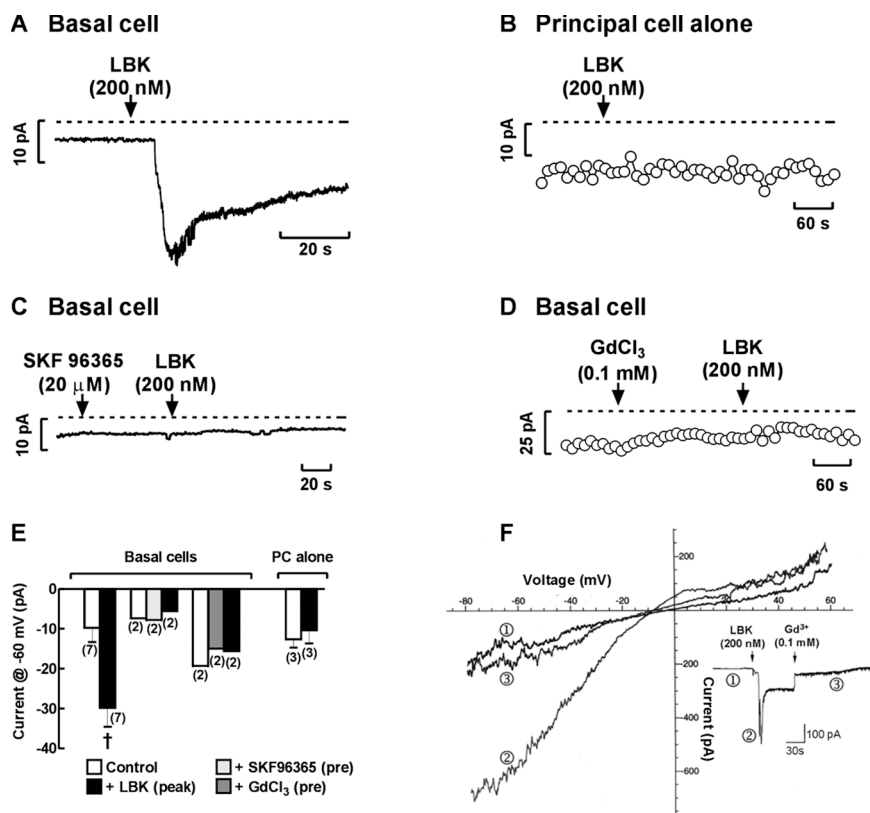


FIGURE 6. LBK activates a nonselective current in basal cells. (A) Whole-cell current recorded before and during exposure to 200 nM LBK in an isolated basal cell clamped at -60 mV dialyzed with K^+ -based pipette solution and superfused with normal PSS. (B) Incubation of principal cells with LBK (without basal cells) evoked no current at -60 mV. (C) Pretreatment with SKF96365 (20 μ M) or (D) Gd^{3+} (0.1 mM) prevented the LBK-evoked current in basal cells. (E) Summary of the current mean amplitudes in A–D: control, current measured before stimulation; +LBK (peak), current measured at the peak of LBK response; +SKF (Pre) or + $GdCl_3$ (Pre), current measured after pretreatment with SKF96365 or Gd^{3+} . Number of cells shown in brackets. †, $P < 0.01$, compared with control. (F) Current–voltage relationship of basal cells before and during exposure to LBK (200 nM), then to Gd^{3+} (0.1 mM). The bathing solution was low- Ca^{2+} PSS, and internal solution was Cs^+ -based solution containing 100 nM $[Ca^{2+}]_i$. Data points in B and D are currents taken at 10-s intervals at -60 mV from principal cells and basal cells, respectively. Dotted line indicates zero current level.

with rectification at negative membrane potentials. This is another characteristic of TRP channel family.

