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The Hormonal Control of Uterine Luminal Fluid Secretion and Absorption

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Abstract. The secretion of uterine luminal fluid initially provides a transport and support medium for spermatozoa and unimplanted embryos, while the absorption of uterine luminal fluid in early pregnancy results in the closure of the lumen and allows blastocysts to establish intimate contact with the uterine epithelium. We have established an in vivo perfusion technique of the lumen to study the hormonal control of the events in the peri-implantation period. Fluorescein-labelled dextran was included in the perfusion medium to monitor fluid movements and the concentrations of Na^+ and CI^- ions in the effluent were monitored. Using an established regimen of steroid treatment of ovariectomized rats mimicking early pregnancy, oestradiol caused fluid secretion, while progesterone resulted in an amiloride-sensitive fluid absorption. Fluid absorption peaked at about the expected time of implantation. The effect of progesterone could be inhibited by treatment with a high dose of oestradiol, by the anti-progestin RU486, and by the presence of an intra-uterine contraceptive device. Studies of expression of Na^+ and CI^- channels (ENaC, CFTR) indicated that these channels were subject to tissue-specific regulation within the uterus, but more work is required to determine their role and the factors controlling their abundance and localization in early pregnancy.

Key words: Estradiol — Progesterone — Uterus — EnaC — CFTR — Implantation

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Introduction

The control of the fluid environment of the uterus is essential for a number of key reproductive events, including sperm and embryo transport, development and implantation. Although cyclical changes in the volume and composition of uterine luminal fluid are known to occur, the physiological and molecular mechanisms controlling these are very poorly understood. Under oestrogen dominance, the uterus has an obvious fluid-filled lumen. However, in species with relatively small blastocysts (e.g., rat, mouse, hamster, guinea pig, humans), this fluid is lost in early pregnancy and the uterine lumen "closes" (Martin, Finn & Carter, 1970; Ljungkvist, 1972; Hedlund et al., 1972). Uterine closure results in the loss of the uterine lumen, with the complete apposition of the epithelium of the opposing uterine walls. This provides a mechanism by which embryos establish and maintain contact with the uterine epithelium prior to initiating implantation. In the rat and mouse, the uterus simultaneously passes through a brief period of sensitivity or receptivity to the presence of a blastocyst and the uterine decidual reaction is initiated. In rodents, the closure reaction and implantation may be disrupted by factors such as inappropriate steroid exposure or the presence of an intrauterine contraceptive device (IUCD) (Martin & Finn, 1978). These observations raise the possibility that any disturbances in fluid handling by the uterus may interfere with normal fertility and also provide an important focus for understanding the action of contraceptive steroids and intra-uterine contraceptive devices.

The mechanisms underlying uterine closure are unclear. Our previous studies suggested that uterine glands may play a primary role in the regulation of uterine fluid volume by switching from a secretory to an absorptive function under the appropriate

endocrine stimulation in the preimplantation period (Naftalin et al., 2002). Others have proposed a role for absorption across the luminal epithelium (Clemetson et al., 1977), possibly involving irregular cytoplasmic projections (''pinopodes'') on the luminal surface (Enders & Nelson, 1973; Leroy, van Hoeck & Bagaert, 1976; Parr & Parr, 1974; Nikas et al., 1995; Nardo et al., 2002).

The control of uterine fluid movement is linked to ion transport. When present, uterine luminal fluid has a distinct ionic composition: in both rats and humans there is a high K^+: Na^+ ratio (e.g., rats: $K^+ \sim 20$ mM; $Na^+ \sim 130$ mM), which changes with the steroid environment (Clemetson et al., 1970; Nordenvall, Ulmsten & Ungerstedt, 1989; Casslen & Nilsson, 1984). Endometrial electrolyte absorption in mice is inhibited by amiloride, an inhibitor of epithelial Na^+ channels (Wang & Chan, 2000) and isolated human endometrial epithelial cells show an amiloride-sensitive Na^+ conductance that may be linked to fluid transport (Matthews et al., 1998). Chan et al. (2002) and Yang et al. (2004) reported changes in the mRNA expression of epithelial Na^+ channel (ENaC) subunits and the cystic fibrosis transmembrane conductance regulator (CFTR: a cAMP-activated Cl^- channel) in mice during both the oestrous cycle and in early-pregnancy mice, consistent with the changing ability of the uterus to secrete and absorb fluid. The purpose of the present study was to use a model in which uterine luminal fluid and ion handling could be studied in vivo in the critical period leading up to and encompassing implantation. The rat was chosen as the experimental model, as both the timing and endocrine control of implantation are so well established in this species.

Materials and Methods

Animals and Hormone Treatments

Adult Wistar rats weighing 200–250 g were maintained under 12:12 lighting conditions, with free access to food and water. In experiments involving mating females with vasectomized males, the day of finding the vaginal plug was designated as Day 1 of pseudopregnancy. Ovariectomies and vasectomies were performed under isofluorane anesthesia; ovariectomies were normally performed at least 10 days before initiating any hormone treatment. In some experiments a silk thread to act as an intra-uterine contraceptive device (IUCD) was inserted into one uterine horn at the time of ovariectomy. All procedures were performed under appropriate UK Home Office regulations. Steroids and the anti-progesterone mifepristone (RU38486) were dissolved in arachis oil and injected sub-cutaneously in a volume of 0.1 ml. To ensure that the silk thread induced an IUCD effect, rather than stimulating decidualization in the progestational uterus, females with an IUCD were subjected to three consecutive cycles of 0.2 mg oestradiol daily for 2 days, followed by three days of no treatment (Martin & Finn, 1979). To mimic the hormonal profile in early pregnancy, oestradiol and progesterone were administered according to an established protocol (Kennedy & Lukash, 1982). In brief, the basic protocol involved 2 daily injections of 0.2 mg oestradiol, followed on the third day by of 0.2 mg oestradiol together with 1 mg progesterone, no treatment on the 4th day, 4 mg progesterone on the next 3 days (days 5–7), and finally 0.1 mg oestradiol together with 4 mg progesterone on the remaining days (8–10). In some experiments, 0.2 mg oestradiol alone was given for just the first 3 days. Vehicle-treated animals received 3 or more daily injections of arachis oil. To investigate the effect of a high dose of oestradiol on progesterone-stimulated fluid absorption, rats were administered with an additional 1 mg oestradiol/kg/day on days 5, 6 and 7 of the treatment regimen (Dao et al., 1996). In experiments involving Mifepristone (RU486), the compound was dissolved in arachis oil was given at a dose of 8 mg/kg on days 5, 6 and 7 together with progesterone.

