Regulation of the immunoexpression of aquaporin 9 by ovarian hormones in the rat oviductal epithelium

María C. Brañes,1 Bernardo Morales,2 Mariana Ríos,1 and Manuel J. Villalón1
1Unidad de Reproducción y Desarrollo, Departamento de Ciencias Fisiológicas, Pontificia Universidad Católica, and 2Departamento de Biología, Facultad de Química y Biología, Universidad de Santiago, Santiago, Chile
Submitted 2 October 2003; accepted in final form 10 January 2005

Brañes, María C., Bernardo Morales, Mariana Ríos, and Manuel J. Villalón. Regulation of the immunoexpression of aquaporin 9 by ovarian hormones in the rat oviductal epithelium. Am J Physiol Cell Physiol 288: C1048–C1057, 2005.—The volume of oviductal fluid fluctuates during the estrous cycle, suggesting that water availability is under hormonal control. It has been postulated that sex-steroid hormones may regulate aquaporin (AQP) channels involved in water movement across cell membranes. Using a functional assay (oocytes of Xenopus laevis), we demonstrated that the rat oviductal epithelium contains mRNAs coding for water channels, and we identified by RT-PCR the mRNAs for AQP5, -8, and -9, but not for AQP2 and -3. The immunoreactivity for AQP5, -8, and -9 was localized only in epithelial cells of the oviduct. The distribution of AQP5 and -8 was mainly cytoplasmic, whereas we confirmed, by confocal microscopy, that AQP9 localized to the apical plasma membrane. Staining of AQP5, -8, and -9 was lost after ovariectomy, and only AQP9 immunoreactivity was restored after estradiol and/or progesterone treatments. The recovery of AQP9 reactivity after ovariectomy correlated with increased mRNA and protein levels after treatment with estradiol alone or progesterone administration after estradiol priming. Interestingly, progesterone administration after progesterone priming also induced AQP9 expression but without a change in mRNA levels. Levels of AQP9 varied along the estrous cycle with their highest levels during proestrus treatments. These results indicate that steroid hormones regulate AQP9 expression at the mRNA and protein level and that other ovarian signals are involved in the expression of AQP5 and -8. Thus hormonal regulation of the type and quantity of water channels in this epithelium might control water transport in the oviductal lumen.

The fluid produced and secreted by epithelial cells of the oviduct provides a physiological medium for fertilization and facilitates ovm transport toward the uterus and early embryonic development. Furthermore, the quality and quantity of the oviductal fluid are modified in correlation with fluctuations of estrogen and progesterone (P4) plasma levels during the estrous and menstrual cycle (30). The rate of fluid secretion increases during the estrus about 2–10 times, depending on the species, compared with the luteal phase or pregnancy (23, 30). However, the mechanism by which ovarian hormones regulate water availability from epithelial cells toward the oviductal lumen for mucus hydration is unknown.

In many tissues, water channel proteins known as aquaporins (AQP) have been implicated in transmembrane water transport (2). Eleven mammalian AQP have been cloned, several of which have been related to physiological processes (2, 52). Moreover, specific mutations of AQP are responsible of some human and rodent inherited diseases (27).

AQP have been documented in the reproductive tract of the male and female. In male rats, several AQP have been detected (3, 5, 8, 40, 46, 48), and their expression has been related to the formation of the seminiferous fluid, processes under steroid hormone control (9, 10, 21, 22, 41). In the female, the presence of AQP1 mRNA has been demonstrated in the human uterus (31) and in the frog oviduct (1). AQP9 mRNA is present in the rat oocyte only throughout proestrus (13), AQP-7, -8, and -9 have been detected in rat granulosa cells (34), and AQP1 has been detected in smooth muscle cells of the rat vagina and uterine tube (16). Recently, the differential expression of various AQP and AQP mRNAs in the mouse uterus has been detected after ovariectomy and in response to hormonal replacement (24, 43). To our knowledge, the expression of AQP by oviductal epithelial cells has not been reported; thus elements of the molecular mechanism that control the volume of oviductal fluid in different physiological states remain to be determined.

The work presented herein demonstrates that the oviduct of cycling rats expresses AQP5, -8, and -9 in epithelial cells with differing subcellular localization. Moreover, the expression of AQP5, -8, and -9 is differentially regulated by ovarian hormones. Finally, we present evidence suggesting that the different levels of AQP9 detected in the epithelium of cycling rats correspond to 17β-estradiol (E2) and P4 regulation at the mRNA and translation levels, respectively.

MATERIALS AND METHODS

Animals. Sprague-Dawley rats (200–260 g body wt) were obtained from the Animal House of the Pontificia Universidad Católica (Santiago, Chile). Animals were kept under controlled temperature of 21–24°C and under lighting from 0700 to 2100 h. Water and pelleted food were supplied ad libitum. Vaginal smears were taken daily to determine the stage of the estrous cycle. The care and manipulation of the animals were done in accordance with the ethical guidelines of our Faculty Animal Experimental Committee.

