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Ouabain-Induced Cell Swelling in Rabbit Cortical Collecting Tubule: NaCl Transport by Principal Cells

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Summary. Ouabain had no effect on the volume of intercalated cells of DOCA-stimulated rabbit cortical collecting tubules, but caused principal cells to swell rapidly at an initial rate of 67%/min. Principal cells swelled 133% then activated regulatory volume decrease mechanisms and shrank at an initial rate of −3%/min to a new volume 13% above control. The initial rate of ouabain swelling was completely inhibited by perfusate Na^+ removal or reduced 95% by luminal addition of 10^{-5} M amiloride. Luminal, peritubular, or bilateral Cl^- removal each caused cell shrinkages of 10% and reduced the rate of ouabain swelling by 70, 85, and 99%, respectively. The presence of an apical Cl^- transport step in principal cells was confirmed by increasing luminal K^+ from 5 to 53 mM, which caused cell swelling of 22%. This volume increase was completely blocked by luminal Cl^- removal, but was unaffected by peritubular Cl^- substitution. Perfusion of CCT with 0.1 mM acetazolomide, 0.1 mM DPC or 0.5 mM SITS caused principal cell shrinkages of 7–9% and reduced the rate of ouabain swelling by 60, 70, and 40%, respectively. The initial rate of ouabain swelling was inhibited 70% by bilateral CO_2/HCO_3 removal and 50% by whole animal acid loading. Taken together these results demonstrate that ouabain swelling is due to cellular NaCl accumulation and that Na^+ enters the cell primarily through apical Na^+ channels. Cellular Cl^- entry occurs at least partially through the apical membrane and may be mediated by a Cl^-/HCO_3^- exchanger. Brief (45–90 sec) exposure of principal cells to ouabain is associated with a rapid inhibition of Na^+ and/or Cl^- entry steps, whereas long-term (>5 min) ouabain exposure completely blocks one or both of these transport pathways.

Key Words cortical collecting tubule · principal cell · intercalated cell · NaCl transport · ouabain · cell volume · mineralocorticoids

Introduction

The cortical collecting tubule (CCT) is involved in the final regulation and "fine tuning" of extracellular solute composition and volume. As such, transepithelial transport functions in the CCT are under control of the body's major salt and water regulatory hormones, vasopressin and aldosterone, as well as numerous local renal factors (e.g., prostaglandins, catecholamines, acid-base status, etc.). Attempts to understand the mechanisms of these transport processes as well as their regulation have been greatly hampered by the cellular heterogeneity of the CCT. In the rabbit, the CCT is composed of three morphologically distinct cell types. Principal cells comprise approximately 65% [19] of the epithelium, while the remainder of the tissue is composed of so-called type "A" and type "B" intercalated cells [21]. A great deal of direct electrophysiological evidence has indicated that principal cells are involved in electrogenic Na^+ reabsorption and K^+ secretion via amiloride and Ba^{2+}-inhibitable Na^+ and K^+ channels, respectively [15–17, 23–25]. Indirect evidence suggests that the intercalated cells are involved in Cl^- [31] and K^+ [4, 34] reabsorption, and H^+ or HCO_3^- secretion [3, 21, 31, 32].

Cell volume measurements using quantitative differential interference contrast microscopy have been used previously to elucidate sites of vasopressin action and water transport [37] as well as volume regulatory mechanisms [35] in principal and intercalated cells. Such optical approaches can be particularly valuable for defining cell transport functions in heterogeneous epithelia since they allow direct visualization of the cell of interest. For example, studies of cell volume changes associated with transepithelial current passage and ion substitutions [5] have elucidated transcellular Cl^- transport pathways in mitochondria-rich cells of toad skin. Studies of the mechanisms of ouabain-induced swelling in this tissue have also shown that mitochondria-rich cells possess an apical Na^+ conductance, basolateral Na^+ pump and apical and basolateral passive Cl^- permeabilities [18].

In CCT dissected from control rabbits ouabain causes relatively slow swelling (K. Strange, *unpublished observation*), making studies of solute transport mechanisms responsible for the volume increase difficult. Cortical collecting tubules

dissected from rabbits fed a low Na^+ diet or chronically treated with mineralocorticoids, however, show rapid and dramatic swelling during pump inhibition. The purpose of this investigation was to begin characterizing the solute transport properties of principal and intercalated cells by examining the mechanisms of ouabain-induced swelling in CCT dissected from rabbits chronically treated with deoxycorticosterone acetate.

Materials and Methods

Tubule Perfusion

Eight to nine week old New Zealand white female rabbits weighing 1.5 to 2.0 kg were maintained on Purina rabbit chow and distilled water *ad libitum*. Animals were given subcutaneous injections of deoxycorticosterone acetate (DOCA; Sigma Chemicals, St. Louis, MO) suspended in sesame oil (Sigma Chemicals) at a dosage of 5 mg/kg body weight/day for 9–13 days prior to the start of experiments. Following sacrifice by cervical dislocation, both kidneys were removed immediately and transferred to room temperature control saline (*see below*). Segments of cortical collecting ducts were dissected from kidney slices in a bath chamber maintained at 18°C and gassed with 95% O_2–5% CO_2. Special care was taken to isolate segments with minimal amounts of adherent interstitial tissue.

Tubule perfusion methods were similar to those described in detail previously [36, 37]. Briefly, a single cortical collecting tubule was transferred to a rapid laminar flow-bath chamber [36] and one end was drawn up into a conventional glass holding pipette. The tubule was cannulated and perfused using an inner concentric perfusion pipette. The distal end of the tubule was held in place with another glass holding pipette. Exposed tubule lengths of 400 to 800 μm were used to minimize tubule movements. Once the tubule was in place, bath perfusion was initiated and maintained at 5–7 ml/min for a 1-hr equilibration period. Peritubular solutions were delivered to the bath chamber by gravity feed through a stainless steel, three-way manifold [36, 37]. Solution composition was switched using computer-interfaced solenoid-actuated pinch valves described previously [36, 37]. Bath solution changes were complete in less than 500 msec [36].

Luminal perfusion was maintained at a rate >30 nl/min. Perfusate composition was changed in approximately 30 sec using a conventional exchange pipette arrangement.

Bath temperature was maintained at 36–38°C using water-jacketed solution reservoirs and glass perfusion lines kept at constant temperatures of 43 and 41°C, respectively.

Microscopy

The bath chamber and perfusion apparatus were mounted on the stage of an inverted microscope (Nikon, Diaphot; Nikon Microscope, Garden City, NY) as described previously [35, 36]. The microscope was equipped with differential interference contrast optics, consisting of a Zeiss Neofluar 63× (1.25 N.A.) oil immersion objective lens with a 500-μm working distance and a Leitz 32× (0.40 N.A.) objective-condenser lens with a 6.6-mm working distance.

Principal and intercalated cells were differentiated based on previously described morphological criteria [36, 37]. Intercalated cells were treated as a single cell type, and no attempt was made to differentiate them as so-called "A" and "B" type cells [21].

Cross-sectional images of principal and intercalated cells were recorded from the lateral tubule wall on video disc as described previously [35–37]. A single cell image was recorded twice at each time point to yield an image pair. Three sets of paired control images were recorded over a 10–15 min period before changes were made in luminal or peritubular solution composition.