Uterine Perfusion

The rats were anesthetized with intraperitoneal injection of 0.1 ml (80 mg/kg) ketamine and 0.15 ml (10 mg/kg) xylazine HCl and maintained under anesthesia on a thermostatically controlled heat pad for the duration of the experiment. Body temperature was monitored using a rectal thermometer. At the end of the perfusion period, the animals were killed. Uterine perfusion was achieved after tying in place fine-bore polythene tubing (ID 0.38 mm; OD 1.09 mm), pre-filled with perfusate, in both the anterior and cervical ends of the uterine horn. The cannula had minimal insertion into the lumen but had slightly heat-flared endings to prevent them being readily pulled out by any ongoing uterine contractions. A syringe-driven infusion pump (Harvard Apparatus) was used to deliver perfusion medium into the anterior cannula at a constant rate of 1.6 ml/min. Timed perfusate collections (every 15–30 min for 2 h) were made into small, covered, pre-weighed polythene tubes. The composition of the perfusion medium was based on published data on the composition of rat uterine fluid and reflected the reported low Na^+ and high K^+ concentrations (Clemetson et al., 1972; Casslen & Nilsson, 1984; Nordenvall et al., 1989) and consisted of: (mmol/l) 110 NaCl, 14.3 $NaHCO_3$, 1 $NaHPO_4$ (total Na^+ 125.3), 15 KCl, 0.8 $MgSO_4$, 10 HEPES, 1.8 $CaCl_2$, 5.5 mmol/l glucose pH 7.34. Fluorescein-dextran (F-dextran; MW 450 kDa) was dissolved (1.0 mmol/l) in the perfusion medium to act as a non-absorbable marker for changes in fluid volume (Naftalin et al., 2002). F-dextran concentrations in each sample were measured in triplicate 1:250 dilutions in PBS using a Hitachi F-2000 spectrophotometer. (E_x = 488 nm, E_m = 520 nm). Na^+, K^+ and Cl^- concentrations in each sample were measured in duplicate after appropriate dilutions using a flame photometer and chloridometer.

Protein Expression

At the end of the treatment period, the rats were killed with anesthetic overdose and the uterine horns and kidneys were immediately removed and proteins prepared for Western blotting analysis by homogenization in tissue lysis buffer (100 mM Tris pH 6.8, 1 mM EDTA pH 8.0, 10% v/v glycerol and 10 ml/l protease inhibitor cocktail (Sigma, UK). Samples were subjected to centrifugation (5 min, 250 × *g*) to remove nuclei. Supernatants from each preparation were heated to 94°C for 5 min in denaturing buffer (Novex, Invitrogen UK). 30 mg protein from the uterine and kidney samples were separated using Novex Bis-Tris gels (Invitrogen, UK). Fractionated proteins were transferred to nitrocellulose membrane (Biorad, UK) by electroblotting and immunostained with anti αENaC (ab-cam, UK), β, γ, ENaC (ADI, USA) and CFTR (Ab-Cam, UK) 1:500. Immunostained proteins were visualized by enhanced chemiluminescence (Amersham, UK) and exposure to autoradiographic film. The size of immunostained proteins was calculated by comparison to protein size markers (BioRad, UK) on the same blots.

For immunohistochemistry, uterine horns were fixed in 4% paraformaldehyde for 4 h at 4°C. The tissues were then processed through to wax blocks and 5 mm sections cut. The sections were dewaxed in xylene and re-hydrated. The tissues were incubated in 10% H_2O_2 in methanol to quench endogenous peroxidase activity and incubated overnight with primary antibodies (rat polyclonal IgG) at the following concentrations in PBS; (1:10 for α-ENaC, 1:25 for β-ENaC, 1:100 for γ-ENaC and 1:100 for CFTR) at 4°C in a humidified chamber. The tissues were then incubated with secondary antibody, biotinylated rabbit anti-rat IgG (Amersham) at 1:500 for 1 h at room temperature and with tertiary antibody, streptavidin-horseradish peroxidase (Amersham) at 1:500 for 1 h at room temperature. The sites of antibody binding were visualized with DAB (Diaminobenzidine HCl), which gave a dark-brown stain. The sections were counterstained with hematoxylin for nuclear staining.

Results

Fluid Absorption by the Uterus under Steroid Conditions Mimicking Early Pregnancy

Uterine perfusions were carried out on Day 0 (i.e., before any steroids), and on Days 3, 4, 5, 6, 7 and 10 of the steroid regimen outlined above. Fluid absorption was monitored independently both via the weight of the collected perfusate and the increase in concentration of F-dextran. Although the weight of the fluid collected was inherently more variable, since it at least partly reflected changes in the contractile tone of the uterus, analysis showed that it was still highly correlated with the independent measurement of F-dextran concentration (Fig. 1; $R = 0.921$ $d.f. = 16$; $P < 0.001$), thus confirming the methods examined by Lucas et al. (2005). In view of this, all further results are presented using just the data from the F-dextran measurements. In the untreated uterus, very little fluid movement was detected, but the rate of fluid absorption increased dramatically ($R = 0.98$, $d.f. = 22$, $P < 0.001$) during the first few days of progesterone treatment (Fig. 2), peaking in the peri-implantation period, before dropping back to lower levels with continued progesterone exposure.