RNA preparation. The rat oviductal epithelium was obtained mechanically following the procedure described by Morales et al. (37). Briefly, oviducts were removed and placed in Hanks’ solution at pH 7.4. Oviducts were cut into 4- to 8-mm2 pieces and placed in MEM solution containing 5 mM EDTA for 30–40 min at 37°C. Tissue pieces were transferred to sterile Hanks’ solution, and the epithelium of each tissue piece was mechanically removed from the rest of the tissue. Total RNA was isolated using acid guanidium thiocyanate-phenol-chloroform extraction as described by Chomczynski and Sac-
chi (6). Total RNA was quantified by spectrophotometry, and its quality was checked by electrophoresis in agarose gels. mRNAs were isolated from total RNA by standard procedures using an oligo(dT) cellulose affinity column (Sigma Chemical; St. Louis, MO).

**Osmotic water permeability assay.** The method used is based on real-time quantitative imaging described by Zhang et al. (54). Oocytes at stages V and VI were harvested from Xenopus laevis and defolliculated by incubation for 1–3 h in collagenase (1.5 mg/ml type I, Worthington Biochemicals; Freehold, NJ) in the presence of trypsin inhibitor (0.5 mg/ml type III-0, Sigma Chemical). Oocytes were injected using an automatic Eppendorf microinjection system on the day after isolation with either 100 nl of water or mRNA (2 ng/ml) and incubated at 18°C for 72 h in modified Barth’s buffer solution [in mM: 88 NaCl, 1 KCl, 0.82 MgSO4, 0.33 Ca(NO3)2, 0.41 CaCl2, 2.4 NaHCO3, and 10 HEPES; pH 7.4, 200 mosM]. Oocytes were transfected from 200 to 10 mosM of the modified Barth’s buffer solution at 25°C, and oocyte swelling was monitored by videomicroscopy using a Nikon inverted phase contrast microscope equipped with a Sony video camera operating at a fixed gain. The images were recorded in a computer through Data Translation hardware boards for analysis. The time course of oocytes swelling was obtained by plotting the relative oocyte volume as a function of time. Relative oocyte volume (V/V0) was calculated in 250-ms intervals from the respective oocyte area (A/A0) in the focal plane using the equation V/V0 = (A/A0)1/2. Oocyte area was determined using Imagepro software.

The coefficient of osmotic water permeability (P0, in cm/s × 10−4) was calculated from the initial 15- to 30-s response of oocyte swelling using the equation P0 = [V0 × d(V/V0)/dt]/[S × V × (osm0 − osm0)], where V0 is the initial oocyte volume and equaled 9 × 10−4 cm3; S is the initial oocyte surface area and equaled 0.045 cm2; Vw is the molar ratio of water and equaled 18 cm3/mol, and osm0 is 200 mosM and osm0,tot is 40 mosM. The inhibition of water channel function was determined by incubation of oocytes in Barth’s buffer solution containing HgCl2 (0.3 mM) for 5 min before the swelling assay was performed in the presence of HgCl2. The reversibility of water channel function inhibition was determined by incubation of oocytes with 2-mercaptoethanol (5 mM) for 15 min after the HgCl2 treatment.

**RT-PCR detection of AQP mRNA.** RT was performed using total RNA (2 μg) previously treated with DNase I amplification grade for 15 min at 25°C. DNase activity was stopped by the addition of 1 μl EDTA (25 mM) and heating to 70°C for 15 min. One microliter of oligo-dT (10 μM) was added, and the mixture was heated for 10 min at 70°C. Seven microliters of a solution containing 1 μl dNTPs (10 mM), 2 μl first-strand buffer (×5), and DTT (100 mM) were added. The reaction mixture was heated for 2 min at 42°C, followed by the addition of 100 units of SuperScript II enzyme. RT was achieved by heating the reaction mixture for 50 min at 42°C and then for 15 min at 70°C. The final volume of the RT reaction was 20 μl. An extra reaction mixture without SuperScript II enzyme was used as a control for DNA contamination. PCR experiments for each AQP and β-actin were performed using 2 μl of the RT product plus 23 μl of PCR mix containing specific primers (0.4 μM). Primer sequences were obtained from Ford et al. (13). The PCR amplification cycle consisted of 30 s for denaturing at 95°C, followed by 30 s for annealing with varying temperatures according to the primers used and 45 s for the extension reaction at 72°C. After 40 cycles, final products were extended for 5 min at 72°C. The annealing temperature and the expected product sizes for each cDNA were as follows: AQP2, 56°C and 277 bp; AQP3, 65°C and 645 bp; AQPS, 65°C and 441 bp; AQP8, 60°C and 433 bp; AQP9, 65°C and 374 bp; and β-actin, 56°C and 281 bp. All reagents for RT-PCR were from Gibco-BRL Life Technologies (Gaithersburg, MD). AQP2s and β-actin amplified cDNA fragments were resolved in agarose gels (1.5%) and revealed by ethidium bromide staining, and their electrophoretic migration was compared against a 100-bp DNA ladder (Winkler; Santiago, Chile). Total RNA isolated from the rat kidney, lung, colon, and liver was used as a positive control for the detection of AQP PCR products. PCR products were isolated from a low melting agarose gel and purified using the Wizard PCR Prep DNA Purification System (Promega; Madison, WI). Their identity was confirmed to correspond to fragments from rat AQPs by automated sequencing using an ABI Prism310 sequencer (Perkin-Elmer) as described by Muscillo et al. (38).

