Regulation of electrolyte transport across cultured endometrial epithelial cells by prolactin

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Abstract

The effect of prolactin (PRL) on ion transport across the porcine glandular endometrial epithelial cells was studied in primary cell culture using the short-circuit current technique. Addition of 1 µg/ml PRL either to the apical solution or to the basolateral solution produced a peak followed by a sustained increase in *I*_{sc}, but with a lesser response when PRL was added apically. Basolateral addition of PRL increased the *I*_{sc} in a concentration-dependent manner with a maximum effect at 1 µg/ml and an effective concentration value of 120 ng/ml. The PRL-stimulated *I*_{sc} was significantly reduced by pretreatment with an apical addition of 5-nitro-2-(3-phenylpropylamino) benzoic acid (200 µM), diphenylamine-2-carboxylic acid (1 mM) or 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (200 µM), Cl⁻ channel blockers, but not by amiloride (10 µM), a Na⁺ channel blocker. In addition, pretreatment with bumetanide

(200 µM), a Na⁺-K⁺-2Cl⁻ cotransporter inhibitor, in the basolateral solution significantly reduced the PRL-stimulated *I*_{sc}. Replacement of Cl⁻ or HCO₃⁻ in the bathing solutions also decreased the *I*_{sc} response to PRL. Pretreatment of the monolayer with AG490 (50 µM), an inhibitor of JAK2 activity significantly inhibited the PRL-induced increase in *I*_{sc}. Western blot analysis of the porcine endometrial epithelial cells revealed the presence of short isoform of PRL receptor (PRLR-S) that could be regulated by 17β-estradiol. The results of this investigation showed that PRL acutely stimulated anion secretion across the porcine endometrial epithelial cells possibly through PRLR-S present in both apical and basolateral membranes. The PRL response appeared to be mediated by the JAK2-dependent pathway.

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Introduction

Endometrial epithelial cells play an important role in the regulation of fluid and electrolyte volume and composition within the uterine cavity, providing an optimal intrauterine environment for implantation and embryo development. The transport-related activities of the surface and glandular epithelial cells have been shown to be regulated by several hormones, growth factors, cytokines, and a number of signaling molecules. Electrophysiological studies of cultured human endometrial epithelial cells (Matthews *et al.* 1993) and the intact porcine endometrial epithelium (Vetter & O'Grady 1996) have provided direct evidence for the regulation of Na⁺ absorption and K⁺ secretion. In the primary culture of mouse and porcine endometrial epithelial cells, prostaglandins (PGs) especially PGE₂, adrenaline, ATP, and UTP were found to activate anion secretion (Chan *et al.* 1997, Fong *et al.* 1998, Deachapunya & O'Grady 1998, Palmer-Densmore *et al.* 2002). These epithelial cells also exhibited Na⁺ transport that was activated by insulin and insulin-like growth

factor, and inhibited by epidermal growth factor (Deachapunya *et al.* 1999, Deachapunya & O'Grady 2001).

Prolactin (PRL) is synthesized and secreted from the anterior pituitary gland, as well as the extrapituitary tissues including myometrium, deciduas, and mammary epithelial cells (Freeman *et al.* 2000). It exerts a wide variety of biological actions, such as the regulation of water and electrolyte balance, growth of mammary gland, milk production, and secretion. Recently, PRL has been reported to stimulate the intestinal Ca²⁺ absorption (Jantarajit *et al.* 2007), especially under conditions of high calcium demand such as pregnancy and lactation (Charoenphandhu & Krishnamra 2007).

In human endometrium, PRL receptors (PRL-R) and its mRNA have been identified in glandular epithelial and stromal cells (Jabbour *et al.* 1998, Tseng & Zhu 1998). PRL-R belongs to the superfamily of the cytokine class-1 receptor (Kelly *et al.* 1991). Several isoforms, i.e. short, intermediate, and long isoforms and the soluble PRL-binding protein have been identified in many tissues (Clevenger & Kline 2001). Binding of PRL to its transmembrane receptors induces receptor

dimerization, tyrosine phosphorylation, and activation of the JAK, which leads to phosphorylation of other associated regulatory proteins especially the STAT proteins. The phosphorylated STAT proteins dimerize and translocate to the nucleus to bind to the PRL-responsive genes, resulting in target gene transcription and biological responses. Other signaling pathways involving mitogen-activated protein kinase (MAPK), insulin-receptor substrate (IRS-1), phosphoinositide 3 (PI-3) kinase, PLC, PKC, and intracellular Ca^{2+} have also been reported to mediate PRL actions (Bole-Feysot *et al.* 1998, Gubbay *et al.* 2002).

