Regulatory mechanism underlying cyclic changes in mouse uterine bicarbonate secretion: role of estrogen

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Abstract

Our previous study has demonstrated that bicarbonate in the uterine fluid plays an indispensable role in sperm capacitation. However, the cellular mechanisms underlying the formation of bicarbonate-rich uterine fluid and the regulatory mechanism remained largely unknown. In this study, the expression profiles of bicarbonate transport/production proteins, the cystic fibrosis transmembrane conductance regulator (CFTR), SLC26A6, carbonic anhydrase 2 (CA2, CA2) and CAR12 (CA12), throughout the estrous cycle, were examined in the mouse uterus by western blot. The results showed that the maximum expression levels of the proteins examined were observed at estrus. Luminal surface pH measurements showed that the resting uterine surface pH at estrus was significantly higher than that at diestrus, which could be reduced significantly by CFTR blocker, diphenylamine-2,2'-dicarboxylic acid, SLC26A6 inhibitor, 4',4'-diisothiocyanostilbene-2',2'-disulfonic acid, and CA inhibitor, acetazolamide. In ovariectomized mice and primary culture of endometrial epithelial cells, estrogen could upregulate CFTR, SLC26A6, CA2, and CAR12 expression with a corresponding increase in the bicarbonate-dependent short-circuit current (I_{sc}) and endometrial surface pH. The present results have demonstrated dynamic changes in uterine bicarbonate secretion and expression of the proteins involved in bicarbonate secretion during the estrous cycle and suggested a novel role of estrogen in regulating uterine bicarbonate transport, which may be important for successful reproduction.

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Introduction

The fluid environment in the female reproductive tract is considered of physiological significance for a number of reproductive events, including sperm transport and capacitation, fertilization, embryo transport, development, and blastocyst implantation (Aitken 1979, Chan *et al.* 2002). Disturbance of the fluid microenvironment in pathological conditions, such as cystic fibrosis (Kopito *et al.* 1973) or hydrosalpinx (Mukherjee *et al.* 1996), is known to result in infertility. Cyclic changes in the uterine fluid volume and composition were observed 80 years ago (Evans & Long 1922). It has been proposed that higher uterine fluid production facilitates sperm transport at estrus, and reduced fluid volume at metestrus or diestrus enables closure of the uterine lumen for embryo implantation. The molecular basis for the cyclic changes in uterine fluid volume has been provided by the observed cyclic changes in the expression of uterine cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-dependent anion channel known to provide the driving force for transepithelial fluid

secretion, during the estrous cycle (Chan *et al.* 2002). The high expression level of CFTR correlates with the maximum uterine fluid volume at proestrus/estrus and the low expression level of CFTR with the minimum uterine fluid volume at metestrus/diestrus, and these correlate with levels of estrogen and progesterone as well as estrogen and progesterone effects on regulation of CFTR expression (Rochwerger & Buchwald 1993, Rochwerger *et al.* 1994, Mularoni *et al.* 1995). Obviously, the ovarian hormones play an important role in regulating the uterine fluid volume; however, their role in regulating the composition of the uterine fluid, i.e. the secretion of particular ions, has not been elucidated.

It was recognized more than 40 years ago that uterine fluids contain two- to four-fold higher bicarbonate content than plasma (Vishwakarma 1962, Murdoch & White 1968). Accumulating evidence indicates a major role of bicarbonate in a series of reproductive processes such as sperm motility, hyperactivation, capacitation, and acrosome reaction as well as embryo development and differentiation

(Kane 1975, Lee & Storey 1986, Visconti *et al.* 1999, Suarez & Ho 2003). However, the molecular mechanisms underlying the formation of bicarbonate-rich fluid in the female tract and its regulation remain largely unknown. A recent study in our laboratory has demonstrated that the secretion of bicarbonate through the apical membrane of the endometrial luminal epithelium is mediated by CFTR and a $\text{Cl}^-/\text{HCO}_3^-$ exchanger (Wang *et al.* 2003), the identity of which is unknown but is likely to be a member of SLC26A6 (Markovich 2001). Our previous studies have also shown that bicarbonate can be accumulated in endometrial epithelial cells either by a $\text{Na}^+/\text{HCO}_3^-$ co-transporter (Wang *et al.* 2002) across the basolateral membrane or by production through intracellular CO_2 hydration catalyzed by the carbonic anhydrases (CAs; Sterling & Casey 2002). Although mounting evidence suggests the importance of bicarbonate in regulating sperm function in the female reproductive tract, the expression profile of bicarbonate transport/production proteins, except CFTR, during estrus cycle and possible involvement of ovarian hormones in regulating their expression have not been reported.