Amiloride Sensitivity of Uterine Fluid Absorption

To investigate whether the progesterone-stimulated fluid absorption was sensitive to amiloride, the uteri of rats on day 7 of the steroid treatment were perfused with medium containing amiloride at various concentrations. The results showed that 100 mM amiloride reduced fluid absorption by more than 50% and very high concentrations of amiloride in the perfusion medium blocked fluid absorption almost completely (Fig. 3) (apparent $K_i = 30.1 \pm 4.9$ μM; $R = 0.90$; $d.f. = 14$; $P < 0.001$). While these concentrations of amiloride in the initial perfusing medium seem high, they should be viewed in the context both of the very slow perfusion rate and of the large surface area of the tubular uterine lumen with extensive deep folds and glands in the endometrium, with amiloride absorption from the perfusate reducing the luminal amiloride concentrations in the distal parts of uterine lumen. The effects of amiloride on uterine luminal ion transport and fluid absorption were investigated in more detail in four selected conditions: untreated and vehicle-treated females, and in animals after either 3 days of oestradiol treatment alone or on day 7 of the steroid treatment regimen (i.e., under progesterone domination). Amiloride was delivered either intraluminally (500 mM), or as a single intravenous injection (10 mg/kg; via tail vein; Runci et al., 1989)

The results are summarized in Fig. 4 and confirmed that progesterone treatment induced net fluid absorption from the uterus, while oestradiol caused net fluid secretion. Both intraluminal and intravenous administration of amiloride produced very similar results: a significant reduction in the fluid absorption from uteri under progesterone domination associated with a significant reduction in the Na^+ concentration in the perfusate leaving the uterus. Amiloride had no significant effect on the small amounts of fluid absorption found in either the untreated or vehicle-treated animals, nor did it affect the fluid secretion seen in oestradiol-treated animals. The suppressive effect of amiloride on fluid absorption was also evident in intact rats studied on day 4.5 of pseudopregnancy (controls: 0.35 ± 0.03 ml/min; + amiloride: 0.08 ± 0.02 ml/min; $n = 4$ per treatment, $P < 0.01$).

The rates of ion transport were calculated using the data on the changes in ion concentration between the inflow and effluent perfusate (Fig. 5). These data clearly show that treatment with oestradiol alone causes net fluid secretion and is unaffected by amiloride, while the fluid absorption induced by prolonged progesterone treatment is associated with amiloride-sensitive Na^+ and Cl^- absorption. The calculated Na^+ and Cl^- concentrations of the absorbate (based on the amount of fluid absorbed) indicate that the absorbate is hypertonic and that this hypertonicity is reduced by amiloride treatment (Table 1).

The Effect of Inhibitors of Implantation on Fluid Movements within the Uterus

This series of experiments was undertaken to investigate whether procedures known to interfere with implantation (the presence of an IUCD, treatment with a high dose of oestradiol or with the anti-progestin RU486) would affect fluid handling by the uterus. All experiments were carried out on day 7 of the steroid treatment regimen when fluid absorption is normally high (Fig. 1*a*). All three treatments significantly inhibited fluid absorption and/or stimulated fluid

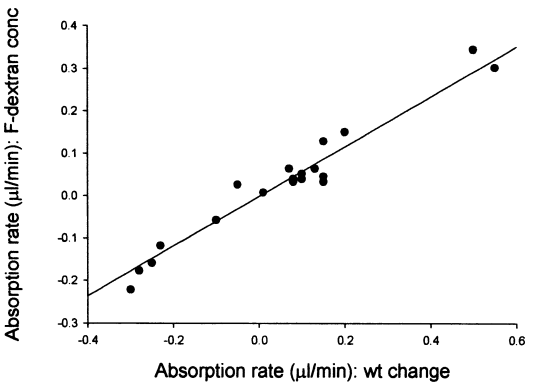

Fig. 1. Correlation (R^2 = 0.921) between the independent measurements of absorption rate (changes in the concentration of the impermeable fluorescein-dextran marker and changes in fluid flow) in perfused rat uterine horns. Each point represents the mean value of each set of observations (n = 4 rats per point).

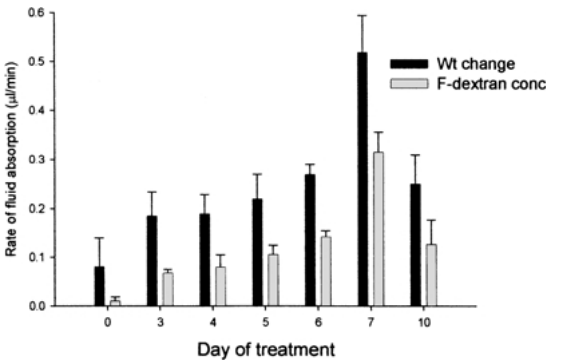

Fig. 2. Rates of uterine luminal fluid absorption (µl/min) in perfused rat uteri in situ detected by weight changes in the perfusate and changes in the concentration of the impermeable marker F-dextran. Ovariectomized rats were treated with oestradiol after day 0 and with progesterone from day 3 (*see* Methods for details). Each point represents the mean value (± SEM) of each set of observations (n = 4 rats per point).

secretion (Fig. 6). Ionic changes in the uterine effluent were monitored in the high-dose oestradiol and RU486 treatment groups and both treatments significantly reduced the absorption of all three ions (Fig. 7).

Protein Expression

Western blotting of protein isolated from rat uteri indicated the presence of α and γ ENaC subunits and CFTR. We were unable to demonstrate β-ENaC protein in the uterus, although a protein of predicted size was detected in kidney (Fig. 8). In the uterus, the immunostained α-ENaC protein product was larger than the ~90 kDa product detected in kidney control samples. We detected two immunostained γ-ENaC proteins common to both the kidney and the uterus of approximately 85 and 65 kDa and a larger product

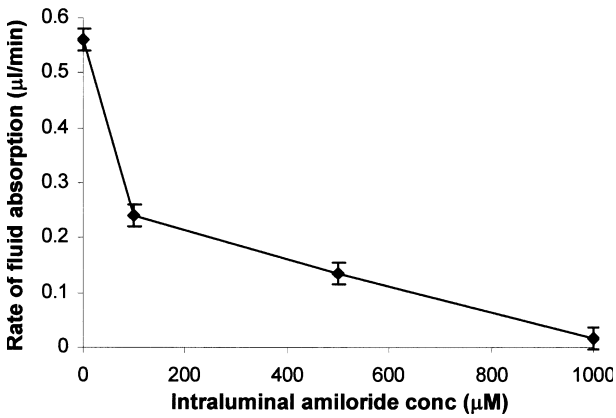

Fig. 3. The effects of increasing dose of amiloride in the perfusate on the rates of uterine luminal fluid absorption (µl/min) in perfused rat uteri in situ detected by changes in the concentration of the impermeable marker F-dextran. Ovariectomized rats were treated with oestradiol followed by progesterone (*see* Methods for details). Each point represents the mean value (± SEM) of each set of observations (n = 4 rats per point).

specifically in the uterus. Protein products of similar size for CFTR were detected in both uterus and kidney (Fig. 8).

