Functional expression of cystic fibrosis transmembrane conductance regulator in rat oviduct epithelium

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The aim of this study was to investigate the functional expression of cystic fibrosis transmembrane conductance regulator (CFTR) with electrophysiological and molecular technique in rat oviduct epithelium. In whole-cell patch clamp, oviduct epithelial cells responded to 100 µM 8-bromo-adenosine 3',5'-cyclic monophosphate (8-Br-cAMP) with a rise in inward current in Gap-free mode, which was inhibited successively by 5 µM CFTR(inh)-172, a CFTR specific inhibitor, and 1 mM diphenylamine-2-carboxylate (DPC), the Cl⁻ channel blocker. The cAMP-activated current exhibited a linear current-voltage (I-V) relationship and time- and voltage-independent characteristics. The reversal potentials of the cAMP-activated currents in symmetrical Cl⁻ solutions were close to the Cl⁻ equilibrium, 0.5±0.2 mV (n=4). When Cl⁻ concentration in the bath solution was changed from 140 mM to 70 mM and a pipette solution containing 140 mM Cl⁻ was used, the reversal potential shifted to a value close to the new equilibrium for Cl⁻, 20±0.6 mV (n=4), as compared with the theoretic value of 18.7 mV. In addition, mRNA expression of CFTR was also detected in rat oviduct epithelium. Western blot analysis showed that CFTR protein is found in the oviduct throughout the cycle with maximal expression at estrus, and immunofluorescence and immunohistochemistry analysis revealed that CFTR is located at the apical membrane of the epithelial cells. These results showed that the cAMP-activated Cl⁻ current in the oviduct epithelium was characteristic of CFTR, which provided direct evidence for the functional expression of CFTR in the rat oviduct epithelium. CFTR may play a role in modulating fluid transport in the oviduct.

Keywords  oviduct; cystic fibrosis transmembrane conductance regulator (CFTR); patch clamp; estrus cycle

Possessing complex physiological function, the oviduct is an important part of the female reproductive system. It is well known that oviduct epithelial cells secrete a variety of nutrient substances and factors, which provide an appropriate environment for a series of reproductive events, including capacitation of sperm, maturation of oocyte and embryo development [1,2].

The mammalian oviduct is capable of active fluid secretion from the blood into the lumen that is driven by electronegenic Cl⁻ secretion across the oviduct epithelium [3]. Cystic fibrosis transmembrane conductance regulator (CFTR) plays a critical role in the regulation of ion transport in a number of exocrine epithelia, including the lungs, intestine, pancreas and sweat gland duct [4–7]. Although CFTR mRNA was detected in murine oviduct and cystic fibrosis (CF) mouse oviduct exhibited defective cAMP-mediated Cl⁻ secretion [8–10], no direct evidence of the electrophysiological characterization of CFTR as a cAMP-activated Cl⁻ channel in the oviduct has been provided with patch clamp technique. Therefore, one of the aims of our present study was to investigate the electrophysiological properties of CFTR in rat oviduct epithelium using the whole-cell patch clamp technique.

Additionally, we investigated the cyclic variations in the
expression of CFTR in rat oviduct, as the number of spermatozoa migrating through the oviduct is significantly higher in estrus than in other stages [11]; the high migration rates may result from fluid accumulation, which is highest during estrus [12]. CFTR expression may regulate uterine fluid production to facilitate sperm transport in different stages. We examined the cyclic variations using Western blot analysis.

Materials and Methods

Animals
Immature and mature female Sprague-Dawley rats were purchased from the Animal Center of Sun Yat-sen University (Guangzhou, China). Animals were housed and fed according to the guidelines of the Sun Yat-sen University Animal Use Committee; all procedures were approved prior to each experiment. Animals were kept in a room with a constant temperature (20 ºC) with a 12L:12D photoperiod and were allowed to access food and water ad libitum.

Medium and drugs
Hanks’ balanced salt solution, penicillin/streptomycin, Dulbecco’s modified Eagle’s medium/F12 (DMEM/F12), 0.25% trypsin and SuperScript One-Step reverse transcription-polymerase chain reaction (RT-PCR) with PlatinumTaq were purchased from Invitrogen and Gibco (Carlsbad, USA). Fetal bovine serum was from Hyclone (Logan, USA). Collagenase, 8-bromoadenosine 3′,5′-cyclic monophosphate (8-Br-cAMP), CFTR inhibitor (inh)-172 and diphenylamine-2-dicarboxylic acid (DPC) were purchased from Sigma (St. Louis, USA). The primers for CFTR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were synthesized by the Sangong Company (Shanghai, China).

