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Cell Biology of H^+ Transport in Epithelia

Contents

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Abstract

Cell homeostasis of H^+ ions has been an object of wide interest in the last two decades, which has led to extended knowledge about a considerable number of membrane transport mechanisms responsible for keeping cell pH within physiological limits. Among these mechanisms the most important are Na^+/H^+ exchange, the vacuolar H^+-ATPase, the H^+-K^+-ATPase, Cl^-/HCO_3^- exchange and Na^+/HCO_3^- cotransport. The present review covers both cellular function and molecular aspects of these transporters, starting from a discussion of the methods used for the determination of cell pH and epithelial H^+ transport, and analysing their molecular constitution, cloning and known isoforms, as well as their functional role in the maintenance of cell pH and epithelial transport.

Keywords: pH, H^+ transport, H^+ homeostasis, Na^+/H^+ exchange, H^+-ATPase, H^+-K^+-ATPase, Cl^-/HCO_3^- exchange, Na^+/HCO_3^- cotransport.

Introduction

H^+ ion transport has been described for a large number of structures, both subcellular, such as lysosomes and other vesicular organelles, as well as whole tissues, particularly epithelia such as gastric mucosa and renal tubules. The object of this transport mechanism is the acidification of the interior of organelles allowing for the hydrolysis of protein and other molecules (Brown & Breton, 1996), the acidification of the stomach supporting the digestion of proteins (Hersey & Sachs, 1995), and of the renal tubule lumen, a mechanism to eliminate excess acid from the body (Malnic, 1989).

The production of H^+ ions in the animal organism is tightly bound to the production of CO_2 by cell metabolism. In aqueous medium, CO_2 reacts with water forming carbonic acid:

$$CO_2 + H_2O \Leftrightarrow H_2CO_3 \Leftrightarrow H^+ + HCO_3^- \quad (1)$$

$$H_2O \Leftrightarrow H^+ + OH^- \qquad OH^- + CO_2 \Leftrightarrow HCO_3^- \quad (2)$$

These reactions represent equivalent and alternative forms of the interaction of CO_2 with water to form H^+ ions.

The reactions involving CO_2 are catalysed by the zinc-containing enzyme carbonic anhydrase, which accelerates these steps by about 10,000 times. This enzyme exists in many epithelial cells, such as HCl secreting cells from the gastric epithelium (oxyntic cells) and renal epithelial cells, mainly from proximal tubule

and in intercalated cells from collecting duct. In these cells, the enzyme is present in the cytoplasm and in the cell membranes. In proximal tubule, high concentrations have been detected in brush border membranes (Loennerholm & Ridderstrale, 1980). On the other hand, in distal convoluted tubule cells and in collecting duct principal cells the enzyme is present almost exclusively on the basolateral membrane (see Figure 1). This distribution has been taken to indicate that oxyntic cells in gastric mucosa, and proximal and intercalated cells in the kidney, are important sites for epithelial H^+ ion generation and transport.

The physiological and pharmacological properties of carbonic anhydrase have been studied extensively by several authors, among them particularly by Maren, Wistrand and collaborators (Maren, 1967; Wistrand & Knuuttila, 1989). A series of sulfonamide inhibitors of carbonic anhydrase have been develloped, the most known of them being acetazolamide (Diamox ®).