Although PRL is known as an important regulator of water and electrolyte transport in lower vertebrates (Bern 1975, Sakamoto & McCormick 2006), there were very few reports on its transport-related effect in the mammalian epithelial cells. Several studies using the everted intestinal sac technique have demonstrated the stimulatory effect of PRL on fluid and NaCl absorption in the rat, hamster, and guinea pig jejunum, but not in guinea pig ileum or rat colon (Mainoya *et al.* 1974). In the rabbit mammary glands, PRL decreased epithelial membrane permeability to sucrose, suggesting a decrease in the permeability of the tight junction (Linzell *et al.* 1975). From the studies using the mouse mammary epithelial cells grown on floating collagen gels, PRL treatment for 3 days was found to increase the short-circuit current (Isc) and transepithelial potential difference (PD), which indicated an increase in net active Na^+ absorption, probably with some Cl^- secretion (Bisbee *et al.* 1979). In the mouse mammary epithelial cell line HC11, PRL acutely increased Cl^- transport through the JAK-STAT system (Selvaraj *et al.* 2000). Eventhough PRL seemed to play an important role in the regulation of transepithelial ion transport in a variety of epithelia, its effect on the ion transport function of the endometrial epithelium has not been investigated. Since we have previously shown that the primary cultured porcine endometrial epithelial cells possessed the transport machinery capable of Na^+ absorption and Cl^- secretion (Deachapunya & O'Grady 1998, Deachapunya *et al.* 1999), the objectives of the present study were to investigate the regulatory mechanism of PRL on the ion transport across these epithelial cells.

Materials and Methods

Materials

PRL, insulin, amiloride, 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB), diphenylamine-2-carboxylic acid (DPC), 4,4'-diisothiocyantostilbene-2,2'-disulfonic acid (DIDS), bumetanide, acetazolamide, PGE₂, 8-chloro-phenylthio-3',5'-cyclicmonophosphate (8cpt-cAMP), non-essential amino acids, and high-purity grade salts were obtained from Sigma Chemical Co. Dulbecco's modified Eagle's medium (DMEM), Dulbecco's PBS (DPBS), fetal bovine serum (FBS), collagenase (type 1), kanamycin, penicillin-streptomycin, and fungizone were purchased from Gibco (Grand Island, NY).

Cell isolation and culture

Porcine uterine tissues collected from 5- to 6-month-old pig were obtained from the Metropolitan slaughterhouse, Klongtoey, Bangkok, under the supervision of the Department of Livestock Development, Ministry of Agriculture and Cooperatives, Thailand. The tissue was placed in an ice-cold porcine Ringer solution containing (mM): 130 NaCl, 6 KCl, 3 CaCl_2 , 0.7 MgCl_2 , 20 NaHCO_3 , 0.3 NaH_2PO_4 , and 1.3 Na_2HPO_4 (pH 7.4). After removal of the serosal muscle layer, the tissue fragments were cut into small pieces and digested overnight with collagenase. The epithelial glands were then isolated as described previously (Deachapunya & O'Grady 1998), and suspended in DMEM supplemented with 3.7 g/l NaHCO_3 , 10% FBS, 850 nM (5 $\mu\text{g}/\text{ml}$) insulin, 1% non-essential amino acid, 5 $\mu\text{g}/\text{ml}$ fungizone, 100 U/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, and 100 $\mu\text{g}/\text{ml}$ kanamycin (standard media). They were then plated onto the cell culture dishes and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Culture medium was changed after 24 h and then every 2–3 days. After 80% confluence (within 2–3 days), the epithelial cells were subcultured onto 24 mm (4.5 cm^2) transparent permeable membrane filters (Costar, Cambridge, MA, USA). Using this method of isolation, the purity of epithelial cells was greater than 90% as assessed by staining the isolated cells with cytokeratin (Deachapunya & O'Grady 1998). Cell monolayers were fed every 2 days and maintained in the standard media for about 7 days before the beginning of the experiment.

