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Alkaline pH- and cAMP-induced V-ATPase membrane accumulation is mediated by protein kinase A in epididymal clear cells

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Abstract

In the epididymis, low luminal bicarbonate and acidic pH maintain sperm quiescent during maturation and storage. The vacuolar H⁺-ATPase (V-ATPase) in epididymal clear cells plays a major role in luminal acidification. We have shown previously that cAMP, luminal alkaline pH, and activation of the bicarbonate-regulated soluble adenylyl cyclase (sAC) induce V-ATPase apical accumulation in these cells, thereby stimulating proton secretion into the epididymal lumen. Here we examined whether protein kinase A (PKA) is involved in this response. Confocal immunofluorescence labeling on rat epididymis perfused *in vivo* showed that at luminal acidic pH (6.5), V-ATPase was distributed between short apical microvilli and subapical endosomes. The specific PKA activator *N*⁶-monobutryl-3'-5'-cyclic monophosphate (6-MB-cAMP, 1 mM) induced elongation of apical microvilli and accumulation of V-ATPase in these structures. The PKA inhibitor myristoylated-PKI (mPKI, 10 μM) inhibited the apical accumulation of V-ATPase induced by 6-MB-cAMP. Perfusion at pH 6.5 with 8-(4-chlorophenylthio)-2-*O*-methyl-cAMP (8CPT-2-*O*-Me-cAMP; 10 μM), an activator of the exchange protein activated by cAMP (Epac), did not induce V-ATPase apical accumulation. When applied at a higher concentration (100 μM), 8CPT-2-*O*-Me-cAMP induced V-ATPase apical accumulation, but this effect was completely inhibited by mPKI, suggesting crossover effects on the PKA pathway with this compound at high concentrations. Importantly, the physiologically relevant alkaline pH-induced apical V-ATPase accumulation was completely inhibited by pretreatment with mPKI. We conclude that direct

stimulation of PKA activity by cAMP is necessary and sufficient for the alkaline pH-induced accumulation of V-ATPase in clear cell apical microvilli.

Keywords

vas deferens; Epac; protein kinase inhibitor

Environmental pH regulation is essential to the function of many biological systems. While spermatozoa mature and are stored along the epididymal tubule, they are kept in a quiescent state in part by the maintenance of a luminal acidic pH of 6.5–6.8 and a low bicarbonate concentration of 2–7 mM. (28, 29). It has been hypothesized that a defect in epididymal luminal acidification might impair sperm maturation and fertility (35). Epithelial cells known as clear cells are important for the acidification capacity of the epididymis. The vacuolar H⁺-ATPase (V-ATPase) is highly expressed in these clear cells, both at their luminal plasma membrane and in intracellular apical vesicles, and is responsible for net luminal acidification in the cauda epididymidis and vas deferens (8). The potential importance of clear cell-mediated luminal acidification is underscored by the fact that mice lacking the transcription factor Foxl1 fail to express the V-ATPase B1 subunit and carbonic anhydrase (CA) II in clear cells, have a higher luminal epididymal pH, and are infertile (3).

The V-ATPase is expressed ubiquitously in eukaryotes, where it can be found in organelles requiring luminal acidification, such as lysosomes, the Golgi apparatus, endosomes, and secretory vesicles (45). In addition to clear cells, the V-ATPase is expressed in the plasma membrane of some specialized cells, such as kidney intercalated cells, osteoclasts, and CA-rich α -cells of the turtle bladder (10). Proton secretion in these specialized acid-secreting cells is actively regulated by environmental cues that include CO₂, bicarbonate, and hormonal stimuli (45).