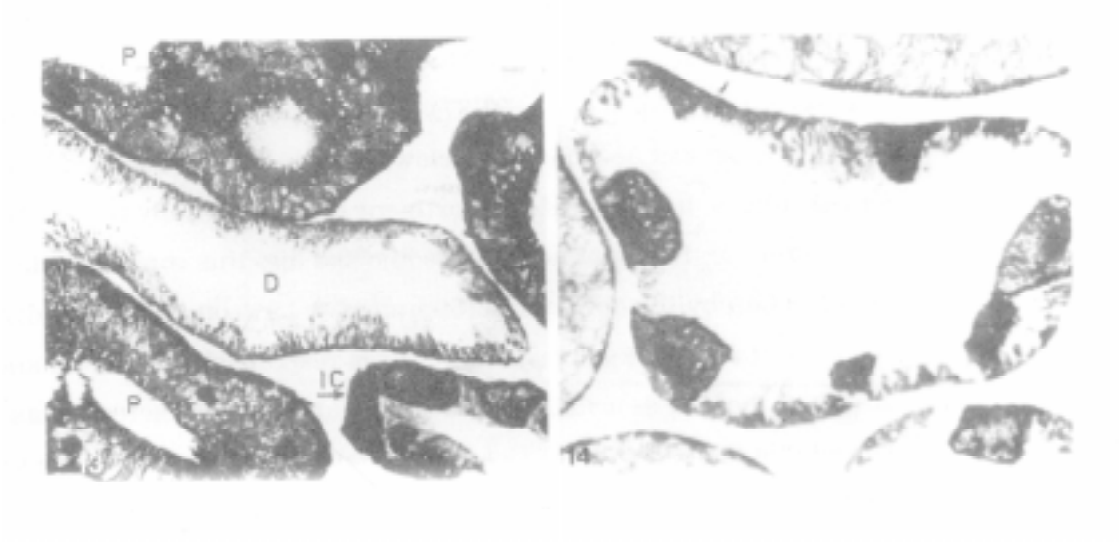

Figure 1. Histochemical localization of carbonic anhydrase in kidney cortex. Black areas correspond to Cobalt sulfide precipitate due to carbonic anhydrase. P, proximal tubule; D, distal convolute tubule; at center of picture at right, cortical collecting duct with intercalated cells (dark) and principal cells (only basolateral staining). From Loennerholm & Ridderstrale (1980).

In recent years, several isoforms of carbonic anhydrase have been cloned, and their distribution analysed by immunocytochemical methods. In kidney, it has been found that the isoform CA II is mostly cytoplasmatic, while the isoform CA IV is found in cell membranes. In red blood cells, on the other hand, isoforms CA I and II are found in cytoplasm, while CA IV is attached to the external membrane surface (Henry, 1996; Bastani & Gluck, 1996). When carbonic anhydrase (CA II) is lacking in the mammal, such as in natural occurrence of the genetic defficiency of the

corresponding gene, leading to a form of renal tubular acidosis, or in gene knock-out mice, marked alterations in urinary acidification are found (Brechue et al., 1991; Schwartz et al., 1993; Brion et al., 1997). When there is a defficiency of one isoform, the expression of others may increase, as has been observed in CA II knock-out mice, where an increase in CA-IV carbonic anhydrase was observed (Brion et al., 1997).

Methods for the Study of H^+ Cell Biology

There has been a number of classic methods for the study of acidification by epithelia. In gastric mucosa and in toad and turtle bladder, one of the most widely used methods was the use of an Ussing chamber, where the epithelium separates two chambers, the mucosal and the serosal compartments (Debellis et al., 1994). The flux of H^+ may be measured electrically, which is the case in turtle bladder, where H^+ transport occurs via a H^+-ATPase by an electrogenic mechanism. When Na^+ transport is eliminated, e.g. by amiloride, which blocks apical Na^+ channels, or by ouabain, an inhibitor of the basolateral Na^+/K^+ ATPase, the only remaining current is that of H^+, which can be measured by the short circuit current technique (Green et al., 1970; Al-Awqati et al., 1977). In gastric mucosa, where H^+ transport is not electrogenic, as will be explained below, H^+ fluxes can be determined by the pH-stat method, where the mucosal compartment is automatically titrated so as to keep its pH constant, and the amount of base injected into this compartment is a measure of acid secretion by the epithelium (Steinmetz & Lawson, 1971; Sanders et al., 1973).

In the kidney, an organ that is responsible for the balance of fixed acid (as against the volatile acid CO2) of the body, overall acid excretion has been measured determining the excretion of bicarbonate and titratable acid by the urine. Clearance techniques have been used for many years, from the time they have been introduced and applied to acid-base function of the kidney by Homer Smith (Smith et al., 1945), Robert Pitts (Pitts, 1948) and others. Using this method, Pitts was the first to demonstrate that the reabsorption of base (HCO_3^-, HPO_4^{2-}) was unable to explain urinary acidification, since in an experiment in dog undergoing phosphate infusion, more acid phosphate was excreted than filtered, implying that part of the filtered alkaline phosphate had to be coverted to acid phosphate by addition of H^+ ions to the urine, that is, by H^+ ion secretion and not only by the reabsorption of alkaline phosphate:

$$Na_2HPO_4 \text{ (filtered)} + H^+ \text{ (secreted)} \Rightarrow NaH_2PO_4 \text{ (excreted)} + Na^+ \text{ (reabsorbed)}$$

An additional method that has been used to evaluate H^+ ion secretion, particularly in the terminal nephron segments, such as the collecting duct, is the determination of PCO_2 in urine of bicarbonate loaded subjects. CO_2 will be formed from secreted H^+ and filtered HCO_3^- in the lumen, and is therefore an approximate way to evaluate the rate of collecting duct H^+ secretion. In this way, it has been possible to detect the inability of distal nephron H^+ secretion in several forms of distal renal tubular acidosis (DuBose Jr. & Caflisch, 1985), and to show that mineralocorticoids stimulate distal nephron acidification (see Fig 2) (Damasco et al., 1989).

