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# Expression of Aquaporin 9 in the Adult Rat Epididymal Epithelium Is Modulated by Androgens[1]

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## ABSTRACT

**Reabsorption of fluid and solutes across the epithelium lining the male excurrent duct is important for adequate sperm maturation, concentration, and storage. Water channels contribute to water movement across epithelia in many tissues. Aquaporin 9 (AQP9) is abundantly expressed in the apical membrane of principal cells that line the epididymis, and in reabsorptive and secretory epithelial cells of the male reproductive tract. In this study we show that the nonsteroidal antiandrogen flutamide, given to adult rats at a dose of 50 mg $kg^{-1}$ $day^{-1}$ for 2 wk via osmotic minipumps significantly decreased the amount of AQP9 in the epididymis. This down-regulation was observed by immunofluorescence of cryostat tissue sections and by Western blotting of epididymal brush border membrane preparations. In addition, castrated adult rats showed lower levels of epididymal AQP9 compared with adult controls, whereas systemic testosterone treatment of castrated adult rats induced a recovery of the expression of AQP9 to control levels. These data indicate that the expression of AQP9, a likely candidate for apical transepithelial fluid and solute transport in several regions of the male reproductive tract, is modulated by androgens in the adult rat epididymis.**

*AQP9, castrated rats, epididymis, flutamide, orchiectomy*

## INTRODUCTION

Spermatozoa develop motility and the ability to fertilize ova as they pass through the male excurrent ducts, including the epididymis, and the composition of the luminal environment of these organs is tightly regulated [1–5]. Significant fluid reabsorption occurs in the efferent duct and epididymis [6–10], and the concentration of sperm in the lumen increases significantly [2–5]. In the efferent duct, isotonic fluid reabsorption with net sodium and chloride reabsorption occurs [6, 11–14]. This process requires various transport proteins, including basolateral Na/K-ATPase and the apical Na/H exchanger, NHE3 [15–18]. In the cauda epididymidis, a significant proportion of fluid transport depends on luminal sodium and is inhibited by amiloride and ouabain, indicating a role for Na/H exchange and Na/K-ATPase activity in the more distal region of the epididymis [19].

In addition to ion and solute transport proteins, water channels (aquaporins, AQPs) contribute to transepithelial water movement in many tissues, including the kidney and the efferent ducts [20, 21]. In the efferent ducts, where 50%–90% of the seminiferous tubule fluid is reabsorbed [6–8, 11], there is abundant expression of AQP1 [22, 23]. In this respect the efferent ducts resemble the renal proximal tubules, which reabsorb up to 80% of the glomerular ultrafiltrate and also express abundant AQP1 [24, 25]. The ampulla of the vas deferens also contains AQP1 [22]. Another aquaporin, AQP2, is present in the vas deferens where it is constitutively expressed on the apical membrane of principal cells [26], in contrast to its vasopressin-regulated membrane expression in the kidney collecting duct [27].

Recent reports have shown that AQP9, a broadly selective neutral solute and water channel [28–30], is abundant in the brush border membrane (BBM) of epididymal principal cells [31, 32], as well as in the efferent ducts and vas deferens [32]. These findings identified AQP9 as a major apical aquaporin in these epithelia, providing a potential route via which transepithelial fluid and solute transport could occur.

Reabsorption of luminal fluid in some regions of the male reproductive tract is under steroid hormone control, and both androgens and estrogens may be involved. Compared with wild type mice, fluid reabsorption is markedly decreased in the efferent ducts of mice that lack the estrogen receptor-α (ERKO) [33, 34]. In addition, chronic treatment of rats with the estrogenic compound diethylstilbestrol (DES) can cause alterations in morphology and in the expression of epithelial proteins in the male reproductive tract [35]. Levels of the AQP1 water channel are significantly lower in the efferent ducts of these animals [23]. A recent study showed that estrogen, acting through both ERα and ERβ, modulates fluid reabsorption in the adult mouse efferent duct [15]. In addition to estrogens, androgens also affect many functions of the male excurrent duct epithelia, including protein synthesis, potassium secretion, and ion transport [9, 36–38]. Fluid reabsorption is reduced in castrated rats, and this effect is reversed by testosterone administration [9]. In addition, treatment with the nonsteroidal antiandrogen flutamide can elevate both pH and bicarbonate concentration in the lumen of the epididymis in situ, leading to the conclusion that luminal acidification in the rat epididymis is also under androgen control [39].