At the cellular level, V-ATPase-mediated proton secretion can be regulated by several mechanisms, including: 1) V-ATPase isoform subunit expression, 2) reversible dissociation of the membrane-associated V₀ and the catalytic V₁ sectors of the V-ATPase, 3) modulation of the coupling between ATP hydrolysis and proton pumping, and 4) modulation of recycling and targeting of V-ATPase-containing vesicles (reviewed in Ref. 5). Indeed, an increase in V-ATPase surface expression and in apical surface area (including microvilli) in these specialized cells correlates with an increase in proton secretion (reviewed in Ref. 40). We have shown previously that, as in other specialized V-ATPase-expressing cells, V-ATPase-containing vesicles in epididymal clear cells recycle between intracellular vesicles and the apical plasma membrane, which indicates that these cells also utilize this regulatory mechanism to control their rate of proton secretion (1, 34).

Extracellular pH changes have been implicated in exocytosis of channels and organelle trafficking (20, 25, 27). It has been shown previously that cAMP stimulates proton secretion in a number of epithelial cell types involved in acidification, including the kidney medullary collecting duct cells (13, 18, 21, 22). In addition cAMP is involved in regulating the exocytosis of many actively recycling membrane transporter, such as aquaporin 2 (AQP2),

aquaporin 8 (AQP8), the cystic fibrosis transmembrane conductance regulator (CFTR), and the glucose transporter (GLUT4) (9, 17, 24, 26).

In the male reproductive tract, we have shown previously that V-ATPase accumulates in the plasma membrane of clear cells in response to cAMP, alkaline luminal pH, and to increases in luminal bicarbonate concentration (34). In addition, we demonstrated that bicarbonate-regulated soluble adenylyl cyclase (sAC), a chemosensor that induces a bicarbonate-dependent elevation of intracellular cAMP, mediates the pH-dependent V-ATPase recycling in epididymal clear cells (34). In the same study, a cell-permeant analog of cAMP by itself induced the apical translocation of V-ATPase in these cells even at acidic luminal pH (34).

PKA is a major mediator that transmits an increase in cAMP to effector and target proteins in cells. Phosphorylation of proteins by PKA is thought to be a major step in cAMP-mediated exocytosis (reviewed in Ref. 38). However, an alternative cAMP-dependent signaling pathway that is independent of PKA has also been identified and involves the exchange protein directly activated by cAMP (Epac). Epacs are guanine nucleotide exchange factors for the Ras-like small GTPase Rap1, and they are insensitive to PKA inhibitors and function even in the absence of an active PKA pathway (14).

In the present study, we investigated the role of Epac and PKA *in vivo* on the regulation of alkaline pH-induced and cAMP-mediated V-ATPase redistribution in rat epididymal clear cells. Our data suggest that PKA activation is both necessary and sufficient to induce V-ATPase accumulation in the apical membrane of epididymal clear cells.

MATERIALS AND METHODS

Reagents and chemicals

All chemicals used in these experiments were obtained from Fisher Scientific unless otherwise noted, including the perfusion solution of PBS (in mM: 10 sodium phosphate, 2 potassium phosphate, 137 NaCl, and 2.7 KCl). Horseradish peroxidase (HRP) was obtained from Sigma (St. Louis, MO). The cell permeant cAMP analogs 8CPT-2-*O*-Me-cAMP and *N*⁶-monobutyl-3'-5'-cyclic monophosphate (6-MB-cAMP) and the specific PKA inhibitor myristoylate PKI (mPKI) were obtained from Biomol (Plymouth Meeting, PA) and were all dissolved in water. Paraformaldehyde was obtained from Electron Microscopy Sciences (Hatfield, PA).

Tissue fixation and immunofluorescence staining

Animal protocols were approved by the Institutional Animal Care and Use Committee of the University of Pittsburgh School of Medicine. Adult male Sprague-Dawley rats were anesthetized with pentobarbital sodium (65 mg/kg body wt ip) and perfused via the left ventricle with PBS (pH 7.4), followed by a phosphate-buffered solution containing 4% paraformaldehyde, 10 mM sodium periodate, 70 mM lysine, and 5% sucrose (PLP). They were processed for immunofluorescence as described previously (6, 7, 33, 34). Briefly, the epididymis and vas deferens (VD) were harvested and further fixed by immersion in PLP overnight and cryoprotected in a solution of 30% sucrose in PBS overnight at 4°C. These tissues were embedded in OT Compound (Tissue TEK, Sakura Finetek, Torrance, CA) and