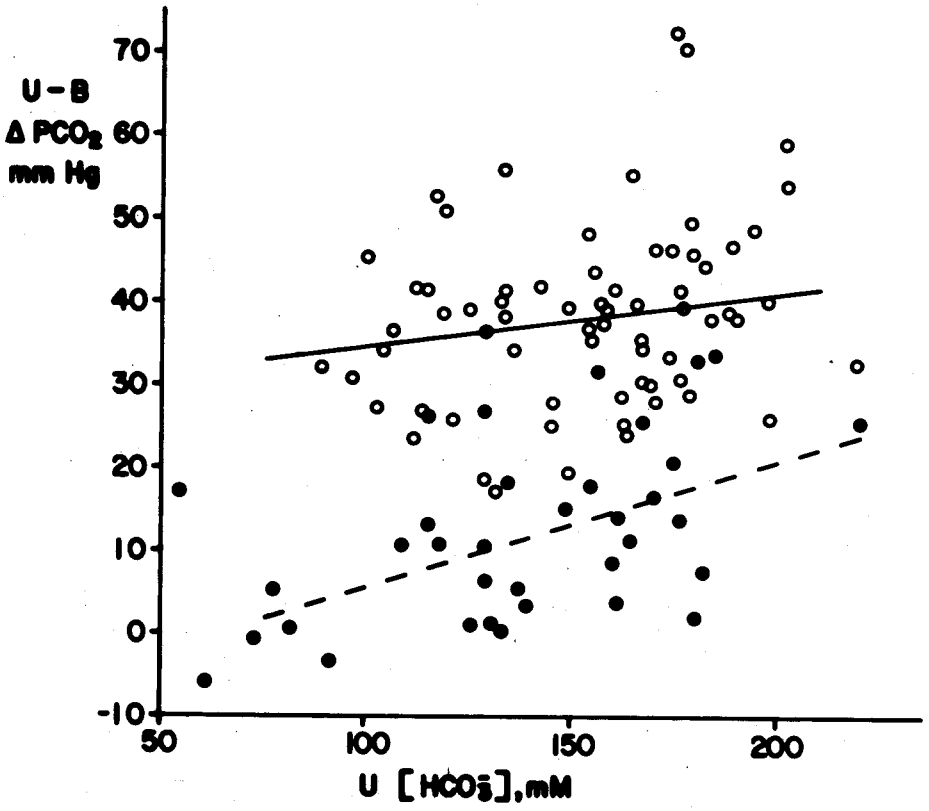

Figure 2. Urine minus blood (U-B) PCO_2 differences at increasing urine bicarbonate concentrations. Open circles, control rats. Black dots, adrenalectomized rats, in which H^+ secretion into urine is decreased. From Damasco et al. (1989).

Micropuncture and microperfusion techniques have contributed extensively to localize the most important sites of H^+ ion secretion along the nephron, and to elucidate the mechanisms of these processes in different nephron segments. Micropuncture experiments have demonstrated, using quinhydrone microelectrodes, that bicarbonate concentrations started to fall in the most early segments of the proximal tubule (Gottschalk et al., 1960), and that tubule pH as measured by antimony microelectrodes fell below plasma pH along the proximal tubule, reaching a level of 6.8 by the end of this segment, and of 6.5 in cortical distal tubule (Vieira & Malnic, 1968).

A stationary microperfusion technique has been used to determine the kinetics of renal tubule bicarbonate transport (Malnic & Mello-Aires, 1971). This technique is based on continuous determination of tubule pH after injection of a bicarbonate containing solution into the tubule lumen, blocking the injected fluid column by oil. Figure 3 shows that after this injection, bicarbonate is reabsorbed, and the rate of this secretion may be calculated evaluating the rate of reduction of bicarbonate concentration with time. That bicarbonate reabsorption is mediated by H^+ secretion was demonstrated by finding a so-called "disequilibrium pH" in proximal and distal tubule lumen, that is, the pH measured by means of microelectrodes (glass or antimony) in the lumen is lower than that measured at equilibrium in the same fluid sample outside the tubule, which indicates sustained secretion of H^+ into a bicarbonate containing solution, causing the maintenance of continuously elevated H_2CO_3 concentrations in the tubule lumen (Rector et al., 1965; Vieira & Malnic, 1968). By these techniques, it has been possible to study properties of bicarbonate reabsorption in proximal tubule, including the effect of peptide hormones (Nascimento-Gomes et al., 1995) and the signalling pathways involved in its regulation (Rebouças & Malnic, 1996), and also the mechanisms and regulation of bicarbonate transport in cortical distal tubule (Fernandez et al., 1994; Barreto-Chaves & Mello-Aires, 1996).