Immunohistochemistry indicated that αENaC protein was localized to the luminal epithelium at low levels in untreated uteri, and at higher levels after progesterone treatment. We did not detect αENaC in the luminal epithelium after oestrogen treatment (Fig. 9*A* and *B*). We also showed that γENaC was present in the luminal epithelium of all our treated tissue samples but more intense staining was seen after progesterone treatment (Fig. 9*A*). γENaC was also present in the glandular epithelia using fluorescence immunostaining (*data not shown*) and in the stroma. Immunohistochemistry showed that CFTR was found in both uterine luminal epithelia and stroma (Fig. 9*A*), but was highest in the stroma. In the epithelia of oestrogen-treated rats, CFTR appeared to be present in the intracellular region of some epithelial cells with a low abundance at the apical membrane (Fig. 9*C*). CFTR protein was apparent at low levels in the uterine luminal membrane after progesterone treatment, with less evidence of intracellular staining (Fig. 9*D*). We did not observe significant changes in stromal staining between the three treatment groups.

Discussion

This is the first reported study of using perfusion to study fluid and ion movements within the uterus. The results clearly demonstrate that these are hormonally controlled, with fluid absorption dominating at the expected time of uterine closure in the peri-implan-

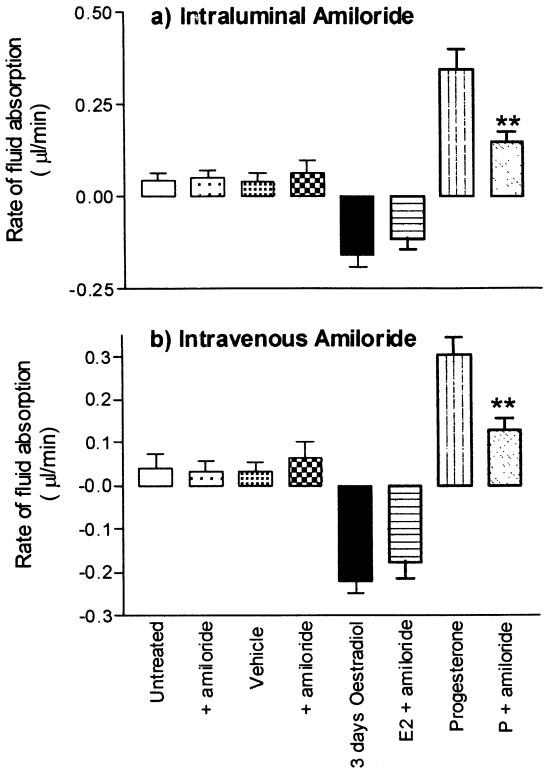

Fig. 4. The effects of intraluminal or intravenous amiloride on the rates of uterine luminal fluid absorption (µl/min) in perfused rat uteri in situ detected by changes in the concentration of the impermeable marker F-dextran. Ovariectomized rats were untreated, or treated with vehicle, oestradiol or oestradiol followed by progesterone (*see* Methods for details). Each point represents the mean value (± SEM) of each set of observations (n = 4 rats per point). $^{**}P < 0.01$.

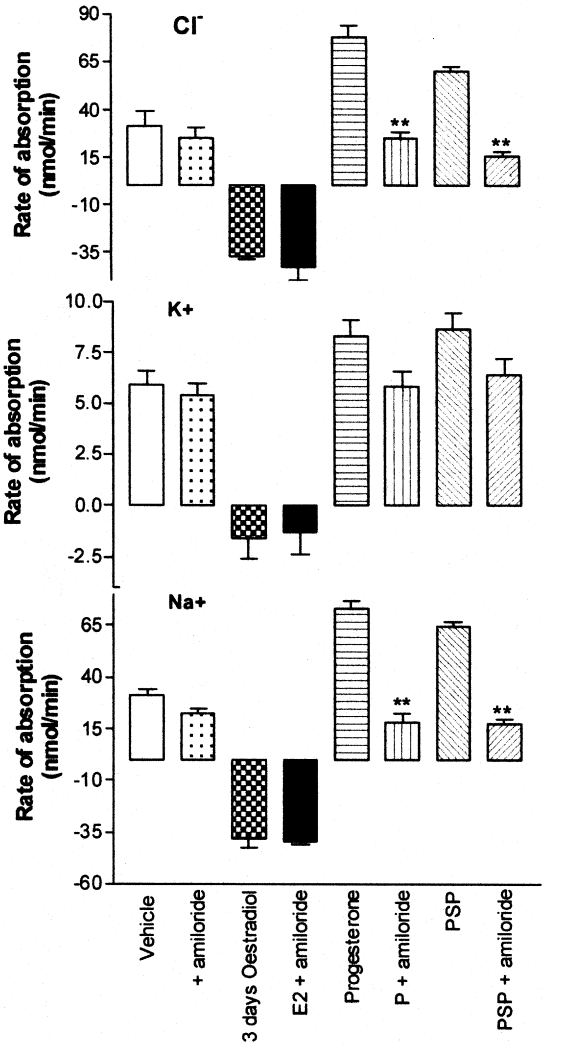

Fig. 5. The effects of intraluminal amiloride on the rates of absorption of Na, K, and Cl ions (nmol/min) in perfused rat uteri in situ. Rats were treated with vehicle, oestradiol or oestradiol followed by progesterone (*see* Methods for details), or intact on day 4.5 of pseudopregnancy (PSP). Each point represents the mean value (± SEM) of each set of observations (n = 4 rats per point). $^{**}P < 0.01$.

tation period. It also demonstrates that inhibitors of early pregnancy can interfere with normal ion transport and fluid movements.