Culture of rat oviduct epithelium
Immature female Sprague-Dawley rats weighing 100–120 g were killed by CO2 inhalation. Their lower abdomens were opened, and the oviducts were isolated and microdissected under sterile conditions to remove fat and connective tissues. The tissues were cut into small segments, incubated for 2 h in Ca2+-free Dulbecco’s PBS solution containing 1 mM ethylene glycol tetraacetic acid (EGTA) for 10–15 min to separate the cells. The cells were then moved to a 1 ml cell culture flask that was fixed on the X-Y axis stage of an Olympus BX51WI immersing lens microscopy system (Tokyo, Japan). Recording was performed at room temperature using a Multiclamp700A amplifier and the Digidata 1322 series interface (AXON Instrument, Foster City, USA). Signals were filtered at 10 kHz. Pelamp 9.0 software system (AXON Instrument) was used for data recording and analysis.

Using a horizontal puller P-97 (Sutter Instrument, Novato, USA) and glass pipettes, we pulled patch pipettes (1.2 mm outside diameter and 0.5 mm inside diameter). Ionic current was recorded using the conventional whole-cell patch clamp technique. The pipettes were filled with a solution containing 120 mM CsCl and 20 mM tetraethyl-ammonium-Cl (pH adjusted to 7.2 with CsOH). The bath solution contained 135 mM NaCl, 1.2 mM Na2HPO4, 2 mM MgCl2, 10 mM HEPES and 10 mM glucose (pH 7.4). The low Cl− (70 mM) bath solution contained 69 mM Na-glutamate, 66 mM NaCl, 1.2 mM Na2HPO4, 2 mM MgCl2, 10 mM HEPES and 10 mM glucose (pH 7.4). The resistance of the patch pipettes was approximately 2–7 MΩ. Positive pressure was applied in the patch pipette before it was immersed in the bath solution. When the tip of the pipette attached to the cell surface, pressure was withdrawn, and then a giga-seal between the pipette and cell membrane formed normally. After being sucked from the pipette, the whole cell configuration was confirmed. The oviduct cell

Immunofluorescence of cytokeratin
Paraformaldehyde-fixed primary rat oviduct epithelial cells were washed in phosphate-buffered saline (PBS) and incubated with 1% bovine serum albumin (BSA) for 15 min before incubation with the primary mouse anti-rat cytokeratin antibody (Cat. No. BM0030; BOSTER, Wuhan, China) at room temperature for 90 min. After three washes with PBS, cells were incubated for 60 min with the secondary anti-mouse IgG-fluorescein isothiocyanate (FITC) conjugate (BOSTER) in the dark, followed by three washes with PBS. Cells were then mounted onto glass slides using a 1:1 mixture of Vectashield medium (Vector Laboratories, Burlingame, USA) and 0.3 M Tris solution (pH 8.9). Cells were then viewed using TCS SP2 Confocal Imaging System (Leica Microsystems, Wetzlar, Germany).
was held at its resting potential $-30 \text{ mV}$ in the episodic recording mode, and the voltage was clamped from $-120 \text{ mV}$ to $+100 \text{ mV}$ in $20 \text{ mV}$ increments. In the Gap-free recording mode, the cells were held at $-70 \text{ mV}$ throughout the period of recording. The pipette potential and liquid junction potential were auto-compensated by the Multiclamp700A amplifier before the electrode touched the cell.

**Reverse transcription-PCR**

Total RNA was isolated using TRIzol reagent (Life Technologies, Gaithersburg, USA), and $2 \mu$g was used to amplify CFTR using Superscript II One-Step RT-PCR with Platinum Taq (Invitrogen). The primers for CFTR were: sense $5'-\text{GACAACATGGGAACACATCCCTCG-3'}$ (corresponding to nucleotides 2514–2537) and antisense $5'-\text{TCTCGTTCGTTTCACAGTCGGTGAG-3'}$ (corresponding to nucleotides 2771–2747), which yielded a PCR product of 258 bp. The primers for GAPDH were sense $5'-\text{ACTGGCGTCTTCACCACAT-3'}$ and antisense $5'-\text{TCCACACACCTGTGCTGA-3'}$, which yielded a PCR product of 300 bp. Reactions were carried out with the following parameters: denaturation at $94 \degree C$ for 1 min, annealing at $60 \degree C$ for 45 s, and extension at $72 \degree C$ for 1 min, for a total of 45 cycles. PCR products were analyzed by agarose gel electrophoresis and visualized by staining with ethidium bromide.