Measurement of Intracellular Calcium Using Fluo-3

To substantiate the argument that the cationic current in the basal cells described above is related to calcium influx, we measured intracellular Ca^{2+} level ($[Ca^{2+}]_i$) in isolated basal cells loaded with Fluo-3 in the presence or absence of extracellular Ca^{2+} . Fig. 7 shows when Ca^{2+} (2.5 mM) was added to a bath solution originally Ca^{2+} free, there was little increase in fluorescence intensity ($[Ca^{2+}]_i$) in the basal and principal cells. However, subsequent addition of LBK (100 nM) caused a marked increase in $[Ca^{2+}]_i$ in the basal cells only. The Ca^{2+} signal was sustained and was attenuated by 0.1 mM Gd^{3+} , which blocks nonselective calcium channels (Fig. 7). No increase in Ca^{2+} was observed in the principal cells stimulated by LBK. Pretreatment of the basal cells with SKF96365 (20 μ M) abolished the calcium response (Fig. 7).

Interaction between Basal and Principal Cells in Short-term Coculture

Attempts were made to study the interaction between isolated basal cells and principal cells by coculturing them in a dish for 8 h. Principal cells are known to constitutively express CFTR, a cAMP-activated chloride channel that under whole-cell patch configuration can

be identified as macroscopic chloride current inducible by cAMP (Huang et al., 1993; Gong and Wong, 2000). In this study, NMDG-Cl was used in both internal and bath solutions so that the main permeant ion was Cl^- . In addition, 5 mM EGTA was added to the internal solution to suppress any Ca^{2+} -activated Cl^- currents. Under these experimental conditions, CFTR Cl^-

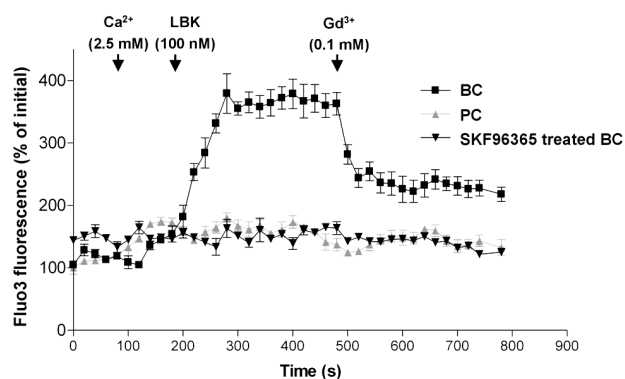


FIGURE 7. Measurement of intracellular Ca^{2+} , $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ measurement in isolated basal cells (squares) or principal cells (upright triangles) using Fluo3 as probe. Addition of Ca^{2+} (2.5 mM) to the cells did not significantly increase $[Ca^{2+}]_i$. Basal cells, but not principal cells, responded to LBK (100 nM) by an increase in $[Ca^{2+}]_i$, which was blockable by Gd^{3+} and prevented by pretreatment with SKF96365 (20 μ M) (inverted triangles). Each point shows the mean \pm SEM of five experiments.