A variety of methods to study the volume and ionic changes in uterine fluid in rodents have previously been used, with almost all the studies being done in rats at times equivalent to the events of the normal cycle. The relatively small volume of uterine luminal fluid obtained by direct aspiration has limited previous analyses, except at pro-estrus when the uterus balloons with secretion (Armstrong, 1968). Under the influence of progesterone after ovulation, there is an immediate loss of the bulk of this fluid, mostly via the cervix (Kennedy & Armstrong, 1975). Studies after this time have largely relied upon interpreting the results from analyzing uterine washes, but the relatively small amounts of fluid in the uterus compared to the wash volumes raise questions concerning the reliability of this data. In addition, attempts to flush the uterus with large volumes of fluid delivered at high pressure can cause tissue damage (Milligan & Martin, 1984). Nevertheless, such studies have consistently indicated that oestradiol causes secretion of fluid into the uterine lumen, while progesterone causes luminal fluid absorption (e.g., Clemetson et al., 1972; Tantayaporn et al., 1974). The results relating to changes in ion concentration of the luminal fluid have been more variable: Tantayaporn et al. (1974) reported that the Na^+ concentration in untreated, ovariectomized rats was 100 mEq/l; this increased to 128 mEq/l after oestradiol and decreased to 119 mEq/l after progesterone, consistent with progesterone causing Na^+ absorption. In contrast, Nordenvall et al. (1989), using an intra-uterine microdialysis probe, reported that progesterone caused an increase in Na^+ ion concentration (124 to 134 mEq/l) and a decrease in K^+ concentration (22 to 18 mEq/l).

Table 1. Calculated concentrations (means ± SEM) of Na^+ and Cl^- in the absorbate I from rat uterine luminal fluid in ovariectomized and progesterone-treated (Day 7 of steroid regimen) animals in the absence or presence of intraluminal amiloride

	Na^+ mM	Cl^- mM
Perfusate	125	137
Vehicle	157 ± 2.6	162 ± 6.5
Vehicle + amiloride	148 ± 2.4	152 ± 5.1
Progesterone	173 ± 4.0	174 ± 2.5
Progesterone + amiloride	138 ± 5.8	158 ± 3.8

This present study, using a very slow, in vivo perfusion system, confirmed that while there is relatively little fluid movement in ovariectomized animals, oestradiol induces secretion, while progesterone induces absorption. Similar results were obtained in both ovariectomized females treated with progesterone and in pseudopregnant females. In both cases, absorption occured at a time equivalent to the expected time of uterine closure in intact animals. This closure of the uterus normally occurs after the developing blastocysts have entered and been distributed along the uterus, with the consequence that each blastocyst is sandwiched between the opposing uterine walls, allowing the small, otherwise free-floating, embryos to establish intimate contact with the uterine epithelium. The blastocysts are then able to initiate the complex embryo-maternal signaling processes and morphological changes associated with implantation (e.g., rat, mouse, and hamster) (Martin et al., 1970; Hedlund et al., 1972; Ljungkvist et al., 1972; Hoversland & Weitlauf, 1981). This uterine closure is very tight: the microvilli on the opposing epithelia become tightly interdigitated, resulting in the complete obliteration of a lumen even at a level observed with electron microscopy (Martin et al., 1970). The few observations that have been made in humans and primates suggest there is a similar reduction or loss of uterine luminal volume: Casslen (1986) estimated human luminal fluid volume was only 5–35 ml in the luteal phase of the cycle, consistent with the observations of Maier and Kuslis (1988) and Shaw, Azar & Moyer (1975).

The mechanism underlying fluid absorption in the peri-implantation period has been the subject of some debate. The simultaneous appearance of luminal epithelial cell endocytosis and uterine closure in rats led to the suggestion that the uterine luminal fluid may be removed by the endocytotic activity of the epithelial cells (Parr, 1983). Characteristic irregular cytoplasmic projections (''pinopodes'') appear on the luminal surface of epithelial cells in humans and rats at around the time of uterine receptivity and have been suggested to play a role in fluid absorption (Enders & Nelson, 1973; Parr & Parr, 1974; Leroy et al., 1976; Murphy, 2000). However, regardless of the role that such morphological features may have in fluid absorption, there is now considerable evidence for more traditional explanations for the fluid and ion movements. Chan and coworkers have undertaken a number of studies on cultured murine endometrial epithelia, particularly in relation to the regulation of HCO_3^- and Cl^- secretion and Na^+ absorption (Chan et al., 1997, 1999, 2002; Wang & Chan, 2000; Wang et al., 2003; Yang et al., 2004). These in vitro studies showed an electrogenic NaCl absorption across cultured mouse endometrial epithelial cells that could be inhibited by 10 mM amiloride, an inhibitor of epithelial Na^+ channels (Wang & Chan, 2000). Isolated human endometrial epithelial cells also show an amiloride-sensitive Na^+ conductance that may be linked to fluid absorption (Matthews et al., 1998). All these findings are in accord with the progesterone-stimulated, amiloride-sensitive, Na^+ absorption seen in vivo in the perfused rat uterus in the present study.