Cell volume changes were quantified by digitizing images recorded on video disc using an image processing computer board with 512 × 512 × 8-bit resolution (Model FG-100-AT; Imaging Technology, Woburn, MA) and an IBM AT computer. Digitized video images were displayed on a 19-inch high resolution video monitor (Model 8831; Aydin Controls, Fort Washington, PA). Cell borders were traced on the monitor using a 12 × 12 inch graphics tablet (SummaSketch; Summagraphics, Fairfield, CT). Cross-sectional areas of the traced regions were quantified using image analysis software (Image-Pro 100; Media Cybernetics, Silver Spring, MD). Each image of an image pair was traced and the values were averaged to yield a mean cross-sectional area. As described previously [35], this image acquisition and analysis system allows detection of cross-sectional area changes with an accuracy of ±2–3%. The relative change in cross-sectional area directly reflects relative changes in cell volume (36, 37).

Initial rates of cell volume change were approximated using linear regression analysis of the first 3–5 points of the volume change curves (e.g., Figs. 2 and 4). In control experiments, these points spanned a time frame of approximately 14–30 sec. The mean (±SE) regression coefficient for 40 randomly chosen experiments was 0.99 ± 0.002 (range = 0.95 – 1.0). As shown previously, rates of volume change can be determined with an accuracy of ±10% [35].

Solution Composition

Tubules were bathed and perfused with a control solution containing (in mM): 118 NaCl, 23 $NaHCO_3$, 2.5 K_2HPO_4, 1.2 $MgSO_4$, 2.0 Ca lactate$_2$, 2.5 glucose, 1.0 Na_3 citrate, and 6.0 L-alanine. Sodium, K^+ and Cl^--free solutions were made by replacing Na^+, K^+, or Cl^- with N-methyl-D-glucamine, Na^+ or isethionate, respectively. Bicarbonate-containing bath and perfusate solutions were gassed with 7% CO_2/93% air and 5% CO_2/95% O_2, respectively [36, 37]. Nominally CO_2/HCO_3^--free solutions contained (in mM): 139 NaCl, 2.5 K_2HPO_4, 1.2 $MgSO_4$, 2.0 Ca lactate$_2$, 5.5 glucose, 1.0 Na_3 citrate, 6.0 L-alanine, and 5.0 N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and were gassed with 100% O_2. In one series of experiments, ouabain swelling was measured in tubules bathed or perfused with Cl^--free solutions containing 6.0 mM Ca^{2+} to compensate for the Ca^{2+}-chelating effects of isethionate. The composition of these solutions was (in mM): 118 Na isethionate, 23 $NaHCO_3$, 2.5 K_2SO_4, 1.2 $MgSO_4$, 2.0 Ca lactate$_2$, 4.0 Ca gluconate$_2$, 5.5 glucose, 1.0 Na_3 citrate, 6.0 L-alanine, 1.0 HEPES.

The following transport inhibitors were used where indicated in this study: acetazolamide (Sigma Chemicals, St. Louis, MO); amiloride (Merck, Sharpe & Dohme, West Point, PA); diphenylamine-2-carboxylate (DPC, Fluka Chemicals, Hauppauge, NY); ouabain (Sigma); 4-acetamido-4'-isothiocyanostilbene-2-2'-disulfonic acids (SITS, Sigma).

Table 1. Effects of 10^{-4} M peritubular ouabain on principal and intercalated cell volume

Cell type	Initial rate of ouabain swelling (%/min)	Maximum cell volume (% control)	Rate of RVD (%/min)	Steady-state regulated volume (% control)
Intercalated cell (*n*)	0 (5;6)	100 ± 1 (5; 6)	—	—
Principal cell (*n*)	67 ± 7 (8; 13)	233 ± 13 (10; 11)	−3 ± 1 (3; 5)	113 ± 4 (3; 4)

All solutions had measured osmolalities ranging between 283–290 mOsm and a pH of 7.4. Sodium- and K^+-free solutions contained immeasurable quantities of Na^+ and K^+ as determined by flame photometry. Chloride concentrations measured by chloridometry in nominally Cl^--free solutions were 0.07–0.09 mM.

Statistical Analysis

Data are expressed as the mean ± SE (n = number of tubules; number of cells). Statistical analyses were performed using a two-tailed Student's t test for independent means. Standard errors were calculated from the number of cells.

Results

Effects of Ouabain on Cell Volume

As shown in Fig. 1 and Table 1, addition of 10^{-4} M ouabain to the peritubular bathing medium had no effect on the volume of intercalated cells. Following a 4–6 min ouabain exposure, the mean change in intercalated cell volume observed was 0 ± 1%. The effect of ouabain on principal cell volume, however, was dramatic. Principal cells began to swell immediately following ouabain application (Figs. 1 and 3). These volume changes, as will be demonstrated below, are due to the continued entry of NaCl into the cell during basolateral Na^+ pump inhibition. The mean initial rate of swelling for principal cells was 67 ± 7%/min (Table 1).

The reversibility of ouabain inhibition of the principal cell Na^+ pump was examined by exposing CCT to brief cycles of ouabain addition and removal or to a single long-term ouabain application. In studies involving short-term pump inhibition, CCT were exposed to 2–3 successive periods of ouabain application. Cells were allowed to recover 10–12 min between each ouabain exposure which lasted 45–90 sec. The effect of two successive ouabain applications on the volume of a single principal cell is shown in Fig. 1. Figure 2 summarizes the results

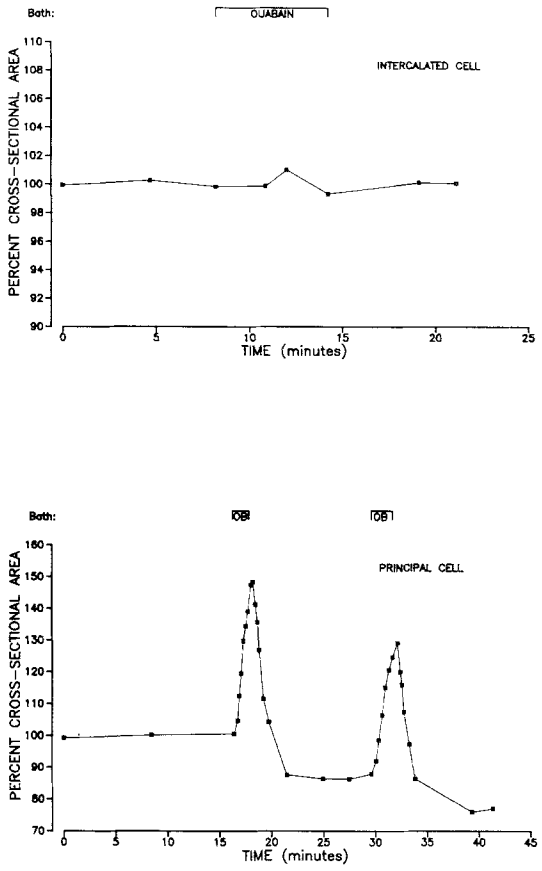

Fig. 1. Effects of ouabain on principal and intercalated cell volume. *Top panel:* Representative experiment showing the effects of peritubular application of 10^{-4} M ouabain on intercalated cell volume. *Bottom panel:* Representative experiment showing the effects 10^{-4} M ouabain exposure on principal cell volume. The initial rates of cell swelling and shrinkage for the first and second periods of ouabain exposure and washout were 47%/min, 26%/min, −35%/min and −35%/min, respectively

of experiments conducted on six isolated, perfused CCT. The mean initial rates of ouabain swelling for the first, second and third ouabain exposures were 65 ± 10%/min, 29 ± 3%/min, and 15 ± 3%/min, respectively. These data demonstrate that succes-