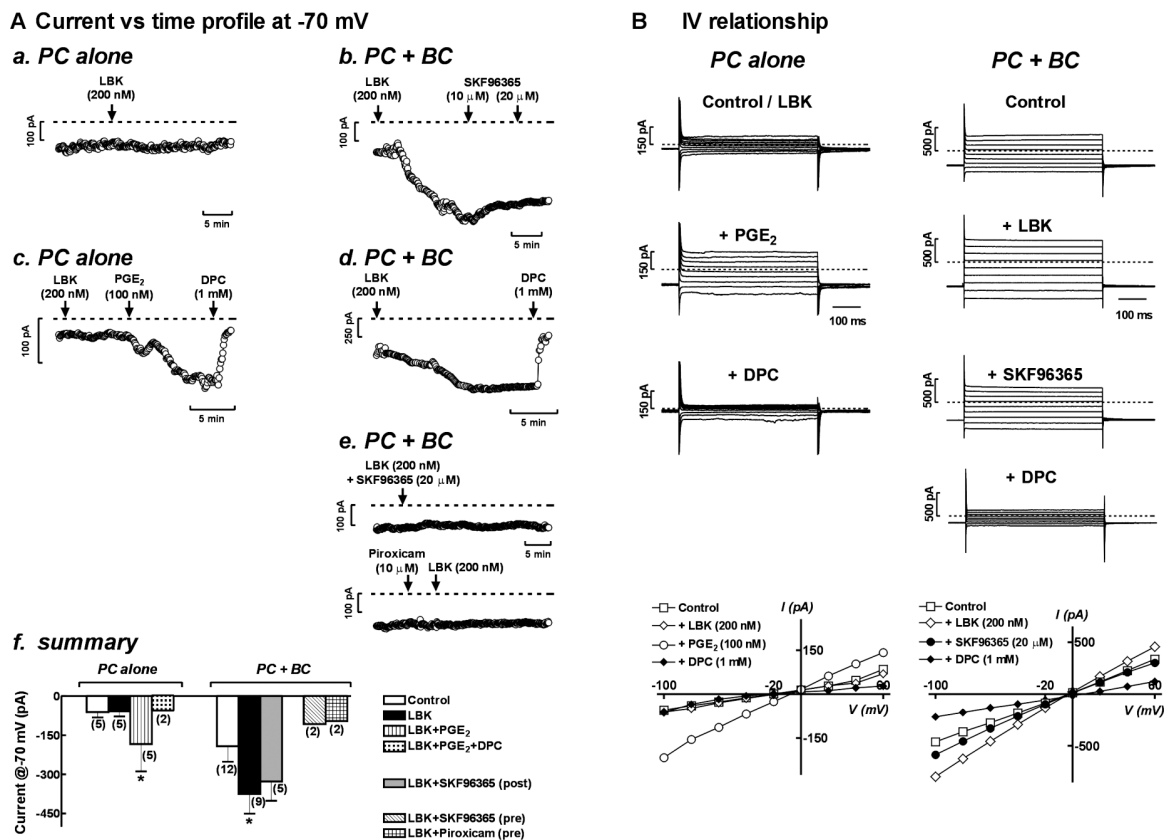


FIGURE 8. Measurement of CFTR-Cl channel activity. Whole-cell recording in isolated principal cells cultured alone or with basal cells. (A) Time profile showing whole-cell currents from principal cells held at -70 mV. Data points are from currents at -70 mV obtained at 10-s intervals. (a) When principal cells were cultured without basal cells (*PC alone*), LBK (200 nM) evoked no current; (c) subsequent addition of PGE_2 (100 nM) developed a current that was abolished by the Cl^- channel blocker DPC (1 mM). When principal cells were cultured with basal cells (*PC + BC*), LBK (200 nM) developed a current that was attenuated by (b) addition of the cation channel blocker SKF96365 (10–20 μM), or by (d) the Cl^- channel blocker DPC (1 mM). (e) SKF96365 (20 μM) added simultaneously with LBK or piroxicam pretreatment (10 μM) prevented LBK to activate the currents. (f) Summary of results from A. Each column shows the mean current amplitudes taken before or after drug addition. The number shows the number of cells studied. *, $P < 0.05$, when compared with unstimulated control. (B) Tracings showing current responses to a series of 500-ms voltage steps between -100 and $+60$ mV from principal cells in the absence (left) or presence of basal cells (right). Corresponding I-V plots are also shown. Note the linear I-V curves and the time independence at each potential level. Dotted line represents zero current level.

channel activity could be isolated and recorded as macroscopic currents in principal cells. We first examined the effect of LBK (200 nM) on isolated principal cells. As demonstrated in Fig. 8 A (a), no current was evoked by incubation with LBK for ~ 20 min. However, addition of PGE_2 (100 nM) evoked an inward current at -70 mV. The current reached a maximum after ~ 10 min and could be blocked by 1 mM DPC, a Cl^- channel blocker (Fig. 8 A, c). This inward current observed at negative potentials could reflect an efflux of Cl^- . As depicted in the left panel of Fig. 8 B, the PGE_2 -evoked current was voltage independent; no time-dependent activation or inactivation and no tail current was observed upon repolarization of the cells. In addition, the I-V relationship was linear. These characteristics are reminiscent of the CFTR- Cl^- current detected in epididymal principal cells stimulated with cAMP (Huang et al., 1993; Gong and Wong, 2000). As shown

