Expression of the rhesus glycoproteins, ammonia transporter family members, RHCG and RHBG in male reproductive organs

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Abstract

The rhesus glycoproteins, Rh B glycoprotein (RHBG) and Rh C glycoprotein (RHCG), are recently identified ammonia transporters. Rhcg expression is necessary for normal male fertility, but its specific cellular expression is unknown, and Rhbg has not been reported to be expressed in the male reproductive tract. This study sought to determine the specific cellular expression of Rhcg, to determine whether Rhbg is expressed in the male reproductive tract, and, if so, to determine which cells express Rhbg using real-time RT-PCR, immunoblot analysis, and immunohistochemistry. Both Rhbg and Rhcg were expressed throughout the male reproductive tract. In the testis, high levels of Rhbg were expressed in Leydig cells, and Rhcg was expressed in spermatids during the later stages of their maturation (steps 13–16) in stages I–VIII of the seminiferous epithelium cycle. In the epididymis, basolateral Rhbg was present in narrow cells in the initial segment, in principal cells in the upper corpus, and in clear cells throughout the epididymis. Apical Rhcg immunolabel was present in principal cells in the caput and upper corpus epididymidis and in clear cells in the middle and lower corpus and cauda epididymidis. In the vas deferens, apical Rhcg immunolabel and basolateral Rhbg immunolabel were present in some principal cells and colocalized with H^+-ATPase immunolabel. We conclude that both Rhbg and Rhcg are highly expressed in specific cells in the male reproductive tract where they can contribute to multiple components of male fertility.

Introduction

The ammonia transporter family, which includes Mep proteins in yeast, AMT proteins in many bacteria and plants, and rhesus (Rh) glycoproteins in mammalian species, is a recently

Declaration of interest

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

identified extended family of integral membrane proteins that mediate critical roles in transmembrane ammonia transport (Winkler 2005, Weiner 2006, Knepper 2008, Weiner & Verlander 2010). Mammals express three ammonia transporter family members, Rh A glycoprotein (RHAG), RHBG, and RHCG (Liu *et al*. 2000, 2001, Marini *et al*. 2000, Weiner & Verlander 2010). Rh glycoproteins are integral membrane proteins with 12 transmembrane segments. They are present in plasma membranes in a homotrimeric form and enable specific transport of ammonia, but no other known solutes, across plasma membranes.

Rh glycoproteins are expressed specifically in tissues in which ammonia transport is necessary for normal biological functioning. They are highly expressed in the kidneys, where ammonia formation and transport are central to renal regulation of acid–base homeostasis (Eladari *et al*. 2002, Quentin *et al*. 2003, Verlander *et al*. 2003, Weiner & Verlander 2011). In liver, Rh glycoproteins are expressed in perivenous hepatocytes, which mediate high-affinity ammonia metabolism (Weiner *et al*. 2003). Sweat contains ammonia in concentrations ~100-fold greater than in plasma (Brusilow & Gordes 1964, 1968, Liu *et al*. 2001), and Rh glycoproteins are highly expressed in sweat glands (Liu *et al*. 2001).

Another tissue that expresses Rh glycoproteins is the male reproductive tract. RHCG is expressed in both the testis and epididymis (Liu *et al*. 2000), and its expression, at least as determined by global knockout studies, is necessary for normal male fertility (Biver *et al*. 2008). However, both the testis and epididymis contain multiple cell types with distinct cellular functions, and the specific cells in these organs that express Rhcg have not been identified. In the studies reporting the initial cloning of mammalian *RHBG*, northern blots did not identify *RHBG* mRNA expression in male reproductive organs (Liu *et al*. 2001). However, we have shown that *RHBG* is expressed in several tissues in which it was not identified in the initial cloning report (Handlogten *et al*. 2005, Han *et al*. 2009). Thus, whether *RHBG* is expressed in the male reproductive tract is unclear.

In view of the importance of RHCG in male fertility, but lack of knowledge regarding its specific cellular expression, and the uncertainty regarding whether RHBG is expressed in the male reproductive organs, the purpose of the current study was to perform a detailed examination of RHCG and RHBG mRNA and protein expression in the testis and epididymis. Our results show distinct cell-specific RHCG and RHBG expression in the testis and cell-specific expression combined with axial heterogeneity in the epididymis and vas deferens. These observations indicate that RHCG and RHBG are likely to be involved in multiple components of male fertility.

Ammonia exists in two molecular forms, NH_4^+ and NH_3, in aqueous solutions. In this manuscript, we use the term 'ammonia' to refer to the combination of these two molecular forms. When referring specifically to one of these molecular forms, we use the terms 'NH_3' or 'NH_4^+.'

Materials and methods

Animals

Adult wild-type C57Bl/6 mice were used in these studies. All animal studies were approved by the University of Florida College of Medicine and the North Florida/South Georgia Veterans Health System IACUC.

Antibodies

Affinity-purified antibodies to RHCG and RHBG generated in our laboratory have been characterized previously (Verlander *et al*. 2003, Weiner *et al*. 2003, Mak *et al*. 2006). Antibodies to the a4 subunit of H^+-ATPase were kindly provided by Dr Fiona Karet (University of Cambridge, UK) and rabbit polyclonal antibody for glutamine synthetase (cat #AB73593) was purchased from Abcam (Cambridge, MA, USA).

mRNA extraction

Adult C57Bl/6 male mice were anesthetized with inhalant isoflurane. After the mice were killed, the kidney, testis, caput, corpus and cauda epididymidis, and vas deferens were removed rapidly, placed into RNAlater (Qiagen), and stored in a −70 °C freezer until used. Total RNA was extracted from kidney, testis, and epididymis using RNeasy Mini Kit (Qiagen) and stored in a −70 °C freezer until used.

Real-time RT-PCR

Real-time RT-PCR amplification of *Rhbg* and *Rhcg* was performed as described in detail previously (Weiner *et al*. 2003, Handlogten *et al*. 2005). Briefly, total RNA was reverse transcribed using SuperScript First Strand Synthesis System for RT-PCR (Invitrogen) and random hexamer primers. For real-time PCR amplification of Rhbg, we used forward primer, 5′-GCCTGCAGAGTGTGTTTCCA-3′; reverse primer, 5′-GAGCTGATACACGGCCTGAGA-3′; and fluorescent probe, 6Fam-TGGCACTCCGCTGACCCTTGG-Tamra. For Rhcg, the forward primer was 5′-GGATACCCCTTCTTGGACTCTTC-3′, the reverse primer was 5′-TGCCTTGGAACATGGGAAAT-3′, and the fluorescent probe was 6Fam-AGCCTCCGCCTGCTCC-CCAAC-Tamra. Real-time RT-PCR was performed on an ABI Prism GeneAmp 7500 Sequence Detection System. We used a two-step cycle protocol including an initial 95 °C denaturation step for 15 s and then 60 °C for 1 min and then 40–50 cycles of alternating temperatures. 18S RNA was amplified in all experiments as an internal control using commercially available primers and probes (Applied Biosystems). All experiments included samples not treated with RT and samples to which RNA was not added as internal controls. All results were confirmed by repeating the experiments on specimens from at least three different mice.

Protein preparation

Animals were anesthetized with inhalant isoflurane, and the testes and epididymides were rapidly removed. The epididymides were dissected cleaned of connective tissues and divided into caput, corpus, and cauda regions. The tissues were frozen in liquid nitrogen and

stored at −70 °C until used. The tissues were homogenized using microtube pestles (USA Scientific, Ocala, FL, USA), and proteins were extracted using T-PER Tissue Protein Extraction Reagent (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's recommended procedures. An aliquot was obtained for protein determination using a BCA assay, and the remainder was stored frozen at −70 °C until used.