In this study, we hypothesize that bicarbonate transport/production proteins, CFTR, SLC26A6, and CAs are involved in the endometrial epithelial bicarbonate transport, are dynamically changed by the estrous cycle, and may be important for successful fertilization. The aim of this study was to investigate the expression profile of CFTR, SLC26A6, and two CA isoforms known to be expressed in the endometrium, CAR2 (CA2) and CAR12 (CA12) (Sterling & Casey 2002), in mouse uterus at different stages of the estrous cycle by western blot analysis. The functional role of the proteins in the uterine bicarbonate secretion was further confirmed by the short-circuit current (I_{sc}) technique and luminal surface pH measurement. The possible involvement of estrogen in regulating the expression of these proteins was studied in ovariectomized (OVX) mice with exogenously administered estrogen and specific antagonist of its receptor.

Results

Expression profiles of bicarbonate transport/production proteins during the estrus cycle

The expression of the bicarbonate transport/production proteins during the estrous cycle was examined by western blot with β -tubulin as the loading control (Fig. 1). There were two bands detected for CFTR, with the upper band being the mature, glycosylated form at 180 kDa, which was only expressed at estrus, and the lower band being the immature, core-glycosylated precursor, which was expressed in other stages of the estrous cycle. The expression of SLC26A6, detected as two bands at 80 and 85 kDa, was the highest at estrus compared with those observed at other stages during the

Figure 1 Western blotting analysis of total homogenate from mouse uteri for CFTR, SLC26A6, CAR2, and CAR12 throughout the estrous cycle. (A) Representative photos of western blot analysis. P (Proestrus), E (Estrus), M (Metestrus), and D (Diestrus). β -Tubulin was used as a loading control. The expression levels of the bicarbonate transport/production proteins are higher at estrus than that of other stages. (B) Quantitation of the western blot analysis of CFTR, SLC26A6, CAR2, and CAR12 expression throughout the estrous cycle ($n=3$ for each group, * $P<0.05$, ** $P<0.01$, and *** $P<0.001$).

estrous cycle. The same pattern was observed for CAR2 expression, detected at 29 kDa, with the maximum level at estrus. Maximal expression level of CAR12, with two bands at 45 and 50 kDa, was also observed at estrus.

Uterine luminal surface pH during the estrous cycle

We then examined whether the cyclic expression patterns of the bicarbonate transport/production proteins correlate with variations in uterine luminal pH at different stages of the estrous cycle. A pH-sensitive fluorescent dye 5-*N*-hexadecanoyl-aminofluorescein (HAF), which attaches to the luminal surface of the endometrium due to its lipophilic characteristic, was used for the measurement of the epithelial surface pH at estrus and diestrus. As shown in Fig. 2A, the fluorescence ratio measured from the uteri of estrus was significantly higher than that of diestrus ($P<0.01$), indicating that the basal uterine luminal pH at estrus was more alkaline than that at diestrus. The uterine luminal surface fluorescence intensity ratio at estrus could be reduced by the inhibitors of CFTR, SLC26A6 and CA,

Figure 2 Comparison of resting uterine luminal surface pH (fluorescence ratio) and the effect of bicarbonate transport proteins at estrus and diestrus. Each data point was obtained from at least four individual mice. Uterine tissues collected at estrus and diestrus were labeled with HAF and were superfused with normal K-H solution. A significantly higher uterine luminal surface pH, indicated by larger fluorescence ratio, at estrus as compared with that at diestrus was observed (** $P < 0.01$, $n = 4$) (A). Decrease in luminal surface pH (fluorescence ratio) could be induced by CFTR blocker, DPC (1 mM; ** $P < 0.01$, $n = 4$), SLC26A6 inhibitor, DIDS (100 μ M; ** $P < 0.01$, $n = 4$), and CA inhibitor, acetazolamide (100 μ M; * $P < 0.05$, $n = 4$), to a significantly larger extent at estrus compared with that at the diestrus (B).

