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REGULATION OF PROTON TRANSPORT IN URINARY EPITHELIA

By QAIS AL-AWQATI, STEPHEN GLUCK, WESTLEY REEVES and CHRISTOPHER CANNON

Departments of Medicine and Physiology, College of Physicians and Surgeons, Columbia University, 630 W 168th St., New York, NY 10032 U.S.A.

SUMMARY

In urinary epithelia, like the turtle bladder, protons are transported by a H^+-translocating ATPase located in the luminal membrane. We have recently discovered that the H^+ pump is stored in small vesicles that lie underneath the luminal membrane. CO_2, a major regulator of H^+ transport, causes these vesicles to fuse with the membrane thereby inserting more H^+ pumps.

We have now isolated these vesicles from the turtle bladder and from beef kidney medulla. Based on inhibitor sensitivity and substrate specificity this proton translocating ATPase is different from the mitochondrial F_0-F_1 ATPase, yeast plasma membrane and the gastric H^+,K^+-ATPase. Solubilization and reconstitution of the enzyme into liposomes shows retention of transport activity and inhibitor sensitivity.

INTRODUCTION

The kidney of vertebrates regulates acid base balance by reabsorption of filtered bicarbonate and by generating sufficient bicarbonate to titrate the acid that is produced during cellular metabolism. It is now accepted that this process is mediated by H^+ secretion rather than by absorption of ionic bicarbonate. Two mechanisms of proton secretion have been identified in the kidney; a passive Na: H exchange and an active H^+ pump. Na: H exchange occurs in more proximal segments of the nephron while the H^+ pump is distributed in proximal and distal segments. To study the cellular mechanisms of urinary acidification Steinmetz & Brodsky and their collaborators used the urinary bladder of the freshwater turtle, which served as an excellent model for the study of the characteristics of the H^+ pump (reviewed in Steinmetz, 1974). Recent studies by renal physiologists using isolated perfused mammalian collecting tubules have now shown that the majority of the processes identified in the reptilian bladder are also present in the collecting tubule.

The turtle urinary bladder has two major cell types, granular cells and mitochondria-rich cells. The latter have a ruffled surface and account for 10–30 % of the luminal surface area. These cells contain virtually all of the carbonic anhydrase of the epithelium and numerous lines of evidence suggest that they are the cells responsible for urinary acidification. A recently appreciated characteristic of these cells is that they contain intracellular vesicles under the luminal membrane which

Key words: H^+-ATPase, urinary acidification, proton pump.

participate in endocytosis and exocytosis, a process which turns out to be of critical importance for the regulation of H^+ transport. Lectins, such as concanavalin A and wheat germ agglutinin, preferentially bind to the luminal membrane of these cells.

THE URINE IS ACIDIFIED BY AN ELECTROGENIC H^+ PUMP

The turtle urinary bladder is a tight epithelium with transepithelial resistances of several thousands Ωcm^2 which is capable of absorbing sodium as well as secreting protons. Inhibition of sodium transport by ouabain or amiloride results in a reversal of the potential difference to a lumen positive orientation. The reversed short-circuit current is equal in magnitude to the rate of H^+ secretion under a wide variety of experimental conditions. Changes in the rate of H^+ secretion are also accompanied by changes in tissue conductance. These results show that the H^+ pump mediates electrogenic transfer of protons across the epithelium. The bladder continues to secrete protons even when the urine pH is far below the cell pH, suggesting that the active step for acidification is located in the luminal membrane (Steinmetz, 1974).

The rate of H^+ secretion in the bladder is exquisitely sensitive to the proton electrochemical gradient across the membrane. A low urine pH and a lumen positive potential both reduce the rate, and their effects for the same electrochemical gradient are equal. The pump stops at an adverse gradient of 3 pH units or 180 mV (Beauwens & Al-Awqati, 1976; Al-Awqati, Mueller & Steinmetz, 1977). When H^+ secretion was measured simultaneously with the rate of glucose oxidation (as an index of ATP hydrolysis) we found that changes in the rate of one was accompanied by changes in the rate of the other, indicating that the two processes were coupled. This coupling was found not only when the rate of transport was changed by CO_2, acetazolamide or aldosterone but also when adverse electrochemical gradients were imposed on the membrane (Beauwens & Al-Awqati, 1976). Thermodynamic analysis of the degree of tightness of coupling revealed that the H^+ pump was operating at a maximal efficiency. These studies suggested that this H^+ pump is sufficiently affected by gradients that the gradient at zero current flow could be considered to be the 'reversal potential' of the pump. The tightness of coupling also predicted that given gradients greater than the reversal potential, the direction of flow can be reversed. Recent studies have shown that such is indeed the case (Dixon & Al-Awqati, 1979).