Immunoblotting procedure

Ten micrograms of protein were electrophoresed on 10% PAGE ReadyGel (Bio-Rad). Gels were then transferred electrophoretically to nitrocellulose membranes, blocked with 5 g/dl nonfat dry milk, and incubated at 4 °C overnight with primary antibody diluted in Blotto buffer (50 mM Tris, 150 mM NaCl, 5 mM Na_2EDTA, and 0.05% Tween 20, pH 7.6) with 5 g/dl nonfat dry milk. Loading and transfer equivalence were assessed with Ponceau S staining. After washing, membranes were exposed to secondary antibody goat anti-rabbit IgG (Millipore, Billerica, MA, USA) conjugated to HRP at a dilution of 1:5000. Sites of antibody–antigen reaction were visualized using ECL (SuperSignal West Pico Substrate, Pierce) and a Kodak Image Station 440CF digital imaging system. Band density was quantified using Kodak 1D, version 5.0, Software (Kodak Scientific Imaging, New Haven, CT, USA). Absence of saturation was confirmed by examining pixel intensity distribution in all immunoblots.

Immunohistochemistry

Following isoflurane anesthesia, testis and epididymis were preserved by *in vivo* cardiac perfusion with PBS (pH 7.4) followed by periodate–lysine–2% paraformaldehyde and then immersed for 24–48 h at 4 °C in this fixative. For light microscopy, 2- or 3 μm-thick sections were cut from paraffin-embedded testis or epididymis.

Immunolocalization was accomplished using standard immunoperoxidase procedures. The sections were deparaffinized in xylene and ethanols, rehydrated, and then rinsed in PBS. Endogenous peroxidase activity was blocked by incubating sections in Peroxidase Blocking Reagent (DakoCytomation, Carpinteria, CA, USA) for 45 min. Sections were blocked for 15 min with Serum-Free Protein Block (DakoCytomation) and then incubated overnight with primary antibody. Sections were washed with PBS and incubated for 30 min with polymer-linked, peroxidase-conjugated goat anti-rabbit IgG (MACH2, Biocare Medical, Concord, CA, USA), again washed with PBS, and then exposed to diaminobenzidine (DAB) for 5 min. Sections were washed in distilled water, then dehydrated with xylene, mounted, and observed by light microscopy. Comparisons of labeling were made only between sections of the same thickness from the same immunohistochemistry experiment. Sections were examined and photographed with a Nikon E600 microscope equipped with differential interference contrast (DIC) optics and interfaced with a DXM1200F digital camera and ACT-1 Software (Nikon). Color correction was performed using Adobe Photoshop CS5 Software (Adobe Systems, Inc.). All immunohistochemistry was performed on tissue sections from at least four different mice.

Double-immunolabeling procedure

Double immunolabeling was accomplished using immunoperoxidase procedures described in detail previously (Kim *et al.* 2009). To distinguish the cell types, tissue sections were labeled with H^+-ATPase using Vector SG (Vector Laboratories, Burlingame, CA, USA) as the chromogen to produce a blue label, as described earlier. The above procedure was repeated with the substitution of a second primary antibody (Rhbg and Rhcg) and the substitution of DAB for Vector SG. This label was easily distinguishable from the blue label produced by the Vector SG for detection of H^+-ATPase. Sections were then washed with glass-distilled water, dehydrated and mounted, and observed by light microscopy. All double-immunolabel immunohistochemistry was performed on tissue sections from at least four different mice.

Statistical analysis

Results are presented as means±S.E.M. Statistical analyses were performed using Student's *t*-test, and *P*<0.05 was taken as statistically significant.

Results

Rhbg and *Rhcg* mRNA expression

We first determined whether *Rhbg* and *Rhcg* mRNA were expressed in the testis and epididymis. To do so, and to enable detection of mRNA with greater sensitivity than the northern blotting techniques used in initial studies of the male reproductive tract, we used real-time RT-PCR with gene-specific primers and TaqMan probe, as we have described previously (Verlander *et al.* 2003, Weiner *et al.* 2003, Handlogten *et al.* 2005, Han *et al.* 2009). We identified expression of both *Rhbg* and *Rhcg* mRNA in both testis and epididymis (Fig. 1A and B). Neither *Rhbg* nor *Rhcg* mRNA was detected in the absence of reverse transcriptase, confirming that mRNA, not genomic DNA, was amplified (not shown).

Because there is known axial heterogeneity of cell types and function in the epididymis, we examined differences in *Rhbg* and *Rhcg* mRNA expression in different regions of the epididymis. Kidney was used as an internal control to assess relative abundance because RHBG and RHCG expression in the kidney is high (Weiner & Verlander 2010, 2011, 2013) and because expression is essential for normal renal ammonia metabolism (Biver *et al.* 2008, Lee *et al.* 2009, 2010, 2013, Bishop *et al.* 2010, 2013). *Rhbg* mRNA in the testis was relatively modest, ~20% of the level in the kidney. In the epididymis, *Rhbg* mRNA expression was maximal in the caput, at ~60% of renal expression. There was a lower level of expression in the corpus and cauda epididymidis (Fig. 1C). *Rhcg* mRNA expression in the testis was ~50% of levels in the kidney. In the epididymis, *Rhcg* mRNA expression was maximal in the caput, where expression level was ~160% of that observed in the kidney (Fig. 1D). Similar to the pattern for *Rhbg*, expression in the corpus and cauda epididymidis was significantly less than that in the caput epididymidis, ~10% of the expression in the caput. Thus, both *Rhbg* and *Rhcg* mRNA are expressed in both the testis and epididymis, their highest levels of expression are in the caput, and there is axial variation in both *Rhbg* and *Rhcg* mRNA expression in the epididymis.

RHBG and RHCG protein expression

We then examined RHBG and RHCG protein expression in the testis and epididymis. Because Rh glycoprotein mRNA expression was the greatest in the caput epididymidis, we specifically examined expression in this region. Immunoblot analysis demonstrated expression of RHBG and RHCG proteins in both the testis and epididymis (Fig. 2). For both RHBG and RHCG, detected molecular weights exhibited slight variations in different portions of the male reproductive tract, consistent with tissue-specific differences in glycosylation as reported previously (Weiner *et al*. 2003, Handlogten *et al*. 2005).

Cellular localization of RHBG and RHCG in testis

We next determined which cells in the testis expressed RHBG and RHCG. RHBG immunolabel was detected only in interstitial cells and not in the seminiferous tubules (Fig. 3A and B). High-power micrographs suggested that the interstitial cells with RHBG immunolabel had a location and morphology suggestive of Leydig cells.

To confirm that RHBG was expressed in Leydig cells, we performed double-immunolabel experiments using antibodies to the Leydig cell-specific protein glutamine synthetase (Holash *et al*. 1993, van Straaten *et al*. 2006). We identified that RHBG immunolabel was found only in glutamine synthetase-positive cells in the testis and that all cells with cytoplasmic glutamine synthetase expression expressed Rhbg (Fig. 4). Thus, RHBG is expressed specifically in Leydig cells in the testis.

Because testis expresses RHCG mRNA and protein, we also examined the cellular pattern of RHCG immunolabel expression. Strong RHCG immunolabel was present in seminiferous tubules, with no detectable expression in interstitial cells (Fig. 3C). High-power micrographs showed RHCG immunolabel exclusively in the head and midpiece of spermatids (Fig. 3D). In addition, spermatids in some tubules were strongly labeled, while there was no labeling in other tubules, suggesting stage-specific variations in RHCG expression. The stages of the seminiferous epithelial cycle where RHCG was expressed were determined using staging criteria described by Meistrich & Hess (2013). RHCG expression was absent in tubules from stage IX through XII, indicating that RHCG was not seen at or before step 12 of spermatid maturation, as all earlier steps of spermatid maturation are present in these stages but RHCG immunostaining was absent. The most apical layer of spermatids stained in tubules between stages I and VIII. This indicates that RHCG expression is confined to steps 13–16 of spermatid maturation, which are the most apical cells during stages I–VIII. Thus, both RHBG and RHCG are expressed in the testis, but their cellular localization differs. RHCG has a highly restricted pattern of expression in seminiferous epithelium and is present in only the more mature spermatids but not in other spermatogenic cells.