diphenylamine-2,2'-dicarboxylic acid (DPC; $P < 0.01$), 4',4'-diisothiocyanostilbene-2',2'-disulfonic acid (DIDS; $P < 0.01$), and acetazolamide ($P < 0.05$; Fig. 2B) respectively to a significant larger extent as compared with that observed at diestrus, indicating more active bicarbonate secretion at estrus.

Effect of exogenously administered estrogen on uterine bicarbonate-dependent current in OVX mice

The serum level of the mouse ovarian hormone, estrogen, fluctuates during the estrous cycle, with a 40% increase from proestrus to estrus and a decline after ovulation at metestrus and diestrus (Fata *et al.* 2001). Since a high level of estrogen at estrus had been demonstrated to be responsible for the maximal expression level of CFTR observed at estrus (Rochwerger & Buchwald 1993, Rochwerger *et al.* 1994, Chan *et al.* 2002), we hypothesized that other bicarbonate transport/production proteins may also be upregulated by the high level of estrogen at estrus. As shown in Fig. 3, the magnitude of the forskolin (10^{-6} M)-induced bicarbonate-dependent I_{sc} , which was measured in Cl^{-} -free bathing solution, increased as the dose of the administered estrogen was increased.

Effect of estrogen on surface pH of primary culture of mouse endometrial epithelial cells

An *in vitro* model of endometrial epithelial cells was established for surface pH measurement to further illustrate the effect of estrogen on the endometrial bicarbonate secretion. Mouse endometrial epithelial cells were seeded onto Transwell filters and cultured for 3 days to confluence with 17β -estradiol (E_2 ; 100 nM) in the culture medium for 24 h prior to the experiment. The surface pH was detected continuously by fluorescent dye HAF. Statistical analysis (Fig. 4) showed that the basal pH of the estrogen-treated endometrial epithelial cells was significantly higher than that of nontreated ones ($P < 0.01$), indicating enhanced bicarbonate transport upon estrogen treatment.

Effect of estrogen receptor antagonist

Major biological effects of estrogen in the uterus are thought to be primarily mediated by nuclear estrogen receptors, ESR1 ($ER\alpha$) and ESR2 ($ER\beta$). To examine whether

Figure 3 Effect of exogenously administered estrogen on the forskolin (10^{-6} M)-induced bicarbonate-dependent I_{sc} in OVX mice. OVX mice were divided into five groups, each received daily i.p. injection of 17β -estradiol of different dosages, 0.5, 1.7, 5, and 17 μ g/kg, with the control group receiving vehicle (1% alcohol in saline) for 2 days. Uterine tissues were collected 24 h after the last treatment for I_{sc} measurement. (A) Representative recordings of I_{sc} responses to forskolin added to the apical side of the endometria from mice treated with different doses of estrogen. Arrowheads indicate the time of forskolin addition. (B) Statistical analysis of forskolin-elicited I_{sc} (μ A/cm²) mainly mediated by bicarbonate ion response to different dose of estrogen in Cl^{-} -free solution. Each point was obtained from at least four individual experiments. Values are means $\pm$ S.E.M. of maximal increase in I_{sc} . * $P < 0.05$, *** $P < 0.001$.