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AQP9 is present in the apical membrane of the epithelial cells of the male excurrent ducts throughout postnatal development in rat [32]. This suggests that AQP9 expression in these epithelia does not absolutely depend on the higher androgen levels that are reached during and after sexual maturation. However, it is possible that AQP9 expression could be induced by androgens present during prenatal and perinatal development in the male, either in the plasma or in situ in the epididymis [40–43]. The aim of the present study was to determine whether androgens have a role in the regulation of AQP9 expression in the adult rat epididymal epithelium. For this purpose, adult rats were either treated with flutamide, which acts by inhibiting androgen uptake or by inhibiting nuclear binding of androgen in the target tissues [39], or were castrated and supplemented with systemic testosterone. The expression of AQP9 in the epididymis was monitored by immunofluorescence in cryostat sections of the proximal cauda epididymidis, where this protein shows high expression, and by Western blotting of BBMs isolated from the entire epididymis.

## MATERIALS AND METHODS

### Experimental Animals

Animal experiments were approved by the Institutional Committee on Research Animal Care of the respective universities, in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals.*

### Flutamide-Treated Adult Rats

Adult Sprague-Dawley male rats (8 wk old, n = 5) were treated (s.c.) with flutamide via implanted osmotic minipumps (Alzet, Cupertino, CA) at a 50 mg $kg^{-1}$ $day^{-1}$ dose for 2 wk. The minipumps were placed under anesthesia using sterile technique. Flutamide was dissolved in polyethylene glycol MW 300 (Acros Organics, Fair Lawn, NJ). The control animals (n = 6) received vehicle alone also via osmotic minipump for the same period of time.

### Castrated Rats with Subsequent Hormonal Treatment

Adult Wistar male rats (12 wk old, n = 9) were anesthetized (i.p.) with sodium pentobarbital (65 mg/kg body weight [BW]), after which they underwent orchiectomy (n = 6). Control rats were sham-operated (n = 3). One week after surgery, 3 castrated animals and the 3 sham-operated rats (controls) were treated with sunflower oil for the following 8 days (0.3 ml/kg BW s.c., once a day), whereas the remaining 3 castrated animals were treated with testosterone enanthate, a long-acting testosterone derivative (RotexMedica GmbH, Trittau, Germany), suspended in sunflower oil (1.5 mg/kg BW s.c. per day) during the same time period.

### Tissue Fixation and Immunocytochemistry

*Tissue preparation..* Rats were anesthetized using sodium pentobarbital (65 mg/kg BW i.p.) and the male reproductive organs were perfused via left cardiac ventricle with 1× PBS pH 7.4, followed by paraformaldehyde-lysine-periodate (PLP) fixation for 5 min. The original PLP recipe [44] was modified to increase the paraformaldehyde concentration from 2% to 4%. The epididymides were removed and placed in PLP buffer at room temperature for 4–6 h or overnight at 4°C. Tissue was then washed three times in PBS and kept in PBS containing 0.02% sodium azide at 4°C prior to use.

*Antibodies..* The AQP9 antibody used for Western blotting and immunocytochemistry was generated by immunization of rabbits with a C-terminal peptide from rat AQP9 [32, 45]. The peptide was coupled to keyhole limpet hemocyanin (KLH), and antibodies were raised in rabbits using standard protocols. The antibody was affinity-purified from whole serum using the SulfoLink Kit (Pierce, Rockford, IL). This antibody has been characterized previously [32].

The $H^+$-ATPase antibody against the C-terminal 14 amino acids of the 31 kDa subunit of the bovine kidney medulla proton pump was raised in chicken, and affinity-purified [17, 32, 46]. The antibody against β-actin was commercially available from Sigma Chemical Company (St. Louis, MO).

*Immunofluorescence..* PLP-fixed tissues were cryoprotected in a solution of 30% sucrose in PBS for at least 2 h at room temperature. They were embedded in OTC Compound 4583 (Tissue-Tek, Miles Inc., Torrance, CA) and mounted on a cutting block. After freezing in a Reichert Frigocut microtome, the tissue was cut at 3–4 μm thickness and sections were placed onto Fisher Superfrost Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Sections were rehydrated in PBS for 5–10 min and pretreated with 1% (w/v) SDS, an antigen retrieval technique that we have previously described [47]. After 3 washes in PBS of 5 min each, slides were preincubated in 1% (w/v) BSA in PBS/0.02% sodium azide for 15 min to block nonspecific staining, after which they were incubated in anti-AQP9 antibody (diluted 1:400 in PBS) for 90 min at room temperature. Sections were washed twice for 5 min in high-salt PBS (2.7% [w/v] NaCl) to reduce nonspecific staining, and once in normal PBS. They were then incubated for 1 h at room temperature with secondary antibody, a goat anti-rabbit immunoglobulin (Ig) G coupled to fluorescent cyanine dye, CY3 (Jackson Immunologicals, West Grove, PA). The sections were again washed as described above. The slides were mounted in Vectashield (Vector Labs, Burlingame, CA) diluted 1:1 with Tris buffer, pH 8.5, and examined using a Nikon E800 epifluorescence microscope. Sections were digitally imaged using a Hamamatsu Orca CCD camera and IP Lab Spectrum software (Scanalytics, Vianna, VA). Final images were imported into Adobe Photoshop and printed.