REGULATION OF H^+ SECRETION BY ENDOCYTOSIS AND EXOCYTOSIS

Three major physiological factors regulate H^+ secretion; the ambient P_{CO_2}, the electrochemical gradient across the membrane and the level of aldosterone. To arrive at a cellular mechanism by which these factors could change H^+ secretion we used an equivalent circuit analysis in which the transport system was modelled by an e.m.f. with a series and a parallel resistance (Al-Awqati *et al.* 1977). The force of the pump, which we termed the protonmotive force, could easily be determined by measuring the adverse gradient needed to nullify the rate of secretion. The slope of the rate of transport on the applied gradient was taken to equal the conductance in the active pathway, assuming of course that the parallel conductance is unchanged. The protonmotive force is a complex function which includes the free energy of hydrolysis

ATP, as well as the stoichiometry of the pump. The active conductance was due to some function of the number of pumps as well as the turnover number of each pump. We found that CO_2, aldosterone, acetazolamide and metabolic inhibitors changed the active conductance rather than the protonmotive force (Al-Awqati *et al.* 1977). Unfortunately this kind of analysis did not lead to any advance in our information, since each of the parameters of the equivalent circuit was a complex function none of whose components was amenable to direct measurement. In one area however, it may have been useful. CO_2 was found to increase the rate of secretion and the active conductance but had no effect on the protonmotive force (PMF). Since the PMF was a function of the proton electrochemical gradient across the membrane, we concluded that the change must have occurred not as a result of large changes in intracellular pH but rather as a result of changes in the kinetic properties of each pump or as a result of an increase in the number of pumps in the membrane.

In subsequent studies we found that the mechanism by which CO_2 causes the change in H^+ transport is by rapid increases in the number of functional proton pumps in the membrane rather than by changing the kinetic properties of each pump (Gluck, Cannon & Al-Awqati, 1982). These rapid increases occur by exocytotic insertion of membranes containing H^+ pumps. We found that the mitochondria-rich cells of the turtle bladder contain numerous acidic vesicles located underneath the luminal membrane. We identified these vesicles using the fluorescent dye acridine orange in living turtle bladders mounted on the stage of a microscope. This dye is a weak base which accumulates in acid compartments. The emission spectrum of this dye is quite sensitive to its concentration because it readily forms multimers. At low concentrations the monomer fluoresces in the green while at higher concentration there is a red shift. We found that the mitochondria-rich cells contained orange vesicles while the granular cells did not. This orange colour disappeared when the bladder was treated with the proton ionophore nigericin, indicating that the accumulated acridine orange was due to a low pH rather than to binding. When we perfused the bladder with a CO_2-enriched medium we found that orange vesicles fused with the membrane, discharging their contents into the luminal medium; this we were able to see because they appeared like puffs of green 'smoke'. We interpreted this to indicate that when the vesicles fused their concentrated acridine orange was suddenly diluted with the consequent spectral shift.

To arrive at a more quantitative estimate of these processes we measured endocytosis, exocytosis and the pH inside these vesicles. Incubation of the turtle bladder with a large molecular weight dextran coupled to fluorescein isothiocyanate (FITC-Dextran) resulted in an uptake of this fluid phase marker into the cell. We were able to show that the uptake was linear over at least 30 min. Fluorescent microscopy showed that the FITC-Dextran was taken up into the mitochondria-rich cells only. Comparison of the rate of uptake of FITC-Dextran in bladders exposed to a high P_{CO_2} with that in bladders exposed to CO_2-free air showed that removal of CO_2 stimulated the uptake significantly (Reeves, Gluck & Al-Awqati, 1983).

We were also able to measure the pH in the vesicles that took up FITC-Dextran. The excitation spectrum of FITC is pH sensitive in the range of 4·5–7·5. By measuring the ratio of the emission intensities after excitation at 490 nm and 458 nm as a function of pH, a calibration curve for measurement of pH could be generated which

is independent of path length or fluorescent dye concentration. In bladders loaded with FITC-Dextran we focused the exciting light on an individual mitochondria-rich cell and passed the emitted light through a microspectrum analyser with the monochromator set to 520 nm, the emission peak of FITC. The cell was excited by two wavelengths sequentially, 490 nm and 458 nm. The ratio of the two intensities allowed calculation of the pH using the calibration curve. We found that the average pH of these vesicles was 5·0. The low pH increased when the bladder was treated with ammonia or proton ionophores. The pH also rose when the bladder was poisoned with metabolic inhibitors, indicating that the acid pH of the vesicles was due to an H^+ pump rather than to a Donnan effect.