Relative levels of AQPs mRNA were measured by semiquantitative RT-PCR using 25 and 24 cycles for AQP2 and β-actin, respectively. For an improved quantitative estimation, PCR products were electrophoresed in 12% polyacrylamide gels (47), revealed by silver staining (Winkler), and scanned with a Bio-Rad model GS-700 imaging densitometer (Bio-Rad; Hercules, CA). The optical density of bands was quantified using NIH Image 1.61 software. Values were normalized against those of β-actin.

**Animal castration and hormonal treatment.** Animals were anesthetized using a mixture of ketamine-xylacine (80/10 mg/kg im), after which they underwent surgical removal of the ovaries. One week after surgery, ovariectomized rats were injected subcutaneously with either 5 μg E2 (Sigma Chemical) in 100 μl propylene glycol, 5 mg P4 (Sigma Chemical) in 500 μl olive oil, 5 μg E2 followed 6 h later by an administration of 5 mg P4, or 5 mg P4 followed 6 h later by another administration of 5 mg P4. Control animals were injected with the respective vehicles. Twenty-one hours after single hormone administration or 15 h after the second hormone administration, anesthetized rats were killed by cervical dislocation, and their oviducts were removed. The dose of 5 μg E2 was selected as it reflected the physiological estrogen plasma levels (11, 44). Because the ratio of the maximal plasma levels of estrogen with respect to that of P4 during proestrus is ~1:1,000 (44), we used 5 mg P4. Furthermore, these doses have been previously reported to produce estrogenic and progesterogen responses in the reproductive tract (20) and other organs (29).

**Tissue fixation, immunohistochemistry, and immunofluorescence.** To obtain the organs used as positive controls, anesthetized rats were perfused via the left cardiac ventricle with 1× PBS (pH 7.4), followed by 3% paraformaldehyde, 75 mM lysine, and 10 mM periodate (PLP) fixation for 15 min. Organs were removed, minced into small pieces, and further fixed with PLP for 1 h at room temperature. Oviducts were removed from anesthetized rats and separated into ampullar and isthmic sections for a dissection microscope. Each segment was fixed in PLP for 1 h at room temperature. Organ pieces were mounted in tissue freezing medium (Electron Microscopy Science), and cryosections (5 μm thick) were obtained. Endogenous peroxidase activity was blocked by incubation for 15 min at room temperature with 5% H2O2 in methanol. Sections were incubated for 10 min at 80°C in 1 mM Tris-HCl and 0.5 mM EDTA (pH 9.5) for antigen recovery. Sections were blocked for 1 h at room temperature with 1% immunoglobulin-free BSA (Sigma Chemical) dissolved in a mixture of 25% rat serum, 25% goat serum, and 50% TCT [Tris-HCl (pH 7.6), 0.7% carragenan, and 0.25% Triton X-100]. Sections were incubated overnight at 4°C with primary antibody dilution (Alpha Diagnostic) in blocking solution. Tissues were washed three times, 10 min each, with 0.02% Triton X-100 in Tris-buffered saline (TTBS) and then incubated for 1 h at room temperature with secondary goat anti-rabbit IgG biotinylated antibody (DAKO). Tissues were washed three times, 10 min each, with TTBS, followed by incubation for 1 h at room temperature with streptavidin-peroxidase complex (DAKO). Tissues were further washed with TTBS, and color development was achieved by incubation for 15 min in 0.5 mg/ml diaminobenzidine-0.1% H2O2 in TBS. The reaction was stopped by washing tissue in tap water, followed by distilled water. Tissues were counterstained with hematoxylin (Merck; Darmstadt, Germany), mounted on glass slides with Permount (Merck) and observed under a Nikon labophot-2 microscope with white light illumination. The specificity of the immuno-reactivity was assessed by preabsorbing each antibody dilution with its corresponding antigenic peptide.
Fig. 1. Characterization of aquaporin (AQP9)–specific recognition by polyclonal serum A and B: immunoreactivity of AQP9 in testis sections using serum A (A) or preabsorbed serum A with antigenic peptide (B). Bar = 30 μm.