Measurement of electrical parameters

Before studying ion transport, the transepithelial resistance of the cell monolayer was measured with an epithelial voltohmmeter (EVOM) coupled to Ag/AgCl 'chopstick' electrodes (World Precision Instruments, Sarasota, FL, USA). Monolayer with high resistance ($\approx 3000 \Omega\text{cm}^2$) was then mounted in Ussing Chamber, bathed in both sides with the standard porcine Ringer solution, which was maintained at 37 °C and bubbled with 95% O_2 –5% CO_2 . Transepithelial PD and Isc were measured with the use of voltage-clamp circuitry (EVC-4000, World Precision Instruments) with Ag/AgCl electrodes connected to the bathing solution via agar bridges. Tissue conductance (G) was calculated using Ohm's law ($G = \text{Isc}/\text{PD}$). The monolayer was continuously short circuited, except for a brief interval of open-circuited readings for PD measurement before and after adding any chemical. Data from the voltage clamp were connected to a MacLab 4S A/D converter and recorded with a 400 MHz PowerPC Macintosh. After mounting, the cell monolayer was equilibrated for at least 20 min to achieve a stable Isc before addition of chemicals. Positive Isc corresponded to the movement of anions in the serosal to mucosal direction or the movement of cations in the mucosal to serosal direction or a combination of both. In the anion replacement experiments, gluconate salts were substituted for chloride

and HEPES were substituted for bicarbonate. The experiment under HCO_3^- free condition was performed in the presence of 100 μM acetazolamide and bubbled with 100% O_2 .

Western blot analysis

Porcine endometrial epithelial cells seeded in the 100 mm cell culture dish were allowed to grow in the standard medium up to 80% confluence. In some experiments, the medium was switched to the serum-free and phenol red-free DMEM alone or supplemented with 10^{-8} M 17 β -estradiol for 48 h. Cells were then harvested and suspended in lysis buffer containing 50 mM Tris-HCl, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EGTA, 1 mM phenylmethylsulfonyl fluoride, 20 μg aprotinin, and 1 mM NaF (pH 7.4). The supernatant was collected and protein concentrations were determined using the BCA protein assay kit (Pierce Biotechnology, Inc., Rockford, IL, USA). Protein samples (20 μg) were separated by 10% SDS-PAGE and electrically transferred to a polyvinylidene difluoride membrane (Hybond-P, Amersham Biosciences) in Tris-glycine buffer. Blotted membranes were washed and then blocked with 5% nonfat powdered milk in Tris-buffered saline for 4 h at room temperature with constant agitation. The membrane was incubated overnight at 4 °C with 1 $\mu\text{g}/\text{ml}$ primary antibody, which is anti-rat PRL receptor monoclonal antibody generated against the extracellular domain of PRL receptor (clone U5, Affinity BioReagents). The membrane was then washed and incubated for 1 h at room temperature with a 1:10 000 horseradish peroxidase (HRP)-conjugated goat anti-mouse secondary antibody (Zymed Laboratories Inc., San Francisco, CA, USA). After washing, the immunoreactive protein bands were visualized using the enhanced chemiluminescence (ECL) detection system (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) according to the manufacturer's instructions. The membranes were exposed to film (Hyperfilm-ECL, Amersham Biosciences) for adequate duration to visualize the chemiluminescent bands. To confirm equal loading, the membranes were stripped and reprobed with a 1:300 000 anti- β -actin monoclonal antibody (clone AC-15, Sigma Co.) followed by a 1:10 000 HRP-conjugated anti-mouse antibody. The intensity of the protein bands was determined using densitometry analysis (Scion Image; Scion Cooperation, Frederick, MD, USA). Band intensity of the PRL-R from each treatment was normalized to the β -actin intensity and expressed as the PRL-R/ β -actin ratio.

Data analyses

All values are presented as mean $\pm$ S.E.M. and n is the number of monolayers from at least three different uterine tissue cultures. The differences between control and experimental means were analyzed using a Student's t -test or ANOVA where appropriate. The difference between treatment and control means following a significant ANOVA was identified by Dunnett's test (Prism 3.0, GraphPad Software, Inc., San Diego, CA,

USA). A value of $P < 0.05$ was considered statistically significant. The effective concentration (EC_{50}) value was determined using a four-parameter logistic function to fit the data (Prism 3.0; GraphPad Software Inc.).