in Fig. 8 A (b), when principal cells were cocultured with basal cells, application of LBK (200 nM) stimulated a Cl^- current displaying CFTR characteristic. This current was partially attenuated by addition of the putative cation channel blocker SKF96365 (Fig. 8 A, b). The LBK-induced current and portion of the basal current were also inhibited by the Cl^- channel blocker DPC (1 mM; Fig. 8 A, d). Pretreatment with SKF96365 (20 μM) and the COX inhibitor piroxicam (10 μM) abolished the Cl^- current evoked by LBK (Fig. 8 A, e).

Measurement of PGE_2 Release

The above experiments demonstrated that upon stimulation with bradykinin, basal cells release a chemical substance that acts on the principal cells to activate CFTR resulting in anion (and secondary water) secretion. It is plausible that such a chemical mediator could

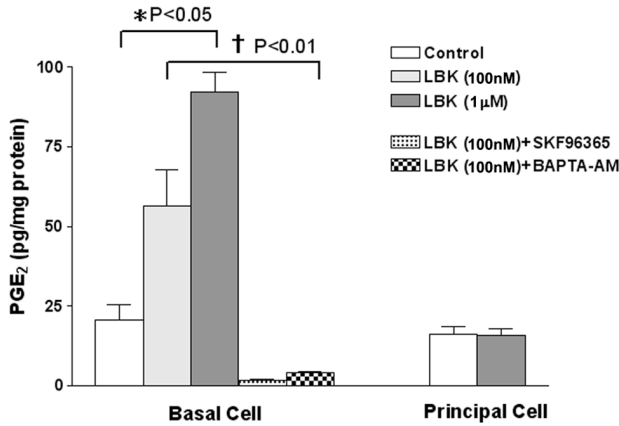


FIGURE 9. Measurement of PGE₂ release. Prostaglandin E₂ (PGE₂) release from isolated basal or principal cells. Significant release of PGE₂ was observed in basal cells but not in principal cells stimulated with LBK (0.1 or 1 μM). PGE₂ release from basal cells was significantly reduced by pretreatment of the cells with BAPTA-AM (0.1 mM, 1 h) or SKF96365 (10 μM, 15 min). Each column represents the mean ± SEM of six experiments. *, P < 0.05, compared with unstimulated control; †, P < 0.01, compared with stimulated control without pretreatment with BAPTA-AM or SKF96365.

be PGE₂. Isolated basal cells or principal cells were incubated in MEM with 1% FBS, and the release of PGE₂ was studied using enzyme immunoassay. The results show that isolated basal cells, like intact epididymal epithelia (Cheuk et al., 2002), release PGE₂ upon stimulation with LBK (0.1 and 1 μM). The release was attenuated by SKF96365 (20 μM) and BAPTA-AM (0.1 mM) pretreatment for 15 min and 1 h, respectively (Fig. 9). Isolated principal cells were found not to release PGE₂ upon stimulation with the hormone (Fig. 9).

Reconstitution of Isolated Basal Cells and Principal cells into Intact Epithelia

Epithelia on millipore filters were reconstituted from principal cells alone or from a mixture of principal and basal cells until confluency was reached. They were then clamped in an Ussing Chamber for measurement of transepithelial potential difference (PD), short-circuit current (*I*_{sc}), and resistance (R) (Wong, 1988a). Epithelia derived from principal cells and basal cells had a PD of 3.76 ± 0.1 mV (n = 6), a basal *I*_{sc} of 5.22 ± 0.13 μAcm⁻² (n = 6), and a measured R of 723 ± 30 Ωcm² (n = 6). By comparison, epithelia derived from principal cells only had a PD of 0.76 ± 0.02 mV (n = 6), a basal *I*_{sc} of 1.18 ± 0.05 μAcm⁻² (n = 6), and a measured R of 642 ± 44 Ωcm² (n = 6). The former epithelia responded to exogenous PGE₂ (500 nM, basolateral application) and bradykinin (100 nM, basolateral application) by an increase in *I*_{sc} of 3.26 ± 0.08 μAcm⁻² (n = 6) and 2.91 ± 0.11 μAcm⁻² (n = 6), respectively. In epithelia derived from principal cells only,