mounted on a cutting block before being frozen in a Reichert Frigocut microtome. Sections were picked up on Super frost Plus slides (Fisher, Pittsburgh, PA). Immunofluorescence staining was performed on 4- μ m cryostat sections after SDS antigen retrieval as previously described (11). Slides were washed in PBS followed by incubation with a blocking solution containing 1% bovine serum albumin in PBS-0.02% sodium azide for 15 min. Slides were then incubated with an anti-Epac antibody (raised in goat, 1:50 dilution) that recognizes the COOH-terminus of both isoforms of Epac1 and Epac2, followed by an antibody against the E subunit of the V-ATPase (raised in rabbit, 1:60 dilution) for 75 min at room temperature (both antibodies from Santa Cruz Biotechnology, Santa Cruz, CA). Sections were then washed twice for 5 min in high-salt PBS (2.7% NaCl) and once in PBS and then incubated for 1 h with donkey anti-goat IgG coupled to FITC and donkey anti-rabbit IgG coupled to CY3 (Jackson Immunologicals, West Grove, PA). After further washes in PBS, the slides were coverslipped after being mounted with Vectashield (Vector Labs, Burlingame, CA). The Epac immunizing peptide used to produce this antibody was employed for peptide inhibition controls using methods previously described (33).

In vivo perfusion of the VD and distal cauda epididymidis and immunofluorescence staining

We have demonstrated previously that activation of V-ATPase-dependent proton secretion in clear cells is proportional to the apical membrane V-ATPase accumulation, which results in a marked elongation of microvilli that contain a higher density of V-ATPase molecules (1, 31). In the present study, as a read-out for the V-ATPase apical accumulation in clear cells, we measured the effects of cAMP downstream effectors on the extension of V-ATPase-labeled microvilli. Adult male Sprague-Dawley rats were anesthetized as described above. An incision was made in the scrotum that exposed the VD and epididymal cauda. The distal VD lumen was cannulated using a microcannula (0.4 mm OD, 0.2 mm ID; Fine Science Tools, Foster City, CA). An additional incision was then made in the distal epididymal cauda as previously described (34). We estimate that in most preparations we perfused 4–5 cm of distal epididymal cauda/VD, of which ~1 cm was proximal VD. Retrograde perfusion was performed from the VD into the distal epididymal cauda and the perfusate exited via the incision made in the distal epididymal cauda. The perfusion rate was 45 μ l/min, using a syringe pump (model 341B, Fisher). The lumen was initially washed free of sperm with PBS adjusted to pH 6.5, as indicated in RESULTS. HRP was added to the perfusate at a concentration of 5 mg/ml to detect endocytosis in the absence or presence of inhibitors and agonists. At the end of the experimental period, the luminal solution was changed to HRP-free ice-cold PBS for 6–9 min (in the continued presence of agonists or inhibitors, as applicable) to wash the lumen free of HRP. The perfused portions of the vas deferens and cauda epididymidis were harvested and fixed by immersion in PLP for 5 h at room temperature or overnight at 4°C. Tissues were then washed in PBS, pH 7.4, and stored in PBS containing 0.02% sodium azide, cryoprotected, embedded, and sectioned as described above. Double immunofluorescence staining was performed using our immunopurified chicken antibody (1:60 dilution) (34) or a rabbit antibody (Santa Cruz, 1:60 dilution) against the E subunit of the V-ATPase combined with an anti-HRP antibody raised in goat at a concentration of 1:300 (Sigma). Washes and secondary antibodies were used as previously described (34). At least three VD and cauda epididymidis were perfused for each

condition described. A minimum of 6–10 cells per tissue were examined by confocal microscopy for a total of at least 30 cells per condition. In Figs. 2, 3, 5–8, only the V-ATPase immunofluorescence staining is shown (green).