Results

Effect of PRL on I_{sc}

Under basal condition, after an equilibrating period of 30 min, the porcine endometrial epithelial monolayer exhibited average I_{sc} , PD (lumen negative), and tissue conductance of $30.68 \pm 2.49 \mu\text{A}$, $-27.22 \pm 3.89 \text{ mV}$, and $1.47 \pm 0.25 \text{ mS}$ ($n=17$) respectively. Addition of 1 $\mu\text{g}/\text{ml}$ PRL to the apical or basolateral solution produced an increase in I_{sc} that reached a peak within 2–3 min before decreasing slightly and was maintained at a level above baseline (Fig. 1A). In some experiments, the PRL-stimulated I_{sc} gradually decreased to the baseline level. The peak I_{sc} response to apical addition of 1 $\mu\text{g}/\text{ml}$ PRL was $3.45 \pm 0.94 \mu\text{A}$ ($n=4$), while the subsequent basolateral addition of 1 and 5 $\mu\text{g}/\text{ml}$ PRL led

Figure 1 Effect of PRL on the basal I_{sc} in the endometrial epithelial monolayers. (A) A representative I_{sc} tracing responded to an addition of 1 $\mu\text{g}/\text{ml}$ PRL to (a) the apical solution followed by 1 and 5 $\mu\text{g}/\text{ml}$ PRL added to (b) the basolateral solution. (B) Concentration–response relationships showing the increase in I_{sc} following basolateral treatment with various concentrations of PRL. The EC_{50} value was 120 ng/ml ($n=6$).

to a peak I_{sc} of $11.00 \pm 0.98 \mu A$ ($n=17$) and $14.36 \pm 1.37 \mu A$ ($n=12$) respectively. Basolateral addition of PRL increased the I_{sc} in a concentration-dependent manner with a maximum response with $1 \mu g/ml$ PRL and a half maximum EC_{50} value of $120 ng/ml$ ($n=6$, Fig 1B).

Ionic basis of the PRL-stimulated I_{sc}

To determine the ionic basis of the I_{sc} response induced by PRL, we examined the effect of PRL in the presence of pharmacological ion channel blockers and ion substitution solutions. An apical application of the Na^+ channel blocker amiloride at $10 \mu M$ inhibited the basal I_{sc} by 35% (Fig. 2A). However, it did not affect the I_{sc} response induced by PRL ($1 \mu g/ml$), which was $12.21 \pm 1.39 \mu A$ ($n=5$), when compared with the control value of $11.44 \pm 1.22 \mu A$ ($n=12$) (Fig. 2E). By contrast, the PRL-stimulated I_{sc} was significantly decreased in the presence of Cl^- channel blockers, NPPB, DPC, and DIDS in the apical solution. NPPB and DPC have been widely used to block CFTR,

Figure 2 Effect of ion channel blockers on the PRL-stimulated I_{sc} . (A) A representative I_{sc} tracing showing that an apical addition of amiloride (Amil, $10 \mu M$) produced a small decrease in basal I_{sc} . A subsequent addition of PRL ($1 \mu g/ml$) into the basolateral solution produced an increase in I_{sc} . (B) An apical addition of NPPB ($100 \mu M$), (C) DPC ($1 mM$), or (D) DIDS ($200 \mu M$) decreased the basal I_{sc} and reduced the PRL response. (E) Bar graph illustrating the average maximal increases in I_{sc} response produced by prolactin alone (control) and in the presence of amiloride, NPPB, DPC, or DIDS. Values represent means $\pm$ S.E.M. * $P < 0.01$ when compared with the control value by ANOVA.

Figure 3 Effect of $Na^+-K^+-2Cl^-$ cotransporter blockers on the PRL-stimulated I_{sc} . (A) A representative I_{sc} tracing showing that a basolateral addition of bumetanide ($200 \mu M$) produced a decrease in basal I_{sc} . A subsequent addition of PRL ($1 \mu g/ml$) into the basolateral solution produced a slightly increase in I_{sc} . (B) Bar graph illustrating the average maximal increases in I_{sc} response produced by prolactin alone (control) and in the presence of bumetanide. Values represent means $\pm$ S.E.M. * $P < 0.01$ when compared with the control value by Student's t -test.