The observations from this and previous studies are consistent with the involvement of the epithelial Na^+ channel (ENaC) in fluid absorption. This would be similar to its proposed role for Na^+ and water absorption in the distal tubule of the kidney (Garty Palmer, 1997), lung (Olver et al., 1986; Folkesson, Norlin & Baines, 1998; Baines et al., 2000) and colonic crypts (Naftalin, Zammit & Pedley, 1995; Naftalin & Pedley, 1999). Uterine ENaC mRNA expression changes during the mouse oestrous cycle (Chan et al., 2002) and increases around the time of implantation (Yang et al., 2004). We have shown that ENaC α and γi subunit proteins were present in uterine tissue. There were some differences in the sizes of these proteins in uterus compared to kidney. However, the described sizes of ENaC subunits vary between species, tissues and cells and have been reported to be products of both differential glycosylation and proteolytic cleavage once expressed in the membrane (Wodopia et al., 2000; Alvarez de la Rosa, Li & Canessa 2002; Masilamani, 2002; Hughey et al., 2003). Thus, the products we detected may indicate uterine-specific processing of these proteins. The significance of the predominant α and γ ENaC subunit expression in this tissue is unknown. The α-subunit is critical for pore formation of the channel, while the β and γ -subunits are known to be important for channel trafficking to the plasma membrane and augmentation of channel function (Firsov et al., 1998; Kosari et al., 1998; Snyder et al., 1998). Although channels comprised of all three subunits exhibit the largest conductance, α and γ subunits can also produce functional amiloride-sensitive Na^+ channels (Canessa, Merillat & Rossier, 1994).

Immunohistochemistry indicated that αENaC protein was localized to the luminal epithelium at the

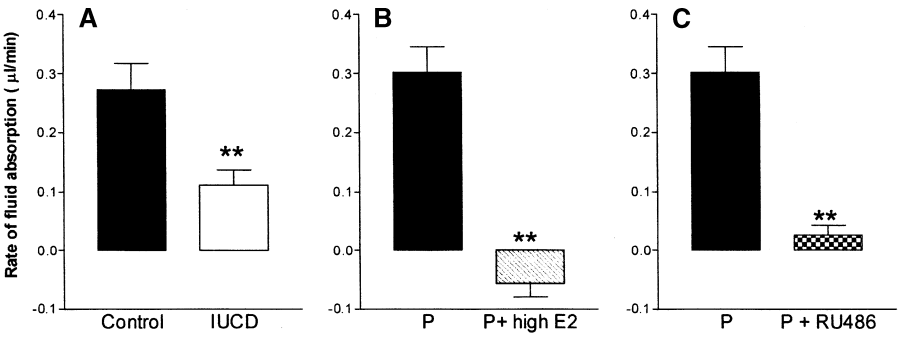

Fig. 6. The effect of potential contraceptive treatments on the rate of uterine luminal fluid absorption in perfused rat uteri in situ. The rate of fluid absorption was determined from changes in the concentration of the impermeable marker F-dextran in the uterine perfusate: (*A*) in the presence on an intrauterine contraceptive device, (*B*) after giving a high dose of oestradiol in combination with progesterone on days 5, 6 and 7, and (*C*) after treatment with 8mg/kg Mifepristone (RU486) (*see* Methods for details of treatments). Each point represents the mean value (± SEM) of each set of observations ($n = 4$ rats per point); $^{**}P < 0.01$ compared to control treatment.

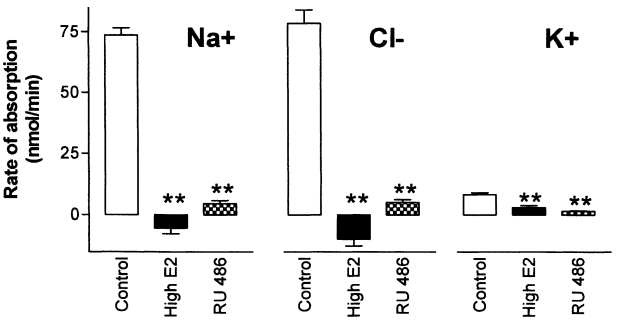

Fig. 7. The effect of potential contraceptive treatments on the rate of ion absorption in perfused rat uteri in situ. The rate of ion absorption was determined from changes in the ionic concentration of the perfusate after giving a high dose of oestradiol in combination with progesterone on days 5, 6 and 7, or after treatment with 8mg/kg Mifepristone (RU486) (*see* Methods for details of treatments). Each point represents the mean value (± SEM) of each set of observations ($n = 4$ rats per point); $^{**}P < 0.01$ compared to control treatment.

highest levels in progesterone-treated animals. We also showed that γENaC was present in the luminal epithelium of all the steroid-treated uteri, but the staining was more intense after progesterone treatment. γENaC was also present in the glandular epithelia using fluorescence immunostaining (*data not shown*). These data suggest that only after progesterone treatment is there a coordinated increase in levels of both α and γ ENaC in the uterine luminal membrane, which could underlie the concurrent increase in amiloride-sensitive fluid absorption. This is supported by the findings of Tsang et al. (2001), who reported that in the endometrium, the expression of γENaC is required for an increase in ENaC current. Conversely, the lack of the pore-forming αENaC subunit in the uterine lumen after oestrogen treatment could underpin the lack of amiloride-sensitive fluid absorption during the secretory phase of the cycle. It is also possible that Na^+/H^+ exchangers in the uterine epithelium may also participate in Na^+ absorption: These exchangers are sensitive to amiloride at high concentration >100 mM. NHE1, NHE2 and NHE4 are present in both the apical and basolateral membranes of the luminal epithelium of mice, and Wang et al. (2003) suggested that the apically located NHEs may play a complementary role in Na^+ absorption. The use of selective inhibitors of NHE would help distinguish the relative roles of ENaC and NHEs. Elucidating the role of NHEs in the uterus is additionally important in view of the reported high concentration of HCO_3^- in uterine luminal fluid (Murdoch & White, 1968).

Studies on cultured mouse endometrial cells have previously suggested that the cystic fibrosis transmembrane conductance regulator (CFTR) may play some role in mediating secretory responses in the uterus (Chan et al., 1909; Matthews et al., 1998). Chan et al. (2002) demonstrated differential regulation of CFTR at different stages of the oestrous cycle in mice. Our own studies indicated that CFTR abundance was highest in the stroma, an observation supported by Yang et al. (2004) who observed CFTR in the stroma was maximal at day 3 post-mating in superovulated mice. The functional significance of CFTR in the stroma is currently unknown. The presence of CFTR has been described in lymphocytes (Krauss et al., 1992), neurons (Weyler et al., 1999) and endothelial cells (Tousson et al., 1998), all of which could be in the stroma. Although CFTR can act as a Cl^- channel, it can also act as a regulator of ATP release (Schwiebert et al., 1999), Ca^{2+}-activated Cl^- channel (CaCC) (Wei et al., 1999), Cl^-/HCO_3^- exchangers (Lee et al., 1999) and gap junctional channel (connexin 45) (Chanson, Scerri & Suter, 1999) which may be important for stromal angiogenesis and decidualization.