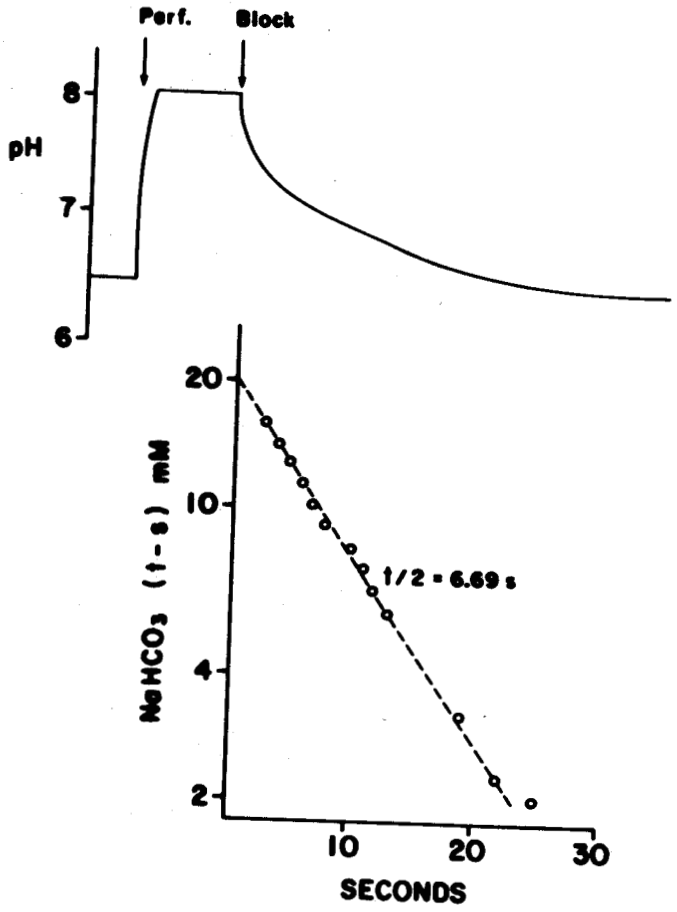

Figure 3. Schematic representation of the stopped-flow microperfusion method for the determination of renal tubule bicarbonate reabsorption. Upper graph, pH measured by H^+-ion sensitive resin microelectrode during perfusion with alkaline, bicarbonate containing fluid (perf) and after blocking with oil. Lower graph, calculated bicarbonate concentrations falling toward their stationary (s) level (t, concentration at time t), indicating half-time of acidification (t/2). Adapted from Fernandez et al. (1994).

A widely used method for the analysis of renal tubule bicarbonate reabsorption, its mechanisms and regulation, has been the picapnotherm technique, which involves injection of a fluid sample collected from the tubule lumen into a chamber in which bicarbonate is transformed into CO_2 by reaction with strong acid, and transfer of this CO_2 by flow of an inert gas such as freon into a second chamber containing a LiOH crystal. The reaction of CO_2 with the crystal forming Li_2CO_3 is exothermic, and the amount of heat liberated is measured with miniature thermistors, being proportional to the amount of CO_2 present in the sample (Vurek et al., 1975). A large number of investigations was performed using this technique (Burg & Green, 1977; Good et al., 1984; Bidet et al., 1992; Levine et al., 1994).

Membrane vesicles: a powerful method for the study of H^+ transporters of cell membranes has been the use of membrane vesicles "in vitro". When a relatively homogeneous tissue is ground, such as kidney cortex, small fragments of cell membranes are produced. These cell membranes will spontaneously form vesicles that may be separated into apical (e.g. brush-border) or basolateral membrane vesicles by differential centrifugation or by electrophoresis (Murer et al., 1976; Kinsella & Aronson, 1980). They may be loaded, by preincubation with corresponding solutions, with specified ion concentrations and pH, and the effect of ion gradients on uptake of isotopic ions may be measured. In this way, e.g. Na^+/H^+ exchange has been detected by analysing the relation between pH gradients across the vesicle membrane and vesicle uptake of $^{22}Na^+$, which was accelerated when the pH inside the membrane was lower than outside, that is, when an inside-out directed H^+ gradient facilitated the movement of Na^+ in the opposite (outside-in) direction.

