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Cadmium Inhibits Vacuolar H^+ATPase-Mediated Acidification in the Rat Epididymis[1]

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ABSTRACT

In rats, an acidic luminal pH maintains sperm quiescence during storage in the epididymis. We recently showed that vacuolar H^+ATPase-rich cells in the epididymis and vas deferens are involved in the acidification of these segments. Treatment of rats with cadmium (Cd) leads to alkalinization of this fluid by an unknown mechanism. Because Cd may affect H^+ATPase function, we examined 1) the in vivo effect of Cd poisoning on H^+ATPase-rich cell morphology and on the abundance and distribution of the 31-kDa H^+ATPase subunit in cells along the rat epididymis, and 2) the in vitro effect of Cd on H^+ATPase activity and function in the isolated vas deferens. Immunofluorescence and immunoblotting data from rats treated with Cd for 14–15 days (2 mg Cd/kg body mass/day) showed that 1) H^+ATPase-positive cells regressed to a prepubertal phenotype, and 2) H^+ATPase was lost from the apical pole of the cell and was redistributed into an intracellular compartment. In experiments in vitro, Cd inhibited bafilomycin-sensitive ATPase activity in isolated total cell membranes and, as measured using a proton-selective extracellular microelectrode, inhibited proton secretion in isolated vas deferens. We conclude that alkalinization of the tubule fluid in the epididymis and vas deferens of Cd-treated rats may result from the loss of functional H^+ATPase enzyme in the cell apical domain as well as from a direct inhibition of H^+ATPase function by Cd.

INTRODUCTION

Recent observations suggest that a disturbing decrease in male fertility has occurred in the human male population over the last 50 yr [1, 2]. A variety of occupational and environmental factors, such as environmental estrogens and antiandrogens, polychlorinated biphenyls, fungicides, and pesticides, may act as endocrine-disrupting agents during fetal development and may also have postnatal effects [3, 4]. In addition, stress, various physical agents, and many chemicals may directly harm the male reproductive tract and decrease fertility [5]. Some heavy metals, such as manganese, lead, and mercury, were found to affect sperm count, shape, and motility, potency, libido, histology of the testes, spermatogenesis, and serum testosterone levels in exposed men and experimental animals. On the other side, cadmium (Cd) and boron alter fertility in various experimental animals, but their effects in men have not been unequivocally established [5].

In adult male rats, acute or chronic treatment with Cd induces a well-documented toxic effect on reproductive organs and production of androgens. Among the reported effects of Cd are a reduction in size and mass of the testes, epididymis, and seminal vesicles [6–8]; necrosis of seminiferous tubules due to vascular damage [9]; decreased sperm concentration in the vas deferens [7, 8, 10, 11]; decreased serum testosterone concentration due to reduced synthesis of testosterone in the testes [12], as well as an increased serum concentration of FSH [8].

Following Cd treatment in the adult male rat, Caflisch and DuBose [6] observed alkalinization of the luminal fluid in the seminiferous tubules and epididymis. The fluid in these tubules is normally acidified by mechanisms that are poorly understood. Compared with the pH in blood (7.4), the normal pH of the luminal fluid in the male reproductive tract is relatively acidic and varies between about 7.0 in the seminiferous tubules and 6.5 in the cauda epididymidis. This acidic pH seems to be involved in maintaining spermatozoa immotile during their passage, storage, and maturation in this organ [6, 13, 14]. We have recently described specialized cells in the epididymis and proximal vas deferens that contain functional vacuolar H^+ATPase (bafilomycin-sensitive proton pump) on their apical plasma membrane [15–18]. This H^+ATPase may thus participate in acidification of the luminal fluid in the epididymis and proximal vas deferens. Inhibition of H^+ secretion by these cells may contribute to the alkalinization of this fluid in Cd-treated rats. The present study was designed to examine the possibility that Cd affects the morphology and function of H^+ATPase-positive cells in the epididymis and vas deferens.

MATERIALS AND METHODS

Animals and Treatment

Adult male Wistar rats were used for studies with Cd in vivo and isolation of total cell membranes for studies in vitro, whereas organs from Sprague-Dawley rats were used for the in vitro studies of H^+ secretion. Rats were treated with Cd by s.c. injections of $CdCl_2$ (2 mg Cd/kg body mass/day) for 14–15 days. This treatment efficiently induces Cd

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poisoning manifested by decreased body mass and nephrotoxic symptoms that include polyuria, proteinuria, and phosphaturia [19, 20]. Control animals were injected with an equivalent volume (0.15 ml) of 0.9% NaCl. During the treatment, animals had standard laboratory food and tap water ad libitum. Animals were sacrificed by decapitation or by exsanguination after Nembutal anesthesia.

Tissue Cadmium

Epididymides from control and Cd-treated animals were removed, trimmed free of fat, and weighed. The specific parts (initial segment plus caput, corpus, cauda) were dissected manually, weighed, and dry ashed at 450°C for 24 h. Ashed samples were dissolved in 2% nitric acid, and Cd concentration was measured by Varian SpectrAA-300 or AA-375 atomic absorption spectrometers in electrothermal (tissue samples from control rats) or flame mode (tissue samples from Cd-treated animals), respectively.

Antibodies

Primary polyclonal antipeptide antibodies raised in rabbit to the C-terminal domain of the vacuolar H^+ATPase 31-kDa (subunit E) and 70-kDa (subunit A) subunits were used. The use of these antibodies in immunocytochemical and immunoblotting experiments has been previously described [20–22]. Secondary antibodies, including fluorescein- or phosphatase-labeled goat antirabbit IgG antibodies, were purchased from Vector Laboratories (Burlingame, CA).