To determine the specificity of the anti-AQP9 antibody, control incubations using antibodies that had been preabsorbed with the AQP9 antigen were performed, as we have previously published [32]. All sections were negative (data not shown).

### Quantification of Mean Pixel Intensity of AQP9-Associated Fluorescence in the Apical Membrane of Principal Cells of the Cauda Epididymidis

The immunofluorescence slides were prepared as described above. At least 2 immunofluorescence incubations were carried out for each animal treatment series. Each incubation included 1 tissue slide from each animal of the control and treatment groups. All slides were treated under identical conditions. Images from each slide were obtained as described above with a digital camera. Proximal cauda epididymidis images were used for quantification. For a given incubation all the images were obtained using identical parameters including exposure time and gain. Each image was then corrected for its own luminal unstained background value. IP Lab Spectrum software was then used to measure the mean pixel intensity of AQP9-associated fluorescence in the apical (brush border) membrane of principal cells. The segmentation function of IP Lab Spectrum was used to set the minimum pixel intensity for inclusion so that the highlighted pixels corresponded to the BBM area of principal cells observed in the original, nonmanipulated image (see *Results*). This part of the procedure was subjective, but by carefully comparing the highlighted BBM segments with the original immunofluorescence, we could produce a reliable and accurate representation of the regions occupied by AQP9-associated fluorescence. We previously used this method to quantify the distribution of AQP2 in kidney collecting duct principal cells [48]. The mean pixel intensity of each segment was measured by the IP Lab Spectrum software, and averaged for each rat. Data were expressed for each treatment group as mean ± standard error of the mean (SEM) for each incubation.

### Immunoblotting (SDS-PAGE and Western Blotting)

Adult rats were anesthetized and perfused through the left ventricle with PBS pH 7.4 containing protease inhibitors. One epididymis from each animal was removed and snap-frozen using liquid nitrogen before storing at −80°C before use. The other epididymis was kept in situ and fixed via left ventricle perfusion as indicated above, and used for immunocytochemistry.

Each epididymis was thawed on ice, cut into smaller pieces with a razor blade, and quickly washed in 1 ml of ice-cold PBS containing Complete protease inhibitors (Roche, Indianapolis, IN). An enriched preparation of BBM was obtained from one epididymidis from each of 3 rats belonging to either the control or treatment groups using the $Mg^{++}$ precipitation technique previously described for kidney proximal tubules and small intestine [49–51]. Briefly, the tissue was homogenized in 30 ml of a buffer containing 250 mM sucrose, 18 mM Tris-HEPES, 1 mM EDTA, and Complete protease inhibitor, pH 7.4, using a PRO 200 homogenizer (20 strokes in a glass/Teflon potter). The homogenate was incubated with 10 mM $MgCl_2$ on ice for 20 min and was then centrifuged at 7700 × *g*

TABLE 1. Epididymis weight, testis weight, and body weight in adult control and flutamide-treated rats.*

| | Control (n = 6) | Flutamide treatment (n = 5) |
|---|---|---|
| Epididymis (g/100 g BW) | 0.122 ± 0.003 | 0.084 ± 0.001† |
| Testis (g/100 g BW) | 0.426 ± 0.013 | 0.417 ± 0.014 |
| Body (g) | 369 ± 6 | 345 ± 11 |

* Data are mean ± SEM.
† $P < 0.001$.

for 15 min. The pellet was discarded and the supernatant was further centrifuged at 20 000 × *g* for 15 min. The pellet was resuspended in a resuspension buffer (150 mM KCl, 5 mM Tris-HEPES and Complete protease inhibitor, pH 7.4) by passing through a 25 5–8 gauge needle and was then centrifuged at 1900 × *g* for 15 min. The pellet was discarded and the supernatant was centrifuged at 30 900 × *g* for 15 min. The supernatant was then discarded and the pellet was resuspended in a small amount of resuspension buffer by passing the mixture through a needle and syringe as above. The protein concentrations of the initial homogenates and the final BBM preparations were measured using the BCA assay (Pierce, Rockford, IL).