Determination of Cell pH: In recent years, the measurement of cell pH has contributed importantly toward the knowledge of the mechanisms that regulate the extrusion of acid or the uptake of base by the cell. Several methods have been used for this purpose over the years. The weak acid DMO (dimethyl-oxazolidine-dione) was used based on the permeability of the cell membranes to its non-dissociated form:

$$DMO\text{-}H \Leftarrow\Rightarrow DMO^- + H^+$$

When added to a tissue "in vitro" or injected intravenously, the concentration of the non-dissociated form of DMO will be equal in extracellular and intracellular fluid due to the permeability of cell membranes to this molecule. When the total concentration of DMO in both fluids is measured, as well as the extracellular pH, it is possible to calculate intracellular pH by the Henderson-Hasselbalch equation applied to this buffer (Boron & Roos, 1976).

The DMO method permits to evaluate mean, overall pH of a large number of cells over relatively long periods of time, since it depends on the analysis (chemical or isotopic) of the tissue being studied. More localized and high frequency pH determinations are not possible by this method. For this purpose, microelectrode determinations have been used. Several models have been used: glass microelectrodes, having tips made out of pH-sensitive glass, which may be soldered into non-sensitive glass (Hinke-type, see Figure 4), or may be included into non-sensitive glass microelectrodes (Thomas-type, recessed tip). Other microelectrodes, made out of materials such as antimony or quinhydrone, have also been used (Lopes et al., 1981; Roos & Boron, 1981). These electrodes have tips of 1 μm diameter or more, and may be used to impale relatively large cells such as those of invertebrates. They are difficult to use in mammalian cells, in general of smaller size, which therefore may be damaged by impalement. On the other hand, microelectrodes allow for continuous determination of cell pH, which is important for studies of physiological aspects of cell pH regulation.

Fluorescence microscopy: in recent years, the determination of cell pH by fluorescence microscopy has been adopted widely due to its relative ease of performance. The cells or tissue (e.g., isolated tubules or cultivated cells in suspension or as confluent layers) are incubated with pH indicators derived from fluorescein, e.g. the fluorescent probe 2',7'–biscarboxyethyl 5,6-carboxyfluorescein

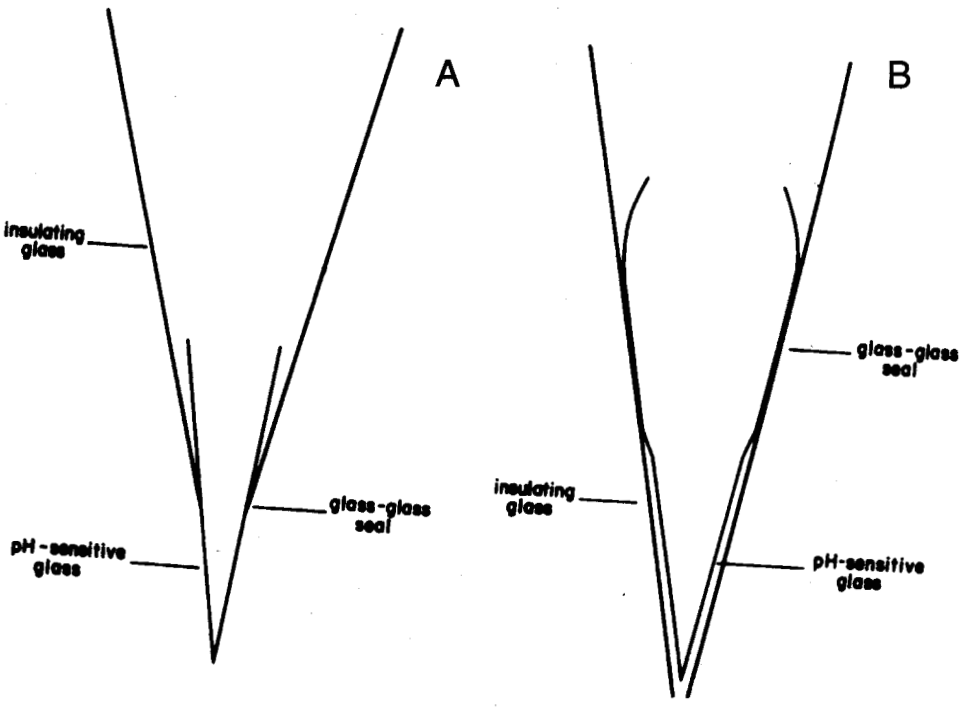

Figure 4. Representation of two types of pH glass microelectrodes. A, Hinke microelectrode, where the pH glass tip is protruding from the insulating glass shaft. B, Thomas microelectrode, where the pH glass tip is soldered into an insulating glass microelectrode ("recessed tip microelectrode") (Meech & Thomas, 1987).