Figure 4 Effect of estrogen on the resting surface pH of cultured mouse endometrial epithelial cells. The primary culture of the endometrium from immature ICR female mice seeded to Transwell filter was cultured for 3–4 days to confluent, then treated with E_2 (100 nM) and vehicle as control in the culture medium for 24 h. With HAF loaded at the luminal surface, the resting surface pH of the endometrium was detected in normal K-HS superfusion. Statistic analysis showed that the basal pH of the endometrium is significantly higher (** $P < 0.01$, $n = 6$) in E_2 -treated group compared with control.

the estrogen-induced responses observed above were through its receptor-mediated action, OVX mice were given a daily injection of E_2 with or without ICI 182 780 (a potent antiestrogen for both ESR1 and ESR2) with the control injected with solvent vehicle for 2 days, and the uterine tissues were collected at 2400 h after the last injection. Uterine expression of CFTR, SLC26A6, CAR2, and CAR12 in OVX mice treated with vehicle, E_2 , or E_2 plus ICI 182 780 was analyzed by western blot. As shown in Fig. 5A and B, the uterine expression levels of the bicarbonate transport/production-related proteins were higher in E_2 -treated OVX mice compared with the controls, which could be suppressed by the treatment with ICI 182 780, confirming a receptor-mediated upregulating effect of E_2 on the bicarbonate-related proteins in the endometrium. The I_{sc} measurement showed that the forskolin (10^{-6} M)-induced bicarbonate secretion was only detected in OVX mice uterus treated with E_2 , but not in those treated with vehicle or E_2 plus ICI 182 780 (Fig. 5C).

Discussion

Estrogen is produced cyclically by the ovaries and plays a crucial role in maintaining and regulating the functions of the female reproductive system. The complex events during the estrous cycle under the influence of estrogen have been a focus of attention; however, how estrogen regulates the uterine fluid environment, especially its composition, remains largely unknown. This study is the first to show that estrogen, in an estrogen receptor-dependent manner, upregulates bicarbonate transport/production proteins in mouse endometrium, providing new insights into our current understanding of the role of estrogen in regulating uterine bicarbonate transport.

Bicarbonate plays a surprisingly important role in a number of reproductive events, including sperm capacitation and embryo development (Lee & Storey 1986, Tajima *et al.* 1987, Visconti *et al.* 1995). It was reported half a century ago that the bicarbonate concentration in

the female reproductive tract varies and closely depends on reproductive events, i.e. increased bicarbonate during fertilization (Blandau *et al.* 1958); however, the molecular mechanism underlying the formation of bicarbonate-rich uterine fluid and its regulation remains largely unknown. Previously, we have established a primary culture of mouse endometrial epithelial cells (Chan *et al.* 1997) and used it to study possible transporters involved in uterine bicarbonate secretion (Wang *et al.* 2002) and its significance in determining the fertilizing capacity of sperm (Wang *et al.* 2003). However, the primary culture has its limitations. First, the epithelial cells have to be isolated from sexually immature mouse uteri in order to enable proliferation in culture, which may not have the same gene expression profile as in mature mice. Secondly, some protein expression or function may be lost during the culture as observed in many cell cultures including human fallopian tubule cells (Downing *et al.* 1997). Most importantly, the culture condition cannot reproduce the cyclic characteristics normally observed *in vivo*, considering the fluctuating levels of ovarian hormones. Therefore, the need to study bicarbonate transport mechanisms *in vivo* is evident.

In this study under physiological conditions, we have found that uterine bicarbonate secretion and the expression of the bicarbonate transport/production proteins exhibit cyclic variations throughout the estrous cycle in mice. We note that the maximal protein expression levels are observed at estrus, which is consistent with the rise of estrogen level at estrus. The expression profile of the bicarbonate transport/production-related proteins agrees well with the results obtained by the I_{sc} and luminal surface pH measurement, showing alkaline pH at the luminal surface at estrus while minimal pH value observed at diestrus. The sensitivity of the alkaline pH to the inhibitors of CFTR, SLC26A6, and CA suggests that these proteins are functional and involved in bicarbonate transport/production in the endometrial epithelium. Taken together, these results indicate more active bicarbonate secretion at estrus and suggest the possible involvement of estrogen in regulating the expression of the bicarbonate transport/production-related proteins and thus the formation of a bicarbonate-rich fluid environment.