Immunoprecipitation and Western blot analysis

Adult rats were anesthetized, and their epididymides and VD were harvested and dissected in PBS pH 7.4 containing Complete Protease Inhibitor cocktail (Roche, Indianapolis, IN) and homogenized as previously described (23, 34). For immunoprecipitation (IP), the VD and distal cauda region were perfused via the lumen using PBS containing Complete Protease Inhibitors to remove the spermatozoa, and the epithelial lining of the vas deferens (which we refer to as the epithelial “sock”) was dissected as previously described, and homogenized as above (41). Electrophoresis and Western blot (WB) were performed as previously described (23). The same anti-Epac antibody used for immunofluorescence, which recognizes both Epac1 and Epac2, was used for IP and WB.

After quantification of the protein concentration using the Bradford assay (Bio-Rad, Hercules, CA), IPs were performed overnight at 4°C on each lysate sample containing 1.0 mg of total protein using either the anti-Epac antibody (0.5 µg/IP) coupled to protein A/G beads or beads alone as previously described (2). After three washes in lysis buffer, the IP samples were then eluted in Laemmli sample buffer and, along with the cell lysate samples, subjected to SDS-PAGE and immunoblotting using previously described protocols (33). Immunoblotting was performed at 1:2,500 dilution of the same anti-Epac antibody in 5% milk in TBS-Tween followed by HRP-conjugated secondary anti-goat antibody (Jackson Immunologicals) at a concentration of 1:10,000. Membranes were then stripped and reprobed with anti-Epac antibody that had been preincubated with the immunizing peptide to demonstrate anti-Epac antibody specificity.

Quantification of V-ATPase apical membrane accumulation

The level of V-ATPase accumulation in the apical membrane of clear cells of the proximal VD and epididymal cauda was quantified from confocal microscopy images as previously described, a quantification method that was validated by immunogold electron microscopy (1, 34). Briefly, confocal images were imported into Metamorph (Molecular Devices, Sunnyvale, CA), the microvilli positive for V-ATPase staining were outlined, and the area occupied by the microvilli was measured. The area measurement for each cell was normalized to the width of the cell between the tight junctions at the apical pole. At least three epididymides were perfused for each condition from different animals, and we examined a minimum of three separate immunofluorescence staining procedures for each tissue. Six to 10 cells were examined per tissue with a total of at least 30 cells per condition used for quantification. These measurements were performed for each image independently by two investigators, who were blinded as to the treatment group. Student's unpaired *t*-tests were performed, and differences were considered significant at $P < 0.05$.

RESULTS

A specific PKA activator induces the apical membrane accumulation of V-ATPase in epididymal clear cells

In an earlier study, we have shown that sAC activation and cAMP stimulate V-ATPase apical membrane accumulation in epididymal clear cells (34). As demonstrated in Fig. 1, clear cells exposed to the control conditions of PBS at pH 6.5 had V-ATPase distributed between short apical microvilli and intracellular apical vesicles, which partially colocalize with HRP containing endosomes (yellow staining), indicating a significant amount of V-ATPase in the endocytic compartment (Fig. 1A). In the presence of 6-MB-cAMP (1 mM), the V-ATPase was concentrated in the apical microvilli of clear cells and only minimal colocalization of V-ATPase and HRP was observed (Fig. 1B). The area occupied by V-ATPase-containing microvilli was measured and normalized to the length of the apical cell membrane, measured at the base of the microvilli (Fig. 1, A–D). Quantitative analysis revealed a significant increase in the normalized area occupied by microvilli in clear cells in tissues exposed to the specific PKA activator 6-MBcAMP (Fig. 1E). These results indicate that specific activation of PKA in clear cells induces the accumulation of V-ATPase in the apical membrane of these cells, even at acidic luminal pH.