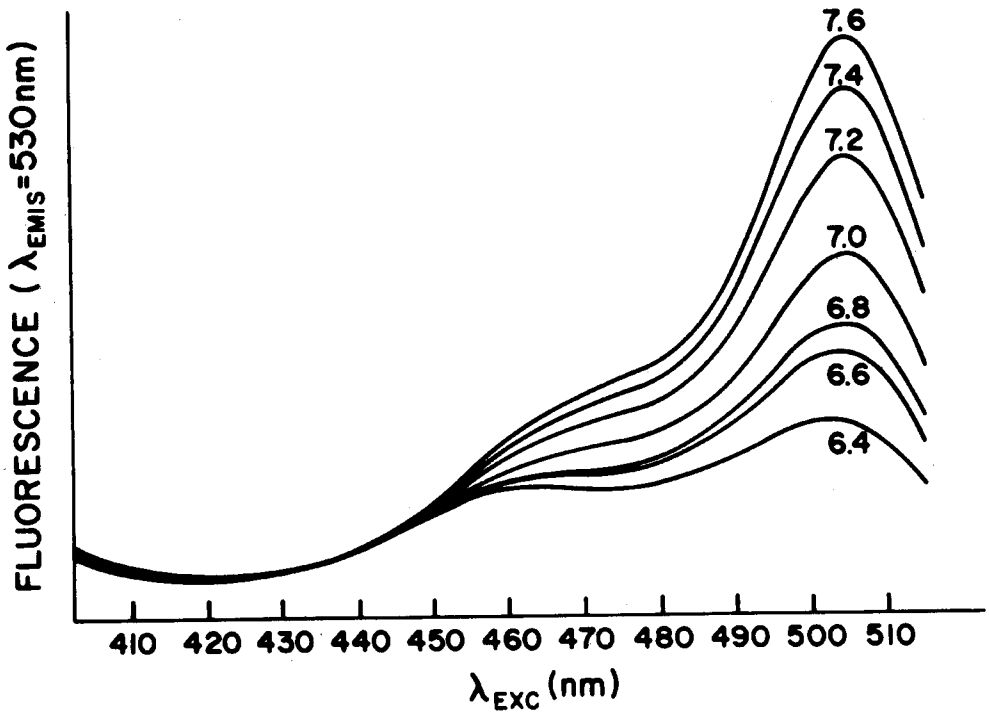

Figure 5. Fluorescence excitation spectrum of BCECF showing pH sensitive part of the spectrum (excitation at 505 nm, emission measured at 530 nm) and isosbestic point at 440 nm. From Bright et al. (1989).

(BCECF), which presents a fluorescence that is dependent on the pH of the medium. Fig. 5 shows the excitation spectrum of this probe, which has a pH-sensitive peak at 495-505 nm, at which the fluorescence increases with the magnitude of the pH of the medium. At this excitation wavelength, light at 530 nm is emitted at an intensity proportional to the pH of the tissue or cell. With excitation at 440 nm, on the other hand, as shown in the Figure, the fluorescence intensity is not proportional to pH. This is called the isosbestic point, and the ratio of intensities at 495 to 440 nm, I_{495}/I_{440}, is used as a measure of pH, which has the advantage of correcting for the loss of probe, which occurs normally during the experiments, since the intensity at the isosbestic point depends only on the amount of probe present (Bright et al., 1987; Rosenberg et al., 1991).

A large number of fluorescent probes has been used for the measurement of different intracellular ion species, such as Na^+, K^+, and Ca^{2+} (Tsien, 1988; Gillis & Gailly, 1994) and also of membrane potential difference (PD) (Miller et al., 1996; Hüser et al., 1996). In order to load cells with a fluorescent probe, several methods have been used. The probe may be injected into the cell by micropipettes (if the cells are large) or by microelectrodes via iontophoresis. But the method that is most widely used depends on the use of molecules made permeable to the cell membrane by using an ester form, such as acetyl-methoxy BCECF (BCECF-AM). This molecule penetrates easily into cells, and there is transformed by cellular esterases back into the ionic form, $BCECF^-$. The cell membrane is only slowly permeable to this

form, so that it is kept inside the cells for prolonged periods of time. In order to calibrate the cell pH measured by this procedure, the method of Thomas (Thomas et al., 1979) is used, which is based on the use of nigericin, a K^+/H^+ ionophore, which equilibrates intra and extracellular pH, so that the intracellular pH will be equal to that of the extracellular fluid with which the cells are incubated. Thus, equilibrating the cells or tissue being used in an experiment with several buffer solutions at known pH, a standard curve can be obtained for the calibration of cell pH.