For Western blotting, the samples were solubilized at 65°C for 15 min in NuPAGE LDS sample buffer (4×) with a final concentration of 10% NuPAGE reducing agent (NuPAGE from Novex, San Diego, CA), as previously described [32]. Protein was added at 20 μg/lane and was separated by SDS-PAGE and transferred to Immun-Blot PVDF membrane (Bio-Rad, Hercules, CA). Membranes were incubated overnight at 4°C in the affinity-purified AQP9 antibody (1:2000 dilution). After several washing steps in PBS, goat anti-rabbit IgG conjugated to horseradish peroxidase (Immuno-Dynamics, La Jolla, CA) was applied to membranes (1:1000 dilution) for 1 h at room temperature. Proteins were detected using Renaissance Western Blot Chemiluminescence Reagent (New England Nuclear, Boston, MA). As a control for loading, the Immun-Blot membranes were also incubated with a monoclonal β-actin antibody (see above) at 1:10 000 dilution overnight at 4°C, and a goat anti-mouse horseradish peroxidase (HRP) antibody conjugated to HRP was used as the secondary antibody (1:500): (Sigma). To confirm the specificity of the AQP9 antibody, some membranes were also incubated with antibody that had been preabsorbed with the immunizing AQP9 peptide. No stained bands were observed under these conditions (data not shown), as we have previously published [32].

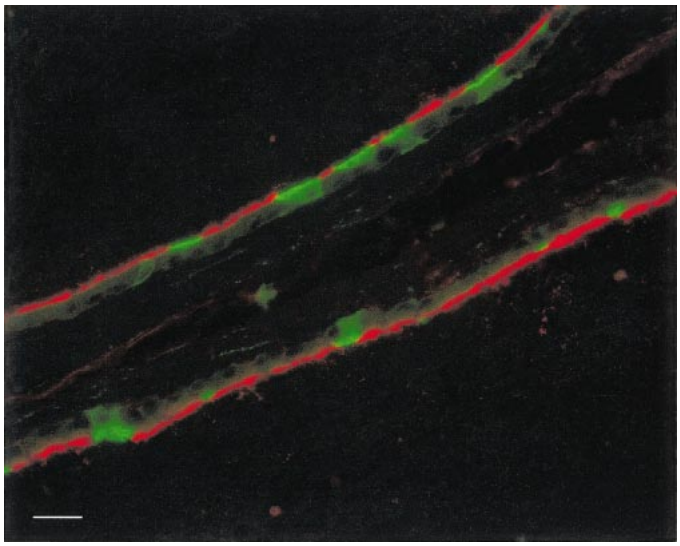

FIG. 1. Double immunofluorescence localization of AQP9 (red) and the $H^+$-ATPase (green) in the cauda epididymidis. AQP9 staining (red) is restricted to the apical microvilli of principal cells. The adjacent clear cells, in which the $H^+$-ATPase (green) is concentrated toward the apical pole, are not labeled with the AQP9 antibody. Bar = 25 μm.

TABLE 2. Epididymis weight and body weight for adult sham-operated control rats, castrated rats, and castrated rats treated with testosterone.*

| | Control + oil (n = 3) | Castrated + oil (n = 3) | Castrated + testosterone (n = 3) |
|---|---|---|---|
| Epididymis (g/100 g BW) | 0.285 ± 0.025 | 0.128 ± 0.007† | 0.201 ± 0.011‡ |
| Body (g) | 460 ± 8 | 412 ± 3† | 436 ± 5‡ |

* Data are mean ± SEM.
† vs. control + oil, $P < 0.05$.
‡ vs. castration + oil, $P < 0.05$.

### *Statistics*

Statistical evaluation of the numeric data was performed using the Student *t*-test and ANOVA at the 5% level of significance.

## RESULTS

### *Efficiency of Treatments*

Administration of flutamide to adult rats reduced epididymal weight compared with controls but had no effect on testis weight (Table 1). The body weights of the rats were not significantly different between the groups (these were posttreatment weights). The epididymal weight reduction induced by flutamide is consistent with previously published studies [39, 52]. In addition, the yield of BBM was decreased by 50% in preparations from the flutamide-treated animals compared with that from control rats (0.7% vs. 1.5% yield of BBM in flutamide-treated vs. control animals).

Castrated animals showed a significant reduction in their epididymal weight compared with the sham-operated controls (Table 2). There was a partial recovery of the epididymal weight in the castrated testosterone-treated animals, which is consistent with results previously published in other studies [36].

### *Immunofluorescence and Quantification of AQP9-Associated Fluorescence in Control and Flutamide-Treated Adult Rats*

Figure 1 shows a high magnification of the cauda epididymidis double-stained for AQP9 and the $H^+$-ATPase. As we have previously described, in the epididymal epithelium, AQP9 staining is present in the apical membrane of principal cells only, whereas clear cells remained unstained [32]. Although the AQP9 apical staining was detected without prior antigen retrieval, the intensity of staining was considerably increased in sections pretreated with SDS [32]. Therefore, all the immunofluorescence data presented in this study were taken from SDS-treated tissue sections. As shown in Figure 2, A and B, the level of AQP9 labeling was significantly decreased in flutamide-treated rats compared to control rats.