A specific PKA inhibitor abolishes cAMP-mediated apical V-ATPase accumulation

Preperfusion with 10 μ M mPKI, a specific PKA pseudosubstrate inhibitor (19), for 20 min before the addition of 6-MB-cAMP (1 mM) prevented the 6-MB-cAMP-induced V-ATPase apical accumulation (Fig. 2, A and B). Quantification of the area occupied by microvilli normalized for the width of the cell measured at the base of microvilli confirmed the inhibitory effect of mPKI (Fig. 2, C and D). These results indicate that specific inhibition of PKA by mPKI prevents cAMP-mediated V-ATPase accumulation in microvilli (Fig. 2E). Importantly, no significant differences in V-ATPase distribution were detectable between tissues perfused with PBS at pH 6.5 and those perfused with 10 μ M mPKI in PBS pH 6.5 (Fig. 3, A and B), respectively. These results indicate that PKA inhibition by mPKI at physiological pH did not alter the baseline distribution of the V-ATPase (Fig. 3C).

Presence of Epac in epididymal epithelium by WB and IP

The actions of cAMP were classically thought to be mediated almost exclusively by PKA. However, there is now a growing body of literature that indicates that cAMP also acts via Epac (15). This family of guanine nucleotide exchange factors mediates Rap activation in the presence of cAMP. We hypothesized that increases in intracellular cAMP in clear cells might also activate Epac in a PKA-independent manner. By immunofluorescence, Epac (Fig. 4, A and D) is detectable in the apical cytoplasm of clear (V-ATPase-rich) and principal cells of the epididymal epithelium (Fig. 4, A–C). No labeling was detectable in epididymis when antibody preabsorbed with the anti-Epac immunizing peptide was used (Fig. 4E). In addition, WB after IP of Epac from enriched epididymal-VD epithelial cell preparation (epithelial “sock”) lysates revealed two bands at ~90 and 110 kDa (Fig. 4F, *left*) that were not present when antibody was omitted from the IP (Fig. 4G, *top*) and were competed away by the immunizing peptide (Fig. 4F, *right* and 4G, *bottom*). These bands probably represent Epac1 and Epac2, which have these expected molecular weights. In Fig.

4F, the band that is detected in the WB after peptide inhibition probably represents the anti-Epac primary antibody (IgG) used in the IP as detected by the secondary antibody in the WB.

PKA inhibition abolishes apical V-ATPase translocation by 8CPT-2-O-Me-cAMP

The cell-permeant cAMP analog 8CPT-2-*O*-Me-cAMP, which has been reported to be a specific Epac activator (15), was used to examine whether Epac plays a role in the trafficking of V-ATPase in clear cells. Cauda epididymidis-VD was perfused as described above with PBS pH 6.5 in the presence or absence of 8CPT-2-*O*-Me-cAMP (34). When 8CPT-2-*O*-Me-cAMP was administered at 10 μ M, a concentration at which this compound has been reported to be specific for Epac (12, 15), there was no apparent difference in V-ATPase distribution (Fig. 5B) compared with cells exposed to PBS pH 6.5 alone (Fig. 5A). Quantification confirmed that this low concentration of 8CPT-2-*O*-Me-cAMP failed to stimulate accumulation of the V-ATPase at the apical membrane of clear cells (Fig. 5C).

However, perfusion at a concentration of 100 μ M 8CPT-2-*O*-Me-cAMP, which is known to activate PKA (15), induced a significant accumulation of V-ATPase in the apical membrane of clear cells (Fig. 6B) when compared with that of controls (Fig. 6A). This difference was confirmed by quantification of the area occupied by the apical microvilli of clear cells exposed to each condition, as described above (Fig. 6C). Preperfusion of tissues with the specific PKA inhibitor mPKI (10 μ M) completely inhibited the V-ATPase apical membrane accumulation induced by 100 μ M 8CPT-2-*O*-Me-cAMP (Fig. 7, A–C), consistent with an activating effect of 8CPT-2-*O*-Me-cAMP on PKA at high concentrations.