In oestrogen-treated rats, CFTR was present in the intracellular region of some epithelial cells, with low abundance at the apical membrane. This could represent an intracellular pool of CFTR channels

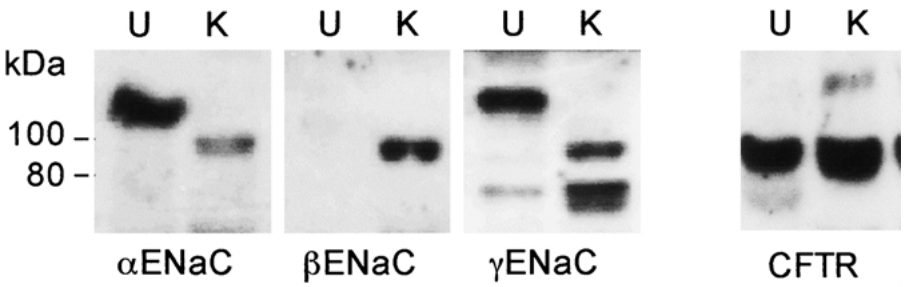

Fig. 8. Western blots of protein isolated from rat uterus (*U*) and control kidney (*K*) tissue samples immunostained with anti-αENaC, βENaC, γENaC or CFTR antiserum. The positions of 80 and 100 kDa protein size markers are shown to the left of the blot.

which has previously been described (Kleizen, Braakman & de Jonge, 2000). After progesterone treatment, CFTR protein became more apparent in the epithelial cell luminal membrane with a reduction in intracellular staining, indicating possible translocation of CFTR to the cell membrane. The role of CFTR in the uterine epithelium is unclear, although the low level of CFTR protein in the epithelium may be important to ensure maximal ENaC activity. Several reports have suggested that ENaC activity is inhibited by functional interaction with CFTR (Stutts et al., 1995; Mall et al., 1998) and activation of CFTR has been shown to decrease ENaC activity in mouse endometrium in vitro (Chan et al., 2001).

Our estimates indicate that the Na^+ and Cl^- concentration of the absorbate during progesterone treatment was hypertonic. In the context of the tight endometrial epithelium (Matthews et al., 1998), hypertonic Na^+ absorption could drive the secondary osmotic absorption of water, thus providing the mechanism of water movement from the lumen to plasma. This would be similar to the situation in colonic glands, in which water absorption is also driven by hypertonic water absorption. Indeed, this raises the question of which are the primary absorptive surfaces in the uterus. While the changes in uterine fluid volume reflect net fluid movement across both the luminal and glandular epithelial surfaces, secretion is generally ascribed to the glands and absorption attributed to the luminal epithelium (e.g., Clemetson et al., 1977; Beier & Beier-Hellwig, 1998). However, we would question whether such a simple division of function is correct and would propose that the uterine glands may play a prominent role in uterine fluid absorption. We have previously used laser scanning confocal microscopy of the distribution of an extracellular marker (F-dextran) within rat uterine glands in excised uteri to show that the endometrial glands change their fluid-handling characteristics under different hormonal conditions (Naftalin et al., 2002). In this system, under progesterone dominance, the glands showed an amiloride-sensitive dextran accumulation, suggesting sodium-dependent fluid absorption; this was absent in the oestrogen-dominated state. The rate of fluid uptake in the progesterone-stimulated gland opening was estimated to be approximately 1×10^{-4} cm s^{-1}, requiring a suction pressure of between 10–20 mmHg at the mucosal surface. Endometrial glands have similarities to colonic crypts: both are blind-ended mucosal tubes surrounding a lumen of 5–20 μm diameter and both mucosae express amiloride-sensitive epithelial Na^+ conductance channels (Naftalin & Pedley, 1999; Chan et al., 2000). A major difference is the absence of any peri-glandular sheath surrounding the endometrial glands: in the colon the myofibroblastic sheath acts as an effective barrier to Na^+ movement and permits build-up of a large osmotic gradient (2–5 atmospheres) across the crypt wall (Thiagarajah et al., 2001a). The absence of a peri-glandular structure in the endometrium means that there can be no large-scale electrolyte accumulation adjacent to the basement membranes of the gland epithelium. The maximal accumulation observed of FITC-dextran within the progesterone-treated glands was only 1.5 times above that in the bathing solution and dextran accumulation in glands was relatively slow ($t_{1/2}$ = 12.6 ± 4.4 min) compared with murine colonic crypts ($t_{1/2}$ of 1–2 min in vitro; 0.3 min in vivo (Thiagarajah et al., 2001a,b). Additional evidence for a lack of any peri-glandular sequestration of ions was the rapidity of action of amiloride ($t_{1/2}$ = 5.3 ± 1 min) on dextran accumulation in the glands from progesterone-treated animals (Fig. 3). In contrast, amiloride treatment in murine colonic crypts reduces accumulation much more slowly ($t_{1/2}$ = 41.7 ± 3.4 min) because of the sequestration of Na^+ in the peri-cryptal sheath (Thiagarajah et al., 2001a).