Using the IP Lab Spectrum software, the AQP9-associated mean pixel intensity of the BBMs of principal cells of the proximal cauda epididymidis was calculated (see *Materials and Methods*). Several independent incubations, each of them including all animals in each group (6 controls and 5 flutamide-treated rats) were used for the quantification. The areas used for quantification are exemplified in Figure 2, C and D. The yellow segments highlight the AQP9-associated pixels of immunofluorescence in the apical membrane. These segments were used to calculate the mean pixel intensity of AQP9-associated fluorescence (Fig.

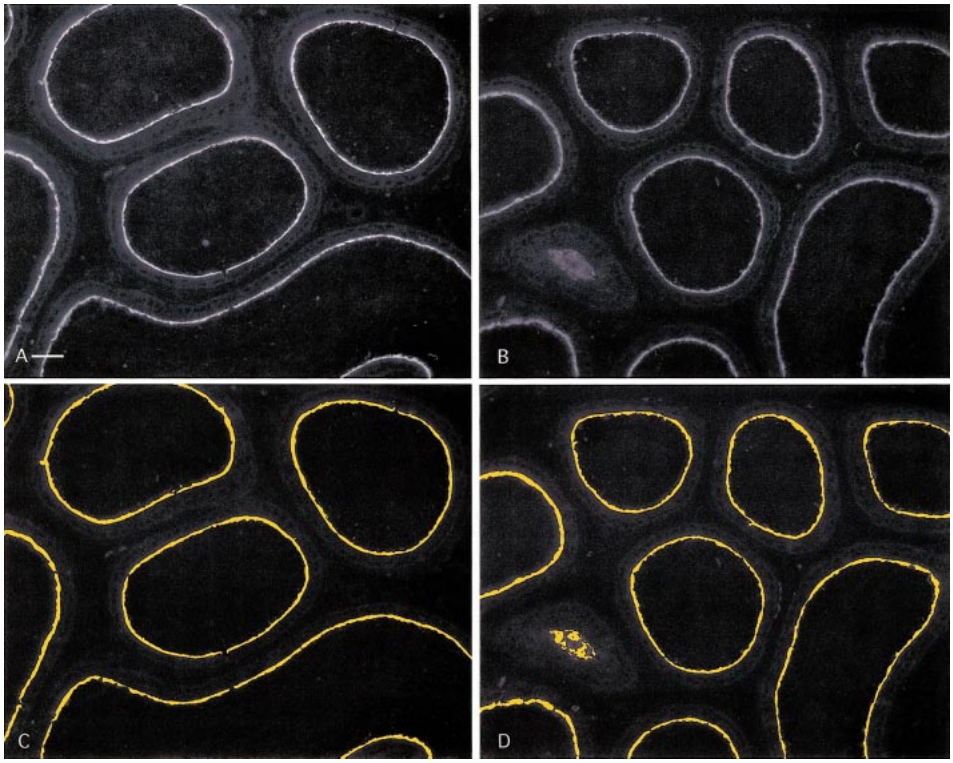


FIG. 2. Down-regulation of AQP9 expression in the cauda epididymidis by the antiandrogen flutamide. Immunofluorescence for AQP9 in the cauda epididymidis from a control rat (**A**) and a flutamide-treated rat (**B**) showing a marked decrease in apical AQP9 staining in the flutamide-treated animal. These digital images were acquired using identical parameters. At this lower magnification, some of the discontinuities in the apical band of staining are attributed to the presence of clear/narrow cells, which are unstained for AQP9 (see Fig. 1). **C** and **D** illustrate the segmentation procedure that was used to select the regions corresponding to the AQP9-associated fluorescence pattern (see *Materials and Methods*), for the images shown in **A** and **B**, respectively. Mean pixel intensity of these regions (shown in yellow) was calculated (see Fig. 3). Bar = 35 μm.

3). The AQP9-associated fluorescence in the epididymal apical membrane of the flutamide-treated animals was significantly lower (~35% decrease; $P < 0.05$) than that of control animals, and this decrease was found consistently in all incubations comparing the same treatment groups.

### *Immunoblotting (SDS-PAGE and Western Blotting)*

Western blotting of BBM preparations from the epididymis of control and flutamide-treated animals detected one main band at 30 kDa using the affinity-purified AQP9 polyclonal antibody (Fig. 4A). Other bands detected by the antibody may represent differentially glycosylated AQP9 products, as described previously [30, 32]. The flutamide-treated animals had significantly weaker AQP9 bands than controls. In contrast, the β-actin bands in BBM samples from control and flutamide-treated animals were similar (Fig. 4B). These results indicate a significant decrease in the amount of AQP9 in BBMs isolated from the whole epididymis, and thus reflect a down-regulation of AQP9 expression in the entire organ.