PKA inhibition abolishes the alkaline pH-mediated V-ATPase accumulation in microvilli of clear cells

Perfusion of epididymis-VD with PBS pH 6.5 followed by a change to PBS pH 7.8 induced a marked accumulation of the V-ATPase in microvilli, as we have described previously (Fig. 8A) (1, 34). Preperfusion with mPKI (10 μ M) in PBS pH 6.5 followed by mPKI in PBS pH 7.8 completely abolished this stimulatory effect (Fig. 8B), suggesting that alkaline pH-induced changes in the V-ATPase distribution in clear cells are also mediated via PKA. Quantification of the normalized area occupied by V-ATPase-labeled microvilli confirmed these results (Fig. 8C).

DISCUSSION

In our previously published studies, we have shown that V-ATPase accumulation in the microvilli of clear cells can be induced by: 1) alkaline luminal pH, 2) cAMP, and 3) luminal bicarbonate (34). We also demonstrated that alkaline pH and bicarbonate-mediated V-ATPase trafficking was blocked by: 1) inhibition of sAC, 2) inhibition of CA, and 3) inhibition of PKC (1, 34). The cell permeant cAMP analog 8-(4-chlorophenylthio)-adenosine-3',5'-cyclic monophosphate was able to overcome the inhibition of sAC and CA, confirming the need for cAMP as the common downstream effector of sAC and bicarbonate (34). The PKA-specific activator 6-MB-cAMP induced the V-ATPase apical membrane accumulation in epididymal clear cells. Furthermore, preincubation of epididymal-VD

epithelium with the specific PKA inhibitor mPKI prevented the 6-MB-cAMP-dependent redistribution of V-ATPase to the apical membrane of clear cells.

The 8CPT-2-*O*-Me-cAMP compound did not induce the apical accumulation of the V-ATPase when used at a concentration (10 μ M) known to be specific for Epac (4, 15). However, when used at the higher concentration of 100 μ M, a concentration that was shown to also induce PKA activation (15), accumulation of V-ATPase in apical microvilli was observed. Preperfusion with mPKI completely inhibited the effect of 100 μ M 2-*O*-Me-cAMP on the redistribution of V-ATPase to the apical membrane, confirming that PKA was activated by this cAMP analog at high concentrations. Our results suggest that the Epac activator 8CPT-2-*O*-Me-cAMP is not specific for Epac at higher concentrations and thus results obtained at these higher concentrations should be interpreted with caution.

Inhibition of PKA with mPKI under baseline conditions (PBS at pH 6.5) did not significantly change the distribution of the V-ATPase in clear cells. This result indicates that PKA is involved in V-ATPase trafficking only under conditions of stimulation (e.g., with high luminal bicarbonate concentrations, alkaline luminal pH, or high intracellular cAMP levels). Because we have reported in earlier studies that the V-ATPase recycles constitutively between intracellular vesicles and the plasma membrane in clear cells, this finding implies that PKA activation is not necessary for this baseline recycling process (34). A similar finding has been reported for another recycling protein, AQP2, whose membrane accumulation is stimulated by a cAMP-PKA-mediated signaling process (9, 16, 32) but whose constitutive recycling pathway is cAMP and PKA independent (30).

We also show here that Epac is present in epididymal epithelium. Epac is an exchange protein activated by cAMP that has been implicated in several cAMP-mediated cellular events, including exocytosis, insulin secretion, cell adhesion, and cell-junction formation (4, 15). As far as the regulation of trafficking of epithelial membrane transport proteins is concerned, Epac has been shown to regulate the Ca^{2+} mobilization and AQP2 exocytosis in the inner medullary collecting duct (47). Although Epac in clear cells may also be activated by intracellular cAMP, our results argue against an obligatory role for Epac in the cAMP-dependent trafficking of the V-ATPase. However, because specific Epac inhibitors are not yet available, we cannot exclude the possibility that Epac may contribute to the response. Our data do suggest, however, that PKA activation by cAMP is both necessary and sufficient to induce the accumulation of V-ATPase in apical microvilli.