For colocalization studies, double immunofluorescence assays were done using serum A (see below) to detect AQP9 and a polyclonal goat anti-rat Mucin 1 antibody (Santa Cruz Biootechnology; Santa Cruz, CA) to label the apical plasma membrane or the oviduct. Immunofluorescence was revealed using cy2-labeled and rhodamine-labeled F(ab′)2 fragments of goat anti-rabbit and donkey anti-goat IgGs, respectively (Jackson Immuno Research Laboratories; West Grove, PA). Tissue sections were mounted in glycerol containing 1 mg/ml DABCO (1,4-diazabicyclo[2,2,2]octane, Sigma Chemical) dissolved in PBS. Samples were observed under a Zeiss LSM 510 confocal microscope equipped with argon and helio/neon lasers. Excitation wavelengths of 488 and 543 nm, and beam path controls BP 505–530 and LP 560 were used for detecting green and red fluorophores, respectively.

**Western blot analysis.** Total membrane preparations from the rat testis and liver were obtained by tissue homogenization in 7.5 mM phosphate buffer containing 0.3 M sucrose, 1 mM EDTA, 3 mM PMSF, 10 μM leupeptin, 0.7 μM aprotinin, and 7 μM pepstatin. Homogenates were centrifuged at 1,000 g for 10 min at 4°C, and the supernatant was then centrifuged at 200,000 g for 1 h at 4°C. The pellet was dissolved in buffer A containing 20 mM Tris-HCl (pH 8), 5 mM EDTA, and 2% SDS by passing it through a 21-gauge syringe several times. Oviductal epithelial cells were collected in the homogenization phosphate buffer at 4°C and centrifuged for 1 min at maximal speed in an Eppendorf centrifuge. The pellet was suspended in buffer A, and proteins were sonicated in position 1 (Microson ultrasonic cell disrupter, Heat Systems; Farmingdale, NY). Proteins were measured in aliquots by the Lowry method (Bio-Rad). After protein samples (200 μg) were mixed with Laemmli buffer, they were boiled immediately for 4 min and resolved by 12% SDS-4 M urea PAGE. Low-range prestained molecular weight standards were used (Bio-Rad). Gels were then blotted onto nitrocellulose membranes at 300 mA for 80 min. Blots were then incubated in 5% nonfat milk in TBS-Tween and then incubated overnight at 4°C either with an affinity purified rabbit anti-AQP9 polyclonal antibody (Chemicon; Temecula, CA) diluted 1:200 in TBS or with a 70 M excess of the antigenic peptide, this label was lost (Fig. 1).

**RESULTS**

Oviductal epithelial cells express mRNAs coding for functional water channels. Osmotic water permeability of oocytes injected with epithelial mRNA (Pf-E) was comparable to that of oocytes microinjected with mRNA from the renal cortex, which was used as a positive control for the expression of AQPs. Figure 2 illustrates that Pf-E was five times greater compared with water-injected oocytes, indicating that epithelial cells express mRNAs that code for water channels. Pf-E was partially inhibited by incubation in 0.3 mM HgCl2. Under this condition, the Pf-E value was similar to that of Pf of skeletal muscle RNA-injected oocytes. The mercurial inhibition was partially reversed by the subsequent incubation in 5 MKAEPSENLEKHELSVIM corresponding to the COOH-terminal amino acids 277–295 of rat AQP9 coupled to hemocyanin (BiosChile Ingenierı ´a Gene ´tica; Santiago, Chile). Serum A was applicable for immunohistochemistry but not for Western blot analysis. When tested in sections of the rat testis, this antibody labeled mainly Leydig cells, as described by Elkjaer et al. (8). After antibody preabsorption with a 20 M excess of the antigenic peptide, this label was lost (Fig. 1).

Fig. 2. Osmotic water permeability (Pf) and sensitivity to Hg2+ inhibition of oocytes microinjected with mRNA isolated from rat oviductal epithelium. Pf was calculated for oocytes microinjected with water or water containing mRNAs isolated from the rat renal cortex, oviductal epithelium, and skeletal muscle as described in MATERIALS AND METHODS. In oocytes injected with oviductal epithelial mRNA, osmotic swelling was also determined after incubation with 0.3 mM HgCl2 (epithelium + Hg) or 0.3 mM HgCl2 followed by incubation with 5 mM β-mercaptoethanol (epithelium + Hg + ME). Each plotted value corresponds to the mean value ± SD obtained in the number of independent experiments indicated in parentheses within each column. *P < 0.01 and **P < 0.05, statistically significant values, respectively, according to Student’s t-test.
mM β-mercaptoethanol (Fig. 2). These results indicate that most of the oocyte swelling observed was mediated by water channels formed mainly by the expression of mercury-sensitive channel proteins.