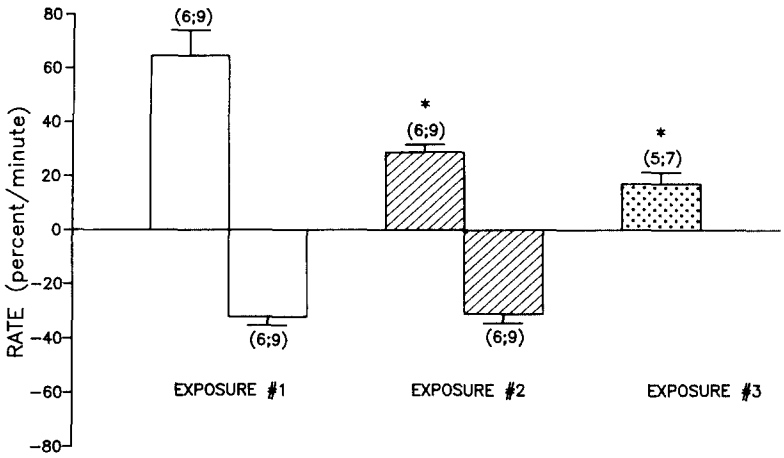

Fig. 2. Mean rates of cell swelling and shrinkage in principal cells during repetitive periods of ouabain exposure and removal. * Value is significantly reduced compared to preceding value ($P < 0.0002$). Rates of shrinkage were not significantly different ($P > 0.4$). n is shown in parentheses

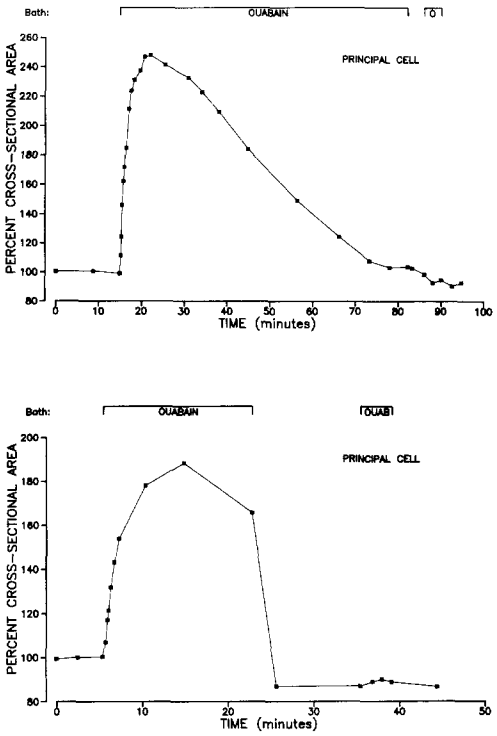

Fig. 3. Effect of long-term ouabain exposure on principal cell volume. *Top panel:* Representative experiment showing volume regulatory decrease in ouabain-treated principal cell and the effects of ouabain removal and readdition to the peritubular bath on cell volume. Note that the second ouabain exposure causes no cell swelling. *Bottom panel:* Representative experiment showing the effects of ouabain removal on principal cell volume following activation of RVD. Ouabain washout causes a rapid cell shrinkage, demonstrating that ouabain binding is at least partially reversible. The second ouabain exposure has no effect on cell volume

sive ouabain applications significantly ($P < 0.0025$) reduce the rate of cell swelling by 40–60% compared to the immediately preceding rate of volume increase. Such a reduction could occur by a reduction in the rate of cellular solute entry or by an irreversible titration of the Na^+ pump with ouabain. To test the latter hypothesis, the rate of cell shrinkage was measured during the period of ouabain washout, which immediately followed the first two ouabain exposures (e.g., Fig. 1). This shrinkage presumably reflects the time required to wash ouabain away from its binding sites and reactivate the Na^+ pump. The first and second ouabain washout periods caused principal cells to shrink to new steady-state volumes of 92 ± 2 and 93 ± 3% (n = 6; 9) of control, at rates of −32 ± 3%/min and −31 ± 3%/min, respectively (Fig. 2). There was no significant difference ($P > 0.4$) between the rates of cell shrinkage. If ouabain was irreversibly titrating the pump then the rate of cell shrinkage should decrease with each period of ouabain exposure and washout. Taken together, these data indicate that successive short-term ouabain exposures and Na^+-pump inhibition cause a concomitant reduction in the rate of cellular solute entry.

Data in Figure 3 show the effects of long-term ouabain exposure on principal cell volume. Principal cells swelled 133 ± 13% above control volume (Table 1). Despite this remarkable volume increase there was no apparent structural damage. Ouabain-swollen cells activated regulatory volume decrease (RVD) mechanisms and shrank at a mean rate of −3 ± 1%/min to a new steady-state volume 13 ± 4% above control (Table 1). Removal of ouabain following attainment of this steady-state volume caused a small shrinkage (e.g., Fig. 3) of −5 ± 1% (n = 3; 4). A second period of ouabain exposure in volume regulated principal cells caused little or no swelling (e.g., Fig. 3). The mean rate of volume increase observed was 1 ± 1%/min (range = 0–3%/min; n = 5; 6).

This apparent nearly total loss of ouabain sensitivity was also observed in cells that had reached a peak volume but had not undergone complete RVD

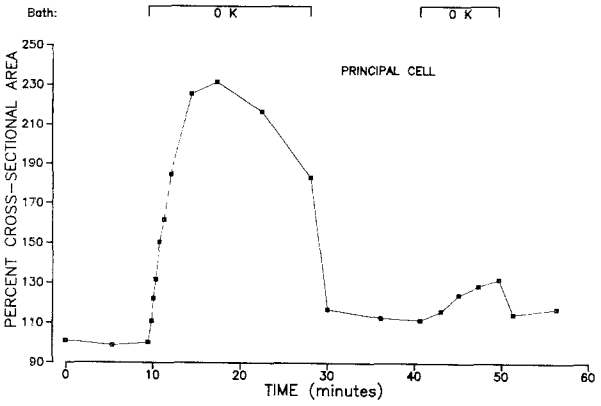

Fig. 4. Representative experiment showing the effects of a K^+-free peritubular bathing medium on principal cell volume. Pattern of cell volume change is similar to that shown in Fig. 3. The cell reaches a maximum swelling volume during pump inhibition and then activates RVD mechanisms. Readdition of K^+ back to the peritubular bath causes a rapid cell shrinkage consistent with pump reactivation. The rates of cell swelling during the first and second exposures to K^+-free bath were 42%/min and 3%/min, respectively