**Protein extraction and Western blot analysis**

Oviduct tissue from mature female rats at different estrus stages were cut into small pieces and disrupted with a Tenbroeck tissue grinder in 3 ml M-PER Mammalian Protein Extraction Reagent, and complete protease inhibitors (Roche Applied Science, Indianapolis, USA). Homogenates were centrifuged for 5 min at 14,000 $\mu$g at 4 $\degree C$. The supernatant was collected and the protein concentration was determined with BCA protein assay kit (Pierce Biotechnology, Rockford, USA). Homogenates were centrifuged for 5 min at 14,000 $\mu$g at 4 $\degree C$ until use. Sections were rinsed in PBS for 5 min and treated with 0.05% Triton X-100 for 1 min. Sections were subsequently washed three times in PBS for 5 min each, incubated for 15 min in 1% ($W/V$) BSA in PBS with 1% sodium azide to prevent non-specific staining and then incubated in the anti-CFTR antibody at room temperature for 120 min. The sections were incubated with PBS alone, which used as negative control. After three 5 min PBS washes, the FITC-conjugated secondary antibody was applied for 1 h at room temperature, and the slides were then rinsed again in PBS three times for 5 min. Slides were mounted in a 1:1 mixture of Vectashield medium and 0.3 M Tris solution (pH 8.9), and then viewed under the TCS SP2 Confocal Imaging System.

**Immunohistochemistry of rat oviduct paraffin section**

Paraffin-embedded 5 $\mu$m sections of paraformaldehyde-fixed oviducts were dewaxed and hydrated. Antigen was retrieved by treatment in 0.01 M citrate buffer (pH 6.0) for 20 min in a sub-boiling water bath. After rinsing with phosphate buffered saline Tween-20 (PBST), sections were incubated in normal blocking serum for 30 min and then with the rabbit anti-rat CFTR antibodies diluted 1:100 with diluting buffer (PBS with 1% BSA, 0.1% gelatine and 0.05% sodium azide) at 4 $\degree C$ overnight. Sections were washed three times with PBST and incubated with biotinylated secondary antibody at 37 $\degree C$ for 30 min. After being washed three times with PBST, the sections were incubated with the horseradish peroxidase conjugated donkey anti-goat IgG antibody for 30 min and finally washed three times with PBST. Visualization was achieved by immersing sections in a peroxidase substrate solution 3,3’-diaminobenzidine-tetrachloride (K4011;
DakoCytomation, Glostrup, Denmark) until desired stain intensity was reached. Slides were rinsed with pure water for 5 min, counterstained with Harris hematoxylin (Sigma), dehydrated, and mounted for observation. Negative controls were incubated with antigen of primary antibody.

**DNA sequencing**

Three 258 bp CFTR PCR products (from separate RNA isolations) were cloned into the pCR 2.1 vector (Invitrogen) using T4 DNA ligase, and they were then used to transform “super competent” Escherichia coli cells, which were grown for 1 d. The DNA was isolated using an isolation kit (Promega, Madison, USA), and the plasmid was sequenced on the ABI PRISM sequencer model 2.1.1 (Foster City, USA) and analyzed by BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). All three sequences obtained were 100% homologous to region 2514–2771 of the rat CFTR gene.

**Statistical analysis**

Results were expressed as mean±SEM, and n indicated the number of experiments. Data were analyzed using Student’s t-test. A P value less than 0.05 was considered to be statistically significant.

**Results**

**cAMP elicited whole-cell currents in cultured oviduct epithelial cells**

Path clamp recordings were applied on rat oviduct epithelial cells after two days of primary culture, which were confirmed by immunofluorescence staining of cytokeratin (Fig. 1), to see whether oviduct epithelial cells functionally exhibit CFTR conductance. The current recordings were obtained using pipette and bath solutions containing symmetrical Cl⁻ concentrations (containing intracellular 140 mM Cl⁻ and extracellular 140 mM Cl⁻, respectively). Under a continuous recording with a −70 mV holding potential, bath application of 8-Br-cAMP (100 µM) elicited inward whole-cell currents [Fig. 2(A)]. Series steps of voltage-clamp stimulation from −120 mV to +100 mV at 20 mV increments were applied to the cell before application of 8-Br-cAMP [Fig. 2(B)]. The cAMP-activated whole-cell currents demonstrated that they were independent of the stimulation time [Fig. 2(C)].