whereas DIDS blocks the Ca^{2+} -activated Cl^- channels with no effect on the activity and conductance of CFTR (Anderson *et al.* 1992). As shown in Fig. 2B and C, pretreatment with $100 \mu M$ NPPB or $1 mM$ DPC reduced the basal I_{sc} by 60 and 75% and decreased the I_{sc} response to PRL to $1.00 \pm 0.71 \mu A$ ($n=4$) and $3.65 \pm 0.95 \mu A$ ($n=5$) respectively. An apical addition of $200 \mu M$ DIDS reduced the basal I_{sc} by 17% and reduced the I_{sc} response to PRL to $6.43 \pm 1.20 \mu A$ ($n=4$) (Fig. 2D). In addition, pretreatment with $200 \mu M$ bumetanide, a $Na^+-K^+-2Cl^-$ cotransporter inhibitor, in the basolateral solution abolished most of the PRL-induced increase in I_{sc} from $11.44 \pm 1.22 \mu A$ ($n=12$) to $2.48 \pm 1.17 \mu A$ ($P < 0.01$, $n=4$) (Fig. 3). Replacement of Cl^- or HCO_3^- in both the apical and the basolateral solutions markedly reduced the maximal I_{sc} response to $1 \mu g/ml$ PRL to $1.63 \pm 1.06 \mu A$ ($n=5$) and $0.9 \pm 0.3 \mu A$ ($n=3$) respectively (Fig. 4).

Intracellular signaling pathways of PRL-induced increase in I_{sc}

The major signaling pathway involved in PRL action is the JAK-STAT pathway which was shown to mediate Cl^-

Figure 4 Effects of anion substitution on the maximal Isc response to PRL. In standard Ringer's solution, PRL (1 μg/ml) produced a mean increase in Isc of 11.44 ± 1.22 μA ($n=12$). Replacement of Cl⁻ and HCO₃⁻ in both the apical and basolateral solutions significantly inhibited the maximal Isc response to PRL by 86% ($n=4$) and 92% ($n=4$) respectively. * $P<0.01$ when compared with the control value by ANOVA.

secretion in the mammary cell line, HC11 (Selvaraj *et al.* 2000). Phosphorylations of JAK2, STAT1, and STAT5 have been demonstrated in response to PRL stimulation (200 ng/ml) in human endometrium (Jabbour *et al.* 1998). To determine whether JAK2 was involved in the PRL-induced increase in Isc, we examined the effect of AG490, an inhibitor of JAK2 activity, on PRL action. As shown in Fig 5A, pretreatment with 50 μM AG490, added to both the basolateral and apical solutions, reduced the basal Isc by 55% from 26.21 ± 4.99 to 11.75 ± 4.01 μA ($P<0.01$, $n=5$). Sequential additions of 1 and 5 μg/ml PRL slightly increased the Isc response by 3.70 ± 3.09 and 0.80 ± 0.66 μA ($n=5$) respectively. However, the presence of AG490 did not affect the Isc response to 100 μM 8cpt-cAMP, which was 22.80 ± 2.14 μA ($n=4$) when compared with the control value of 18.99 ± 1.59 μA ($n=5$), but slightly decreased the Isc response to 3 μM PGE2 to 12.08 ± 0.87 μA ($n=4$), which was not statistically significant from that of control (16.84 ± 1.55 μA, $n=7$, Fig. 5B).

Expression of PRL receptor

To confirm the functional significance of PRL in the regulation of ion transport, the expression of PRL-R was determined using western blot analysis. A representative western blot as presented in Fig. 6A demonstrated the presence of proteins with an approximate molecular mass of 36 kDa in porcine endometrial epithelial cells as well as in the human mammary gland cancer cell MCF-7 and human endometrial cancer cell RL-95. The 36 kDa protein band corresponded to the short form of PRL-R. Replacement of the standard medium of the endometrial epithelial cells with the serum-free and phenol red-free medium reduced the expression of the protein, whereas addition of 17β-estradiol (10^{-8} M) in the serum-free medium up-regulated the PRL

Figure 5 Effect of JAK2 activity inhibitor on the PRL-stimulated Isc. (A) A representative Isc tracing showing that an addition of AG490 (50 μM), an inhibitor of JAK2 activity, to both apical and basolateral solutions completely inhibited both basal and PRL-induced increase in Isc. (B) Bar graph illustrating the average maximal increases in Isc response produced by PRL (1 μg/ml), 8cpt-cAMP (100 μM), or prostaglandin E₂ (PGE, 3 μM) in the absence and presence of AG490 (50 μM). Values represent means ± s.e.m. * $P<0.01$ when compared with the corresponding control value by Student's *t*-test.

protein expression. Based on the densitometry analysis, the PRL-R/β-actin ratio was significantly decreased from 5.67 ± 0.79 in the standard medium to 1.15 ± 0.09 ($P<0.05$, $n=4$) in the serum-free medium. Treatment with 17β-estradiol increased the PRL-R/β-actin ratio by twofold to 2.35 ± 0.12 .