**Expression of AQPs in epithelial tissue of the rat oviduct.** Because functional expression experiments indicated that the oviduct epithelium expressed mRNA coding for Hg\(^{2+}\)-sensitive water channels, we focused our research on AQP2, -3, -5, -8, and -9. We did not include AQP1 because it has been previously detected only in the muscle layer and blood vessels of the rat oviduct (16). We analyzed epithelial RNA samples from proestrus, the stage preceding maximal water content in oviductal fluid. RT-PCR experiments were performed using specific primers for each AQP. Positive control products for each analyzed AQP were detected at the expected size in total RNA isolated from the kidney (AQP2 and -3), lung (AQP5), colon (AQP8), and testis (AQP9) (Fig. 3). RT-PCR products obtained from total RNA isolated from oviductal epithelial cells revealed the presence of mRNA for AQP5, -8, and -9 but not AQP2 and -3 (Fig. 3). We validated the identity of our RT-PCR products by sequencing each isolated band, demonstrating 100% identity to those reported in the NIH databank.

To evaluate whether the detected mRNAs are translated, immunostaining for AQP5, -8, and -9 was analyzed in ampullar and isthmic segments of the oviduct. AQP5, -8, and -9 immunoreactivity was detected in both segments only in epithelial cells (Fig. 4). AQP5 and -8 immunoreactivity was present in the cytoplasm, whereas that of AQP9 was restricted to the apical membrane of epithelial cells (Fig. 4). The plasma membrane localization of AQP9 was confirmed by confocal microscopy using an antibody directed against Mucin-1 as an apical membrane marker (28, 45). Colocalization of the red and green fluorescence was observed widely along the luminal membrane in both segments of the oviduct (Fig. 5). Immunoreactivity of AQP9 was detected along the oviduct during all stages of the estrous cycle (Table 1). However, AQP5 was absent in the ampulla during estrus and proestrus, and AQP8 was absent in the ampulla and isthmus during estrus and in the ampulla during diestrus (Table 1).

The expression of AQP5, -8, and -9 was suppressed by ovariectomy, and AQP9 was restored by estradiol and/or progesterone. To study whether the expression of the detected AQPs is under hormonal control, their immunoreactivity was studied in ovariectomized rats as well as in ovariectomized rats injected with E\(_2\), P\(_4\), or a combination of both hormones. In ovariectomized rats, epithelial cells of the ampulla and isthmus did not present immunoreactivity for AQP5 (not shown), AQP8 (not shown), or AQP9 (Fig. 6, vehicle). The direct correlation between ovariectomy and lack of expression of this subset of AQPs indicated that sexual hormones may influence their expression in the oviduct. To further investigate this hypothesis, different regimens of hormonal replacement were administered to ovariectomized rats (see MATERIALS AND METHODS). Neither AQP5 nor AQP8 were detected in oviducts of ovariectomized rats treated with E\(_2\) and/or P\(_4\) (not shown). However, AQP9 labeling was detected, mainly in the isthmus, after 21 h of a single E\(_2\) administration (Fig. 6, E). In the same time interval, a single P\(_4\) dose did not restore the immunoreactivity for AQP9 (Fig. 9, F). To mimic the hormonal plasma level peaks in cycling rats, a dose of P\(_4\) was administered to ovariectomized rats 6 h after a priming dose of E\(_2\), and samples were obtained 15 h later. Under these conditions, a more intense AQP9 reactivity was observed in both segments, with an even higher intensity in the isthmus (Fig. 6, EP). If the priming hormone was P\(_4\) followed by a second administration of P\(_4\), the immunoreactivity of AQP9 was also reestablished in both oviductal segments (Fig. 6, PP). AQP9 immunoreactivity was negligible 15 h after a single administration of E\(_2\) or not detected after P\(_4\), respectively (not shown). Protein levels of AQP9 detected by Western blot analysis correlated with the immunohistochemical data (Fig. 6, A–C). The highest value of AQP9 expression was obtained after the E\(_2\) and P\(_4\) injections, and no protein was detected after a single P\(_4\) administration. E\(_2\) alone and P\(_4\) followed by P\(_4\) induced only 20% and ~70% of the maximal response on AQP9 expression, respectively. We also observed statistically significant differences on AQP9 levels during the estrous cycle (Fig. 7), with their highest levels during estrus and proestrus.