**Effect of CFTR(inh)-172 and DPC on the cAMP-stimulated Cl⁻ conductance**

The currents were maximally activated by cAMP and were then suppressed by subsequent application of 5 µM CFTR (inh)-172, a specific CFTR channel blocker, and 1mM DPC, the Cl⁻ channel blocker [Fig. 2(A)]. The whole-cell membrane current after cAMP stimulation followed by 5 µM CFTR(inh)-172, 5 µM CFTR(inh)-172 and 1 mM DPC, and 1 mM DPC alone, respectively, in response to square voltage pulses from a holding potential at −30 mV to a potential between −120 mV and +100 mV in 20 mV increments [Fig. 2(D–F)]. The mean percent inhibitions of CFTR(inh)-172 and DPC were 71.5% (n=4) and 75.2% (n=4), respectively. The current-voltage (I-V) relationship obtained from the cell at rest, cAMP stimulation alone, cAMP stimulation followed by CFTR(inh)-172 or cAMP...
The I-V relationship demonstrated that the cAMP-activated whole-cell currents had a linear relationship with gradually increased clamping voltages \[\text{Fig. 4(A)}\]. The reversal potentials of the cAMP-induced currents in symmetrical Cl$^-$ solutions were close to the Cl$^-$ equilibrium, $0.5\pm0.2$ mV \((n=4)\). In order to further identify whether the cAMP-activated whole-cell currents were mediated by Cl$^-$, and not through any non-selective conductance, we performed experiments in which the Cl$^-$ concentration in the bath was reduced from 140 mM to 70 mM, while a pipette containing 140 mM Cl$^-$ was used. The reversal potential shifted to a value close to the new equilibrium for Cl$^-$, 20$\pm$0.6 mV \((n=4)\), as compared with the theoretic value of 18.7 mV calculated according to the Nernst function and based on the present experimental conditions \[\text{Fig. 4(B)}\]. The results suggested that Cl$^-$ mediates currents activated by extracellular cAMP.

Identification of CFTR in epithelial cells of the oviduct by RT-PCR
RT-PCR was used to study the mRNA expression of CFTR in the oviduct \[\text{Fig. 5}\]. The PCR products of CFTR (258 bp) were amplified by RT-PCR from RNA isolated from the oviduct’s epithelium, and the epididymis was used as positive control. No product was detected from choroid...
plexus, which was used as a negative control. GAPDH standard product was expressed in all the tissues. These products were confirmed by sequencing (data not shown).

Detection of oviduct CFTR protein expression by Western blot analysis
The protein expression of CFTR in the oviduct at different
estrus stages was also examined by Western blot analysis. The CFTR antibody raised against a peptide (C) KEETEEEEVQDTRL, corresponding to amino acid residues 1468–1480 of cytoplasm, the C-terminal part of human CFTR. The major immunoreactive band of CFTR and β-actin displayed a molecular mass of about 170 kDa in rat oviduct and 42 kDa in epididymis [Fig. 6(A,B)]. In addition, β-actin was detected in choroid plexus as a negative control, but CFTR was not detected. CFTR protein was detected in the oviduct throughout the estrus cycle and in the expression of estrus and metestrus. However, it was not detected in diestrus, and it was significantly lower in the expression of proestrus [Fig. 6(C)].

**Immunolocalization of CFTR in oviduct**

To investigate the location of CFTR in oviduct, we stained sections from rat oviduct using immunofluorescence and immunohistochemistry technique. The apical surface and cytoplasm of the epithelial cells of rat oviduct were highly stained with CFTR antibody [Fig. 7(A), Fig. 8(A)], and there was no immunoreactivity in negative control [Fig. 7(D), Fig. 8(B)]. Bright field and converged images of CFTR immunofluorescent stained rat oviduct sections were shown in [Fig. 7(B)] and [Fig. 7(C)], respectively.