Discussion

A previous study in mouse mammary epithelial cells demonstrated that the PRL-induced increase in Isc was predominately mediated by Na⁺ absorption (Mainoya *et al.* 1974). However, in another study in the mouse mammary epithelial cell line HC11, it was Cl⁻ transport that was acutely stimulated by PRL (Selvaraj *et al.* 2000). Using cultured porcine endometrial epithelial cells that possessed the machinery for Na⁺ and Cl⁻ transports, we showed that PRL acutely stimulated anion secretion without affecting Na⁺ absorption. This was further supported by the findings that the PRL-induced increase in Isc was blocked by Cl⁻ channel

Figure 6 Expression of PRL receptor protein in porcine endometrial epithelial cells. (A) Western blot analysis of PRL receptor (PRL-R). A 36 kDa band of PRL-R was detected in human mammary gland cancer cell MCF-7, human endometrial cancer cell RL-95, and porcine endometrial epithelial cells (PE) under standard medium (SM), serum-free medium (SF) alone, or supplemented with 10^{-8} M 17β -estradiol (SF + E 10 nM). The internal control of 43 kDa band of β -actin was also detected in all samples. (B) Bar graph illustrating the ratio of PRL-R to β -actin protein expression in the endometrial epithelial cells, based on the densitometry of immunoblots obtained from SM, SF, and SF + E10 nM ($n=4$). Values represent means $\pm$ S.E.M. * $P<0.05$ when compared with the serum-free medium condition by Student's *t*-test.

blockers, NPPB, DPC, and DIDS, but not by Na^+ channel blocker, amiloride. In addition, the basolateral pretreatment with bumetanide, a blocker of $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter, or replacement of Cl^- or HCO_3^- significantly inhibited the PRL-stimulated *I*_{sc}. PRL added to the basolateral solution of the high-resistance monolayer produced a greater increase in the *I*_{sc} response than when added to the apical solution, suggesting that PRL receptors were predominately located at the basolateral membrane. Western blot analysis revealed the expression of a 36 kDa protein band, which corresponded to the short form of PRL-R, indicating the role of this PRL receptor isoform in the mediation of transepithelial anion secretion in the porcine endometrial epithelium.

The basal electrical properties of cultured epithelial cells used in the present study have been described previously (Deachapunya & O'Grady 1998). Under the basal condition, these cells exhibited substantial *I*_{sc} that was due to a greater Cl^- secretion than Na^+ absorption. Application of PRL produced a concentration-dependent increase in the anion transport with a maximal effect seen at 1 $\mu\text{g}/\text{ml}$ PRL and an EC_{50} value of 120 ng/ml. In addition, the maximal PRL