### *Immunofluorescence and Quantification of Epididymal AQP9 Expression in Control, Castrated, and Testosterone-Treated Castrated Rats*

Using immunofluorescence techniques, AQP9 expression in the apical membrane of principal cells of the proximal cauda epididymidis of castrated rats was greatly decreased compared with that of sham-operated control animals (Fig. 5, A and B). Treatment of castrated rats with testosterone for 8 days rescued AQP9 expression to a level that was comparable to that of sham-operated controls (Fig. 5C). Using the segmentation protocol described above, the mean pixel intensity of AQP9-associated fluorescence in digital images from control, castrated, and testosterone-treated castrated rats was determined (Fig. 6). The results clearly illustrate a significant decrease of the AQP9-associated fluorescence following castration (~65% decrease; $P < 0.05$) and its recovery to levels that were comparable to controls after castrated rats had been treated with testosterone.

## DISCUSSION

Fluid and electrolyte transport processes in cells lining the epithelium of the male reproductive tract significantly modify the luminal environment in which spermatozoa mature and are stored. Fluid reabsorption begins in the rete

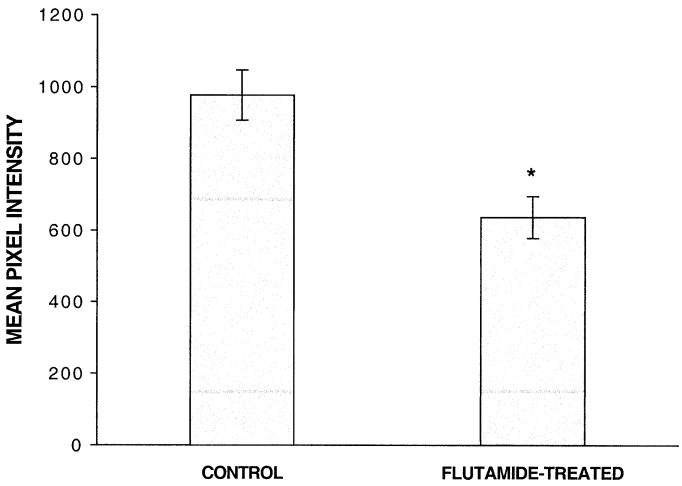


FIG. 3. Quantification of the AQP9-associated fluorescence in the cauda epididymidis of control and flutamide-treated rats. The mean pixel intensity of AQP9-associated fluorescence in the apical membrane of principal cells of the cauda epididymidis was measured in tissue sections from 6 control and 5 flutamide-treated animals. Digital images were acquired using identical parameters and analyzed using the IP Lab Spectrum software. The intensity of apical membrane AQP9 staining was markedly decreased following flutamide treatment. Data are expressed as mean ± SEM. *$P < 0.05$ compared with control rats.

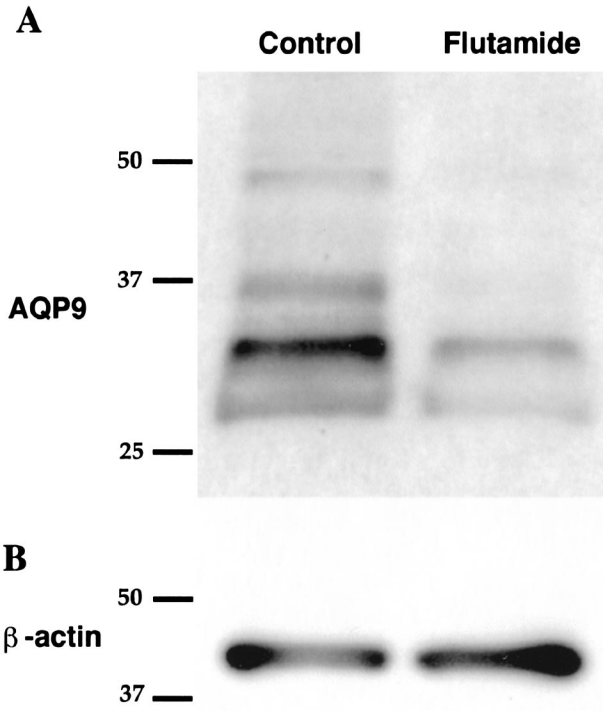


FIG. 4. Western blot showing the reduction in the amount of AQP9 in epididymal BBM preparations by the antiandrogen flutamide. A strong band at around 30 kDa is present in the epididymal BBM preparation of control rats (**A**). Additional, higher molecular weight bands probably represent different glycosylation states of AQP9. The flutamide-treated adult rats had weaker bands than controls, whereas similar β-actin bands are observed in both groups, showing that a similar amount of protein was loaded in both wells (**B**).