To test for exocytosis we loaded bladders with FITC-Dextran in the absence of CO_2. With the bladders mounted in Ussing chambers we pumped fluid through the mucosal side and passed the effluent through a fluorimeter. In this set-up we were able to monitor simultaneously the rate of H^+ secretion and fusion of vesicles; the latter would appear as a secretion of fluorescent material. Stimuli to H^+ secretion, such as an increase in ambient P_{CO_2} or an increase in urine pH, caused fusion of these vesicles with the luminal membrane. The time course of this fusion was identical to that of the change in the H^+ current. Hence this fusion resulted in an insertion of H^+ pumps into the luminal membrane and consequently an increase in transepithelial H^+ transport. Both the release of FITC-Dextran and the rise in current were inhibited by pre-treatment with colchicine, an inhibitor of exocytosis in many cells.

The results show that CO_2 stimulates exocytosis (Gluck *et al.* 1982) and inhibits endocytosis (Reeves *et al.* 1983). Hence, the membrane must expand; recent morphometric and electrical studies suggest that this is indeed the case. Whether this process is the same as that of membrane recycling or more similar to exocytosis in the nerve terminal remains to be investigated. There is increasing evidence for a role of endocytosis and exocytosis in the regulation of transport across epithelia, suggesting that such a process may act as a fundamental mechanism of cellular regulation of transport of a variety of solutes yet to be discovered.

THE H^+ PUMP IS A REVERSIBLE ATPase

There are a variety of proton pumps in nature. These include H^+-ATPases, redox pumps and light-driven proton pumps. To distinguish among these types we imposed electrochemical gradients across the membrane in bladders depleted of ATP by metabolic poisons. Gradients larger than the reversal potential produced net ATP synthesis which was blocked by dicyclohexyl carbodiimide and proton ionophores (Dixon & Al-Awqati, 1979). These results constituted strong evidence for a reversible proton-translocating ATPase as the agent which mediates urinary acidification. It should be noted that the use of inhibitors of ATPases in epithelia to probe the mechanism of acidification is fraught with ambiguity since such agents may inhibit H^+ secretion by other mechanisms, such as depleting cellular energy stores or inhibiting fusion.

To confirm the finding of a proton-translocating ATPase we fractionated the turtle bladder on sucrose density gradients and obtained a plasma membrane fraction that contained an H^+ pump (Gluck, Kelly & Al-Awqati, 1982). Two assays were used f

[illegible]s process, H^+ transport in vesicles and a DCCD-sensitive ATPase activity. DCCD, of course, inhibits many ATPases, but the assay conditions were fixed in such a way as to nullify the contribution of the oligomycin-sensitive mitochondrial ATPase, the Na^+,K^+-ATPase and the Ca^{2+}-ATPase. We found that a plasma membrane fraction of the turtle bladder transported protons on external addition of ATP. This was measured as the uptake of the fluorescent dye, acridine orange, assayed in a dual wavelength spectrophotometer. This weak base is a convenient probe for the analysis of H^+ transport in vesicles. Its protonated form is much less permeable than the free base. The dye readily forms stacks when present in concentrations greater than 10 μM and the absorption and emission spectra of the chromophore are consequently shifted. With the spectrophotometer tuned to the absorption of the monomer, a useful semiquantitative assay for H^+ secretion can be obtained.

Using this assay we found that the turtle bladder plasma membrane vesicles contained an electrogenic proton-translocating ATPase (Gluck *et al.* 1982). Addition of ATP caused acidification of the interior of the vesicles which was seen as uptake of acridine orange. This was completely reversed by nigericin, the addition of a $K^+:H^+$ exchanger. The initial rate of uptake in sodium-containing media was indistinguishable from that in potassium-containing media. Hence, this ATPase is different from the gastric H^+,K^+-ATPase.

The rate of acidification was accelerated by the addition of valinomycin in the presence of potassium. This behaviour suggests that during H^+ pumping a membrane potential develops which is positive inside and hence will tend to slow the rate of pumping. Valinomycin, by increasing the potassium conductance, collapses this potential leading to acceleration of the rate of transport. Complementary experiments to this were performed using the ATPase assay, and we found that collapsing the pH and potential gradients enhanced the ATPase activity.