Tissue Fixation and Immunocytochemistry

Rats were anesthetized with Nembutal (65 mg/kg body mass, i.p.) and the circulation was perfused via the left ventricle of the heart, first with 50–60 ml aerated (95% O_2/5% CO_2; 37°C) PBS to remove circulating blood, followed by 200 ml PLP fixative (2% paraformaldehyde, 10 mM sodium periodate, 75 mM lysine). The reproductive organs were removed, kept overnight in the same fixative at 4°C, washed with PBS, and kept in PBS containing 0.02% sodium azide at 4°C until further use.

Tissue cryosections (4 μm thick) were collected on gelatin-coated microscope slides, rehydrated in PBS for 10 min, incubated for 5 min with 1% SDS (in PBS) in order to enhance antibody labeling [23], and extensively washed with PBS. Sections were then kept in 1% BSA (in PBS) for 15 min, incubated with anti-31-kDa antibody (immune serum diluted 1:200) overnight at 4°C, washed, incubated with fluorescein-labeled goat antirabbit IgG antibody (8 μg/ml) at room temperature for 1 h, washed, incubated for 30 sec in 0.01% Evan's blue to achieve a red background in color images, washed, mounted in a fluorescence fading retardant (Vectashield; Vector Laboratories Inc., Burlingame, CA), and examined with a fluorescence microscope. The images were photographed using Kodak Ektachrome Elite 400 slide film. Slides were scanned using a Polaroid SprintScan 35 Plus scanner. Scans were imported into Adobe Photoshop 4.0 software and printed on an Epson Stylus Color 600 printer.

SDS-PAGE and Immunoblotting

To determine the abundance of H^+ATPase subunits in total cell membrane preparations that are rich in plasma membranes, caudae epididymides from control and Cd-treated rats were removed. To obtain enough material, tissues from either two control or four Cd-treated rats were pooled and further processed as one sample. The tissue was finely minced with scissors. To remove spermatozoa, the minced material was washed three times with 15 ml homogenizing buffer (HB: 300 mM mannitol, 1 mM PMSF, 10 μM benzamidine, 0.1 μg/ml antipain, 5 mM EGTA, 12 mM Tris/HCl, pH 7.4). The tubule fragments were dispersed in 15 ml HB and homogenized for 1 min with a Polytron homogenizer (setting 5). The total homogenate was centrifuged at 2500 × *g* for 15 min. The pellet was discarded, whereas the supernatant (henceforth referred to as the homogenate) was centrifuged at 10 000 × *g* for 15 min. The mitochondria-rich pellet was discarded; the supernatant was further centrifuged at 48 000 × *g* for 30 min. The resulting supernatant was saved, whereas the pellet (total cell membrane fraction that was rich in plasma membranes) was dissolved in HB. All steps of the isolation procedure were performed in cold (ice baths, chilled buffers) and in a refrigerated high-speed centrifuge. Protein was determined by a dye-binding assay [24] using BSA as the standard.

The proteins were denatured in a sample buffer (1% SDS, 12% v/v glycerol, 5% β-mercaptoethanol, 30 mM Tris/HCl, pH 6.8) at 95°C for 5 min, separated by 12% mini-SDS-PAGE (40 μg protein per well), and transferred to Immobilon membrane (Millipore, Bedford, MA). The membrane was blocked in blotting buffer (5% nonfat dry milk, 0.15 M NaCl, 1% Triton X-100, 20 mM Tris/HCl, pH 7.4), incubated at 4°C overnight in the same buffer containing anti-31-kDa or anti-70-kDa H^+ATPase subunit antibody (immune sera diluted 1:500), washed, incubated for 1 h with phosphatase-labeled goat antirabbit IgG antibody (0.5 μg/ml blotting buffer), washed, and stained for alkaline phosphatase activity with the BCIP/NBT kit (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The protein bands were scanned with an LKB Ultroscan XL Enhanced Laser Densitometer (Bromma, Sweden). The scanned peak area of each band was printed on paper, excised, weighed, and expressed in units (%) relative to the mass of the largest peak area in the respective blot.

H^+ATPase Assay

Total cell membranes from the cauda epididymidis were prepared as described above, except that the final membrane pellet was dissolved in KCl buffer that contained (in mM): 150 KCl, 5 $MgSO_4$, 50 HEPES-Tris, pH 7.0. Bafilomycin-sensitive ATPase activity in the total cell membranes was measured by a phosphate (P_i) liberation assay as described previously in detail [25]. Membrane preparations were used either intact or following treatment with Tween 20 (final concentration 0.05% v/v) in an ice bath for 10 min in order to open vesiculated membranes and expose total H^+ATPase activity. In preliminary experiments, the presence of membrane vesicles was tested by acridine orange fluorescence quenching by monitoring ΔpH-dependent signals generated by the potassium gradient-driven diffusion potential, as described previously [26]. With this method, in the absence of detergent, freshly prepared total cell membranes from control cauda epididymidis exhibited a small fluorescence quenching signal (5–10% of the total fluorescence), indicating that only a small proportion of the membranes was vesiculated (data not shown). Following preincubation of membrane preparations with 0.05% Tween 20 at 0°C for 10 min, the fluorescence signal was completely abolished, indicating a loss of vesiculated mem-

TABLE 1. Organ mass and tissue concentration of cadmium in the epididymides of vehicle-treated (control) and cadmium-treated rats.