E\(_2\), but not P\(_4\) upregulates AQP9 by increasing the levels of AQP9 mRNA. Because hormone administration to ovariectomized rats induced the synthesis de novo of AQP9, we studied the effect of these hormone combinations on its mRNA levels. Using semiquantitative RT-PCR, we measured the relative levels of AQP9 mRNA versus β-actin mRNA. After 15 h treatment with E\(_2\) or E\(_2\) followed by P\(_4\), AQP9 mRNA levels were statistically significantly higher than in animals injected with vehicle (Fig. 8). However, AQP9 mRNA levels did not increase after a single or two consecutive injections of P\(_4\) (Fig. 8). The latter treatment did not increase AQP9 mRNA levels even at earlier time intervals (12, 9, or 6 h after the second administration; data not shown).

![Fig. 3.](http://ajpcell.physiology.org/) mRNA identification of Hg\(^{2+}\)-sensitive AQPs expressed by epithelial cells of the oviduct. Total RNA obtained from the oviductal epithelium of rats in proestrus was used for the RT-PCR amplification of AQP2 (lane 2), AQP3 (lane 4), AQP5 (lane 8), AQP8 (lane 10), AQP9 (lane 15), and β-actin (lanes 6, 13, and 17) mRNAs. Total RNA obtained from the rat kidney was used as a positive control for the amplification of AQP2 (lane 1) and AQP3 (lane 3) mRNAs. The rat lung, colon, and liver were used as positive control tissues for AQP5 (lane 7), AQP8 (lane 9), and AQP9 (lane 14) amplification products, respectively. The β-actin amplification band from the rat kidney (lane 5), lung (lane 11), colon (lane 12), and liver (lane 16) is also shown. MW, lanes containing the 100-bp DNA ladder.
DISCUSSION

In the present study, we identified three water channel proteins (AQP5, -8, and -9) in the rat oviduct using molecular and immunological techniques. AQP5, -8, and -9 are expressed by the oviductal epithelium, and AQP9 is restricted to the apical membrane. We also report that AQP5, -8, and -9 are under the control of the ovary, because their immunoreactivity was lost after ovariectomy. However, E2 and/or P4 administered to ovariectomized rats only restored the expression of AQP9. Furthermore, this effect is mediated by E2 upregulation of AQP9 mRNA levels, whereas P4 would increase protein expression without a change in its mRNA levels.

Herein, we report, for the first time in the mammalian oviduct, the expression of AQP5, -8, and -9 in epithelial cells. The presence of AQP9 in the membrane suggests that it may be, at least, one of the responsible components of water movement toward the lumen of the oviduct. Thus its detection unravels a possible mechanism to the still-unexplained phenomenon of oviductal fluid formation. A similar role in water transport has been suggested for epithelial AQPs in the uterine fluid secretion (24) and for apical AQPs in exocrine glands and respiratory epithelial cells (2). Although AQP2 and -3 have been proposed as responsible for water movement in the uterus (24), our results indicate that this role in the oviduct may rely...
mainly on AQP9 and probably on AQP5 and -8. This differential pattern of expression of AQPs between the epithelium of the oviduct and uterus is similar to the heterogeneity observed within different segments of the renal (39), respiratory (27), and gastrointestinal epithelium (32). Other epithelial proteins, such as MUC-1, have a varying pattern of expression along the reproductive tract epithelium and during the menstrual cycle, suggesting a correlation with its putative antiadhesive function (25). In a similar manner, AQP5 and -8 are present or absent in the oviduct at different stages of the estrous cycle, and AQP9, although always present, has varied expression levels also within the cycle. As demonstrated in other organs (39, 18, 53), epithelial oviductal cells express more than one AQP. This is possibly related to their differential permeability as AQP5 and -8 are water-selective channels, whereas AQP9 is also permeable to glycerol and other small neutral solutes (50, 2). Apical localization of AQP9 channels and their permeability properties may indicate that their function is related not only to water transport but also to metabolite secretion or absorption from the luminal fluid to maintain the appropriate environment for reproductive functions (26). Alternatively, each AQP may be sorted to different cell membrane compartments to accomplish transcellular water transport, as has been demonstrated in kidney epithelial cells (39). In oviductal epithelial cells, AQP9 localization to luminal plasma membrane is equivalent to that found in epithelial cells along the male reproductive tract (41). Microtubule disruption in the epididymus does not affect its cell surface localization, suggesting that AQP9 membrane insertion is constitutive and does not involve a regulated vesicle trafficking mechanism (41). Currently, AQP5 has been detected only in the apical membrane of epithelial cells in various organs (27, 4). On the other hand, AQP8 has been

Fig. 5. AQP9 is located in the apical plasma membrane of the oviduct. Immunoreactivity to Mucin-1 (A and D) and AQP9 (B and E) was detected by double indirect immunofluorescence and confocal microscopy in sections of the ampulla (A–C) and isthmus (D–F). The respective merge images are shown in C and E. Bar = 50 μm.