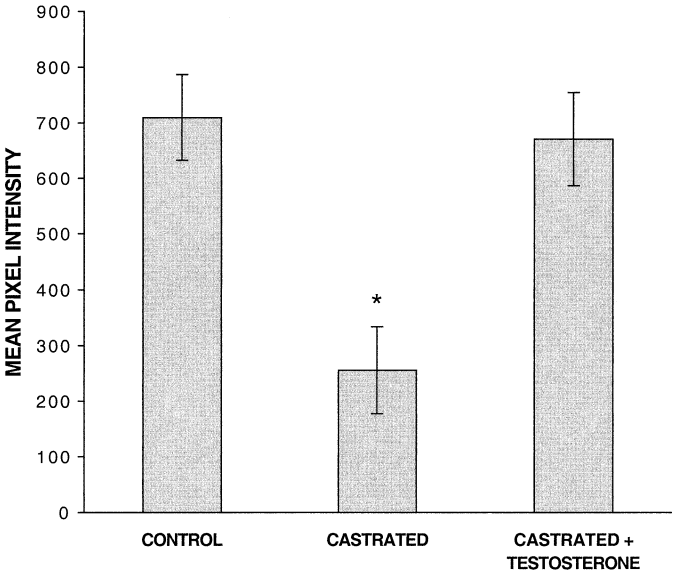


FIG. 6. Quantification of the down-regulation of AQP9-associated fluorescence in the cauda epididymidis of adult castrated rats, and recovery of AQP9 expression by exogenous testosterone treatment. The mean pixel intensity of AQP9-associated fluorescence in the apical membrane of principal cells of the cauda epididymidis was measured in tissue sections from 3 sham-operated control rats, 3 castrated rats, and 3 castrated rats that received testosterone supplementation. The intensity of apical membrane AQP9 staining was markedly decreased in castrated animals and was restored to normal levels following testosterone supplementation. Data are expressed as mean ± SEM. $*P < 0.05$ in comparison to control rats.

testis, but most of it occurs in the efferent ducts and more distal segments of the excurrent duct system, including the epididymis [6–8, 34]. This transepithelial fluid movement is probably facilitated by aquaporins, which are present in specific segments of the male reproductive tract. AQP1 is present in the efferent ducts, the region of the excurrent duct where up to 90% of the fluid reabsorption occurs, and in the ampulla of the vas deferens [22], whereas AQP2 is constitutively expressed in the apical pole of distal vas deferens cells [26]. AQP9, a ''promiscuous'' water channel that allows the passage not only of water but also of neutral solutes, is abundantly expressed in the male reproductive tract [31, 32], where it probably represents an apical pathway for transmembrane water and neutral solute flow [28–30]. AQP9 staining is present in the apical pole of epididymal principal cells, whereas the adjacent clear/narrow cells, which are positive for the $H^+$-ATPase, are not labeled with anti-AQP9 antibodies [32].

Reabsorption of luminal fluid in some regions of the male reproductive tract is regulated by steroid hormones [19, 33–35]. During fetal and postnatal development, androgens play a crucial role in the development of the male reproductive tract [36]. When testosterone interacts with functional androgen receptors, the biosynthesis of many macromolecules in these tissues increases [5]. Therefore, the purpose of the present study was to examine the regulation of AQP9 expression by androgens in the adult rat epididymis. Our study provides evidence that AQP9 expression is regulated by androgens, as demonstrated by the decrease in the immunofluorescence staining in the apical membrane of principal cells of the proximal cauda epididymidis in flutamide-treated adult animals and through confirmation of this result by Western blotting of BBM preparations. In addition we show that the decrease in AQP9 expression seen in adult castrated rats can be reversed by exogenous administration of systemic testosterone.

We previously described that AQP9 was detectable in some cells of the developing epididymis as early as 1 wk postnatally [32]. A progressive increase in AQP9 expression was seen during postnatal rat development, and by 5 wk of age, the expression levels of AQP9 were almost in-

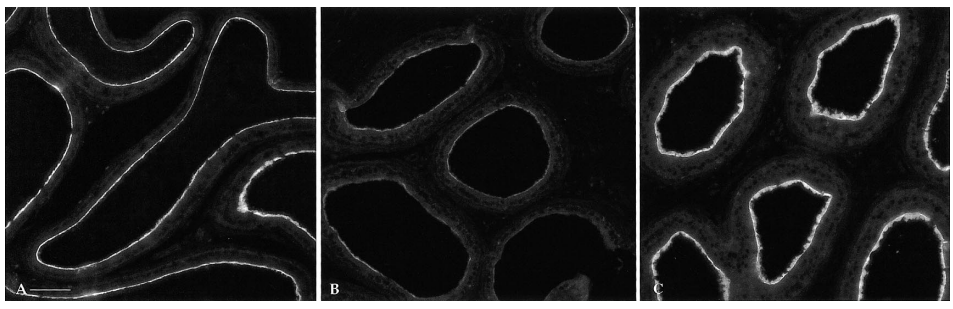


FIG. 5. Down-regulation of AQP9 expression in the cauda epididymidis of adult castrated rats, and recovery of AQP9 expression by exogenous testosterone treatment. In comparison with sham-operated animals (**A**), immunofluorescence staining shows a significant decrease in the expression of AQP9 in the apical membrane of principal cells of castrated rat epididymis (**B**), whereas systemic testosterone supplementation restores AQP9 staining to control levels (**C**). Bar = 50 μm.