Parameter	Control (n)[a]		Cadmium (n)[a]	
Organ mass (g)	1.19 ± 0.63 (5)		0.47 ± 0.02 (8)[d]	
Tissue cadmium				
Initial segment plus caput	8 ± 0.6 (3)[b]	71 ± 5.3[c]	11.30 ± 0.98 (5)[d,e]	101 ± 8.7[f]
Corpus	12 ± 3.0 (3)[b]	107 ± 26.7[c]	8.03 ± 1.11 (5)[d,e]	71 ± 9.9[f]
Cauda	15 ± 8.7 (3)[b]	133 ± 77.4[c]	5.87 ± 0.38 (5)[d,e]	52 ± 3.4[f]

[a] Shown are means ± SEM of the data measured in tissues from (n) number of animals. Cadmium was measured by atomic absorption spectroscopy in the ashed tissues.
[b] Tissue Cd measured as ng/g wet mass.
[c] Tissue Cd measured as nmol/L, calculated under rough assumption of equal distribution of Cd in the tissue (1 g of tissue mass = 1 ml volume; atomic mass of Cd = 112).
[d] Versus respective data in control animals, $P < 0.001$.
[e] Tissue Cd measured as μg/g wet mass.
[f] Tissue Cd measured as μmol/L.

branes. Simultaneously, the bafilomycin-sensitive ATPase activity increased 40–50%. However, in membrane preparations from Cd-treated rats, the quenching signal was not observed even in the absence of a detergent, and bafilomycin-sensitive ATPase was not elevated by detergent treatment, indicating that these membrane preparations contained no detectable vesiculated membranes (data not shown).

The standard ATPase mixture [25] contained KCl buffer, various inhibitors and ionophores, and either 10 μg (intact membranes) or 15 μg protein (detergent-treated membranes). Under these conditions the reaction of bafilomycin-sensitive P_i liberation was linear for up to 30 min (data not shown). Bafilomycin-sensitive ATPase was measured as the difference in ATPase activity in the absence and presence of 1 μM bafilomycin A_1 (a gift from Dr. K. Altendorf, Osnabrück, Germany). Vesicles were preincubated with the inhibitors and ionophores at 37°C for 15 min before ATP was added to start the reaction. The reaction was terminated 15 min later and processed as described previously [25].

Proton Flux

Apical proton (H^+) fluxes were detected in isolated vas deferens using an extracellular H^+-selective, self-referencing electrode, as described previously [15, 18, 27]. The proximal portion of the vas deferens of adult Sprague-Dawley rats (300–350 g body mass) was dissected and most of the surrounding connective and muscular tissue was removed. The vas deferens was opened by a longitudinal incision, the apical surface of the epithelium was exposed, and the whole tissue was bathed in low PBS (2 mM phosphate) as described in our previous reports [15, 18].

Other Materials

Nembutal was from Abbot Laboratories (North Chicago, IL), paraformaldehyde, lysine, Evan's blue, PMSF, benzamidine, antipain, Triton-X-100, and Tween-20 from Sigma (St. Louis, MO), sodium periodate from Fisher Scientific (Springfield, NJ), $CdCl_2$ and mannitol from Merck (Darmstadt, FRG), SDS from Serva (Heidelberg, FRG), PAGE-electrophoresis reagents and equipment from Bio-Rad (Hercules, CA). Other chemicals were p.a. grade and were purchased commercially.

Presentation of the Data

The figures showing immunofluorescence and immunoblotting data represent observations in two independent experiments with three to four animals in each experimental group. The numeric data are means ± SEM statistically evaluated by the two-tailed unpaired or paired *t*-test at a 5% level of significance using Microsoft Excel version 5.0 software.

RESULTS

Experiments In Vivo

Efficiency of Cd treatment. In rats treated s.c. with 2 mg Cd/kg body mass/day for 2 wk we observed a significant atrophy of the epididymides; their mass was 60% lower than in control animals (Table 1). Furthermore, whereas epididymides from control rats contained only trace amounts of Cd (8–15 ng/g tissue wet mass), epididymal tissue accumulated very high concentrations of Cd after 2 wk of Cd treatment. The levels were 390 (cauda), 670 (corpus), and 1400 (initial segment plus caput) times higher than in the respective epididymal segments of control animals (Table 1). Assuming equal distribution of Cd in the tissue, a rough calculation indicates that in Cd-treated rats the tissue Cd reached at least 50, 70, and 100 μmol/L in the cauda, corpus, and initial segment plus caput, respectively (Table 1).

Immunocytochemistry. Immunocytochemical inspection of the epididymis in intact rats revealed widely opened ducts, usually full of spermatozoa, with an epithelium that was tall columnar in the initial segment (Fig. 1A), low columnar in the caput (not shown) and corpus (Fig. 1C), and cuboidal toward the cauda (Fig. 1E). In accordance with our previous reports [15, 17], H^+ATPase-positive cells were located in the initial segment (apical or narrow cells), caput and corpus (low columnar clear cells), and cauda (cuboidal clear cells). The H^+ATPase-positive cells in all segments exhibited strong immunostaining at the apical pole and a weak intracellular staining. This result indicates that the majority of H^+ATPase is localized in or close to the luminal membrane of these cells, a finding supported by our previous immunoelectron microscopic data [17]. H^+ATPase-positive cells, morphologically similar to those in the cauda epididymidis, were also found in the proximal part of the vas deferens [15].