Table 1. AQPs immunoreactivity associated to oviductal epithelium in cycling rats

<table>
<thead>
<tr>
<th>Estrus</th>
<th>Diestrus 1</th>
<th>Diestrus 2</th>
<th>Proestrus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ampulla</td>
<td>Isthmus</td>
<td>Ampulla</td>
</tr>
<tr>
<td>AQP5</td>
<td>–</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>AQP8</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>AQP9</td>
<td>+</td>
<td>–</td>
<td>+</td>
</tr>
</tbody>
</table>

Oviduct sections obtained from rats in different days of the estrous cycle were prepared for immunohistochemical detection of aquaporin (AQP)5, -8, and -9 as described in MATERIALS AND METHODS. + and –, positive and negative immunoreactivity, respectively. At least three sections of the ampulla or isthmus from four different animals for each stage of the estrous cycle were analyzed.
detected in the basolateral and apical membranes (53, 4) and also in cytoplasmic vesicles in different tissues (15, 7). Recently, it has been demonstrated that in hepatocytes, cytoplasmic AQP8 may redistribute to the plasma membrane after stimulation with glucagon or cAMP (33, 17), suggesting that regulated trafficking to the plasma membrane might also occur in oviductal epithelium. In our system, although it remains to be determined the precise localization of AQP5 and -8 within the epithelial cell, it is possible according to previous reports that AQP5 might localize mainly in the apical membrane (27, 4) and that AQP8 may localize either in the basolateral or apical membrane (4, 33, 53). Furthermore, the variable presence of AQP5 and -8 compared with the permanent expression of AQP9 along the oviductal epithelium during the estrous cycle (Table 1), together with their distinctive permeability properties, suggest a different role for each AQP channel in fluid formation in the oviduct.

Differential levels of AQP9 were detected throughout the estrous cycle with the highest levels detected in estrus and proestrus. The high AQP9 levels correlate with those stages where water content in the oviductal fluid is elevated (30, 23). Cyclic changes in AQP9 levels could result in variations of water permeability of the apical membrane of the oviductal epithelium. This could explain the higher rate of fluid secretion...
into the lumen of the oviduct detected in estrous compared with the luteal phase in many mammalian species (23). Furthermore, fluid accumulation reaches its maximum around the estrus, but it declines after ovariectomy (23). However, the latter effect can be counteracted by a systemic administration of E2 (35). In our study, we detected that the expression of epithelial AQP5, -8, and -9 was lost in ovariectomized rats. Only AQP9 expression was restored after a specific combination and timing of E2 and/or P4 administrations, further supporting a role for AQP9 in fluid formation. The latter suggests that sex-steroid hormones control AQP9 expression and that other molecules produced by the ovary or by other organ in an ovary-dependent manner could control AQP5 and -8 expression. Similar posttranscriptional mechanisms have been proposed to occur in the estrous cycle-dependent expression of c-Fos in the rat uterine epithelium (36). Also, a posttranscriptional regulation of the E2- and P4-dependent expression of microtubule-associated protein-2 was observed (41). Other authors have also reported that not all AQP proteins or mRNAs lost after ovariectomy are restored (43) and that different AQPs are expressed after ovarian hormone replacement (24). These observations indicate that each AQP may need a specific combination or sequence of ovarian hormones to reproduce the physiological conditions required for their expression. However, it remains to be addressed whether water channels formed by de novo synthesis of AQP9 after E2, P4, or combined hormone replacements are functional channels. Interestingly, hormonal regulation of AQPs only occurs in epithelial cells of the uterus but not in other cell types of this organ also expressing AQPs (24). This suggests that the control of water transport relies on cells lining the tubal lumen. Thus further clarification of the contribution of each AQP channel to the transcellular water transport through the genital tract epithelium is needed to understand the variations in water volume at different stages of the reproductive cycle.

The increased AQP9 levels detected under hormonal replacement treatments following ovariectomy did not always parallel changes in mRNA levels, suggesting that E2 and P4 treatments triggered different mechanisms for AQP9 upregulation. We observed that, whereas similar mRNA levels were detected after the administration of E2 alone or of E2 followed by P4, protein levels were further elevated in the latter treatment. Moreover, two consecutive administrations of P4 increased protein levels but slightly decreased AQP9 mRNA levels. Therefore, our results suggest that E2 may trigger a mechanism that increases mRNA levels by activating its transcription rate or inhibiting its degradation. On the other hand, P4 administered after priming with E2 or P4 may trigger a mechanism to activate AQP translation. Similar posttranscriptional mechanisms have been proposed to occur in the estrous cycle-dependent expression of c-Fos in the rat uterine epithelium (36). Also, a posttranscriptional regulation of the E2- and P4-dependent expression of microtubule-associated protein-2 was observed (41). Other authors have also reported that not all AQP proteins or mRNAs lost after ovariectomy are restored (43) and that different AQPs are expressed after ovarian hormone replacement (24). These observations indicate that each AQP may need a specific combination or