Cellular localization of RHBG in the efferent ductules

We next determined the expression of these ammonia transporter family members in the efferent ductules. No significant expression of either RHBG or RHCG immunolabel was detectable (Fig. 5A and B). Intact antigenic preservation was demonstrated by showing normal localization and expression of H^+-ATPase immunolabel (Fig. 5C and D).

Cellular localization of RHBG in the epididymis

Because our mRNA and protein analysis studies of tissue homogenates demonstrated for the first time, to our knowledge, that RHBG is expressed in the epididymis, we determined its specific localization using immunohistochemistry. Because of axial variations in the epididymis, we paid specific attention to RHBG expression in the different anatomic regions. Figure 6 shows representative findings. Basolateral RHBG immunolabel was expressed in all regions of the epididymis. In the initial segment and caput epididymidis, only a subpopulation of cells showed basolateral RHBG immunolabeling; the proportion of cells expressing RHBG appeared to be greater in the initial segment than in the caput epididymidis. In the upper corpus, all cells expressed basolateral RHBG, with varying expression in different cells. In the middle and lower corpus epididymidis, the cauda epididymidis, and the vas deferens, only some cells exhibited basolateral RHBG immunolabel. No immunolabel was observed when primary antibody was omitted from the immunohistochemistry procedure as a control (data not shown).

Double immunolabeling of H$^+$-ATPase and RHBG in the epididymis

The heterogeneous expression of RHBG in the epididymis suggested cell-specific differences in its expression. To test this, we performed double-immunolabel immunohistochemistry using antibodies to RHBG and to H$^+$-ATPase. Figure 7 shows representative results. Throughout the epididymis and vas deferens, RHBG immunolabel was found in H$^+$-ATPase-positive cells. Thus, RHBG is present in narrow cells and some principal cells in the initial segment, principal cells, and clear cells in the upper corpus epididymidis and in clear cells throughout the epididymis. Table 1 summarizes RHBG immunolabel expression in the epididymis.

RHCG immunolocalization in the epididymis

We next determined the cell-specific expression of RHCG in the epididymis. Figure 8 shows a low-power micrograph of RHCG immunolabel in the epididymis. RHCG immunoreactivity was greatest in the caput epididymidis, intermediate in the upper corpus epididymidis, and lowest in the middle and lower corpus epididymidis and vas deferens.

Higher-power micrographs identified significant cellular heterogeneity in RHCG expression in the epididymis. In the caput and upper corpus, most, but not all, of the cells had significant RHCG immunolabel (Fig. 9). In contrast, only a minority of cells in the middle and lower corpus had detectable RHCG immunolabel. In both the cauda epididymidis and the vas deferens, only a small population of cells expressed RHCG. In contrast to RHBG, which exhibited exclusively basolateral immunolabel, RHCG immunolabel was exclusively apical.

Double immunolabeling of H$^+$-ATPase and RHCG in epididymis

Because only a subpopulation of epididymal cells expressed RHCG immunolabel, we used double immunolabeling to determine the specific cell types that expressed RHCG. To do so, we colocalized RHCG with the vacuolar H$^+$-ATPase. Figure 10 shows representative findings. In the initial segment, significant RHCG immunolabel was not detected, either in H$^+$-ATPase-positive or H$^+$-ATPase-negative cells. In the caput and upper corpus

epididymidis, strong apical RHCG immunolabel was present only in H^+-ATPase-negative cells. In the middle and lower corpus and cauda epididymidis and vas deferens, apical RHCG immunolabel colocalized with H^+-ATPase.

Double immunolabel of RHBG with RHCG

To further confirm the cellular expression of RHCG in these more distal epididymal regions, we colocalized it with RHBG (Fig. 11). Clear cells, identified by basolateral RHBG (see results from Fig. 7), expressed apical RHCG immunolabel; principal cells had neither basolateral RHBG nor apical RHCG immunoreactivity. Table 1 summarizes these findings. Thus, apical RHCG immunolabel exhibits both axial and cell-specific heterogeneity in the epididymis.

Discussion

The current studies identify for the first time, to our knowledge, the cell-specific expression of RHBG and RHCG in the male reproductive tract and provide several new and important findings. First, we demonstrate for the first time, to our knowledge, that RHBG mRNA and protein are expressed throughout the male reproductive tract. In the testis, high levels of Rhbg are expressed in Leydig cells. In the epididymis, basolateral RHBG immunolabel is present in H^+-ATPase-positive cells throughout the epididymis and also in H^+-ATPase-negative cells in the upper corpus epididymidis. Thus, RHBG is present in male reproductive organs and it is expressed in specific cells known to have important roles in male fertility. RHCG expression exhibits both axial and cellular heterogeneity in the epididymis. This expression pattern of RHBG and RHCG in the male reproductive tract suggests important roles for these ammonia transporter family members in male fertility.

The first major finding reported here is that RHBG is expressed in both the testis and epididymis and that its expression demonstrates highly specific cellular expression in the male reproductive tract. We show this by demonstrating RHBG mRNA and protein expression in both testis and epididymis homogenates, by demonstrating heterogeneous RHBG protein expression in these organs using immunohistochemistry, and by identifying the specific cell types that express RHBG using double-immunolabel immunohistochemistry.

In the testis, we show for the first time, to our knowledge, that Leydig cells express RHBG, as demonstrated by the histological distribution of RHBG immunoreactivity consistent with Leydig cell distribution and confirmed by double-immunolabeling of RHBG with the Leydig cell-specific marker, glutamine synthetase. We speculate that RHBG-mediated ammonia transport may regulate Leydig cell steroidogenesis. Leydig cells are known to produce both testosterone and estrogen (Raeside *et al.* 2006, Carreau *et al.* 2007, 2011, Svechnikov *et al.* 2010), and humans with chronic liver disease often have elevated plasma ammonia levels in conjunction with altered sex steroid hormone levels (Chopra *et al.* 1973, Galvao-Teles *et al.* 1973). Another potential role of Rhbg in Leydig cells may be to facilitate ammonia metabolism. Leydig cells express high levels of glutamine synthetase, an enzyme that mediates the reaction of ammonia with glutamate to form glutamine, thereby decreasing ammonia concentration (Holash *et al.* 1993, van Straaten *et al.* 2006). The combined

expression in the same cell of RHBG, an integral membrane ammonia transporter, with glutamine synthetase could enable high-affinity ammonia metabolism; this expression pattern is also present in the perivenous hepatocytes in the liver (Weiner *et al*. 2003), cells that mediate a critical role in hepatic ammonia metabolism (Haussinger 1983, Haussinger *et al*. 1985).

The testis also expresses RHCG, although in different cell populations than those that express RHBG. In particular, RHCG is expressed only during the later stages (steps 13–16) of spermatid maturation, leading to stage-specific differences in its expression during the seminiferous epithelial cycle. This expression of RHCG is consistent with the expression of *Rhcg* mRNA in the seminiferous epithelium that was reported in studies using *in situ* hybridization (Liu *et al*. 2000) but further indicates that expression is confined to one cell type during one specific maturational period. These results suggest that that RHCG-mediated transport of NH_3 could play a role in spermatid maturation during the later stages of spermatid development. Alternatively, as the yeast ammonia transporter family member, Mep2, can regulate cellular function through mechanisms independent of ammonia transport (Lorenz & Heitman 1998, Biswas & Morschhauser 2005), it is possible that Rhcg may also regulate spermatid maturation and/or function through mechanisms not involving ammonia transport.