In Cd-treated rats, the height of the epithelium in ducts of the initial segment was not visibly different from that in control animals (Fig. 1B), whereas in the caput (not shown), corpus (Fig. 1D), and cauda (Fig. 1F), the epithelium became considerably thicker. The epithelial cells were transformed into tall columnar cells that were morphologically similar to those in the initial segment. In the caput

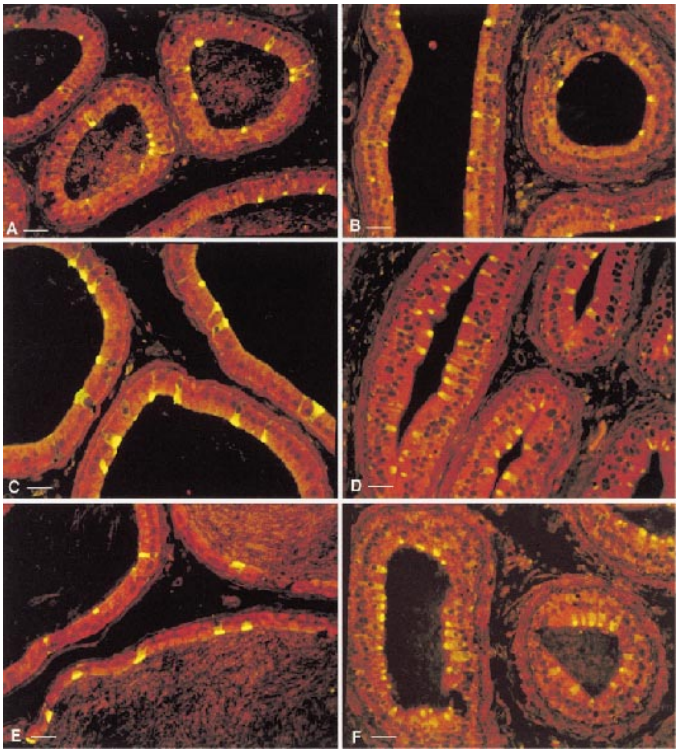


FIG. 1. Immunostaining of H⁺ATPase-positive cells with the anti-31-kDa subunit antibody (yellow fluorescence) in the initial segment (**A, B**), corpus (**C, D**), and cauda (**E, F**) of epididymidis in control (**A, C, E**) and Cd-treated rats (**B, D, F**). In control rats, the duct epithelium is tall columnar, low columnar, and low cuboidal in the initial segment (**A**), corpus (**C**), and cauda (**E**), respectively. The H⁺ATPase-positive cells in the initial segment (chalicelike apical or narrow cells), corpus (low columnar clear cells), and cauda (low cuboidal clear cells) are stained brightly at the apical cell domain and weakly intracellularly. In Cd-treated rats, the duct lumen in all segments is narrow and the epithelium in the corpus (**D**) and cauda (**F**) is as tall as that in the initial segment. The H⁺ATPase-positive cells in all segments are morphologically similar, e.g., tall columnar with chalicelike shape similar to those in the initial segment. The fluorescence at the apical domain of these cells is largely diminished and shifted intracellularly. A background red color results from counterstaining of sections with Evan's blue. Bar = 15 μm.

(not shown), corpus, and cauda, the ducts were greatly reduced in diameter, with a narrow or closed lumen that was either empty or contained only a few spermatozoa. Staining with the anti-31-kDa subunit antibody showed that, phenotypically, H⁺ATPase-positive cells in all sections of the epididymis became similar: they were high columnar with a narrow, chalicelike shape. However, the intensity of H⁺ATPase staining in the apical domain of these cells was greatly diminished, and many cells exhibited a diffuse or finely granular staining uniformly distributed throughout the cytoplasm. This phenomenon was particularly evident in cells from the cauda epididymidis that underwent the most dramatic transformation from a cuboidal shape with strong apical H⁺ATPase staining in control rats to a tall columnar shape with intracellularly located H⁺ATPase in rats treated with Cd (Fig. 2).

Western blots and bafilomycin-sensitive ATPase. Immunocytochemical data showed that the most prominent differences in cell morphology and staining intensity between control and Cd-treated rats were present in the cauda epididymidis. In order to determine whether Cd treatment in vivo affected the abundance, distribution, and/or the activity of H⁺ATPase in the cell membranes, various samples from the cauda epididymidis were prepared from both animal groups. Immunoblots were performed on tissue homogenates (post-2500 × *g* supernatant), plasma membrane-rich total cell membranes (pellet between 10 000 × *g* and 48 000 × *g*) and respective post-48 000 × *g* supernatants. In addition, the bafilomycin-sensitive ATPase activity was measured in the total cell membranes.

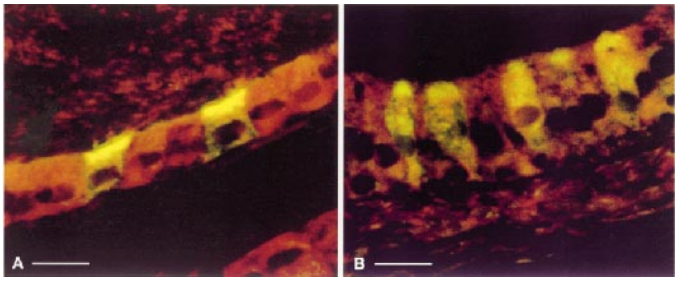


FIG. 2. Immunocytochemical appearance of representative H⁺ATPase-positive cells in the cauda epididymidis in control (**A**) and Cd-treated rats (**B**). In control animals, the cells are low cuboidal with most of the fluorescence (H⁺ATPase) concentrated at the apical pole. In Cd-treated animals, the cells are tall columnar, wine glass-shape, and the fluorescence is distributed intracellularly. The red background color results from staining of the sections with Evan's blue. Bar = 10 μm.