Fig. 7. AQP9 levels along the reproductive cycle. A: Western blot analysis of protein samples from total epithelium proteins of oviducts obtained from rats in estrus (E), diestrus 1 (D1), diestrus 2 (D2), and proestrus (P). Testis (T) and liver (L) total membrane proteins were used as positive controls for the detection of AQP9. B and C: a parallel Western blot analysis was made using the primary antibody preabsorbed with the antigenic peptide (B) or the gel was stained with Coomassie blue (C). D: graph showing relative levels of AQP9 versus total proteins. Bars correspond to mean values ± SD of three independent experiments. All conditions were statistically significant different values (P < 0.05) according to the Kruskal-Wallis test followed by Mann-Whitney analysis for nonparametric data.

Fig. 8. Relative levels of AQP9 mRNA are upregulated by 17β-estradiol but not by progesterone. Semiquantitative RT-PCR analysis was performed using total RNA extracted from oviducts of rats injected with vehicle (C), a single dose of 5 μg 17β-estradiol (E) or 5 mg progesterone (P), or a first dose of 5 μg 17β-estradiol or 5 mg progesterone followed 6 h later by a second injection with 5 mg progesterone (EP and PP, respectively). Rats were killed 15 h after the single or second injection, respectively. Each bar in the graph represents the mean ± SD of the relative band densities of AQP9 versus β-actin mRNA obtained in different experiments (n = 3 rats). *P < 0.05, statistically significant values with respect to C according to the Kruskal-Wallis test followed by Mann-Whitney analysis for nonparametric data.
in hippocampal neurons has been described in ovariectomized rats (42). Although a single P4 administration to ovariectomized rats did not induce AQP9 expression, priming with P4 induced AQP9 synthesis to similar levels compared with those detected after a priming injection of E2. This effect on protein expression was not preceded by mRNA increased levels. We discarded the possibility of missing a previous and transient expression, after two consecutive P4 injections, by analyzing AQP9 mRNA levels at smaller time intervals. In accordance with our results, P4 administered alone reduces water channel function in assays utilizing mRNAs isolated from Bufo arenarum oocytes and urinary bladder (12), possibly through a mechanism that reduces the transcription of yet unidentified AQP mRNAs. This is in agreement with previous observations showing that in ovariectomized ewes, fluid formation was not restored after a single progesterone administration (35). However, it has been established the importance of hormonal priming in protein expression and cellular responses to different hormonal treatments. In fact, similar to our observations on the priming effect of P4, it has been reported that E2 administration induces AQP expression in uteri of ovariectomized mice only after P4 priming (43). Furthermore, P4 priming differentially affects P4 single treatment on the proliferative/differentiative response in breast cancer cells (19). These data support our results that indicate a differential response of epithelial cells of the oviduct after P4 administration alone or after a priming hormone dose.

No sex hormone response elements in the AQP9 gene have been described; however, a binding motif for glucocorticoid receptor has been found (51). Therefore, the possibility that sexual hormones directly regulate the transcription of AQPs needs further analysis. Besides a direct effect of hormones on the promoter activity of AQPs, their effect could be mediated through an indirect pathway that involves the expression of other intracellular mediators. Permeability regulators that vary along the reproductive cycle, such as VEGF receptor transcripts and platelet-activating factor receptor expressed by epithelial cells of the oviduct, are possible candidates to explain this mechanism (14, 49).

In this study, we demonstrated, for the first time, the expression of AQP5, -8, and -9 in the oviductal epithelium and that their presence depends on ovarian signals. Moreover, we demonstrate that steroid hormones control AQP9 expression most probably by regulating mRNA levels and protein translation. Taken together, our results provide new evidence to suggest the involvement of AQP water channels in water transport in the oviductal epithelium. Thus the hormonal fine tuning of oviductal fluid characteristics, mediated in part by the regulation of water channels expression, will determine the success of fertilization and early embryonic development.

ACKNOWLEDGMENTS

The authors thank María E. Ortiz for valuable experience in rat ovariectomy, Nelly Farías for technical assistance in image processing, and Daniel Valdés for assistance with the confocal microscopy imaging.

GRANTS

This study was supported by Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT) Postdoctoral Grant 3030066 (to M. C. Brañas), Fondo de Investigación Avanzada en Áreas Prioritarias, Center in Biomedicine, Postdoctoral Fellowship 13980001 (to M. C. Brañas), and FONDECYT Grants 8980008 (to M. J. Villalón), 1040804 (to M. J. Villalón), 1030315 (to M. Risso), and 1030220 (to B. Morales).

REFERENCES