In the epididymis, both RHBG and RHCG are widely expressed, typically in different epithelial membrane domains in the same cells. The finding of polarized expression of both ammonia transporter family members, RHBG and RHCG, suggests that ammonia is transported by specific epididymal epithelial cell types. Several lines of evidence support this hypothesis. First, intraluminal ammonia concentrations are high in the epididymis. Direct measurements have shown ammonia concentrations of 800-2800 µmol/l in epididymal fluid in a wide variety of species (Johnson *et al*. 1972, Jones 1978). In contrast, normal plasma ammonia levels are 20–30 µmol/l, indicating that the epididymal fluid ammonia concentration is 25- to 100-fold greater than plasma and suggesting epididymal ammonia secretion. Secondly, *Rhcg* deletion lowers epididymal fluid pH (Biver *et al*. 2008), a finding easily explained through inhibition of RHCG-mediated NH_3 transport. Lastly, the coexistence of basolateral RHBG, apical H^+-ATPase, and apical RHCG in clear cells in the middle and lower corpus and cauda epididymidis is similar to that observed in the renal collecting duct (Weiner & Hamm 2007, Weiner & Verlander 2010, 2011), a known site of transepithelial ammonia secretion (Weiner & Verlander 2010, 2011) that requires RHBG and RHCG expression (Biver *et al*. 2008, Bishop *et al*. 2010, Lee *et al*. 2009, 2010). Thus, ammonia secretion by the epididymis involving RHBG and RHCG is likely to play a critical role in both epididymal ammonia secretion and determination of epididymal fluid pH.

The current study is the first, to our knowledge, to identify RHBG expression in the male reproductive tract. A previous study using northern analysis did not detect *Rhbg* mRNA expression (Liu *et al*. 2001), whereas the current study, using RT-PCR, immunoblot analysis, and immunohistochemistry, demonstrates RHBG mRNA and protein expression in testis, epididymis, and vas deferens. The increased sensitivity of real-time RT-PCR compared with northern blot analysis probably explains the different results of these studies. Similar identification of RHBG expression using real-time RT-PCR, immunoblot analysis

and immunohistochemistry in tissues not reported to contain *Rhbg* mRNA by northern analysis has been made for several other tissues, including lung, stomach, small intestine, and colon (Handlogten *et al*. 2005, Han *et al*. 2009).

Our identification of RHCG expression in epididymis provides important new information. We show specific colocalization of RHCG with H^+-ATPase, a pattern identical to that in the kidney, where RHCG and H^+-ATPase are co-expressed in intercalated cells. Intercalated cells have many similarities to epididymal clear cells (Paunescu *et al*. 2004, Pastor-Soler *et al*. 2005, Da Silva *et al*. 2010) and are responsible for renal collecting duct ammonia secretion. Thus, it is likely that RHCG mediates an important role in epididymis ammonia secretion and that this process involves parallel transport by clear cells of both NH_3 and H^+. This finding significantly expands our understanding of the function of these cells by suggesting critical roles not only in epididymal fluid acidification but also in epididymal fluid ammonia secretion. In the initial report identifying RHCG, *Rhcg* mRNA expression was demonstrated in both the mouse and human testis; protein expression was not reported (Liu *et al*. 2000). A subsequent report showed apical RHCG immunolabel in the epididymis but did not determine the cell-specific expression pattern (Biver *et al*. 2008). Thus, the current study is consistent with these previous studies and provides important new information regarding RHCG expression in the male reproductive tract.

In summary, the current study provides the first characterization, to our knowledge, of RHCG and RHBG cellular distribution in the male reproductive organs, especially testis and epididymis, by real-time RT-PCR, immunoblot analysis, and immunolocalization. RHBG is present in Leydig cells in the testis and in the basolateral membrane of H^+-secreting cells in the epididymis. RHCG demonstrates both axial and cell-specific heterogeneity in the epididymis and is also expressed in sperm. Ammonia transport by RHCG and RHBG is likely to mediate critical roles in multiple components of male fertility.

Acknowledgments

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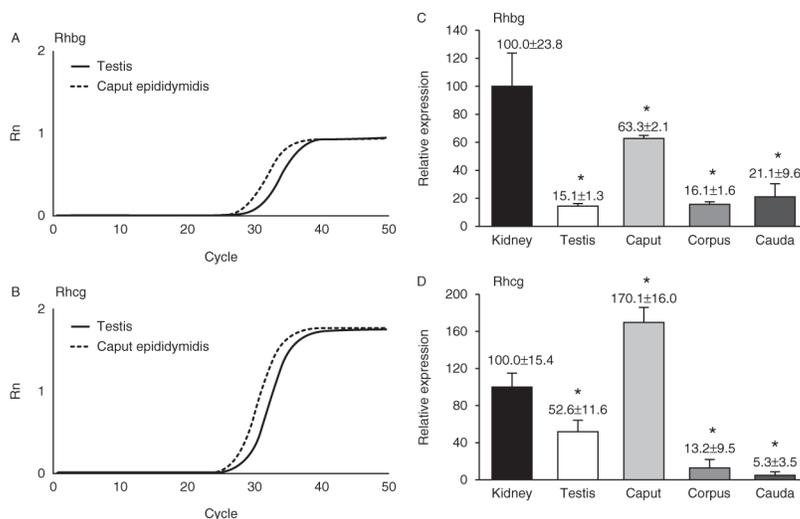
Figure 1.
Real-time RT-PCR relative expression of *Rhcg* and *Rhbg* mRNA in the mouse. mRNA was reverse transcribed and amplified using gene-specific primers and TaqMan probe for Rhcg and Rhbg. (A) Amplification curves for Rhbg in the testis and the caput epididymidis. No amplification was identified in the absence of RT or when sample mRNA was not included. (B) Amplification curves for Rhcg in the testis and the caput epididymidis. No amplification was identified in the absence of RT or when sample mRNA was not included. (C) Relative expression levels of *Rhbg* mRNA in the testis and different regions of the epididymis. Rhbg is present in all male reproductive tract regions tested, with axial heterogeneity in its expression levels in different portions of the epididymis. *Rhbg* mRNA is expressed relative to renal cortical mRNA determined simultaneously. (D) Relative expression levels of *Rhcg* mRNA in the testis and different regions of the epididymis. Rhcg is present in all male reproductive tract regions tested, with axial heterogeneity in its expression levels in different portions of the epididymis. *Rhcg* mRNA is expressed relative to renal cortical mRNA determined simultaneously. Values are means±S.E.M. *P<0.05 vs kidney. *n*=3 for each sample. Kidney cortex mRNA was used as a positive control.
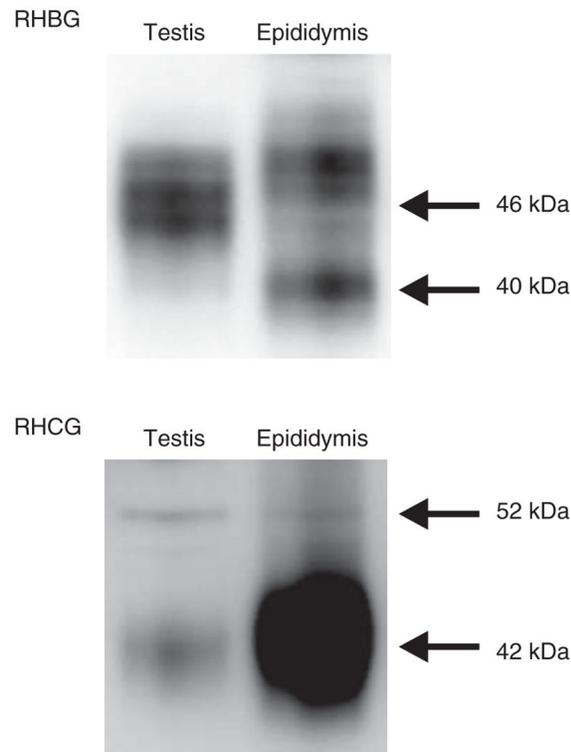
Figure 2.
Immunoblot analysis of RHCG and RHBG in the male reproductive tract. (Upper panel) Immunoblot analysis of RHBG protein expression. RHBG is present in both testis and epididymis.