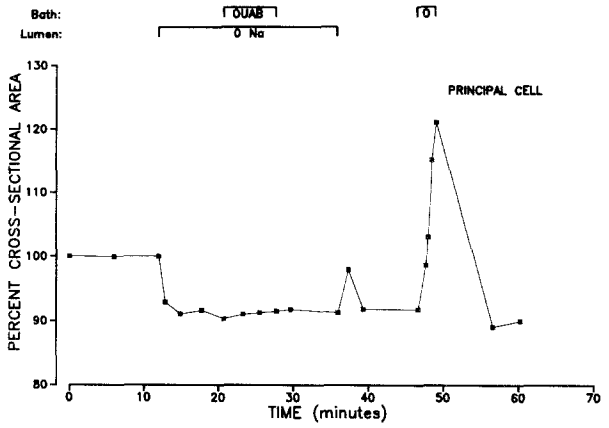

Fig. 5. Representative experiment showing the effects of luminal Na^+ removal and readdition on principal cell volume and ouabain-induced swelling

(e.g., Fig. 3). In six tubules principal cell RVD shrinkages of 2–23% were observed before ouabain was removed from the peritubular bath. Ouabain washout caused these cells to shrink rapidly (e.g., Fig. 3) to a new steady-state volume equal to 87 ± 5% (n = 6; 7) of control volume. A second ouabain exposure following a 10–15 min recovery period had little effect on principal cell volume, causing the cells to swell at a mean rate of 1 ± 1%/min (range = 0–6%/min; n = 6; 7).

The apparent loss of ouabain sensitivity described above can again be explained by an inhibition of solute entry or by an irreversible pump blockage during prolonged ouabain exposure. Data in Fig. 3 argue against the latter hypothesis. The rapid cell shrinkage observed during ouabain removal indicates that the pump is still active and that ouabain binding is at least partially reversible following prolonged exposure to this inhibitor. To test this idea further, however, experiments were conducted in which the Na^+ pump was inhibited by complete removal of peritubular K^+. Tubules exposed to a K^+-free bathing medium for 12–18 min swelled rapidly at an initial rate of 32 ± 5%/min (n = 3; 7) (Fig. 4) and reached a peak volume which was 78 ± 16% (n = 3; 7) greater than control volume. Both the rate and maximum swelling volume were significantly ($P < 0.01$) lower than observed in ouabain-swollen cells. The reasons for the slower rate of swelling and smaller peak volume are uncertain. Removal of peritubular K^+ will alter the electrochemical gradient driving K^+ movement and favor basolateral K^+ efflux. An increased rate of K^+-anion loss would blunt both the rate and magnitude of volume increase during pump inhibition. Alternatively, the Na^+ pump may not be completely inhibited due to the presence of small amounts of K^+ in lateral interspace and basal membrane infolding unstirred layers.

Attainment of a peak swelling volume in a K^+-free bathing medium is followed by activation of RVD mechanisms (e.g., Fig. 4). Principal cells exhibited volume decreases of 10–70% before K^+ was added back to the peritubular bath. Reactivation of the Na pump caused a rapid cell shrinkage to a new steady-state volume equal to 99 ± 9% (n = 3; 7) of control. After a 10-min recovery CCT were again exposed to a K^+-free bath, which caused the cells to swell at a greatly reduced rate of 3 ± 1%/min (range = 2–6%/min; n = 3; 7). These data demonstrate clearly that the apparent loss of ouabain sensitivity is not due to an irreversible binding of this inhibitor to the Na^+/K^+ ATPase and instead is due to an inhibition of solute entry mechanisms.

In summary, the data discussed above indicate that brief Na^+-pump inhibition results in a rapid reduction in the rates of cellular solute entry. With prolonged pump blockage, principal cells reach a peak swelling volume which is associated with an almost complete cessation of solute entry and activation of RVD mechanisms.

Mechanisms of Ouabain Swelling

The nature of the solute entry pathways responsible for ouabain swelling were examined by conducting ion substitution and transport inhibitor drug experiments. Complete replacement of luminal Na^+ with N-methyl-D-glucamine caused principal cells to shrink −12 ± 2% (n = 5; 7) and completely blocked ouabain swelling (e.g., Fig. 5). Readdition of Na^+

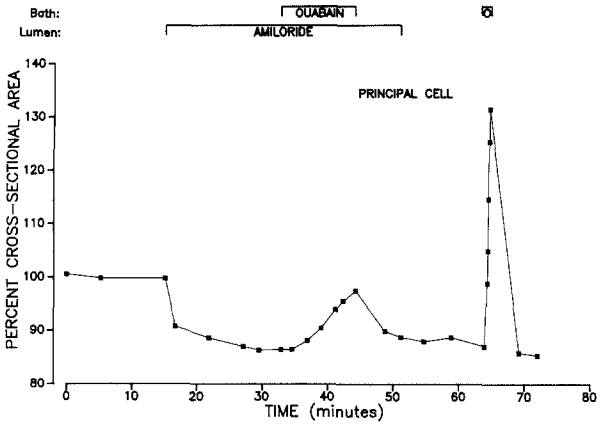

Fig. 6. Representative experiment showing the effects of luminal application of 10^{-5} M amiloride on principal cell volume and ouabain-induced swelling

back to the lumen caused a small transient cell swelling (e.g., Fig. 5) of 11 ± 3% (n = 4; 6). Ouabain addition after cells had reached a new steady-state volume caused swelling at a rate of 15 ± 3%/min (n = 5; 7).

Since the bulk of Na^+ reabsorption in the rabbit CCT appears to be mediated through an amiloride-inhibitable Na^+ channel [24], the effects of amiloride on ouabain swelling were examined. Addition of 10^{-5} M amiloride to the luminal perfusate caused principal cells to shrink −8 ± 3% (n = 7; 12) and reduced the rate of ouabain swelling approximately 95% to a mean value of 3 ± 1%/min (n = 7; 12) (e.g., Fig. 6). The swelling observed in the presence of luminal amiloride was peculiar in that there was a noticeable time lag of 2–3 min before cell volume increased in response to peritubular ouabain addition (e.g., Fig. 6). The reason for this delayed swelling is uncertain. Following amiloride removal, addition of ouabain to the peritubular bath caused principal cells to swell at a mean rate of 31 ± 8%/min (n = 6; 8) (e.g., Fig. 6).

In order for electroneutrality to be preserved and cell swelling to occur, an anion must accompany inward Na^+ movement. Replacement of luminal Cl^- with isethionate caused principal cells to shrink −9 ± 1% and reduced the rate of ouabain swelling by approximately 70% to a mean value of 20 ± 2%/min (Fig. 7). Addition of Cl^- back to the luminal perfusate following ouabain removal caused a 7 ± 3% (n = 4; 6) increase in cell volume and significantly ($P < 0.005$) increased the rate of ouabain swelling to a mean value of 35 ± 3%/min (n = 5; 9). Tubules perfused with Cl^--free saline containing 6.0 mM Ca^{2+} (*see* Materials and Methods) swelled at a rate of 15 ± 2%/min (n = 2; 5). This value was not significantly different ($P > 0.05$) from that observed in tubules perfused with Cl^--free saline containing 2.0 mM Ca^{2+}.