This study has further established a positive link between the circulating serum estrogen levels and uterine bicarbonate secretion as demonstrated by a dose dependency of the uterine forskolin-activated bicarbonate-dependent I_{sc} on exogenously administered estrogen to the OVX mouse. This effect of estrogen on uterine bicarbonate secretion appears to be mediated by upregulation of bicarbonate transport/production proteins as demonstrated by the observation that the primary culture of endometrium epithelial cells treated with estrogen exhibits higher luminal surface pH than the control, with upregulated bicarbonate

Figure 5 Effect of estrogen and estrogen receptor antagonist on uterine bicarbonate transport and related proteins' expression in OVX mice uteri. (A) Representative photo of western blot analysis on CFTR, SLC26A6, CAR2, and CAR12 expression in the uteri from OVX mice treated with vehicle, E₂, or E₂ plus ICI 182 780 (ICI). β -Tubulin was used as loading control. Lane 1: OVX mice treated with vehicle. Lane 2: E₂ plus ICI. Lane 3: E₂. Treatment of OVX mice with E₂ alone produced an obvious increase in CFTR, SLC26A6, CAR2, and CAR12 protein level as compared with the OVX mice treated with vehicle or E₂ plus ICI respectively ($n=4$ for each group). (B) Quantitation of western blot analysis of estrogen effect on CFTR, SLC26A6, CAR2, and CAR12 expression. * $P<0.05$, ** $P<0.01$, *** $P<0.001$. (C) Representative traces of I_{sc} response to forskolin (10^{-6} M) in chloride-free solution. The OVX mice were given daily injection of E₂ with or without ICI with the control injected with vehicle for 2 days, and uterine tissues were collected at 24 h after the last injection and subjected to I_{sc} measurement. The forskolin-induced bicarbonate secretion was only detected in OVX mice uterus treated with E₂ (middle trace), but not in those treated with vehicle (left trace) or E₂ plus ICI (right trace). Arrowheads indicate the time of forskolin addition.

transport/production proteins' expression. These findings are consistent with the previous finding that administration of estrogen to immature and OVX mature female rats leads to the induction of CFTR expression (Rochwerger *et al.* 1994). As CFTR has been shown to conduct bicarbonate (Poulsen *et al.* 1994, Hug *et al.* 2003) and demonstrated to be involved in uterine bicarbonate secretion (Wang *et al.* 2003), the presently observed co-expression of CFTR with other bicarbonate transport/production genes in response to estrogen stimulation further supports the important role of CFTR in bicarbonate secretion and its regulation by estrogen. It should be noted that major biological effects of estrogen in the uterus are thought to be primarily mediated via a process that involves estrogen binding to nuclear estrogen receptors, ESR1 or ESR2 in target cells, which leads to gene transcription and, subsequently, to a modification of cellular responses. Using the OVX mouse model administered with both E₂ and estrogen receptor antagonist ICI 182 780, we show in this study that the upregulated bicarbonate secretion and related proteins expression are indeed stimulated by estrogen and mediated by estrogen receptor, although the specific receptor subtype involved remains to be elucidated.

It should be noted that there are at least two ways for bicarbonate to accumulate in the endometrial epithelial cells. Previous studies on the primary culture of mouse endometrial epithelial cells have demonstrated that bicarbonate can be taken into the cells via Na⁺-HCO₃⁻ co-transporter (Wang *et al.* 2002). This study suggests that bicarbonate production through CO₂ hydration is also important because two isoforms of CAs expressed in the endometrial epithelial cells are upregulated at estrus and by estrogen. Although a Cl⁻/HCO₃⁻ exchanger has been implicated in uterine bicarbonate secretion previously (Wang *et al.* 2003), its molecular identity has not been demonstrated. This study is the first to examine the expression of a potential candidate, SLC26A6, in the endometrial epithelial cells and the co-expression of SLC26A6 with CFTR and CAs in response to estrogen supports its involvement in uterine bicarbonate secretion. In addition to a direct role of CFTR in mediating bicarbonate exit of the cells, it may also work with SLC26A6 as a Cl⁻ channel, providing a Cl⁻ recycling pathway in order to sustain the operation of the Cl⁻/HCO₃⁻ exchanger, as established in pancreatic bicarbonate secretion (Hug *et al.* 2003). The enhanced production of bicarbonate, as indicated by upregulated CAs expression, and increased bicarbonate transport, as indicated by the upregulation of the transporter proteins and results of the I_{sc} and pH measurement, either at estrus or in response to estrogen stimulation, suggest an important role of estrogen in stimulating uterine bicarbonate secretion at estrus, a time immediately prior to ovulation. As bicarbonate is known to promote sperm motility and capacitation, a process by which sperm acquire their ability to fertilize the egg, the