The predominant expression of RHBG is at ~46 kDa in the testis and 40 and 49 kDa in the epididymis, consistent with tissue-specific variations in glycosylation. (Lower panel) Immunoblot analysis of RHCG protein expression. The predominant expression of RHCG is at 42 and 52 kDa in the testis and at 42 kDa in the epididymis. These variations are consistent with tissue-specific variations in RHCG glycosylation.
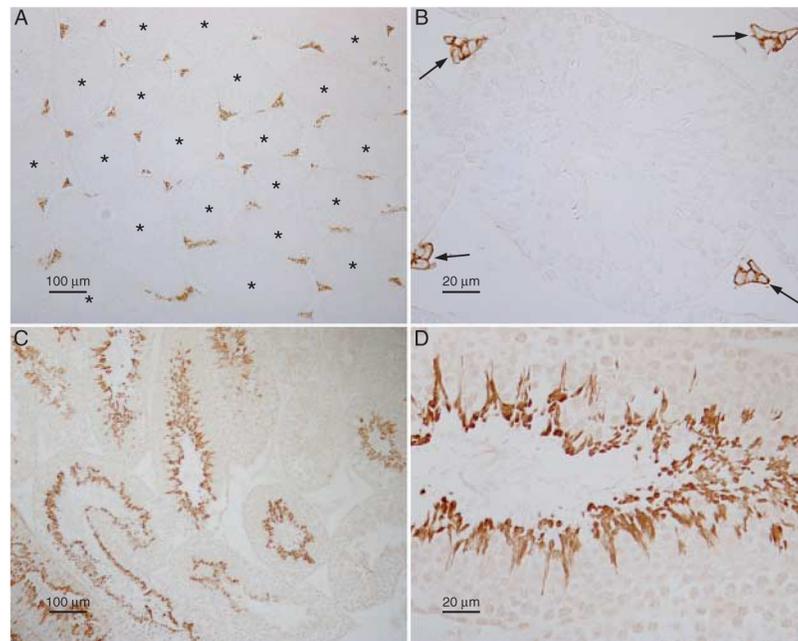
Figure 3.
RHBG and RHCG localization in the testis. (A and B) Low- and high-power micrographs respectively of RHBG immunolabeling in the testis. No RHBG immunolabel is present in seminiferous tubules (asterisks). However, strong plasma membrane RHBG immunolabel is present in interstitial cells with morphology and localization consistent with identification as Leydig cells (arrows).