distinguishable from the adult pattern. Thus, AQP9 expression did not seem to absolutely depend on the higher androgen levels that are reached during and after sexual maturation in rats. However, because androgens are present in male rats during both prenatal and postnatal development, it is possible that AQP9 expression is modulated by these hormones, and that the lower androgen levels in prepubertal rats are sufficient to trigger a progressive expression of the protein [5, 40–42]. In addition, the peripubertal rat epididymal cells have the capacity to synthesize other androgens from testosterone [40]. As reviewed by Hinton [53], the epididymis and maturing spermatozoa can receive androgens via at least three different routes: 1) from the testis, bound to the androgen binding protein produced by the Sertoli cells [54]; 2) from the systemic circulation [55]; or 3) by synthesis in the epididymal epithelium [56, 57]. In the present study, exogenous systemic testosterone treatment to castrated rats was sufficient to restore AQP9 expression to control levels. It therefore appears that a normal expression of AQP9 can be achieved in the absence of testis-derived luminal factors.

The down-regulation of AQP9 expression induced by flutamide treatment or castration could partially be the result of a general regression of the epithelium to an androgen-depleted state [36, 58]. Such regression was indicated in the present study by a smaller yield of BBMs from the epididymides of flutamide-treated animals compared with controls. However, Western blot analysis revealed that the amount of AQP9 protein per milligram of BBM protein was smaller in the flutamide-treated rats than it was in control rats, indicating a direct effect of this drug on the expression of AQP9. In addition, the presence of a putative steroid receptor binding site in the promoter region of the human AQP9 gene [30] suggests that androgens may indeed upregulate the expression of AQP9 in the epididymis. Thus, altogether, our data suggest that AQP9 down-regulation probably results from a combination of the following mechanisms: 1) general regression of the epididymis leading to underdeveloped BBM, and 2) direct down-regulation of the expression of the AQP9 protein.

The trend in the onset of expression of AQP9 is similar to that of other epithelial proteins, such as the $H^+$-ATPase, because cells (narrow and clear cells) that are rich in $H^+$-ATPase reach their peak of expression just before spermatozoa appear in the epididymal lumen [59]. These findings initially were interpreted as showing that the expression of $H^+$-ATPase was not regulated only by androgens, because cells rich in $H^+$-ATPase appeared at times when androgen production was low. Subsequently, however, we found that neonatal treatment with the antiandrogen flutamide resulted in fewer $H^+$-ATPase-rich cells in the peripubertal rat [60]. This result indicates that androgens play a major role in the differentiation of these specialized cells, and that prepubertal androgen levels are sufficient to initiate this process.

On the other hand, fluid reabsorption in the male excurrent ducts has been shown to be influenced by estrogens [34]. It is interesting that the expression of AQP1 in efferent ducts and fluid reabsorption in these tubules depend on exposure to an appropriate level of estrogens [23, 34, 61]. Although a considerable amount of fluid reabsorption occurs in the efferent ducts, significant transepithelial water reabsorption also occurs in more distal regions of the excurrent duct system, including the epididymis. Our data now show that AQP9, which was recently described as an abundant apical membrane protein in all regions of the epididymis [31, 32], is under the influence of androgens, and that systemic supplementation of castrated rats with testosterone is sufficient to restore the expression of this protein. However, the higher level of down-regulation of AQP9 that we observed following castration, compared with the level induced by flutamide, indicates the potential involvement of other androgen-dependent modulators in the regulation of AQP9. For example, because testosterone can be metabolized to estrogens in the male reproductive tract by the P-450 aromatase [62, 63], it remains possible that some of the effects seen in the castrated animals are mediated by a reduction in estrogens as well as androgens. The complete recovery of AQP9 expression observed in castrated rats treated with testosterone enanthate further indicates that estrogens might be involved, in addition to androgens, because this testosterone derivative can be metabolized into estrogens. Nevertheless, as flutamide acts exclusively at the androgen receptor level [64], our results show that androgens are involved, at least partially, in the regulation of AQP9 expression.

In summary, our data show that AQP9, a major apical water channel expressed in principal cells of the epididymis, is regulated by androgens in adult rats. Although this aquaporin could represent an important apical pathway for transmembrane water movement, it could also serve as a conduit for other solutes, based on its known promiscuity [29]. The role of other steroid hormones, such as estrogens, in the expression of AQP9 in the male reproductive tract in adulthood and during development remains to be determined.

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