response observed within 3–5 min after application, implied a non-genomic action. At PRL concentration of 1 $\mu\text{g}/\text{ml}$, which could be considered a hyperprolactinemic level, PRL was also found to maximally stimulate Cl^- transport in the mammary epithelial HC11 cells (Selvaraj *et al.* 2000). This effective concentration of PRL was comparable with the circulating levels during pregnancy and lactation in the human and the rat (Handwerger & Freemark 1987, Arbogast & Voogt 1998). Like in many other epithelia, Cl^- secretion across the endometrial epithelial cells requires activation of the apical membrane Cl^- channels and the basolateral membrane K^+ channels with the basolateral $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporters serving as the Cl^- loading step. Since two types of Cl^- channels, cAMP-activated cystic fibrosis transmembrane conductance regulator (CFTR) and Ca^{2+} -activated Cl^- channels have been identified in a variety of epithelia including endometrial epithelial cells (Deachapunya & O'Grady 1998, Palmer-Densmore *et al.* 2002), NPPB and DPC, inhibitors of anion channels including CFTR, and DIDS, an inhibitor of Ca^{2+} -activated Cl^- channels, were used to elucidate PRL action. As the present results showed that the PRL-induced increase in *I*_{sc} was nearly completely inhibited by DPC and NPPB, it was most likely that CFTR was the primary target of the PRL-stimulated Cl^- secretion. However, the 40% inhibition of the PRL-stimulated *I*_{sc} by DIDS indicated that the Ca^{2+} -activated Cl^- channels may also be partially involved in the PRL activation of Cl^- secretion. In addition, the PRL-stimulated increase in *I*_{sc} was also diminished by bumetanide, an inhibitor of $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter, which was not surprising since the bumetanide-sensitive Cl^- uptake probably served as the Cl^- loading step for PRL-stimulated Cl^- secretion. The finding that *I*_{sc} response to PRL was abolished in the Cl^- free solution further confirmed the PRL activation of Cl^- secretion. Since the electronic HCO_3^- secretion used the cAMP- and Ca^{2+} -activated Cl^- channels (Illek *et al.* 1999), a marked reduction of *I*_{sc} response to PRL in the HCO_3^- free solution suggested a substantial contribution of HCO_3^- to the PRL-induced increase in *I*_{sc}. Altogether, the results suggest a possible involvement of $\text{Cl}^- - \text{HCO}_3^-$ exchangers as well as $\text{Na}^+ - \text{Na}^+ - \text{HCO}_3^-$ cotransporters in the PRL-dependent HCO_3^- secretion. However, it was noted that the replacement of HCO_3^- itself, could have produced the intracellular acidification, which could affect the transport pathways or the signaling mechanisms that regulate anion secretion. Taken together, it could be stated that PRL-induced anion secretion by stimulating the apical Cl^- efflux through CFTR as the major channel type and through some Ca^{2+} -activated Cl^- channels, concurrently with the increase in the basolateral Cl^- uptake through the bumetanide-sensitive $\text{Na}^+-\text{K}^+-2\text{Cl}^-$ cotransporter. Although not being investigated in this study, the basolateral K^+ channels that provide the driving force for Cl^- exit across the apical membrane, could also be a target of PRL action, and are subject to further investigation.

Several signaling pathways mediating the multiple actions of PRL have been demonstrated in a variety of tissues with the

JAK-STAT pathway being most extensively studied. Previous evidence of the phosphorylation of JAK2, STAT1, and STAT5 in response to PRL stimulation in the human endometrium (Jabbour *et al.* 1998) and the PRL-stimulated Cl^- transport through the JAK2 cascade pathway in mouse mammary epithelial cell line HC11 (Selvaraj *et al.* 2000) suggested that JAK2 is a likely mediator of PRL-stimulating effect on the anion transport in the porcine endometrial epithelial cells. In the present study, pretreatment with AG490, an inhibitor of JAK2 activity inhibited both the basal Isc and the PRL-induced increase in Isc. Regarding the basal Isc, since the basal Isc of the cultured porcine endometrial epithelial cells has been shown to be generated mainly by Cl^- secretion (Deachapunya & O'Grady 1998), the fact that AG490 could inhibit the basal Isc within 10 min suggested that a constitutive JAK2 activity was responsible for the basal active Cl^- secretion. Although no direct association between JAK2 activity and Cl^- transport mechanism has been reported, the fact that tyrosine-phosphorylated proteins could regulate the basal Cl^- secretion in human colonic epithelial cell line, T84 (Uribe *et al.* 1996) suggested that the JAK2 inhibitors inhibited Cl^- secretion by interfering with the tyrosine phosphorylation of the regulatory transport proteins. Furthermore, based on the findings that i) Cl^- secretion could still be activated by 8cpt-cAMP in the presence of JAK2 inhibitor, ii) the inhibition of the basal Isc did not affect the PGE2-stimulated Isc response, and iii) the PGE2-stimulated Cl^- secretion was via the cAMP-dependent pathway (Deachapunya & O'Grady 1998), it is likely that the cAMP-dependent Cl^- secretion did not involve JAK2 pathway.

In contrast to the PGE2-stimulated Cl^- secretion, the PRL-stimulated Cl^- secretion was probably mediated by the JAK2 pathway because the PRL-induced increase in Isc was significantly inhibited by AG490. These findings were consistent with a report of AG490 blocking phosphorylation of STAT5 and PRL-induced Cl^- secretion, but not the PGE1-induced Cl^- secretion in mouse mammary cell line (Selvaraj *et al.* 2000). The effect of AG490 was more specific to PRL action, since tyrosine kinase inhibitor genistein had no effect on the PRL-stimulated Isc (data not shown).