The downstream targets that are phosphorylated by PKA to regulate V-ATPase membrane accumulation in clear cells (e.g., the V-ATPase itself or accessory proteins involved in its trafficking) have not yet been identified. In mammalian cells, it has been reported that the B subunit of the V-ATPase from bovine brain is phosphorylated, although the role of this phosphorylation on V-ATPase activity has not been fully elucidated (31). In addition, none of the subunits of the V-ATPase have been found to be phosphorylated by PKA in mammals (42, 45). We have shown previously that the cAMP-mediated V-ATPase apical accumulation in clear cells requires the remodeling of the actin cytoskeleton (1). We have suggested that an indirect mechanism might be involved in the cAMP-induced apical accumulation of the V-ATPase, as it is in the case of other transporters (36, 37, 39, 43, 46).

Recently, the C subunit of the V-ATPase has been found to be phosphorylated by PKA in insects, suggesting that this kinase may act directly to modulate V-ATPase accumulation in subcellular compartments (44). We believe that further characterization of this PKA-dependent pathway is warranted to identify novel targets for the treatment of male infertility or for the development of a male contraceptive.

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Fig. 1.

Protein kinase A (PKA) activation with *N*⁶-monobutyl-cAMP (6-MB-cAMP) induces vacuolar H⁺-ATPase (V-ATPase) apical accumulation in clear cells. Confocal images of double immunofluorescence labeling for V-ATPase (green) and horseradish peroxidase (HRP) (red) of clear cells from cauda epididymides perfused with PBS pH 6.5 (A) or with PBS pH 6.5 containing 6-MB-cAMP (1 mM) (B). In the absence of 6-MB-cAMP, the V-ATPase is distributed between apical vesicles and short apical microvilli, and the yellow staining indicates partial colocalization of HRP-containing endosomes with V-ATPase containing vesicles. In contrast, in the presence of 6-MB-cAMP, V-ATPase is located mainly in apical microvilli (green). Arrows point to the base of the microvilli, often visible under the microscope as a dark line between the apical microvilli and the subapical region. The lumen of the epididymis is in the top part of each panel. In C and D, the level of V-ATPase accumulation in microvilli was quantified by measuring the area occupied by V-ATPase-labeled microvilli (enclosed in the white line), normalized for the width of the cells at the apical pole (blue line) for each cell. E shows the quantification of the surface occupied by V-ATPase-labeled microvilli of clear cells normalized by the apical width of each cell under the conditions shown in A and B. Data shown are the means ± SE from three tissues and at least 30 cells per condition (**P* < 0.05). Scale bar = 5 μm.

Fig. 2.

The PKA inhibitor myristoylated PKI (mPKI) prevents V-ATPase apical accumulation in clear cells by the PKA activator 6-MB-cAMP. Confocal images of V-ATPase distribution (green) in clear cells perfused with 6-MB-cAMP (1 mM) at pH 6.5 (A) or 6-MB-cAMP (1 mM) with 10 μ M mPKI at pH 6.5 (B), after preperfusion with PBS pH 6.5 containing 10 μ M mPKI. Arrows demarcate the base of the microvilli in clear cells. In C and D, as also shown in Fig. 1, the level of V-ATPase in microvilli was quantified by measuring the area occupied by V-ATPase-labeled microvilli (enclosed in the white line) normalized for the width of the cells at the apical pole (blue line) for each cell. Pretreatment with mPKI prevented the elongation of apical microvilli observed upon exposure to 6-MB-cAMP. Quantification of V-ATPase accumulation in apical microvilli is shown in E, as described in the legend of Fig. 1. Data shown are means $\pm$ SE (* P < 0.05). Scale bar = 7.5 μ m.

Fig. 3.

The PKA inhibitor mPKI has no effect on the baseline V-ATPase apical distribution in clear cells at pH 6.5. Confocal images of V-ATPase distribution (green) in epididymal clear cells perfused with PBS pH 6.5 (A) compared with PBS pH 6.5 containing 10 μ M mPKI (B). Arrows point to the base of the microvilli. C: quantification of V-ATPase accumulation in apical microvilli of clear cells normalized by the width of each clear cell exposed to the above conditions. Data shown are means $\pm$ SE. Scale bar = 7.5 μ m.

Fig. 4.