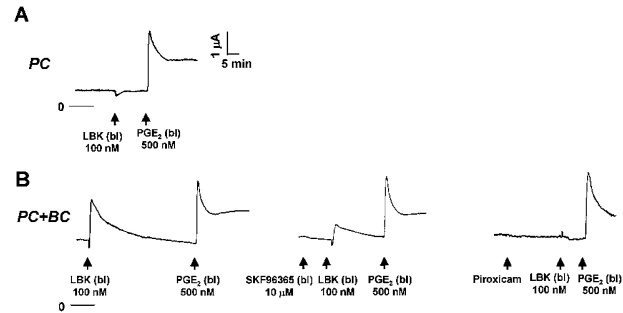


FIGURE 10. Reconstitution of epithelia from cells isolated from rat cauda epididymis. (A) Short-circuit current (*I*_{sc}) recording in epithelia reconstituted from isolated principal cells (10⁵ cells/ml) or (B) from a mixture of principal cells (0.7 × 10⁵ cells/ml) and basal cells (0.3 × 10⁵ cells/ml). Epithelia reconstituted from principal cells only responded to LBK (100 nM) poorly although they responded to PGE₂ (500 nM) with a significant increase in current. Epithelia reconstituted from principal cells plus basal cells responded to PGE₂ as in principal cell only-derived epithelia but responded to LBK with a marked increase in current. This LBK response in PC+BC epithelia was reduced and abolished by pretreatment with SKF96365 (10 μM) and piroxicam (10 μM), respectively. Each tracing is representative of six experiments.

the corresponding Δ*I*_{sc} after PGE₂ was 3.32 ± 0.05 μAcm⁻² (n = 6) (insignificantly different from basal + principal cell-derived epithelia), whereas Δ*I*_{sc} after bradykinin was only 0.22 ± 0.06 μAcm⁻² (n = 6) (significantly different from the corresponding value for basal + principal cell-derived epithelia at P < 0.01) (Fig. 10). The responses of the basal + principal cell-derived epithelia to bradykinin were reduced by SKF96365 (10 μM). Δ*I*_{sc} after SKF96365 was 1.20 ± 0.03 μAcm⁻² (n = 6) (significantly different from LBK-stimulated control at P < 0.01) (see also Fig. 3). The responses of PC+BC-derived epithelia to LBK were abolished by piroxicam (10 μM) pretreatment (Fig. 10). Cell suspensions containing basal cells only did not successfully form epithelia with measurable transepithelial potential difference and resistance.

DISCUSSION

The epithelium lining the epididymal duct is a pseudostratified epithelium consisting of several cell types. Among them, the principal cells are most abundant and their functions most extensively studied. There are other cell types such as the narrow cells, clear cells, halo cells, and basal cells, which are scattered along the duct albeit in lesser number (Robaire and Hermo, 2002). Although these minority cell types have not been studied to the same extent as the principal cells, it is conceivable that their presences are essential to the functions of the epididymis. Recently, we have been interested in the basal cells. They are dome-shaped cells adhering to the basement membrane forming contact (Gregory et al., 2001; Dufresne et al., 2003) with the

principal cells above them. Their hemispherical cell bodies give rise to thin attenuated processes that wrap around a good part of the circumference of the epididymal tubule. Recent morphological studies have provided evidence that basal cells are equipped with Golgi apparatus and possess abundant secretory granules, indicating that the cells are actively involved in secretion (Robaire and Hermo, 2002). Because of their proximity to the principal cells, it is speculated that basal cells may interact with the principal cells to bring about some integrated functions of the epithelium.