**Discussion**

The oviduct provides an electrolyte environment for ovum pickup, sperm transport, ovum capacitation, sperm capacitation, ovum fertilization, zygote development and zygote transport [13–15], but the regulation and mechanism of fluid movement across the epithelium remain poorly understood. The present study demonstrated that cAMP stimulated an inward current in cultured rat oviduct epithelia in whole-cell patch clamp. Several lines of evidence suggested that the cAMP-activated Cl− current in the oviduct epithelial cell was characteristic of CFTR [6,16–20]. First, elevating cellular cAMP stimulates whole-cell Cl−-selective conductance and exhibited time- and voltage-independent characteristics. Second, this conductance has a linear I-V relationship. Third, the cAMP-activated Cl− current is suppressed in a voltage-independent manner by 5 µM CFTR(inh)-172, a specific CFTR channel blocker [21,22]. In support of these functional data, the detection of CFTR mRNA and protein immunolocalization in rat oviduct confirm earlier reports [8,23], which suggested the presence of this channel in the oviduct epithelium.

**Fig. 5 The mRNA expression of CFTR in the oviduct epithelium**

Positive control with rat epididymis complementary DNA indicated the expected size of the amplified fragment (258 bp). Lane 1, choroid plexus CFTR (negative control); lane 2, choroid plexus GAPDH (300 bp); lane 3, epididymis CFTR (258 bp); lane 4, epididymis GAPDH (300 bp); lane 5, oviduct GAPDH (300 bp); lane 6, oviduct CFTR (258 bp). M, DNA marker. CFTR, cystic fibrosis transmembrane conductance regulator.

**Fig. 6 Western blot analysis of the expression of oviduct CFTR protein throughout the estrus cycle**

Western blot results of CFTR (170 kDa) (A) and β-actin (42 kDa) (B) in rat oviduct. Lane 1, choroid plexus as negative control; lane 2, proestrus; lane 3, estrus; lane 4, metestrus; lane 5, diestrus; lane 6, epididymis as positive control. Experiments were repeated three times, and the first experiment was shown. (C) Relative expression of oviduct CFTR protein normalized with the internal marker β-actin. Values are expressed as mean±SEM (n=3). **P<0.001 compared with proestrus; *P<0.01 compared with proestrus; *P=0.05 compared with proestrus. CFTR, cystic fibrosis transmembrane conductance regulator.
**Fig. 7 Immunofluorescence of CFTR in rat oviduct**  
(A) Fluorescence, (B) bright field and (C) converged images of CFTR immunofluorescent-stained rat oviduct sections are shown. Arrows indicate that immunoreactivity of CFTR was detected in the apical membrane of the epithelial cells.  
(D) Negative control. CFTR, cystic fibrosis transmembrane conductance regulator; L, tubular lumen. Bar=50 µm.

**Fig. 8 Immunohistochemistry of CFTR in rat oviduct**  
(A) Immunoreactivity of CFTR was detected in the apical membrane of the epithelial cells. (B) Control oviduct section with primary antibodies pre-absorbed with CFTR antigen. CFTR, cystic fibrosis transmembrane conductance regulator; L, tubular lumen. Bar=50 µm.
As a cAMP-activated Cl− channel, CFTR plays a critical role in electrolyte and fluid secretion. When it was stimulated by intracellular cAMP, the channel opened and allowed Cl− efflux. In addition, the estrus cycle-dependent protein expression of CFTR in the present study was consistent with observed cyclic changes in CFTR mRNA expression [8]. Moreover, estrogen may function as a physiological regulator of CFTR in the oviduct [23]. Therefore, enhanced expression of CFTR at estrus may result in a higher rate of chloride secretion and thus greater fluid accumulation.

The presence of functional CFTR in rat oviduct epithelial cells revealed in our study should help us better understand its role in oviduct secretion and in the pathophysiology of cystic fibrosis. Previous reports indicated that females with CF have reduced fertility probably due to thick, dense cervical mucus that presents a barrier for sperm penetration [24]. However, CFTR mutant in endometrium and oviduct, which results in abnormal electrolyte secretion [25,26], may be an important cause of infertility in CF female patient.

In summary, this study showed that the cAMP-activated Cl− current in the oviduct epithelium was characteristic of CFTR, which provided direct physiological evidence for the expression of CFTR in the rat oviduct epithelium. The present results also suggested the capability of the oviduct to secrete chloride ion at different stages of the estrus cycle. Cyclic variations in the expression of CFTR likely alter the composition of oviductal fluid to permit successful reproductive events at different times. The functional expression of CFTR indicates that CFTR may play a role in modulating fluid transport in the oviduct.

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