Understanding the mechanisms controlling fluid-handling by the uterus has huge potential significance in terms of understanding the basis of normal fertility, the causes of infertility, and potential target sites for contraceptive action. In women, there is considerable individual variation in uterine fluid contents and volumes recovered by flushings and 20–44% of women with unexplained infertility have retarded endometrial development. Luteal phase flushings from such women show disturbances in protein composition (Hamilton et al., 1998). Women with hydrosalpinx have reduced fertility as a result of the presence of excessive fluid in the uterine cavity (Strandell & Lindhard, 2002). Disruption of fluid absorption may also contribute to the mode of action of contraceptives in otherwise fertile women. The

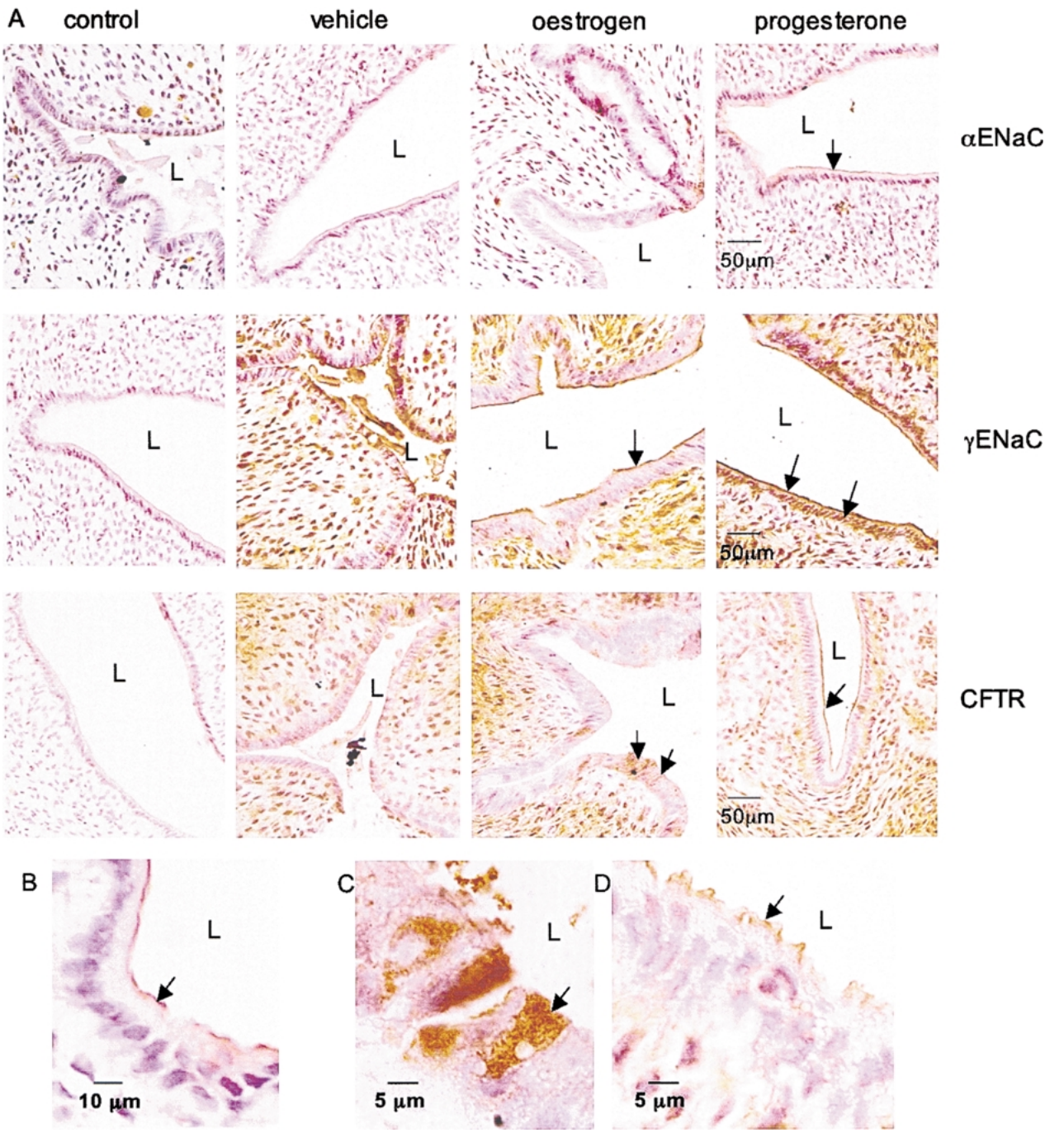

Fig. 9. (*A*) Immunohistochemical localization of αENaC, γENaC and CFTR in the uterus of oestrogen- and progesterone-treated ovariectomized rats. The pictures are representative images showing the distribution of αENaC, γENaC and CFTR in the luminal epithelia. Bound antibody is stained dark brown and marked with arrows ($n = 3$ animals per treatment group). The control image represents no incubation with primary antibody, and the vehicle control represents uterine sections from non-steroid-treated ovariectomized animals. *L* = lumen. Larger magnification images of αENaC/progesterone (*B*), CFTR/oestrogen (*C*) and CFTR/progesterone (*D*) are also shown.

profile of oestradiol and progesterone in the preimplantation period is critical for establishing the endometrial receptivity for the embryos and disturbances of this profile, for example by contraceptive steroids, post-coital steroids, or the anti-gestogen RU486, may disrupt endometrial receptivity and implantation (Dao et al., 1996; Chaudhury & Chaudhury, 1976; Huang et al., 2005). We have shown that administering a high dose of oestradiol or RU486 disrupts normal fluid absorption in the progesterone-dominated uterus. These observations indicate that one mechanism of action of such contraceptives may be by inhibiting fluid absorption and uterine closure. Similarly, IUCDs prevent uterine

receptivity and implantation (Spinnato, 1997) and, in mice at least, they prevent closure of the uterine horn both in the vicinity of the IUCD and in distant regions of the uterine horn (Martin & Finn, 1978). In humans, Tauber, Cramer and Zaneveld (1993) suggested that IUCDs actually stimulated luminal fluid secretion. Our studies again suggest that the effects of IUCDs may be caused by disturbances in uterine fluid absorption.

Our observations support the involvement of ENaC channels in the regulation of fluid absorption in the uterus. More work is required to fully elucidate and quantify the effects of oestrogen, progesterone and the presence of an IUCD on the abundance and localization of these channels in the uterine epithelium. The uterine luminal environment has to fulfil a number of essential functions, particularly in relation to the transport and support of rapidly developing embryos. In view of the critical importance of this stage of embryo development and the need for successful implantation, it is surprising that so many gaps still exist in our knowledge of the underlying physiological processes regulating uterine luminal fluid.

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