Transport of electrolytes and water in the epididymis is subjected to regulation by neuro, hormonal, paracrine, and autocrine factors. These factors interact with G protein-coupled receptors linked to various signal transduction pathways converging on the CFTR-Cl⁻ channels located in the apical membrane of the principal cells. Bradykinin, angiotensins, endothelin, vasopressin, and serotonin stimulate anion secretion through *de novo* formation of prostaglandins as their actions are blocked by cyclooxygenase inhibitors (for references, see INTRODUCTION). Other neurohumoral factors such as ATP (Wong, 1988b), noradrenaline (Leung and Wong, 1994b), adrenaline (Wong, 1988a), CGRP (calcitonin gene-related peptide) (Leung et al., 1992), and secretin (Chow et al., 2004) stimulate anion secretion in the epididymis without involving prostaglandins. The COX isoform responsible for the actions of the former group of agents has been found to be COX-1 but not COX-2 (Wong et al., 1999). Since the only epididymal cell type expressing COX-1 are the basal cells (Wong et al., 1999), it transpires therefore that the basal cells mediate the neurohumoral control of electrolyte and fluid transport in the epididymis.

The present work shows that basal cells uniquely express TRPC3 and TRPC1, which in many cells serve as nonselective cation channel with a moderate permeability to calcium. These channels therefore provide a pathway for calcium influx (Clapham et al., 2001). Basal cells and principal cells isolated from the rat epididymis using antibody-coated Dynabeads are amenable to patch-clamp study. They are readily distinguishable from each other by their cell shape and size (Fig. 1). Basal cells can be further identified by their exclusive COX-1 expression (Fig. 1). Under whole-cell patch-clamp conditions, basal cells, but not principal cells, developed a cation current when stimulated with bradykinin (Fig. 6 A). This current displayed a nonlinear I-V relationship with rectification at negative membrane potentials and is blockable by La³⁺, Gd³⁺, or SKF96365. These characteristics are reminiscent of the TRPC current recorded in heterologous cells expressed with cloned human TRPC genes or in intact smooth muscle and other cells (Zhu et al., 1996; Kamouchi et al., 1999;

McKay et al., 2000; Ng and Gurney, 2001). Since most of the TRPC channel isoforms in other cellular systems have been shown to have similar electrical properties (Halaszovich et al., 2000; Jung et al., 2002), we cannot ascertain unequivocally that TRPC3 protein or TRPC1 protein alone is the structural entity responsible for the nonselective cation current detected in the basal cells, nor can we preclude the possibility that this whole-cell current is indeed the result of a heteromeric complex formed by the interactions of several TRPC proteins, such as between TRPC1 and TRPC3, as coassembly of these two proteins has been shown to generate diacylglycerol-sensitive cation channels (Hofmann et al., 2002; Lintschinger et al., 2000). It does appear, however, that TRPC3 protein is the only isoform exclusively immunolocalized in the basal cells (Fig. 4 B). In agreement with the notion that TRPC channels function as calcium influx pathways, isolated basal cells, but not principal cells, were found to increase intracellular calcium upon stimulation with bradykinin (Fig. 7). Calcium influx like the cationic current induced by bradykinin was blocked by Gd³⁺ and SKF96365.