As shown in Figure 3, the 31-kDa protein band was stronger in homogenates from Cd-treated rats in comparison with that from control animals. In tissue homogenates from eight control and eight Cd-treated rats the measured band density was 62.5 ± 2.9 and 92.5 ± 3.2 ($P < 0.001$) relative units, respectively. This apparent increase in total 31-kDa protein content (approximately 50%) may be attributed to the Cd-induced atrophy of the epididymis that might lead to an increase in the number of epithelial cells per unit volume. In the total cell membranes, the 31-kDa protein band was not significantly different between Cd-treated rats and control (68.6 ± 5.6, n = 8, vs. 74.9 ± 5.9, n = 8, relative units, respectively), whereas the intensity of the respective band in the post-48 000 × *g* supernatant was increased about 70% in samples from Cd-treated rats (84.1 ± 5.7, n = 8; $P < 0.001$) compared to controls (50.4 ± 3.9, n = 8). These results were confirmed using immune

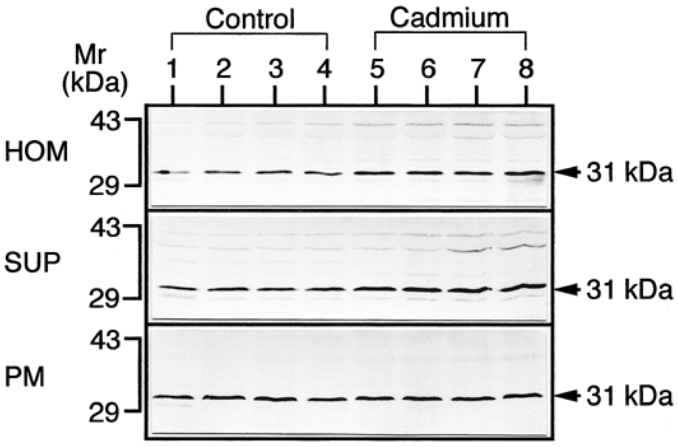


FIG. 3. Immunoblot showing abundance of the 31-kDa H⁺ATPase subunit in tissue samples following differential centrifugation of the homogenized cauda epididymides from control (control) and Cd-treated (cadmium) rats. Data represent four different membrane preparations in each experimental set. The respective protein band was stronger in tissue homogenates (HOM) and supernatants (SUP) from Cd-treated rats, whereas in total cell membranes (PM) from both animal groups the band was similar.

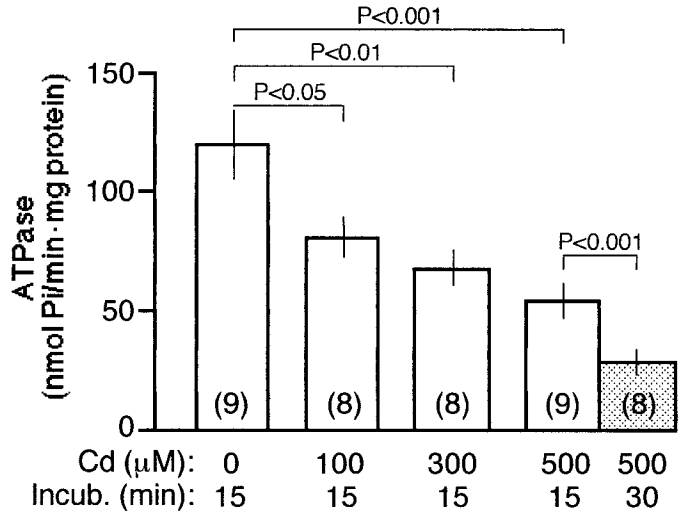


FIG. 4. In vitro inhibition of bafilomycin-sensitive ATPase activity by Cd in detergent-treated plasma membranes from the cauda epididymidis. Membranes were preincubated with the indicated concentrations of Cd at 37°C for 15 or 30 min before adding ATP. The inhibition of bafilomycin-sensitive ATPase increased with increasing concentration of Cd and with time of preincubation with Cd. Each bar represents the mean ± SEM of measurements in n different membrane preparations.

serum against the 70-kDa H⁺ATPase (catalytic) subunit (data not shown).

Bafilomycin-sensitive ATPase activity (nmol P_i/min mg protein) in intact total cell membranes from organs in control rats was 66 ± 11.6 (n = 4), whereas the activity in intact membranes from organs in Cd-treated rats was higher, 134 ± 23.4 (n = 4, vs. control; $P < 0.05$). However, the activity of this ATPase in Tween-20-treated membranes from control animals was 95 ± 16.5 (n = 9), e.g., about 45% higher than that in intact membranes from the same animals, indicating that a portion of the ATPase activity in intact membranes from control rats was masked in membrane vesicles. The presence of intact vesicles in these membrane preparations was confirmed by acridine orange fluorescence quenching (see *Materials and Methods*; data not shown). In detergent-treated membranes from Cd-intoxicated rats the bafilomycin-sensitive ATPase activity was 138 ± 22.7 (n = 8), a value not significantly different from that in intact membranes from Cd-treated rats and from that in the detergent-treated membranes from control rats. The absence of a detergent effect indicates that the intact membranes from Cd-treated rats were not significantly vesiculated, as also found by the method of acridine orange fluorescence quenching (see *Materials and Methods*, data not shown).