Since luminal Cl^- removal did not completely block ouabain swelling, it is possible that some cellular Cl^- uptake occurs through the basolateral membrane. Replacement of bath Cl^- with isethionate caused principal cells to shrink −10 ± 2% and reduced the rate of ouabain swelling approximately 85% to a mean value of 9 ± 3%/min (Fig. 7). Elevation of Ca^{2+} concentration to 6.0 mM did not significantly ($P > 0.25$) alter this rate of swelling (rate = 7 ± 2%/min; n = 3; 5). The fact that luminal and peritubular Cl^- substitutions are not additive in their inhibition of ouabain swelling indicates that removal of this ion has effects on principal cell function besides simply blocking Cl^- entry steps (e.g., changes in intracellular pH, Ca^{2+} concentration, etc.) and that these effects directly alter the rate of ouabain swelling.

Bilateral replacement of Cl^- with isethionate caused principal cells to shrink −12 ± 1% which was not significantly different ($P > 0.4$) from the shrinkage observed with luminal Cl^- removal alone. This maneuver, however, completely blocked or further reduced the rate of ouabain swelling to a mean value of 1 ± 1%/min (range = 0–3%/min) (Fig. 7).

Cortical collecting tubules from rabbits treated with DOCA actively reabsorb Cl^- from urine to blood [10, 11, 33]. Schuster and coworkers [*reviewed in* 31] have provided strong evidence indicating that intercalated cells are at least partially responsible for transepithelial anion movements in CCT from control rabbits. The cell shrinkage and inhibition of ouabain swelling during luminal Cl^- substitution shown in this study argue for the existence of a Cl^- entry pathway in apical membranes of principal cells and strongly suggest that this cell type is involved in transepithelial Cl^- reabsorption during mineralocorticoid stimulation of the CCT. The significant inhibitory effects of peritubular Cl^- removal on ouabain swelling, however, make this interpretation less certain. As a further test for the presence of an apical Cl^- entry step then, the effects of elevating extracellular K^+ on principal cell volume were examined. Apical and basolateral membranes of these cells have a significant K^+ conductance [16, 17]. Increases in bath or perfusate K^+ will alter K^+ electrochemical gradients such that this ion will be driven into the cytoplasm or its efflux from the cell will be reduced. If an anion accompanies K^+ movement, cell swelling will occur. As shown in Fig. 8, increasing luminal K^+ from 5 to 53 mM caused principal cells to swell 22 ± 2%. Elevation of peritubular K^+ from 5 to 53 mM after this volume change was complete (ca. 2–3 min) resulted

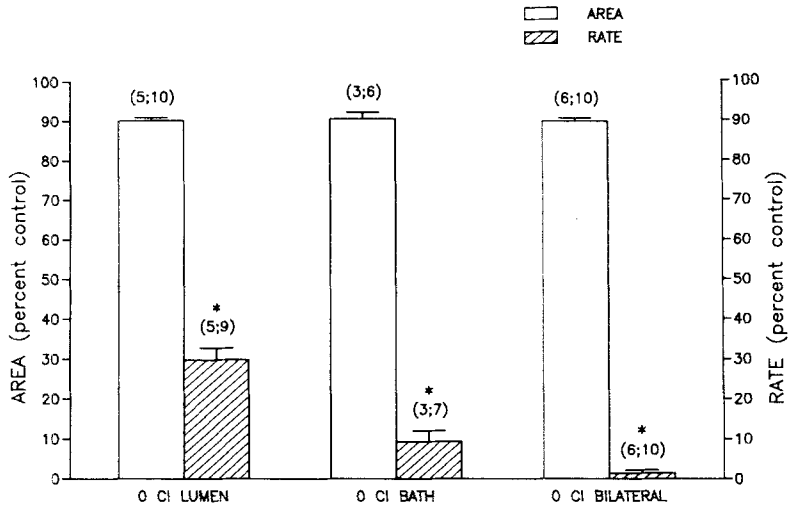

Fig. 7. Effects of perfusate and/or bath Cl^- removal on mean relative principal cell volume and rate of ouabain swelling. Open bars show the relative cell volume measured 2–3 min after lumen, bath or bilateral replacement of Cl^- with isethionate. Hatched bars show the relative rate of ouabain swelling. * Values are significantly less than control ($P < 0.0005$). n is shown in parentheses

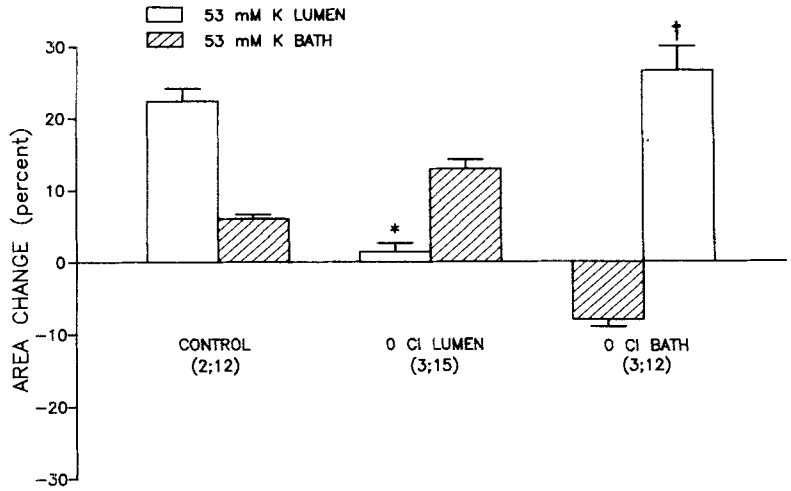

Fig. 8. Effect of luminal or bath Cl^- removal on K^+-induced swelling in principal cells. * Value is significantly less than control ($P < 0.0005$); † value is not significantly different from control ($P > 0.1$). n is shown in parentheses

in an additional swelling of 6 ± 1%. These results are qualitatively similar to those described for CCT dissected from control rabbits [35].

If Cl^- is the anion responsible for K^+-induced swelling and if Cl^- enters the cell through the apical membrane, then elevating luminal K^+ in the absence of Cl^- should prevent the volume increase. When CCT were perfused with a Cl^--free 53 mM K^+ saline, the mean change in principal cell volume observed was 1 ± 1% (Fig. 8).[1] Elevation of peritubular K^+ under these experimental conditions increased principal cell volume 13 ± 1% (Fig. 8).

The effect of peritubular Cl^- removal on K^+-induced swelling was also examined. If the basolateral membrane is the only site of cellular Cl^- permeation, then removal of bath Cl^- should prevent cell swelling during increases in perfusate K^+. This idea was tested in two groups of CCT. In the first group, tubules were bathed in a Cl^--free saline containing 53 mM K^+. The change in cell volume was measured 15–20 min after the bath solution switch. As shown in Fig. 8, this experimental maneuver caused a −8 ± 1% principal cell shrinkage typical of peritubular Cl^- removal; no K^+-induced swelling was observed. A second group of tubules were exposed first to a Cl^--free bath saline which also caused principal cells to shrink −8 ± 1% (*data not shown*). Elevation of luminal K^+ in the absence of bath Cl^- increased principal cell volume 27 ± 3% (Fig. 8). While this volume change was not significantly ($P > 0.1$) different from that observed in control CCT, the time required to reach a new steady-state volume was considerably longer (ca. 10–15

[1] Similar results have been observed in principal cells of CCT dissected from control rabbits (K. Strange, *unpublished observations*), suggesting that this cell type may mediate transepithelial Cl^- movements under a variety of experimental conditions.