PRL-R and its mRNA have been identified in human glandular epithelial and stromal cells (Jabbour *et al.* 1998, Tseng & Zhu 1998). Two isoforms of PRL-R, short and long, have been identified in several rat tissues including liver, ovary, thymus, and spleen (Gunes & Mastro 1996, Telleria *et al.* 1997). In the present study, we examined the expression of PRL-R protein in the endometrial epithelial cells by western blot analysis. The monoclonal antibody used in the present study detected protein with molecular mass of about 36 kDa, which corresponded to the short form of the PRL-R, similar to the predominant short isoforms expressed in the rat spleen and brain (Shingo *et al.* 2003). By contrast, the long form of PRL-R is the major receptor isoforms in the rat liver and mammary gland (Jahn *et al.* 1991, Selvaraj *et al.* 2000). Although at least two isoforms of PRL-R mRNA have been found in the reproductive tissues (Telleria *et al.* 1997), the present data indicated that the short form PRL-R was the functional

PRL-R in the porcine endometrial epithelial cells, and that the PRL-stimulated Cl^- secretion in the porcine endometrial epithelial cells was mediated through the short form of PRL receptors located predominately at the basolateral membrane.

Generally, PRL is synthesized by the decidualized endometrial stromal cells during the late secretory phase of the menstrual cycle and throughout pregnancy. The level of PRL is apparently much higher in the blood and amniotic fluid during pregnancy (Golander *et al.* 1978, Daly *et al.* 1983). After conception, a continuous increase in PRL production in the decidual cells leads to an accumulation of PRL in the amniotic fluid up to 2–3 $\mu\text{g}/\text{ml}$ (Golander *et al.* 1978, Daly *et al.* 1983). Concomitantly, the PRL receptor expression and its mRNA are up-regulated toward the secretory phase of the menstrual cycle (Jabbour *et al.* 1998, Jones *et al.* 1998) and maintained throughout pregnancy (Maaskant *et al.* 1996). The level of PRL receptor mRNA is much higher in the glandular cells than in the stromal cells (Jabbour *et al.* 1998, Jones *et al.* 1998). Although the exact role of PRL in the human endometrium remains to be clarified, the pattern of secretion and expression supports a role of PRL in implantation and placentation. In agreement with those reports, the presence of PRL-R protein that was up-regulated by 17β -estradiol (Fig. 6) in the cultured porcine endometrial epithelial cells strongly suggested the physiological role of PRL in pregnancy. This speculation was consistent with the report that the blastocyst implantation and the maintenance of pregnancy were impaired in the PRL and PRL-R knockout mice (Jikihara *et al.* 1996). The next question is what is the exact role of PRL in the pregnant uterus. It is known that specific concentrations of electrolytes and pH within the uterine lumen are important for implantation and embryo development. In the rhesus monkey, PRL has been shown to regulate the amniotic and fetal extracellular fluid and electrolyte balance by decreasing the water flux from the amniotic side of the fetal membrane (Josimovich *et al.* 1977). Concomitant with the present finding of PRL role in the stimulation of anion secretion across the endometrial epithelial cells, and that active secretion of Cl^- and HCO_3^- provides the driving force for fluid secretion and the regulation of luminal fluid pH, it is likely that PRL exerts endocrine and paracrine actions to regulate the volume and composition of the fluid within the uterine cavity, thus providing an optimal condition for implantation and development of the embryo.

In conclusion, this study shows for the first time the regulation of the transepithelial anion secretion by PRL in the endometrium. The results showed that PRL acutely stimulated anion secretion across the porcine endometrial epithelial cells through the short isoform of PRL receptor and the JAK-STAT-dependent pathway. The PRL-stimulated anion secretion was mostly a result of the activation of DPC- and NPPB-sensitive Cl^- channels, and bumetanide-sensitive $\text{Na}^+ - \text{K}^+ - 2\text{Cl}^-$ cotransport. Further investigation is required to define the physiological and pharmacological significance of PRL action in the endometrium. In addition, the PRL-signaling mechanisms, i.e. intracellular Ca^{2+} , cAMP, or other signaling molecules remain to be elucidated.

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