enhanced uterine bicarbonate secretion at estrus is a necessary process to enable sperm to acquire their fertilizing capacity and thus important for fertility. Therefore, in addition to its well-known effect on proliferation of the endometrium, estrogen may also play an important role in regulating uterine fluid environment, both in volume as suggested previously (Chan *et al.* 2002) and bicarbonate content as demonstrated by the results of this study. The upregulated CFTR at estrus appears to play a dual role. On the one hand, as a Cl^- channel it promotes Cl^- secretion and thus providing the driving force for fluid secretion, leading to a large fluid volume increase normally observed around estrus, which may facilitate sperm transport. On the other hand, being involved in bicarbonate transport, either directly as demonstrated previously (Wang *et al.* 2003) or indirectly working in parallel with the $\text{Cl}^-/\text{HCO}_3^-$ exchanger, possibly SLC26A6, as in the pancreas (Hug *et al.* 2003), CFTR plays an indispensable role in uterine bicarbonate secretion on which sperm function and reproduction depend. It should be noted that while sperm capacitation is traditionally considered to take place in the oviduct, the present finding, as well as the demonstration reported previously (Wang *et al.* 2003), suggests that the process may also begin in the uterus to prepare sperm for their travel through the oviduct, as hyperactivation is one of the consequences of capacitation.

In summary, this study has demonstrated for the first time the differential expression of CFTR, SLC26A6, CAR2, and CAR12, and their involvement in HCO_3^- secretion in the murine uterus during the estrous cycle, with demonstrated effect of estrogen on upregulation of their expression and HCO_3^- secretion. Together, with its well-known action on proliferation of the endometrial lining and follicle development, estrogen also appears to exert its influence on sperm through uterine bicarbonate secretion necessary for sperm motility and capacitation. Therefore, the present findings suggest that the precise and coordinated control of estrogen-regulated HCO_3^- secretion is essential to the success of reproduction.

Materials and Methods

Chemicals

DPC was obtained from Riedel de Haen Chemicals (Hannover, Germany). Forskolin, DIDS, and acetazolamide were purchased from Sigma-Aldrich Co. Stock solutions of all the chemicals were dissolved in DMSO. Final DMSO concentrations never exceeded 1% (v/v). Preliminary experiments indicated that the vehicle did not alter any baseline electrophysiological or fluorescence parameters (data not shown).

Animals

Female imprinting control region (ICR) mice were provided by the Laboratory Animal Service Center of the Chinese University of Hong Kong. They were maintained in an air-conditioned room with controlled temperature of $24 \pm 2^\circ\text{C}$ and humidity of $55 \pm 15\%$, in a 1200 h light/darkness cycle regulation and were fed laboratory chow and water *ad libitum*. Ethics committee approval was obtained before this study, and all animal experiments were conducted in accordance with the University Laboratory Animals Service Center's guidelines on animal experimentation with approval from the Animal Ethics Committee of the University. Three- to four-weeks-old immature female mice were used for endometrial epithelial cell culture. Eight- to ten-weeks-old sexually mature female mice were used for protein and functional study.

Determination of the estrous cycle

Stages of the estrous cycle encompassing proestrus, estrus, metestrus, and diestrus were determined by cytological evaluation of vaginal smears on 8–10-weeks-old sexually mature ICR mice as described previously (Rugh 1980). Mice undergoing normal estrous cycle changes over at least two cycles were included in further studies. Vaginal smears were observed twice daily at 0900 and 1200 h, and those exhibiting a stage change at noon were divided into diestrus, proestrus, or metestrus groups and were killed immediately. Thus, all samples collected are representative of the initial onset of each stage, and this regimen minimized the expected intrastage variation. As mice enter estrus in the early hours of the night, vaginal smears were obtained at 2100 and 0900 h, and those mice that entered estrus from proestrus overnight were euthanized immediately.