No immunolabel was observed in experiments in which primary antibody was omitted (data not shown). (C and D) Representative low- and high-power micrographs respectively of RHCG immunolabel in the testis. RHCG exhibits significant variation in immunolabel intensity in different seminiferous tubules, consistent with stage-dependent variations in RHCG expression. High-power micrographs (D) spermatids, but not Leydig cells, display RHCG immunolabel. Controls in which primary antibody was omitted had no detectable immunolabel. Results are representative of findings from at least four mice.
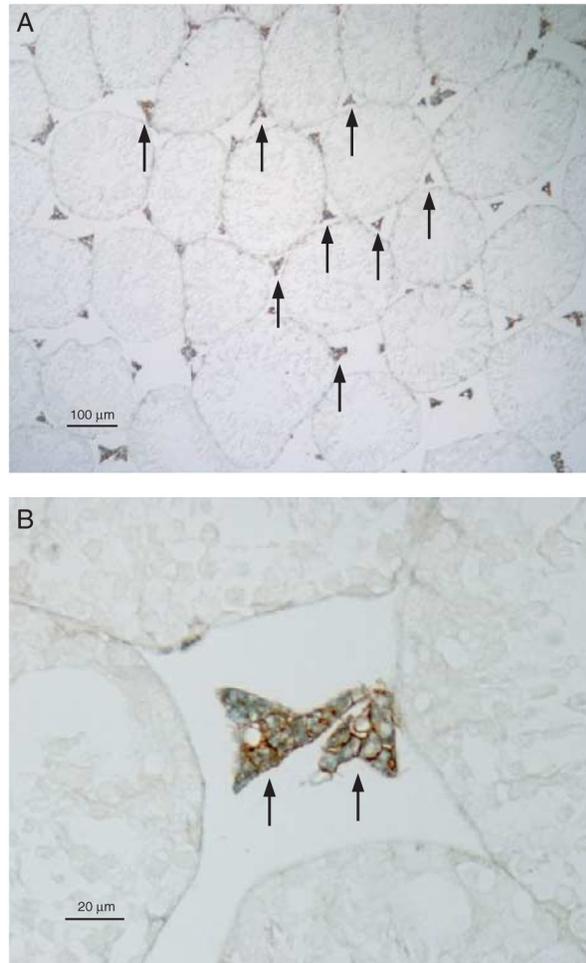
Figure 4.
Double-immunolabel for RHBG (brown) and glutamine synthetase (blue) in the testis. Glutamine synthetase immunolabel was used as a Leydig cell-specific marker. (A) Low-power micrograph and (B) High-power micrograph. All cells with RHBG immunolabel (brown) also exhibited cytoplasmic glutamine synthetase immunolabel (blue), and all cells with glutamine synthetase immunolabel expressed RHBG immunolabel (arrows). These findings indicate RHBG immunolabel expression in Leydig cells in the testis. Results are representative of findings from at least four mice.
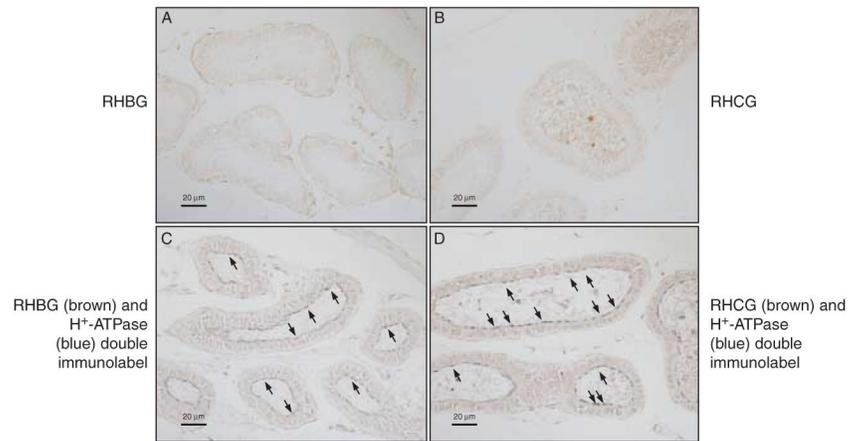
Figure 5.
RHBG and RHCG immunolabel expression in the efferent ductules. (A) Efferent ductules stained for RHBG immunolabel expression. No significant immunolabel is detectable. (B) Efferent ductules stained for RHCG immunolabel. No significant immunolabel is detectable. (C) Double-immunolabel of RHBG (brown) with H^+-ATPase (blue) to confirm retention of antigenic sites. Apical H^+-ATPase immunolabel is present (arrows), but no RHBG immunolabel is detectable. (D) Double-immunolabel of RHCG (brown) with H^+-ATPase (blue) to confirm retention of antigenic sites. Apical H^+-ATPase immunolabel is present (arrows), but no RHCG immunolabel is detectable. Results are representative of findings from at least four mice.
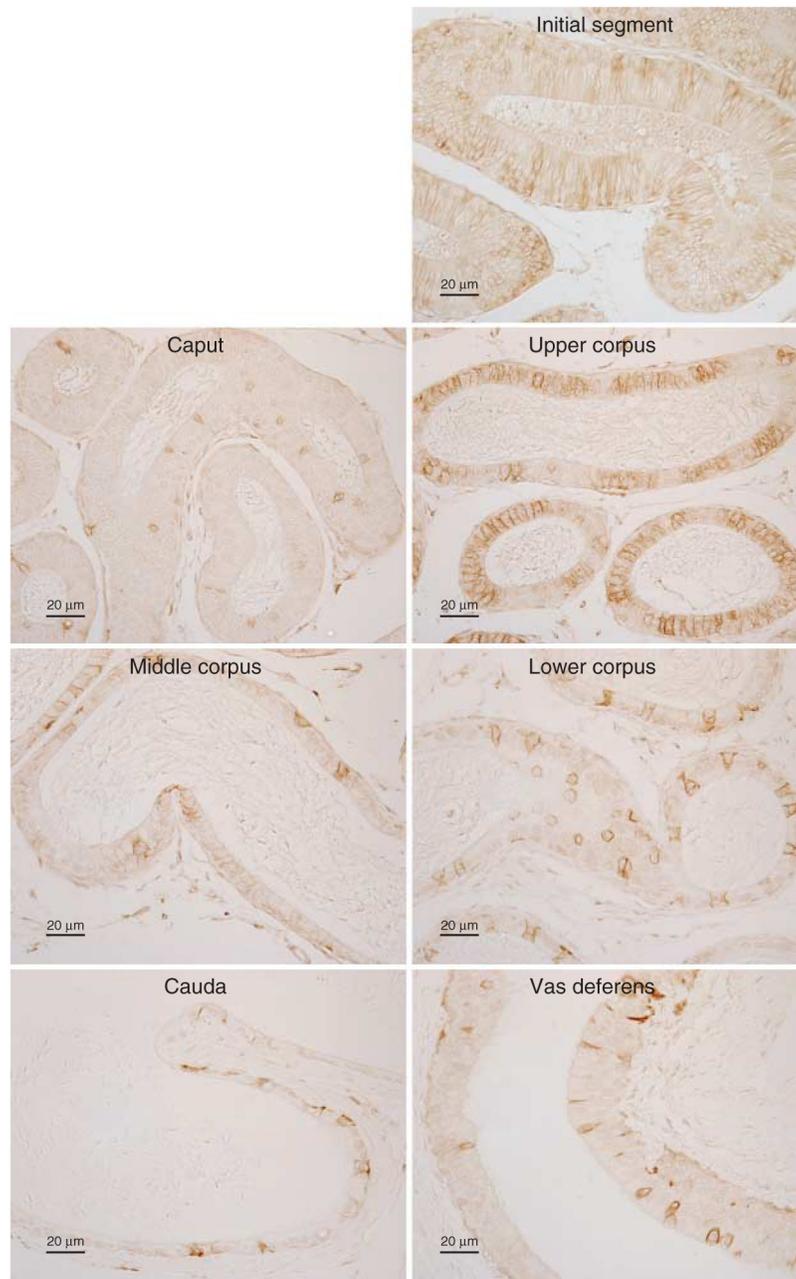
Figure 6.
RHBG immunolabel in the epididymis. In the caput epididymidis, a small population of cells expressed moderate-intensity basolateral RHBG immunolabel; the majority of cells, however, did not express detectable immunolabel. In the upper corpus epididymidis, all epithelial cells exhibited basolateral RHBG immunoreactivity, albeit at varying levels of immunolabel intensity. In the middle and lower corpus and cauda epididymidis, and vas deferens, basolateral RHBG immunolabel was present in a minority of epithelial cells. Results are representative of findings from at least four mice.
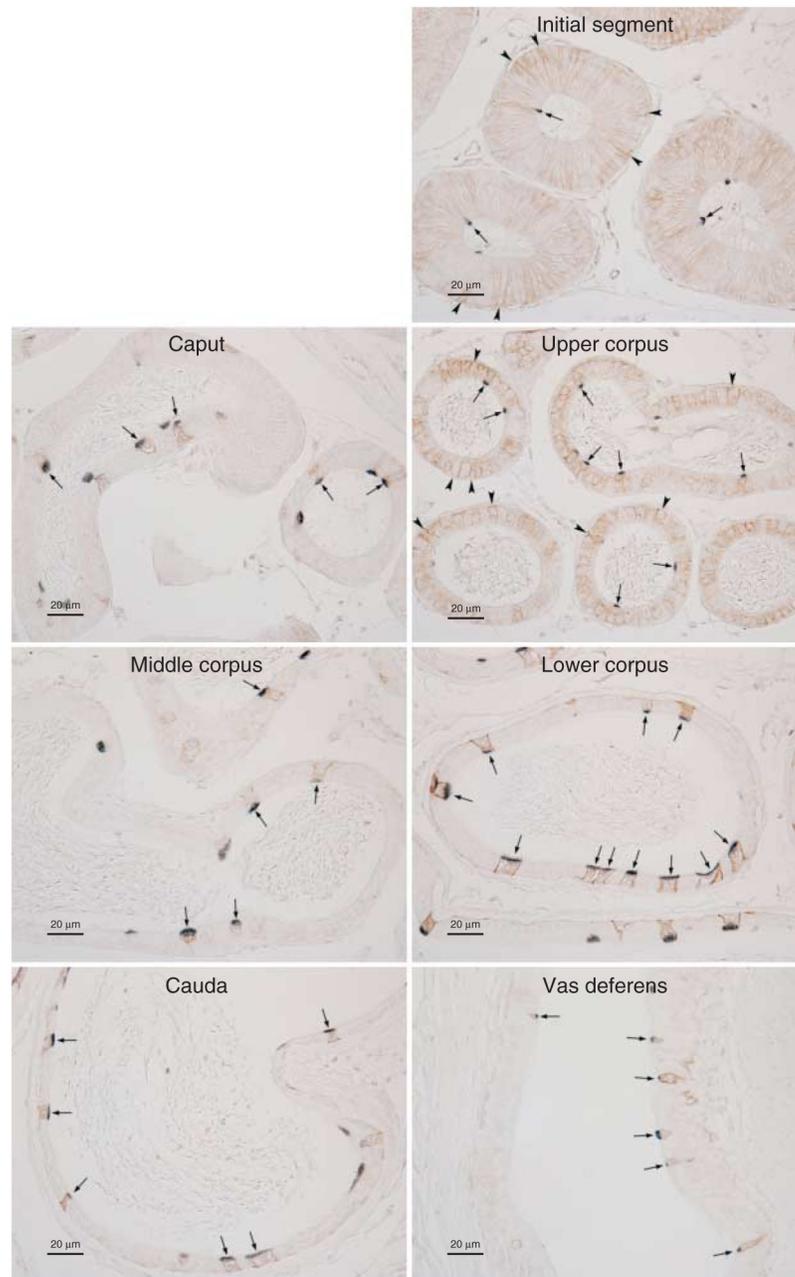
Figure 7.
Colocalization of RHBG (brown) with H$^+$-ATPase (blue) in the epididymis. In order to determine specific epididymal cell types expressing RHBG, we colocalized RHBG with H$^+$-ATPase. In the initial segment, basolateral RHBG immunoreactivity (brown) is present in H$^+$-ATPase-positive narrow cells (arrows) and H$^+$-ATPase-negative principal cells (arrowheads). In the caput epididymidis, basolateral RHBG immunoreactivity is present exclusively in H$^+$-ATPase-positive clear cells (arrows). In the upper corpus, both H$^+$-ATPase-positive clear cells (arrows) and a subset of H$^+$-ATPase-negative principal cells (arrowheads) expressed basolateral RHBG immunolabel. In the middle and lower corpus