Molecular Biology of Membrane Transporters: In the last two decades, large progress has been obtained concerning the structure of membrane transporters related to H^+ ion transport, which has been achieved by cloning of these molecules, determination of their mRNA expression by Northern blot and other techniques, and by measurement of their protein abundance by Western blot. The devellopment of molecular biology techniques in these years has been enormous, and we will touch only lightly onto this area. These studies have led to the determination of the primary (amino-acid sequence) and secondary structure of a large number of membrane transporters. The latter involves the investigation of their two dimensional disposition, particularly by hydropathy analysis, based on the determination of the hydrophilic and hydrophobic properties of the amino-acids of the primary structure, and leading to proposals of the insertion of these molecules into the cell membranes, as shown in Figure 8 below (Stevens & Forgac, 1997). By these techniques it has also been possible to produce artificial mutations, i.e., to clone molecules in which certain amino-acid segments were deleted or others were introduced, leading to knowledge about their specific role in the function of these transporters, e.g. determining which phosphorylation sites interact with protein kinase A or C, and which sites interact with cellular Ca^{++} or calmodulin, and in this way establishing the cellular signalling path for different physiological effects or hormone action (Wakabayashi et al., 1994; Wakabayashi et al., 1997).

Another method that introduced important advances to the knowledge of the role and functional importance of membrane transporters is the gene knock-out technique, by which mice defficient of the expression of specific transporters can be raised. It has been frequently observed that these animals survive reasonably well after the deletion of a gene that might be thought of as essential for life, which has been shown to be due to compensatory mechanisms such as the expression of other isoforms of the molecule or the taking over of the function of one renal tubule segment by other downstream segments or other parallel mechanisms. Nevertheless, important knowledge concerning the role of these molecules was obtained by this means (Nakamura et al., 1999; Spicer et al., 2000). This technique has, in addition, increased the interest in the renal physiology of the mouse (Meneton et al., 2000).

H^+ Transporters in Cell Membranes

Na^+ /H^+ Exchangers: H^+ transporters in cell membraneshese are the most ubiquitous of H^+ transporters, found in most mammalian cells as well as in a large range of cells in general. Figure 6 shows how this transporter may be detected. Cells are acid-loaded by means of superfusion with NH_4Cl containing Ringer's solution; this solution contains a low concentration of NH_3, a gas, that is lipossoluble and therefore penetrates easily into the cell across the lipid bilayer membrane. Inside the cell the following reaction occurs:

$$NH_3 + H^+ \Leftrightarrow NH_4^+$$

The reaction of NH_3 with cellular H^+ ions alkalinizes the cell, leading to the abrupt alkalinization seen in the Figure. However, when thereafter NH_4Cl Ringer is substituted by normal Ringer solution, NH_3 leaves the cell and the above reaction is reverted, leaving H^+ ions inside the cell, thereby causing its acidification. Following the acid pulse, the cell recovers its normal pH by extruding acid via H^+ ion transporters in its cell membrane. This extrusion is markedly slowed when the cells are superfused with solution devoid of Na^+, indicating that the presence of external Na^+ is essential for H^+ transport out of the cell (Fernández & Malnic, 1998). In MDCK cells, a cell line derived from a dog renal tumor, in the absence of external Na^+ other mechanisms, which will be discussed below, still remain able to extrude H^+, although at a smaller rate than Na^+ dependent H^+ extrusion, that is, Na^+/H^+ exchange.

Na^+/H^+ exchange has been first conclusively demonstrated in renal brush-border membrane vesicles by Kinsella and Aronson (Kinsella & Aronson, 1980; Kinsella

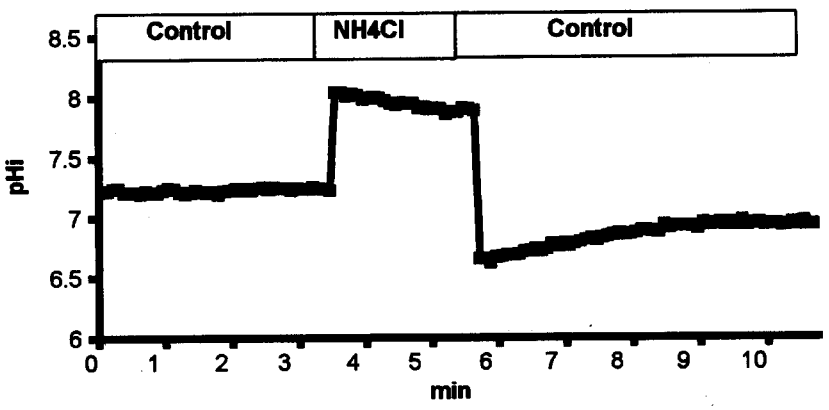

Figure 6. Cell pH in T84 colon cancer cells. NH_4^+, superfusion with NH_4Cl Ringer, followed by normal Ringer causing cell acidification, after which Na^+ dependent pH recovery is observed. From Ramirez et al. (1999).