Ovariectomy and estrogen treatment

Eight- to ten-weeks-old sexually mature mice were bilaterally OVX under ketamine (75 mg/kg i.p.) and xylazine (10 mg/kg i.p.) anesthesia and left to recover for 3 weeks. Mice were then divided into six groups; each received daily i.p. injection of E_2 (100 μl , Sigma-Aldrich) of different dosages, 0.5, 1.7, 5, and 17 $\mu\text{g/kg}$ with the control group receiving vehicle (1% alcohol in saline) for 2 days. When mice were treated with E_2 plus ICI 182 780 (Sigma-Aldrich), a potent antiestrogen for both ESR1 and ESR2, 17 mg/kg of ICI 182 780 was injected 30 min before E_2 (1.7 $\mu\text{g/kg}$) injection. Animals were killed 24 h after the treatment and their uteri were removed for I_{SC} or frozen in liquid nitrogen and stored at -70°C for western blot analysis.

Endometrial epithelia cell culture

Endometrial epithelial cells were enzymatically isolated from the mouse uterus according to the method described by McCormack & Glasser (1980) with slight modifications, which produced pure epithelia identified by epithelial cell markers (Chan *et al.* 1997). Samples of uteri were obtained from 3.5- to 4-week-old immature ICR mice to avoid the complication of the endometrial cycle. Animals were killed by placing them in

a CO₂-gassed chamber for 3 min. Uteri were removed and placed into a Petri dish containing sterile PBS (without Ca²⁺ and Mg²⁺). After washing with PBS and trimming off the remaining fatty and connective tissues, the uteri were sliced longitudinally. The sliced uteri were incubated in PBS supplemented with 7.5 mg/ml trypsin (Gibco Laboratory), 25 mg/ml pancreatin (Gibco Laboratory), 100 U/ml penicillin, and 100 µg/ml streptomycin (Gibco Laboratory) at 0 °C for 60 min and then at room temperature for another 60 min. After the enzyme digestion, the test tube containing PBS and the tissues was shaken gently for 30 s. Uterine tissue was carefully removed and the crude cell solution was passed through a 70 mm fluorocarbon mesh filter (Spectra Mesh; Spectrum, Houston, TX, USA). The filtrate was centrifuged at 1000 *g* for 5 min. The supernatant was discarded and the cell pellet was re-suspended in 12 ml PBS. The cells were allowed to settle for 5 min, and then the top portion (about 2 ml) of the cell suspension was discarded. The cell suspension was centrifuged again at 1000 *g* for 5 min. The washing procedures were then repeated once more. After centrifugation, the cell pellet was re-suspended in phenol-red-free Ham's F12-DMEM culture medium (Gibco Laboratory) containing 10% (v/v) charcoal/dextran-treated fetal bovine serum (Hyclone, Logan, UT, USA), 1% (v/v) nonessential amino acids, 100 U/ml penicillin, and 100 mg/ml streptomycin. The isolated endometrial cells were plated at 1.5 × 10⁶ cells/ml on Transwell-Col membranes with pores of 0.45 mm (Corning Incorporated, Corning, NY, USA). Cultures were incubated at 37 °C in 95% O₂/5% CO₂ and reached confluence in 3 days. Epithelial luminal surface pH was measured on the third day of culture and E₂ was added to some wells 24 h prior to apical pH measurement.

Western blot analysis

Snap-frozen uterine tissues were ground thoroughly with a pestle in liquid nitrogen cooled condition. Uteri from six sexually mature mice at the same estrous cycle were mixed together. RIPA lysis buffer and protease inhibitors were added then. The whole cell lysates were obtained by centrifugation at 13 000 *g* for 30 min at 4 °C. After the protein concentration was determined, the cell lysates containing equal amounts of protein were loaded onto 8–12% SDS-PAGE. Blots were incubated with monoclonal mouse anti-mouse CFTR antibody by 1:500 dilution (Alexis Biochemicals Corporation, San Diego, CA, USA) and rabbit anti-mouse β-tubulin by 1:2000 dilution (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Polyclonal rabbit anti-mouse CAR2 (1:200 dilution) and CAR12 (1:200 dilution) antibodies were kindly supplied by Prof. W S Sly and J Grubb from St Louis University, USA. Monoclonal rabbit anti-mouse SLC26A6 (1:200 dilution) antibody was kindly supplied by Prof. Z H Wang from the University of California, USA. ECL was visualized by film development.