Experiments In Vitro

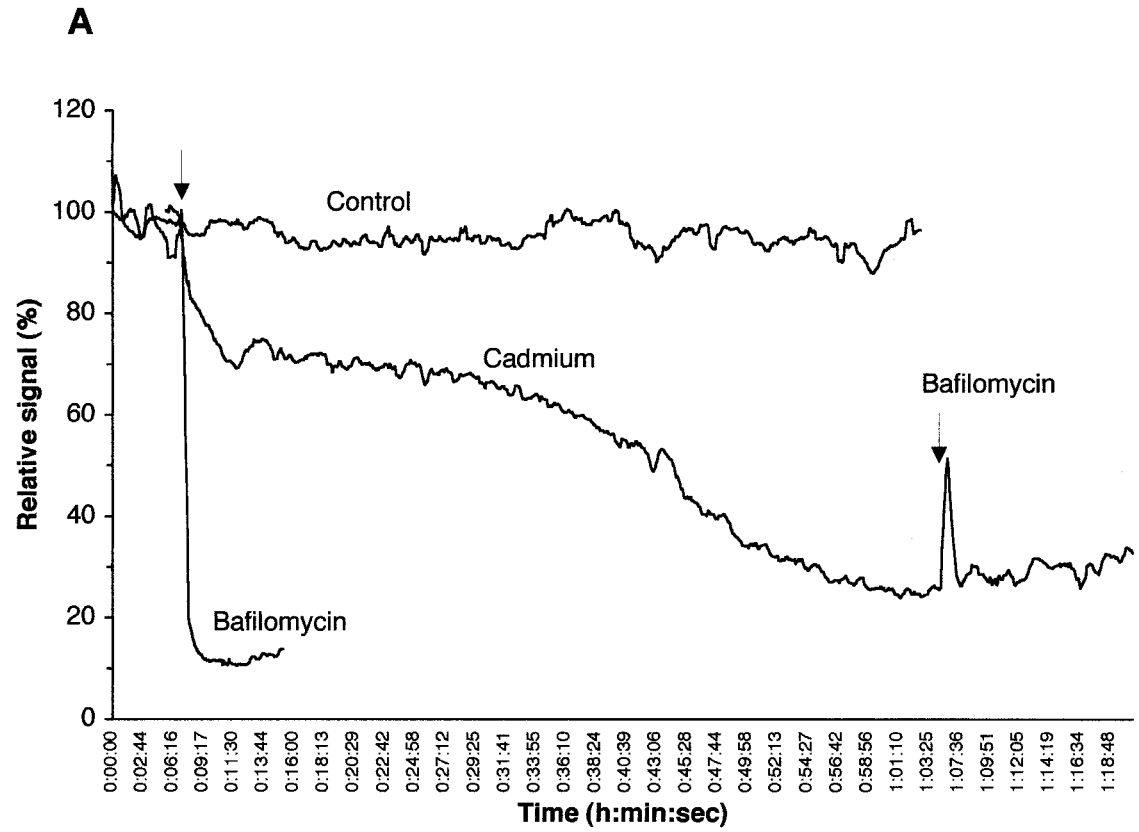
Inhibition of bafilomycin-sensitive ATPase by Cd in total cell membranes. To test the possibility that Cd may directly influence membrane-associated H⁺ATPase activity in the male reproductive tract, detergent-treated total cell membranes from the cauda epididymidis from control rats were preincubated with 0–500 μM $CdCl_2$ at 37°C for 15 min before the ATPase reaction was initiated by adding ATP. As shown in Figure 4 (open bars), Cd inhibited bafilomycin-sensitive ATPase activity in a concentration-dependent manner; at 500 μM Cd the inhibition reached 56% after 15 min of preincubation. The inhibition was also time dependent; following preincubation with 500 μM Cd for 30 min, the inhibition increased to 80% (Fig. 4, hatched bar). The concentration of Cd that, after 15 min preincubation, caused 50% inhibition of the enzyme activity (IC_{50}) was estimated from the individual curves at 338 ± 42.3 μM (n = 8).

Inhibition of H^+ flux by Cd in vas deferens. In an in vitro model of H⁺ secretion [15, 18], the proximal vas deferens from control animals was incubated in a weakly buffered medium in the absence (control) and presence of Cd and/or bafilomycin, and H⁺ fluxes from proton-secreting cells were monitored for 60–80 min using a self-referencing, proton-selective microelectrode. In control conditions (absence of Cd and bafilomycin), the H⁺ flux by these cells was steady for at least 60 min (Fig. 5A, control). By adding 1 μM (final concentration) bafilomycin to the medium, the H⁺ flux rapidly decreased, indicating that an H⁺ATPase is involved in the measured H⁺ secretion (Fig. 5A, bafilomycin). In the presence of 500 μM (final concentration) Cd in the medium, a steady, time-dependent decrease of H⁺ flux was recorded (Fig. 5A, cadmium). When 1 μM bafilomycin was applied ~60 min later, only a limited additional inhibition of the H⁺ flux was detected. As summarized in Figure 5B, 60 min following addition of Cd the H⁺ flux was inhibited 61 ± 5% (n = 8, vs. control, $P < 0.005$) and, by adding bafilomycin, it was additionally inhibited by 11 ± 3% (n = 8, vs. cadmium, $P < 0.05$).

DISCUSSION

It is well established that a high H⁺ concentration [13, 14, 28, 29] and a low HCO_3^- concentration [30] in the tubule fluid along the mammalian epididymis and vas deferens keep spermatozoa immotile during their storage and maturation and protect from premature activation of acrosomal enzymes [31, 32]. Luminal acidification in the epididymis is partially Na^+ dependent and may be related to apical Na^+/H^+ exchange [29, 33]. Recent work from our laboratory has shown, however, that a major contributor to H⁺ secretion may be a specialized population of epithelial cells that express a high level of both functional H⁺ATPase in their apical plasma membrane [15, 17] and carbonic anhydrase type II in their cytoplasm [18, 34–36].