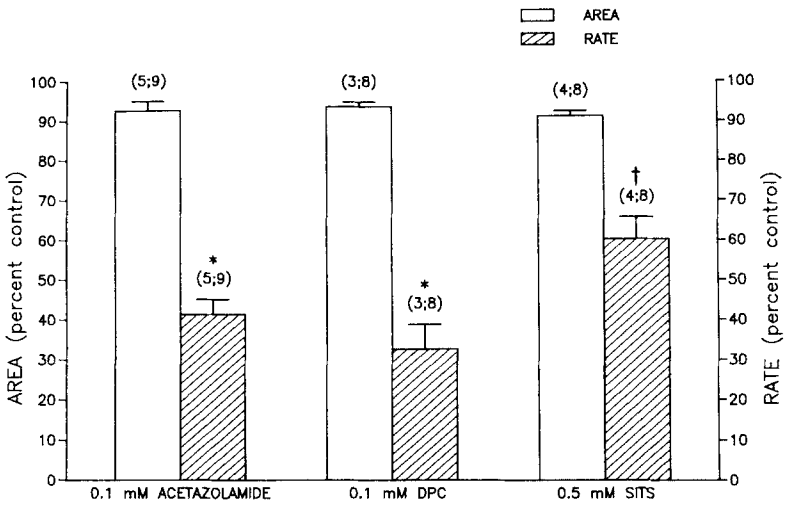

Fig. 9. Effects of perfusing CCT with various anion transport inhibitors on principal cell volume and rate of ouabain swelling. Tubules were perfused with the inhibitors for 10–15 min prior to peritubular ouabain application. Open bars show the relative steady-state cell volume after perfusion of the tubule with the inhibitor. Hatched bars show the relative rate of ouabain swelling. * Value is significantly less than control ($P < 0.0005$); † value is significantly less than control ($P < 0.005$)

min). The reasons for this reduced rate of swelling are uncertain, but may be due to changes in electrochemical gradients driving K^+ and/or Cl^- movements.

Taken together, the above results provide strong direct evidence for an apical Cl^- transport step in principal cells and demonstrate that this cell type plays a role in transepithelial Cl^- reabsorption. To investigate the mechanism of apical Cl^- entry, the effects of a variety of anion transport inhibitor drugs on principal cell volume and ouabain swelling were examined. Tubules were perfused with these drugs for 10–15 min prior to ouabain application. As shown in Fig. 9, addition of the carbonic anhydrase inhibitor acetazolamide at a concentration of 0.1 mM to the luminal perfusate reduced the rate of ouabain swelling by approximately 60%. Acetazolamide also caused a significant shrinkage of principal cells of $-7 \pm 2\%$. This effect was extremely variable, however, with observed volume changes ranging from 0 to −26%. Higher concentrations of acetazolamide (1.0 mM) caused no further cell shrinkage or inhibition of ouabain swelling (K. Strange, *unpublished observations*). Perfusion of CCT with 0.1 mM DPC or 0.5 mM SITS, both potent inhibitors of anion channels and Cl^-/HCO_3^- exchangers [12, 26, 31], caused consistent cell shrinkages of $-7 \pm 1\%$ and $-9 \pm 1\%$ and reduced the rate of ouabain swelling by approximately 70 and 40%, respectively (Fig. 9).

In addition to increasing Na^+ reabsorption across the CCT, DOCA stimulates Cl^- reabsorption and HCO_3^- secretion [7, 10, 11, 14, 33]. Studies on isolated CCT dissected from DOCA-treated rabbits suggest strongly that HCO_3^- secretion is at least partially coupled to Cl^- reabsorption via a Cl^-/HCO_3^- exchanger [7, 33]. Electrophysiological studies suggest that the only conductive ion movements across apical membranes of principal cells are those for Na^+ and K^+ [25, 27, 28]. Given these observations plus the inhibitor studies described above, it seemed plausible that apical Cl^- movement in principal cells may be mediated by an anion exchanger. To test this idea, the effects of bilateral CO_2 and HCO_3^- removal on ouabain swelling were examined. In these studies tubules were first luminally perfused with a nominally CO_2-free saline for 10–15 min followed by exposure to a nominally CO_2-free bathing saline for an additional 5–10 min. As shown in Table 2, removal of luminal CO_2 and HCO_3^- caused a cell shrinkage of $-13 \pm 2\%$. The effect of peritubular CO_2 removal on principal cell volume was quite variable, causing a marked swelling of up to 10% in some tubules. Overall, however, there was no significant ($P > 0.1$) additional change in cell volume (Table 2). The rate of ouabain swelling in the absence of exogenous CO_2 and HCO_3^- was inhibited significantly ($P < 0.0005$) by approximately 70% compared to that seen in control tubules (Table 2).

Garcia-Austt et al. [7] have shown that net HCO_3^- secretion in CCT from DOCA-treated rabbits can be completely inhibited by in vivo acid loading. Rabbits were acid loaded in these studies by feeding them rat chow and allowing them free access to drinking water containing 50 mM NH_4Cl for 7–8 days, which reduced urine pH to 5.3 [7]. If principal cell Cl^- transport is at least partially coupled to HCO_3^- movement then it seemed likely that whole animal acidosis should inhibit ouabain swelling. Table 3 shows the effects of acid loading on urine pH and ouabain swelling. Ammonium chloride loading alone (50–75 mM NH_4Cl plus 100 mM glucose-containing drinking water for 8 days) had

Table 2. Effects of luminal and peritubular CO_2/HCO_3 removal on principal cell volume and rate of ouabain swelling

Cell volume, CO_2-free lumen (% control) (*n*)	Cell volume, CO_2-free lumen and bath (% control) (*n*)	Initial rate of ouabain swelling, CO_2-free bath and lumen (%/min) (*n*)
88 ± 2 (6; 12)	92 ± 3 (5; 9)	21 ± 3 (6; 12)

Table 3. Effect of NH_4Cl loading on urine pH and rate of principal cell ouabain swelling

Experiment	Urine pH	Initial rate of ouabain swelling (%/min)
Control (*n*)	8.05 ± 0.11 (7)	67 ± 7 (8; 13)
50–75 mM NH_4Cl plus 100 mM glucose drinking water for 8 days (*n*)	7.74 ± 0.20 (4)	60 ± 9 (4; 4)
75 mM NH_4Cl plus 100 mM glucose drinking water with fasting for 3 days (*n*)	5.16 ± 0.08 (6)	33 ± 4 (6; 9)

only a small and statistically insignificant ($P > 0.1$) effect on urine pH, reducing it from a control value of 8.05 ± 0.11 to 7.74 ± 0.20. The rate of principal cell swelling in CCT dissected from these animals was 60 ± 9%/min, which was not significantly different ($P > 0.4$) from control (Table 3). When animals were given 75 mM NH_4Cl drinking water containing 100 mM glucose and fasted for 3 days, urine pH dropped dramatically to a mean value of 5.16 ± 0.08. This experimental maneuver significantly ($P < 0.0005$) inhibited the rate of ouabain swelling by approximately 50% (Table 3).