It appears therefore that in basal cells, mechanisms are in place to regulate intracellular Ca²⁺ and that Ca²⁺ influx through TRPC channels may contribute to a rise of intracellular calcium induced by bradykinin. It is conceivable that a rise in cell calcium activates PLA₂, which hydrolyzes membrane lipids (PIP₂) to arachidonic acid, which, in the presence of COX-1, forms prostaglandins. The regulation of prostaglandin synthesis by intracellular calcium via PLA₂ has been proposed for other cell systems (McAllister et al., 1993; Frearson et al., 1995; Wise and Jones, 2000). The prostaglandins formed by the basal cells would then act as paracrine factors controlling principal cell transport of ions and water. Previous work has shown that among the COX products, PGE₂ predominately stimulates anion secretion in cultured epididymal epithelia via EP₂/4 receptors (Wong et al., 1999). Furthermore, PGE₂ is released from intact epididymal epithelia stimulated with bradykinin (Cheuk et al., 2002). This study further ascribes the source of the release to the basal cells as isolated basal cells, but not principal cells, release PGE₂ upon stimulation with bradykinin (Fig. 9). The release of PGE₂ is further shown to be dependent on intracellular calcium as BAPTA-AM, an intracellular calcium chelator, and SKF96365, a blocker of nonselective cation channels, attenuated the release (Fig. 9).

The contention that PGE₂ is the chemical messenger that mediates the control of principal cell secretion of electrolytes and water by the basal cells is supported by the coculture experiments. Basal cells and principal cells were cocultured in a dish for 8 h, after which time principal cells were subjected to whole-cell patch-clamp recording of CFTR-Cl⁻ channel activity. It has been es-

tablished that CFTR is expressed by the rat and human epididymal cells (Huang et al., 1992, 1993; Leung and Wong, 1994a; Leung et al., 1996; Patrizio and Salameh, 1998; Gong and Wong, 2000; Leung et al., 2001a). Addition of bradykinin to the cocultures stimulated in the principal cells an anion current with CFTR characteristics, i.e., linear I–V relationship, time and voltage independence, and blockade by diphenylamine-2-carboxylic acid (DPC), a known chloride channel blocker (Huang et al., 1993; Nilius and Droogmans, 2003). This current, however, was not observed in the principal cell-only cultures, which, however, responded to PGE₂ with an inward current with similar characteristics (Fig. 8). The LBK-stimulated current recorded from the principal cells in the presence of basal cells could be due to PGE₂ released from the basal cells as this current was blocked by piroxicam, inhibitor of PGE₂ synthesis (Fig. 8). In the intact tissue, the released PGE₂ then stimulates principal cells to secrete chloride via the apically placed CFTR.

The importance of the basal cells in the functional integrity of the epithelium was highlighted by reassembly of the isolated cells into intact epithelia. Dispersed epithelial cells from the rat (Cuthbert and Wong, 1986; Chan and Wong, 1996), mouse (Leung et al., 1996), and human (Leung et al., 1992) epididymis were shown to form confluent cell monolayer cultures capable of active electrolyte transport, which can be measured as *I*_{sc}. Epithelial cultures from principal and basal cells together developed higher transepithelial PD and basal *I*_{sc} compared with those formed from principal cells only. Both epithelia responded to PGE₂ by an increase in *I*_{sc} of similar magnitudes. However, only the epithelia derived from a mixture of basal and principal cells responded to bradykinin with a significant increase in current, indicating that basal cells are essential to the regulation of ion transport by bradykinin. The LBK-stimulated *I*_{sc} was abolished by piroxicam (Fig. 10), suggesting the involvement of PGE₂ synthesis. These, together with the observation that PGE₂ is released from the basal cells in a calcium-dependent manner (BAPTA-AM and SKF96365 inhibit) and that calcium influx can be observed in isolated basal cells most probably through TRPC proteins (immunodetection of TRPC1 and TRPC3 in basal cells) lend supports to the hypothesis that basal cells regulate principal cell functions through the release of PGE₂. This process involves a calcium signaling pathway and COX-1 in the basal cells.

In conclusion, this work demonstrated for the first time that basal cells are essential for the integrated function of the epididymis. They regulate principal cell electrolyte transport by releasing paracrine factors. In this way, basal cells resemble the endothelial cells whose regulation of vascular smooth muscle tone via

chemical mediators is well known. In both the epididymal basal and vascular endothelial cells, regulation of cell Ca²⁺ and biosynthesis of prostaglandins appear to be the common features in both cell types. It is concluded that cell–cell interaction underlies the formation of the epididymal environment in which maturing spermatozoa are bathed.

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