Impaired acidification in the epididymis and vas deferens might result in deficient sperm maturation and motility, leading to lower male fertility and is a potential consequence of exposure to various occupational and environmental pollutants that reduce male fertility in humans and animals. Indeed, Caflisch and DuBose described that Cd, a well-known environmental pollutant and toxin to the kidney and male reproductive tract [5, 7–11, 19, 20], causes alkalinization of the fluid in the seminiferous tubules and epididymis in the adult male rat [6]. As the mechanism of alkalinization of the tubule fluid along the male reproductive tract in Cd-treated rats was not known, we hypothesized that Cd may affect the function of H⁺ATPase-positive (H⁺-secreting) epithelial cells in the epididymis and vas deferens. Our data show that treatment of male rats with Cd for 2 wk leads to 1) atrophy of epididymides and accumulation of Cd in the tissue; 2) decrease in the diameter of the duct lumen and thickening of the duct epithelium, mainly in the caput, corpus, and cauda epididymidis; 3) a morphological change of H⁺ATPase-positive cells from low columnar (caput, corpus) and cuboidal (cauda) into tall columnar in these segments; and 4) loss of H⁺ATPase protein from the apical cell domain and its redistribution into an intracellular compartment in the H⁺ATPase-positive cells. In experiments in vitro, Cd directly inhibited bafilomycin-sensitive ATPase activity in preparations of total cell mem-



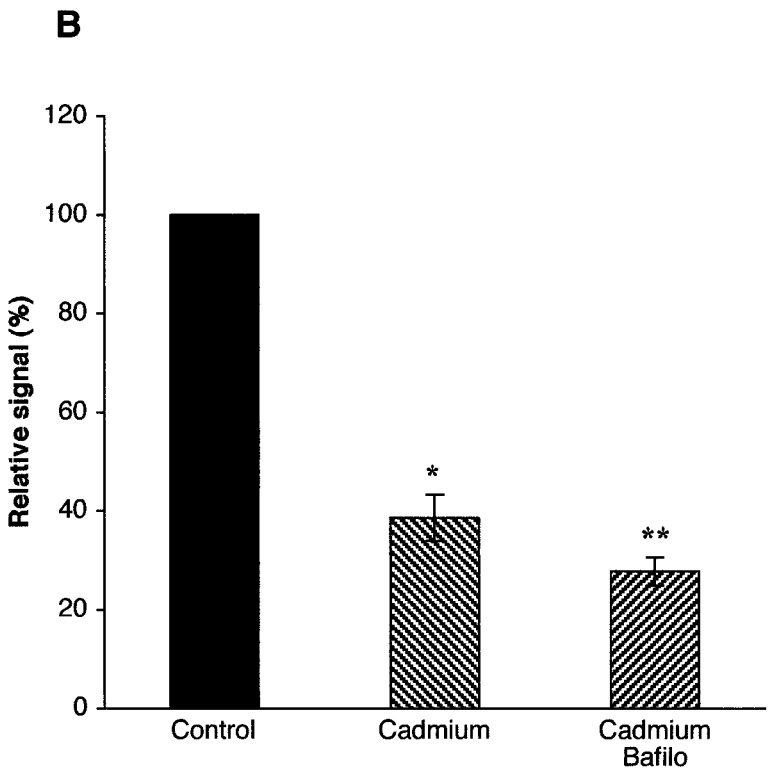


FIG. 5. Effect of Cd on H^+ fluxes in the vas deferens from control rats as monitored in vitro by an extracellular self-referencing proton-selective microelectrode. **A**) Representative tracings of H^+ fluxes in control conditions (no additions) and following addition of 1 μM (final concentration) bafilomycin or 500 μM (final concentration) $CdCl_2$ to the bath. **B**) Bars represent relative signals (mean ± SEM) at 60–70 min from a series of eight preparations of vas deferens. *Versus control, $P < 0.001$; **versus cadmium, $P < 0.05$.

branes from the cauda epididymidis, and inhibited bafilomycin-sensitive H^+ flux in the vas deferens.

Atrophy of the epididymal duct shown in this study, manifested by the loss of duct lumen and thickening of the epithelium, as well as morphological transformation of the H^+ATPase-positive cells in Cd-treated rats, may be related to the level of circulating testosterone in these animals. As shown previously, rats treated with Cd exhibit a dramatically reduced production of testosterone in the testis [12], a lower concentration of testosterone in the serum [7, 8], and an increased serum concentration of FSH [8]. The general morphology and reabsorptive processes in the epididymis seem to be androgen dependent. Wong and Yeung showed that, following castration of the adult male rat, the internal diameter of the duct and the reabsorption of fluid in the epididymal tail become reduced, and this reduction can be ameliorated by replacement therapy with testosterone [37]. In the adult rat, the tall columnar shape of the epithelium and H^+ATPase-positive cells is retained only in the initial portion (initial segment) of epididymis, whereas cells in the caput, corpus, and cauda differentiate into low columnar and cuboidal, respectively, at the onset of puberty [34, 38]. These studies furthermore indicated that while the presence of H^+ATPase-positive cells in the epididymis is not dependent on androgens, the differentiation of these cells in the caput, corpus, and cauda epididymidis into adult forms (low columnar and cuboidal, respectively) may be androgen dependent. Finally, in adult male rats, in vivo treatment with an antiandrogen, flutamide, led to the atrophy of the epididymis and increased pH and bicarbonate concentration in the tubule fluid along the epididymis, thus indicating that the size of organ and acidification of the intraluminal milieu along the male reproductive tract may be regulated by androgens [13]. The present study shows that the morphological appearance of the epithelium and H^+ATPase-positive cells along the epididymis in Cd-poisoned rats is similar to that described in 2- to 3-wk-old (prepubertal) animals [34]; atrophied tubules in Cd-treated rats also exhibit a thick epithelium and tall columnar H^+ATPase-positive cells along the entire epididymis. Because the production and concentration of serum testosterone in Cd-treated adult rats is strongly diminished, the transformation of the epithelium into a uniformly tall columnar shape may represent dedifferentiation of the cells into a morphological and functional state that is characteristic of prepubertal or castrated rats. It thus seems that, at least in the rat, Cd may act as a hormonal disruptor analogous to a variety of other occupational and environmental hazards that have been implicated in the reduction of fertility in man and animals [5].