Discussion

Sodium Transport

Sodium reabsorption in CCT of mineralocorticoid-treated rabbits appears to be mediated largely by an amiloride-inhibitable Na^+ channel [24]. Extensive electrophysiological studies indicate that this Na^+ absorptive pathway is localized to the principal cell (16, 17, 23). Data in Fig. 6 are in complete agreement with these findings. Luminal application of 10^{-5} M amiloride inhibited the rate of ouabain swelling by 95%. The small amount of ouabain swelling that did occur during perfusion with amiloride began 2–3 min after ouabain application. The reasons for the time lag are uncertain. This swelling could, however, be blocked by luminal Na^+ removal (K. Strange, *unpublished observation*), suggesting that it was mediated by an apical Na^+ entry step. Whether Na^+ is entering through channels incompletely blocked by amiloride or via an amiloride-insensitive pathway remains to be determined.

Principal cells swell at a mean relative rate of 67%/min when exposed to ouabain (Table 1). The *initial* change in the concentration of osmotically active solutes which must occur in these cells to cause a given *isosmotic* volume change during Na^+ pump inhibition can be estimated by:

$$\Delta C_o = -\frac{C_o\, x'}{\Delta V + 1 - x} + C_o \qquad (1)$$

where C_o is the cellular concentration of osmotically active solutes prior to ouabain addition, ΔC_o is the *initial* change (i.e., before water influx) in the concentration of these solutes, ΔV is the fractional cell volume change, and x and x' are the fractions of the osmotically inactive and active cellular volume, respectively. Assuming that $x' = 0.8$ [35, 37], $x = 0.2$, and $C_o = 285$ mOsm, and given that $\Delta V = 0.67$, then $\Delta C_o = 130$ mOsm. Thus, in order to swell at a rate of 67%/min (Table 1), there must be a net gain of 130 milliosmoles of solute per liter of initial principal cell water per minute. Since cell volume changes only occur during net salt accumulation or loss there is a net gain of 65 millimoles of Na^+ per liter of initial principal cell water per minute during pump inhibition. This value can be used to estimate a *minimum* principal cell Na^+ reabsorptive rate. Implicit in this estimate is the assumption that no other net salt transport processes (e.g., apical or basolateral KCl movement) contribute to the observed ouabain-induced volume change and that all Na^+ influx contributes to cell swelling (i.e., that there is an equivalent influx of anions for each millimole of Na^+ entering the cell during ouabain application). Using a mean principal cell volume of 1708 μm^3 (K. Strange, *unpublished observation*) and a cell water content of 80% [35, 37], the rate of principal cell Na^+ reabsorption is 0.1 pmol/cell/min. The average lumen-to-bath Na^+ flux in CCT from DOCA-treated rabbits is 88.8 pmol/mm/min [17]. Assuming that there are 507 cells/mm of CCT [8] and that 65% of

these are principal cells [19], a Na^+ reabsorption rate of 0.3 pmol/cell/min can be calculated. The excellent agreement between these two values as well as the marked inhibitory effect of luminal amiloride on ouabain swelling (Fig. 6) provide further strong evidence to support the contention that the major pathway for Na^+ reabsorption in the mineralocorticoid-treated CCT is via apical membrane Na^+ channels in principal cells [16, 17, 24].

Chloride Transport

During the last 10 years a number of detailed studies of Cl^- transport in the rabbit CCT have been conducted [*reviewed in* 31]. From these various investigations it is clear that DOCA treatment stimulates net Cl^- reabsorption against chemical and electrical gradients [10, 11, 33]. Star et al. [33] have provided strong evidence indicating that at least part of this transepithelial Cl^- movement is directly coupled to active HCO_3^- secretion via a 1:1 Cl^-/HCO_3^- exchanger.

The cellular location and control of anion transport in the CCT is an important focus of research in renal physiology [*reviewed in* 31]. Several investigations have implicated the intercalated cells as being a major site of Cl^- reabsorption and HCO_3^- secretion. For example, studies by Schuster and coworkers on CCT dissected from non-DOCA treated rabbits have shown that Cl^- self exchange and Cl^-/HCO_3^- exchange are stimulated by cAMP and isoproteronol, but not vasopressin [31, 38]. Previous indirect evidence suggested that β agonist receptors were localized to intercalated cells [22]. Based on these observations plus the fact that isoproteronol and vasopressin have different physiological effects on the CCT and yet act through the same intracellular messenger, cAMP [22], Tago et al. [38] suggested that intercalated cells were the site of Cl^-/HCO_3^- exchange. In addition, Schwartz et al. [32] have demonstrated that a subpopulation of intercalated cells acidify when peritubular Cl^- is removed, suggesting the presence of an apical anion exchanger and implicating this cell type in HCO_3^- secretion and Cl^- reabsorption.

Results from this study by no means rule out an important role for the intercalated cell in transepithelial anion movement. The data do, however, indicate that principal cells of DOCA-treated CCT reabsorb Cl^- and suggest that apical Cl^- entry may be mediated by an anion exchanger. Two issues warrant detailed discussion: (i) sidedness of Cl^- entry and (ii) mechanisms of Cl^- transport.

Data in Figs. 5 and 7 demonstrate clearly that ouabain-induced cell swelling is the result of cytoplasmic NaCl accumulation during pump inhibition. While Na^+ entry appears to occur primarily through apical membrane Na^+ channels (Figs. 5 and 6), the location of the Cl^- uptake step is less obvious. Both luminal and peritubular Cl^- removal caused cell shrinkage and markedly inhibited the rate of ouabain swelling (Fig. 7), suggesting that Cl^- could enter through apical and/or basolateral membranes. One possible site of cellular Cl^- uptake is via basolateral membrane Cl^- channels [28]. Equivalent electrical circuit analysis has suggested that the basolateral membrane potential (V_{bl}) is close to E_{Cl^-} in both control and DOCA-stimulated CCT [27]. The normal V_{bl} in CCT from DOCA-treated rabbits is approximately -100 mV [15, 27]. If the resting V_{bl} was indeed close to E_{Cl^-}, then intracellular Cl^- activity (aCl_i) should be 2–3 mM and thus the ouabain-induced depolarization of the basolateral membrane [15, 27] could drive Cl^- influx. Results of this study indicate that aCl_i is considerably higher than 2–3 mM. Luminal, peritubular, or bilateral Cl^- removal caused principal cells to shrink on average -10% (Figs. 7 and 8). Assuming that Cl^- is the only anion leaving the cell under these conditions and that only monovalent cations accompany Cl^- efflux, then a *minimum* intracellular Cl^- concentration of 19 mM can be estimated from Eq. (1). This value is well within the range of intracellular Cl^- levels observed in most epithelial cells (e.g., [2, 6]) and is in excellent agreement with recent electron microprobe studies which demonstrated a Cl^- content of 25 mmol/kg wet wt in principal cells of perfused CCT dissected from control rabbits [29]. Using the value of 19 mM for intracellular Cl^-, the calculated E_{Cl^-} for the basolateral membrane would be approximately -48 mV. During peritubular ouabain addition the basolateral membrane exhibits a rapid spiking depolarization of 10–15 mV, which is complete within 9–12 sec [15, 27]. This is followed by a slower depolarization, which occurs at a rate of approximately 8 mV/min. Membrane depolarization clearly contributes to cell swelling in that basolateral Cl^- exit is slowed during pump inhibition. The basolateral membrane potential, however, does not reach E_{Cl^-} until several minutes after ouabain addition. Inasmuch as cell swelling starts immediately after ouabain application (e.g. Figs. 1 and 3), cellular Cl^- entry cannot occur through the basolateral Cl^- channel.