Inhibitors such as oligomycin, that typically inhibit the mitochondrial ATPase, were found to be without effect on this H^+ pump. Vanadate did not reduce the rate of H^+ secretion nor did it affect the ATPase activity.

These responses confirm that the mechanism of acidification by the turtle urinary bladder is by H^+ secretion rather than by HCO_3^- absorption. The most direct test of the nature of the transported ion species would be completely to remove bicarbonate from the medium and to observe the effect on acidification. Studies on intact bladders could not be carried out in the complete absence of CO_2 since that can only occur in non-metabolizing epithelia. The studies in the vesicles show all the characteristics of the epithelial H^+ pump and yet they were all performed in the complete absence of HCO_3^- and CO_2. Hence they provide compelling evidence that urinary acidification is due to a proton-translocating ATPase.

THE NATURE OF THE H^+-ATPase RESPONSIBLE FOR URINARY ACIDIFICATION

There have been a number of proton-translocating ATPases described in procaryotic and eucaryotic cells. They fall roughly into two classes; the F_0-F_1 type and the E_1-E_2 type. Table 1 lists some of the differences between these two types of [illegible]mps. We have recently started to study the proton-translocating ATPase of urinary

Table 1. *Proton translocating ATPases*

	$F_0 - F_1$	$E_1 - E_2$
Location	Mitochondria, bacteria, chloroplast, chromaffin granule, ?lysosome, ?yeast vacuole, ?plant tonoplast	Gastric H^+, K^+-ATPase, ?yeast plasma membrane, ?Ca^{2+}-ATPase
Phosphorylated intermediate	No	Yes
Vanadate sensitivity	No	Yes
Catalytic portion		
Structure	8–10 subunits	1–2 subunits
M_r	40 000–60 000	100 000
Ultrastructure	'Lollipop'	No visible structure

acidification in some detail and our initial conclusions suggest that it is of the F_0-F_1 type (Gluck, Kelly & Al-Awqati, 1983). We isolated a fraction from bovine kidney medulla which is enriched in ATP-dependent, proton-translocating activity. This fraction was depleted in enzymes that serve as traditional markers of mitochondria (succinic dehydrogenase), basolateral membranes (Na^+,K^+-ATPase), and lysosomes (*N*-acetyl glucosaminidase). This proton pump was not inhibited by oligomycin, rutamycin or efrapeptin, agents that inhibit the mitochondrial F_0-F_1 ATPase. However, it was quite sensitive to *N*-ethyl maleimide (NEM), an agent that had no effect on the mitochondrial pump. We were unable to get H^+ pumping with any nucleotide except ATP, whereas the lysosomal H^+ pump reportedly can use ITP and GTP to support transport. The rate of proton transport was unaffected by the presence of potassium nor was it inhibited by vanadate. It was also electrogenic, in that its rate of acidification was enhanced by collapsing the membrane potential with valinomycin. These findings suggest that it is different from the gastric H^+,K^+-ATPase discussed in detail in another chapter of this volume by George Sachs.

We developed an ATPase assay that shows some of the characteristics identified above. In this assay ATP hydrolysis before and after the addition of NEM in media that contained oligomycin, ouabain and EGTA was used as an enzymatic label for the proton pump. Addition of proton ionophores stimulated the ATPase activity, as would be expected from a proton translocating ATPase. Vanadate did not inhibit the ATPase in the absence of pH gradients but dicyclohexyl carbodiimide and NBD-Cl did. The latter two reagents inhibited H^+ pumping in these vesicles (Gluck *et al.* 1983).

Preliminary studies show that ATP protects the enzyme from inhibition by NEM. We have now performed labelling of these vesicles with tritiated NEM before and after ATP protection (Gluck & Al-Awqati, 1983). In the ATP protected vesicles, ^{3}H-NEM labelled eight bands on SDS-PAGE, none of which had a molecular weight of 100 000 Da. This is further evidence that this enzyme is likely to be of the F_0-F_1 type. The labelled peptides differed in molecular weight from those that were obtained from a purified submitochondrial F_0-F_1 ATPase obtained from bovine kidney. This information suggests that this NEM-sensitive ATPase is different from that of lysosomes, mitochondria and gastric vesicles. Since we have found that the turtle bladder ATPase is packaged in endocytic vesicles and since there is growing eviden

[redacted]t a variety of endocytic vesicles have a low pH, we propose that this proton pump which acidifies the urine also acidifies endocytic vesicles. In urinary epithelia these endocytic vesicles have been subverted to function as transepithelial proton pumps by causing them to fuse with only the luminal membrane.

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