Exchange protein directly activated by cAMP (Epac) is present in epididymal epithelial cells. Confocal immunofluorescence staining image of epididymal epithelium using an anti-Epac antibody demonstrates vesicular structures in the apical cytoplasm of epididymal cells of the cauda epididymidis (A, green). In the same slide, clear cells were labeled with an anti-V-ATPase antibody (B, red). The merged image demonstrates that both clear (V-ATPase-rich) cells and principal cells of the epididymis express Epac (C). Scale bar = 5 μ m. D: lower magnification confocal image of immunofluorescence staining of epididymal epithelium using the anti-Epac antibody on epididymal epithelium. E: preincubation of the anti-Epac antibody with its immunizing peptide abolished the staining. Scale bar = 12.5 μ m. Western blot (WB) of immunoprecipitation (IP) performed with anti-Epac antibody from an enriched

epididymal-VD epithelial cell preparation (epithelial “sock”) (*F, left*, and *G, top, left lane*) are shown. Two bands at ~90 and 110 kDa corresponding to the expected molecular weights of Epac1 and Epac2 were detected with the antibody. In the presence of the immunizing peptide during WB, the Epac-specific bands were no longer detected (*F, right* and *G, bottom, left lane*). No bands were detected when the IP was performed in the absence of the anti-Epac antibodies (*G, top, right lane*).

Fig. 5.

Treatment with 10 μ M 8CPT-2-*O*-Me-cAMP fails to induce V-ATPase apical accumulation in clear cells. Representative images demonstrating the baseline V-ATPase distribution in clear cells from tissues perfused at luminal pH 6.5 (A) and in the presence of PBS pH 6.5 containing 10 μ M 8CPT-2-*O*-Me-cAMP (B). C: quantification of clear cell V-ATPase accumulation in apical microvilli (the base of which is indicated by the arrows) in the absence and presence of 10 μ M 8CPT-2-*O*-Me-cAMP. Data shown are the means $\pm$ SE. Scale bar = 5 μ m.

Fig. 6.

Treatment with 100 μM 8CPT-2-*O*-Me-cAMP induces V-ATPase apical accumulation in clear cells. V-ATPase distribution in clear cells from tissues perfused at luminal pH of 6.5 (A) and V-ATPase apical microvilli accumulation in the presence of 100 μM 8CPT-2-*O*-Me-cAMP (B). Arrows point to the base of the microvilli. C: summary of the normalized V-ATPase accumulation in apical microvilli of clear cells from tissues exposed to Epac activator 8CPT-2-*O*-Me-cAMP (100 μM) compared with controls (PBS, pH 6.5). Data shown are the means ± SE (**P* < 0.05). Scale bar = 5 μm.

Fig. 7.

PrePerfusion with mPKI prevents the 100 μ M 8CPT-2-*O*-Me-cAMP-induced apical V-ATPase accumulation in clear cells. Representative images of the V-ATPase distribution in tissues perfused with 8CPT-2-*O*-Me-cAMP 100 mM in PBS at pH 6.5 (A) and of tissues preperfused with PBS pH 6.5 containing 10 mM mPKI followed by exposure to 8CPT-2-*O*-Me-cAMP still in the presence of mPKI (B). Quantification of V-ATPase accumulation in apical microvilli of clear cells from tissues exposed to 100 μ M 8CPT-2-*O*-Me-cAMP in the presence or absence of PKA inhibitor mPKI is shown in C (* P < 0.05). Scale bar = 5 μ m.

Fig. 8.

PKA inhibition abolishes the alkaline pH-mediated V-ATPase apical accumulation in clear cells. Representative images of the V-ATPase distribution in tissues perfused with either PBS pH 7.8 (A) or in the presence of mPKI, followed by luminal alkalinization with PBS pH 7.8 in the continued presence of mPKI (B). C: quantification of V-ATPase accumulation in apical microvilli at pH 7.8 with or without inhibition of PKA with mPKI. Arrows point to the base of the microvilli. (* $P < 0.05$). Scale bar = 5 μm .