Short-circuit current measurement

*I*_{sc} measurement has previously been described (Ussing & Zerahn 1951). In brief, freshly removed endometrial epithelia from which the serosa and muscular layers had been removed were clamped vertically between two halves of the Ussing

chamber. The epithelia were bathed on both sides with Krebs-Henseleit solution (K-HS) that was maintained at 37 °C. The K-HS had the following composition: 117 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgCl₂, 24.8 mM NaHCO₃, 1.2 mM KH₂PO₄, and 11.1 mM glucose. Cl[−] in Cl[−]-free K-H solution was substituted with gluconate salts, which had the following compositions (mM): Na-gluconate, 117; K-gluconate, 4.7; MgSO₄, 1.2; KH₂PO₄, 1.2; NaHCO₃, 24.8; Ca-gluconate, 2.56; glucose, 11.1. The pH value of normal K-HS and Cl[−]-free K-HS was maintained at 7.4 when gassed with 95% O₂/5% CO₂. Drugs were added directly to the apical side of the epithelium. The transepithelial potential differences exhibited by the epithelia were measured by the Ag/AgCl electrodes (World Precision Instruments, Sarasota, FL, USA) connected to a preamplifier, which was connected to a voltage clamp amplifier (DVC 1000, World Precision Instruments). The change in *I*_{sc} was defined as the maximal rise in *I*_{sc} after agonist stimulation and was normalized as current change per unit area of the uterine epithelium (µA/cm²).

Luminal surface pH measurement

The pH-sensitive fluorescent dye HAF (Invitrogen) molecule consists of two domains, the fluorescein molecule and a palmitoyl chain connected to this fluorescing moiety. Dragsten *et al.* (1981) have shown that this palmitoyl chain inserts into the apical membrane of cells due to its lipophilic character. The sensitivity of HAF to pH changes relies on the presence of dissociable groups in the fluorescing moiety. A section of the uterus was cut open longitudinally. The mucosa was stripped off the serosa and muscular layers with fine forceps, and then the stripped epithelium fixed to the supporting ring was placed in a specially designed microperfusion chamber with the lumen side uppermost. Primary cultures of the endometrial epithelial cells were grown for 4 days on clear Transwell-Col membranes (Corning Incorporated) with pores of 0.45 µm. A final HAF concentration of 15 µmol/l was used to incubate the mucosal side of the endometrial tissue or cultured cells in the dark for 5 min at 37 °C, and then the endometrial epithelial cells were washed thoroughly and perfused with normal K-HS (carbogen gassed, 37 °C) to rinse away unbound dye. The measurement of luminal surface pH was recorded in an inverted Olympus IX70 microscope equipped with a CCD camera. Fluorescence was recorded in the images of the HAF signals that were obtained by excitation wavelength at 440 and 490 nm. The ratio of these two signals was directly proportional to the pH and the images were captured by using MetaFluro from Universal Imaging Corp. (Washington, DC, USA). For calibrations, nigericin (10 µg/l) was added to the calibration buffers containing (in mmol/l) 152 Cl[−], 133.4 K⁺, 25 HEPES, 15 Na⁺, 1.8 Ca²⁺, 0.8 Mg²⁺, and 0.8 SO₄^{2−}. The pH of these solutions was adjusted to a defined pH in the range 6.0–8.0 by different amounts of 1 M KOH. The osmolarity of the buffers was 300 mosmol/l.

Statistical analysis

Results are expressed as means ± s.e.m., and *n* indicates the number of experiments. Statistical analysis was carried out by Prism (GraphPad, Inc., San Diego, CA, USA). Comparisons

between two groups of data were carried out using Student's *t*-test. Comparisons among three or more groups of the data were carried out using one-way ANOVA, followed by Newman–Keul's multiple range test. A *P* value <0.05 was considered statistically significant.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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