The loss of apical polarity of the H^+ATPase 31-kDa subunit as shown by immunocytochemistry indicates that Cd treatment led to the removal of the H^+ATPase from the cell apical domain and to its translocation into an intracellular compartment. This phenomenon may be analogous to the endocytosis of apically located H^+ATPase in renal intercalated cells in metabolic alkalosis that results in a reduction of H^+ secretion [16, 39]. By Western blotting, the H^+ATPase was about 50% more abundant in tissue homogenates from the cauda epididymidis of Cd-treated rats that may be attributed to an increased number of H^+ATPase-rich cells per unit volume due to Cd-induced atrophy of the epididymis. This increased abundance of antigenicity is confined to the cytosolic fraction (post-48 000 $\times$ *g* supernatant) in which H^+ATPase is either free in the cytosol or/and associated with small vesicles that are not pelleted at 48 000 $\times$ *g*. The increase is not seen in the plasma membrane-rich total cell membrane preparation. Our previous immunoelectron-microscopic studies showed that in the H^+ATPase-positive epididymal cells of intact rats, the H^+ATPase is concentrated both on the apical plasma membrane (including microvilli) as well as on tubulovesicular structures beneath the apical membrane [17]. This finding is corroborated immunocytochemically by bright staining of the cell apical domain [17] (data in this paper). In Cd-treated rats, both the Western blotting and immunocytochemical data are consistent with a shift in the localization of H^+ATPase from the plasma membrane to a cytosolic/small vesicle compartment within the cell. Immunoblotting and bafilomycin-sensitive ATPase data indicate that the apparent abundance of H^+ATPase in preparations of total cell membranes is unchanged after Cd exposure, while the amount associated with the total cell homogenate and the cytosolic fraction are both significantly increased. This relative loss of antigen from the plasma membrane-rich total cell membrane fraction could have two explanations. First, atrophy induced by Cd may decrease the active secretory surface (e.g., by endocytosis) at the apical domain of the H^+ATPase-rich cells, leading to a diminished ability to secrete protons. This could occur even if the amount of membrane-associated H^+ATPase was unaltered on a per unit membrane basis. Second, the amount of H^+ATPase protein per unit of apical membrane may be diminished by Cd, a possibility that is supported by the weaker apical staining of these cells detected by immunofluorescence. Furthermore, in the plasma membrane-rich total cell membrane preparation, a possible loss of antigen from the apical plasma membrane may be partially masked by an increased content of H^+ATPase associated with other intracellular membranes/organelles that may contaminate this fraction. Whatever the actual mechanism, the end result would be a cell whose apical membrane is modified such that net secretion of H^+ is diminished.

The Cd-induced transformation of the epididymal H^+ATPase-positive cells to a prepubertal phenotype, as well as the removal of the H^+ATPase units from the apical cell domain suggest that these processes may be at least partially responsible for the observed alkalinization in the epididymis. Similar cellular mechanisms may explain alkalinization of the tubule fluid along the male reproductive tract in rats treated with flutamide [13]. However, in our experiments in vitro Cd also directly inhibited the bafilomycin-sensitive ATPase activity in isolated total cell membranes and its H^+ secretory function in the isolated vas deferens, and this may also contribute to the alkalinization process. Relatively high concentrations of Cd were used to inhibit ATPase activity and H^+ flux in our experiments, but these levels may not be very different from those measured in tissue after 2 wk of Cd treatment. The values for tissue Cd in Table 1 show that Cd concentrations in the initial segment plus caput, corpus, and cauda epididymidis may reach about 100 μM, 70 μM, and 50 μM, respectively. Although the IC_{50} of the bafilomycin-sensitive ATPase for Cd after preincubation for 15 min was high, about 340 μM, the finding that Cd inhibition of H^+ATPase activity and function increased with time suggests that Cd may effectively block H^+ secretion even at lower concentrations upon more prolonged contact with the transporter. This phenomenon may be analogous to the recently described inhibition of H^+ATPase activity and function in renal brushborder membranes and endosomal vesicles in Cd-induced nephrotoxicity [20]. Furthermore, preliminary experiments in-

dicated that the total cell membrane preparations from the cauda epididymidis of Cd-treated rats were less vesiculated than the membranes from control tissues, perhaps indicating greater fragility and, consequently, higher permeability of the plasma membrane to H^+ and other ions in vivo.

In summary, our data indicate that alkalinization of the tubule fluid in the epididymis and vas deferens in Cd-treated rats may result from 1) the loss of functional H^+-pumping ATPase units from the cell apical membrane and their intracellular redistribution in the H^+ATPase-positive cells that, at the same time, undergo morphological transformation to a prepubertal phenotype, and 2) direct inhibition of the H^+ATPase activity and its H^+-secretory function. Although the relevance of this finding to the human population is not known at present, it is worth noting that in comparison with several parts of the reproductive tract of normal male population, epididymis accumulates Cd most efficiently [40].

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