and cauda epididymidis, H$^+$-ATPase-positive clear cells (arrows), but not H$^+$-ATPase-negative principal cells, expressed basolateral RHBG immunolabel. In the vas deferens, basolateral RHBG was present only in H$^+$-ATPase-positive cells (arrows). In all regions, intraluminal sperm did not label for either RHBG or H$^+$-ATPase. Results are representative of findings from at least four mice.
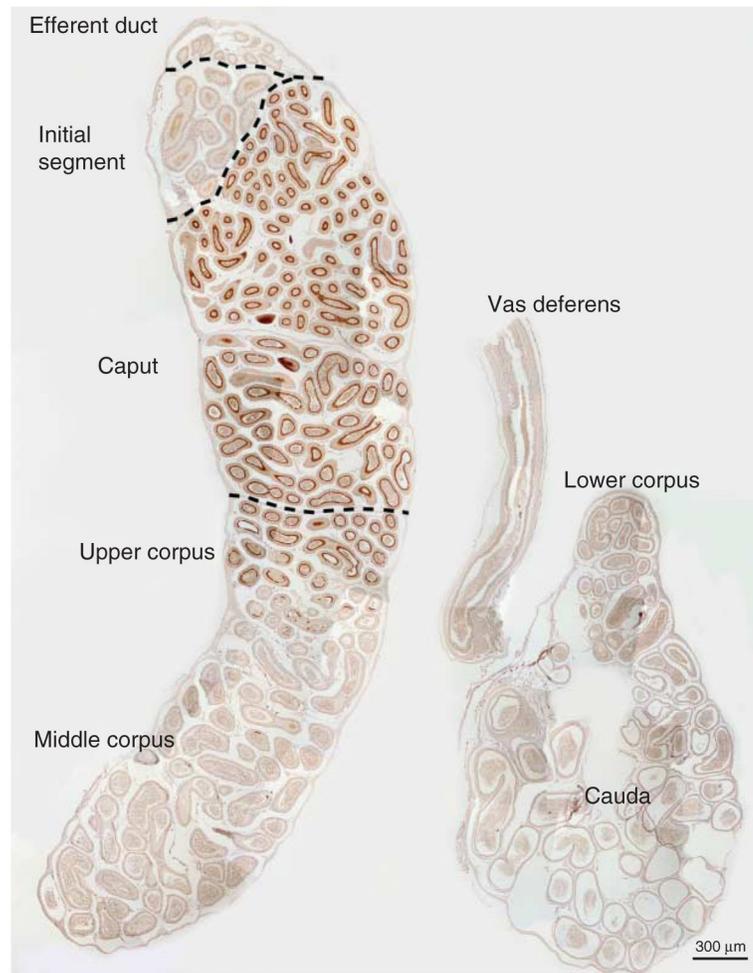
Figure 8.
Low-power micrograph of RHCG immunolabel in the epididymis. In the initial segment, RHCG immunolabel was not identified. High-intensity immunolabeling is present in the caput epididymidis, intermediate-intensity in the upper corpus epididymidis, and only low-intensity immunolabelling in the middle corpus, lower corpus and the cauda epididymidis. No immunolabeling was detected when primary antibody was omitted (not shown). Results are representative of findings from at least four mice.
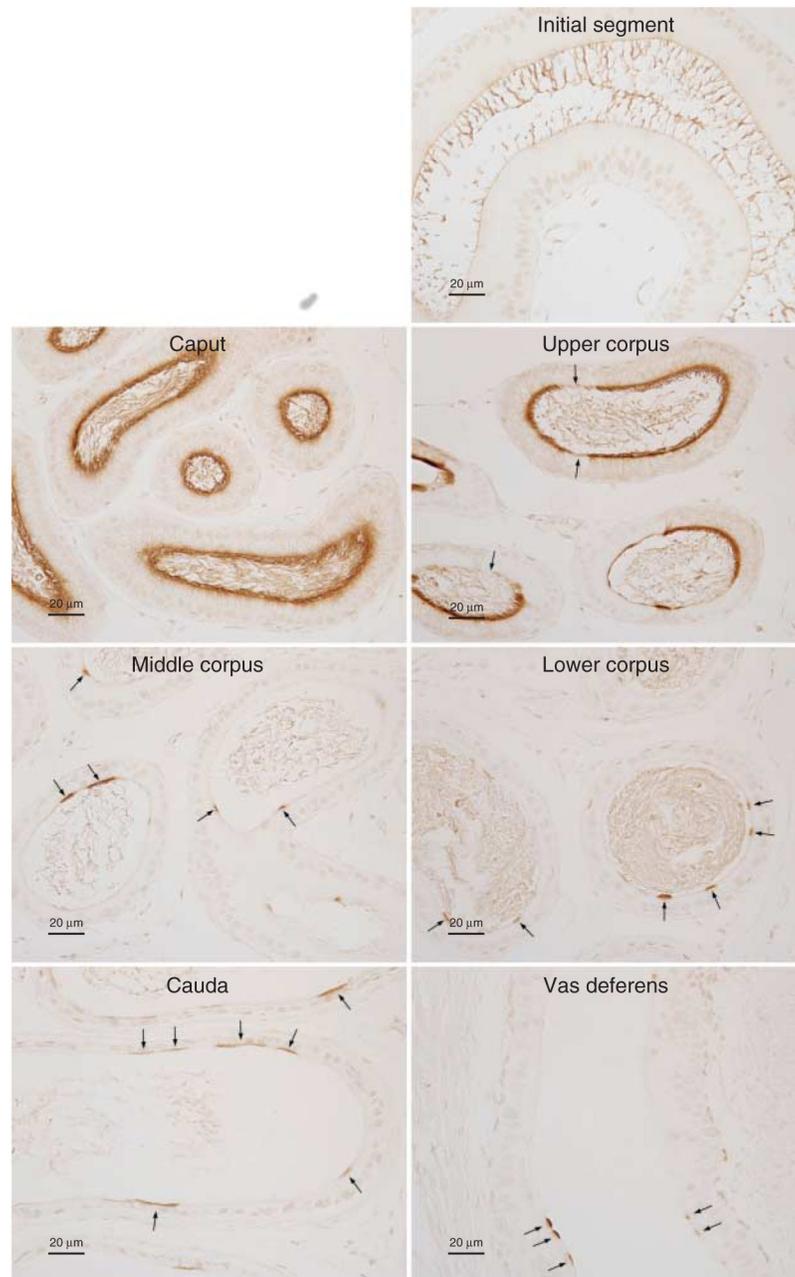
Figure 9.
High-power micrographs of RHCG immunolabel in the epididymis. In the initial segment, RHCG immunolabel was not identified. In the caput epididymidis, the majority of cells exhibit strong apical RHCG immunolabel. In the upper corpus, apical RHCG was expressed by the majority of cells. Again, significant cellular heterogeneity in RHCG immunolabel was present, with some cells exhibiting either very low or undetectable RHCG immunolabel (arrows). In the middle and lower corpus and cauda epididymidis, and the vas deferens, the majority of cells did not immunolabel for RHCG. However, a subpopulation of cells exhibit moderately strong apical RHCG immunolabel (arrows). No immunolabel was detected when