The above contention is further supported by experiments conducted with K^+-free bathing saline (Fig. 4). While the effects of peritubular K^+ removal on the basolateral membrane potential are not known, an initial hyperpolarization of V_{bl} is expected, driving it further away from E_{Cl^-}. Nevertheless, principal cells swelled rapidly and immediately upon exposure to a K^+-free bath (Fig. 4).

At this time the contribution of an electroneutral basolateral Cl^- entry step to ouabain swelling

cannot be ruled out. However, given that the rate of cellular Cl^- uptake must at least be equivalent to the rate of Na^+ influx causing cell swelling (i.e., 0.1 pmol/cell/min), it seems more likely that the bulk of Cl^- entry occurs through the apical membrane. Studies of K^+-induced swelling in the absence or presence of luminal and peritubular Cl^- (Fig. 8) provide for the first time direct evidence for an apical Cl^- entry step and indicate that principal cells mediate a component of transepithelial Cl^- reabsorption in the CCT. In addition, these investigations provide a simple functional explanation for the presence of Cl^- channels in principal cell basolateral membranes [28, 31]. Clearly, a basolateral Cl^- conductance would mediate net cell-to-blood Cl^- efflux and transcellular Cl^- movement.

While elucidation of the mechanisms of principal cell Cl^- transport clearly requires extensive investigation, results of this study indirectly suggest that Cl^- reabsorption may be at least partially mediated by a Cl^-/HCO_3^- exchanger. Star et al. [33] have shown that transepithelial Cl^- reabsorption in CCT dissected from DOCA-treated rabbits is inhibited 75% by bilateral CO_2/HCO_3 removal. The same experimental maneuver inhibits the rate of ouabain swelling by 70% (Table 2). Garcia-Austt et al. [7] demonstrated that whole animal acidosis inhibited DOCA-stimulated bath-to-lumen total CO_2 flux by 85%, whereas acidosis reduces the rate of ouabain swelling by 50% (Table 3). The cell shrinkage and marked inhibitory effects of low luminal concentrations of acetazolamide or DPC on ouabain swelling (Fig. 9) also argues for an apical anion exchanger in principal cells.

Recent studies by Alper et al. [1] have provided strong evidence for the presence of an anion exchanger in the apical membrane of rat CCT principal cells. These investigators cloned a band 3 anion exchanger-related cDNA from mouse kidney, which stimulates Cl^- uptake when microinjected into *Xenopus* oocytes. The hydrophobic region of the gene product is 67% identical to the band 3 protein. Antibodies to the band 3-related protein bind only to the apical membrane of principal cells in the CCT and to apical membranes of all cells in the distal convoluted tubule. Direct functional demonstration of a rabbit principal cell apical anion exchanger requires measurement of intracellular pH and Cl^- under a variety of experimental conditions. Such studies are currently under way in this laboratory.

Cellular Homeostasis

The effects of long-term and brief, repeat periods of Na^+ pump inhibition on principal cell NaCl transport are important and warrant discussion. Repetitive exposure of this cell type to ouabain inhibits the rate of cell swelling during subsequent periods of pump inhibition (Figs. 1 and 2). This effect was clearly not due to an irreversible titration of the Na^- pump with ouabain (Fig. 2). The simplest explanation for the reduction in the rate of ouabain swelling is that pump inhibition results in a rapid inhibition of Na^+ and/or Cl^- entry steps. This inhibition could be mediated by so-called "homocellular" [30] or feedback regulatory mechanisms as has been suggested for *Necturus* gallbladder [13], frog skin [20], and mammalian colon [39]. Alternatively, the inhibition could reflect reduction in the driving forces for inward Na^+ and/or Cl^- movement. Elucidation of the inhibitory effects of pump blockage on NaCl entry steps requires the direct measurement of Na^+ and Cl^- electrochemical potentials.

During prolonged ouabain exposure (>5 min) principal cells reach a maximum volume and activate RVD mechanisms (Fig. 3). A similar pattern of ouabain swelling and volume regulation has been observed in *Necturus* gallbladder [13]. Readdition of ouabain to the peritubular bath following ouabain removal at any point after the maximum swelling volume has been reached has little or no effect on principal cell volume (Fig. 3). This apparent nearly complete loss of ouabain sensitivity cannot be explained by irreversible ouabain binding to the pump. Ouabain removal from the peritubular bath causes rapid and dramatic cell shrinkage if RVD is not complete (Fig. 3) or if it has been inhibited (K. Strange, *unpublished observation*), indicating that ouabain binding is at least partially reversible following prolonged exposure. Furthermore, a similar inhibition of cell swelling was observed when the Na^+ pump was repeatedly inhibited by exposure of CCT to a K^+-free peritubular bathing medium (Fig. 4). Taken together, these data demonstrate that the apparent loss of ouabain sensitivity during prolonged pump inhibition is due to a nearly complete cessation of Na^+ and/or Cl^- entry steps. Cessation of cellular solute entry prevents cellular lysis and allows the cells to activate RVD mechanisms and downregulate their volume. Again, inhibition of Na^+ and/or Cl^- entry could reflect a feedback regulatory process between apical and basolateral cell membranes.

Cellular homeostatic mechanisms play a critical role in the function of epithelial cells, preventing deleterious changes in cell volume and cytoplasmic composition that can occur during variations in transcellular solute and water movement [30]. Such processes might be expected to be particularly important in the physiology of the collecting tubule. This epithelium is responsible for the "fine control" of blood pressure, volume and salt composition,

and its function is constantly changing in response to whole animal salt and water regulatory demands. In this respect it is interesting to note that the ouabain experiments and associated cellular events described in this paper may have a direct physiological correlate. Rapidly accumulating experimental evidence suggests that the hypothalamus produces a high-affinity inhibitor of the Na^+/K^+ ATPase, which functions in a manner similar to ouabain and may play a role in controlling renal salt and water excretion [9]. The responses of the principal cell to pump inhibition observed in this study would prevent cellular lysis and long-term changes in cell salt and water content during the putative natriuresis induced by this hypothalamic factor.

Conclusions

During Na^+ pump blockage principal cells of CCT from DOCA-treated rabbits swell rapidly due to the continued entry of NaCl and water. Sodium influx occurs primarily through an amiloride-inhibitable channel in the apical membrane. Chloride entry occurs, at least in part, through the apical membrane and may be mediated by an anion exchanger. Brief periods of pump inhibition results in a rapid inhibition in one or both of these transport steps. Prolonged Na^+ pump blockage completely inhibits Na^+ and/or Cl^- entry and results in the activation of RVD mechanisms.

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