primary antibody was omitted (data not shown). Results are representative of findings from at least four mice.
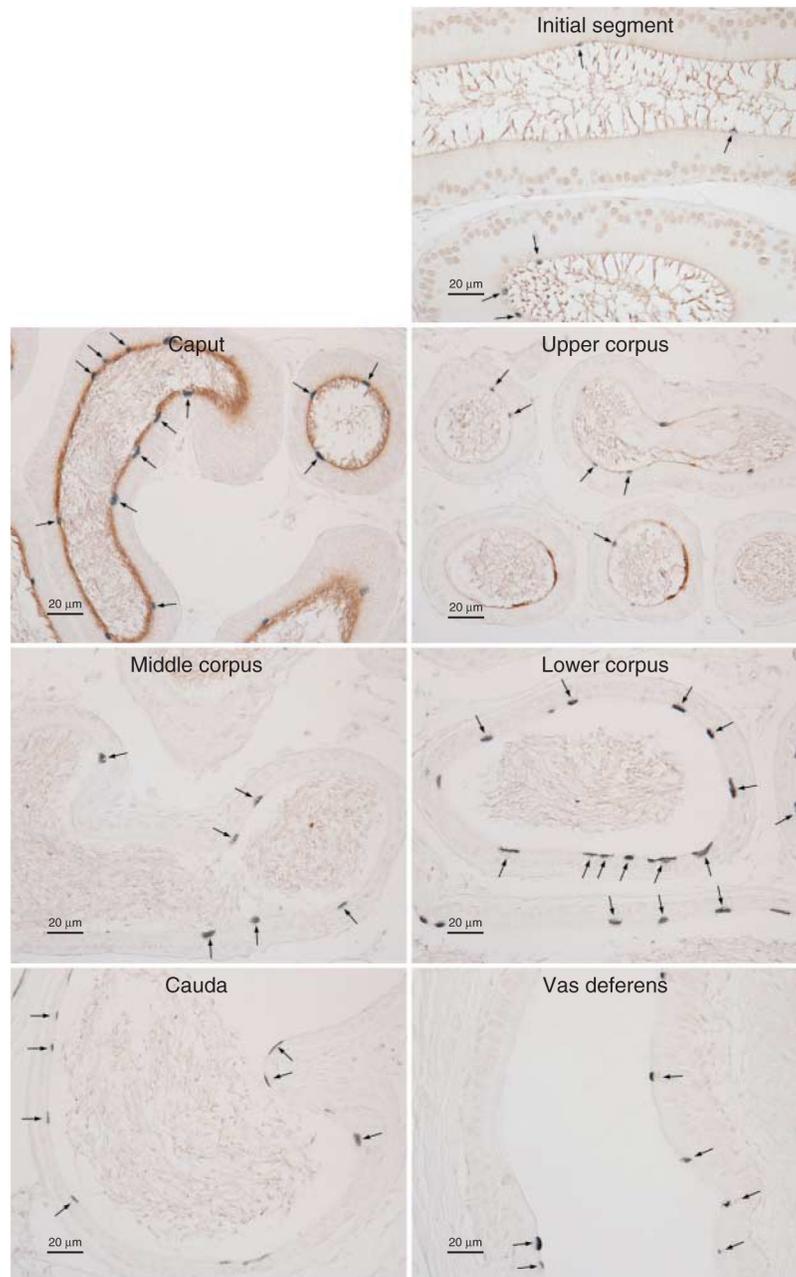
Figure 10.
Double immunolabeling of H^+-ATPase and RHCG in the epididymis. In the initial segment, RHCG immunolabel was not present in H^+-ATPase-positive (narrow) cells (arrows). In the caput and upper corpus, intense apical RHCG immunolabel was limited to H^+-ATPase-negative (principal) cells. H^+-ATPase-positive (clear) cells (arrows) do not exhibit RHCG immunolabel. In the middle corpus, apical RHCG immunolabel was limited to H^+-ATPase-positive clear cells (arrows) and no detectable RHCG immunolabel was found in H^+-ATPase-negative principal cells. H^+-ATPase was expressed on the apical membrane of clear cells. In the lower corpus and cauda epididymidis and in the vas deferens colocalization of

H$^+$-ATPase and RHCG was present (arrows), similar to middle corpus. Results are representative of findings from at least four mice.
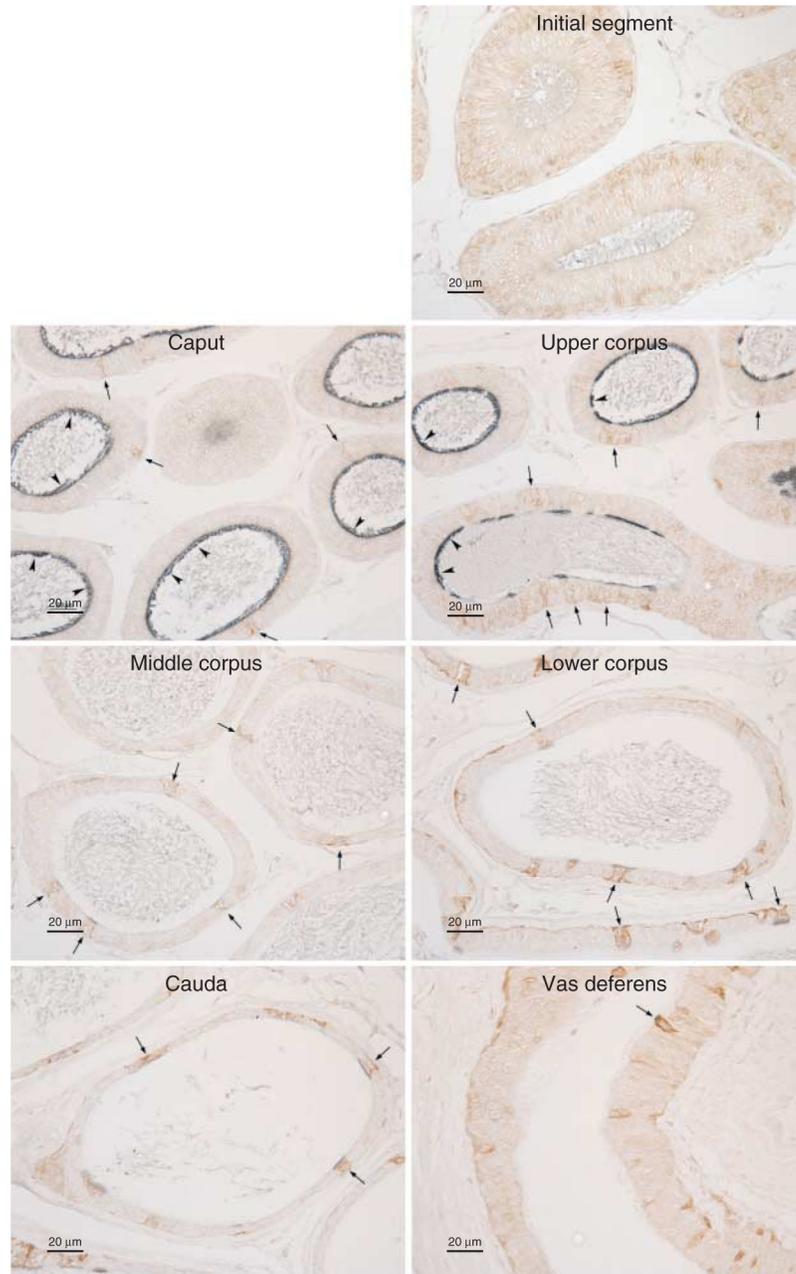
Figure 11.
Double-immunolabel of RHCG and RHBG in the epididymis. In the initial segment, basolateral RHBG immunoreactivity (brown) is present in narrow cells and principal cells. In the caput epididymidis, apical RHCG immunoreactivity (blue) is present in principal cells (arrowheads) and basolateral RHBG immunoreactivity is present in clear cells (arrows). In the upper corpus, apical RHCG immunoreactivity is present in principal cells (arrowheads) and basolateral RHBG immunoreactivity is present in both principal cells and clear cells (arrows). In the middle corpus, apical RHCG immunolabel and basolateral RHBG immunolabel are limited to clear cells (arrows). In the lower corpus and cauda epididymidis

and in the vas deferens, apical RHCG and basolateral RHBG are present (arrows), as in the middle corpus. Results are representative of findings from at least four mice.

Table 1

Summary of expression of RHCG, RHBG, and H⁺-ATPase in the efferent ductules, epididymis, and vas deferens.

| | Efferent duct | | Caput | | | | Corpus | | | | | | Cauda | | Vas deferens |
| | | | Initial segment | | Head | | Upper | | Middle | | Lower | | | | |
	Ciliated cells	Non-ciliated cells	PCs	Narrow cells	PCs	Clear cells	PCs	Clear cells	PCs	Clear cells	PCs	Clear cells	PCs	Clear cells	Epithelial cells
RHCG	−	−	−	−	+	−	+	−	−	+	−	+	−	+	+
RHBG	−	−	+	+	−	+	+	+	−	+	−	+	−	+	+
H⁺-ATPase	−	+	−	+	−	+	−	+	−	+	−	+	−	+	+

PCs, principal cells.