& Aronson, 1982). These authors showed that, in the presence of a pH gradient across the vesicle membrane, with H^+ ion concentration larger inside the vesicle than outside, uptake of $^{22}Na^+$ was markedly accellerated, suggesting the presence of an exchange mechanism, that is, Na^+ uptake driven by H^+ efflux from the vesicle. This transport was not dependent on metabolic energy, since inside the vesicles no ATP producing mechanisms were present, i.e., the vesicle is a preparation consisting purely of the cell membrane, with artificial media inside and outside. Since in a living cell a Na^+ gradient is always present due to the activity of the basolateral Na^+/K^+ ATPase, which extrudes Na^+ from the cell, this gradient will always be available for extrusion of H^+ produced by cell metabolism. Since the extrusion of H^+ is indirectly dependent on the Na^+/K^+ ATPase, which is a primary active transport mechanism, H^+ extrusion is often referred to as a secondary active transport mechanism . It was also demonstrated that Na^+/H^+ exchange was an electroneutral process, since, when an electrical potential difference (PD) was created across the vesicle membrane by establishing a K^+ gradient in the presence of valinomycin, which is a K^+ ionophore, across this membrane, no effect on the exchange rate was observed, as opposed to what is observed for the Na^+/glucose cotransporter, which carries a positive charge (see Figure 7). This behavior shows that Na^+/H^+ exchange is a 1:1, electroneutral mechanism (Kinsella & Aronson, 1982).

Several isoforms of the Na^+/H^+ exchanger have been cloned. The most important are NHE1, NHE2, NHE3 and NHE4. Basically, these molecules have 12

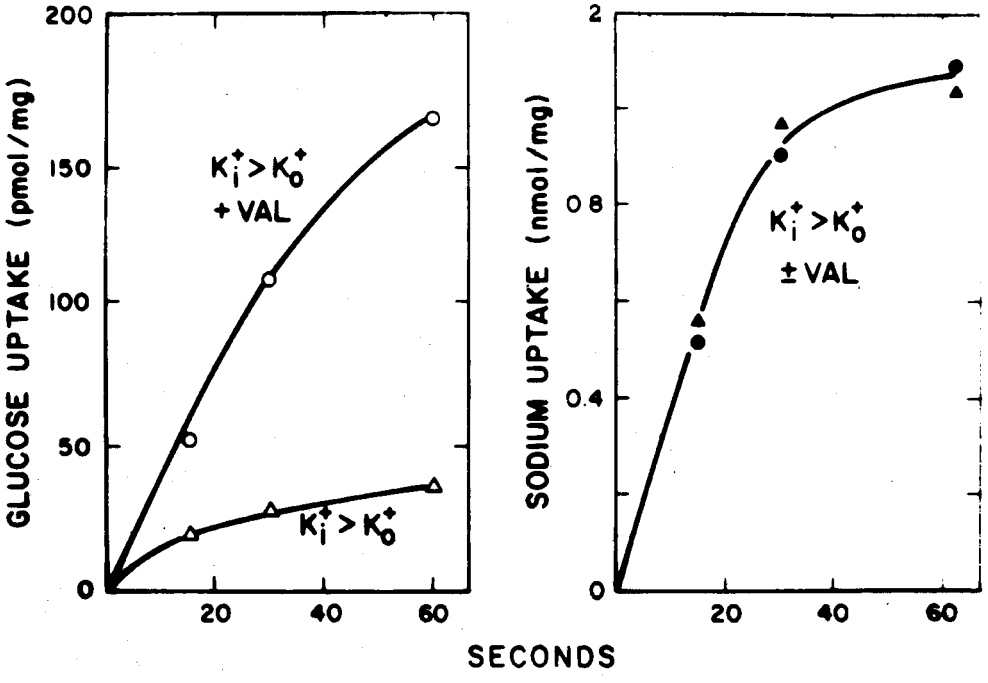

Figure 7. Comparison of Na^+ gradient dependent, electrogenic, glucose uptake into renal proximal tubule brush-border membrane vesicles (stimulated by potassium gradient, valinomycin dependent PD) with H^+ gradient dependent but electroneutral ^{22}Na uptake. From Kinsella & Aronson (1982).

transmembrane loops, and a long cytoplasmic C-terminal end (see Figure 8). The isoform NHE1 is the most ubiquitous, being found on the basolateral cell membrane of most cells, where it is responsible for H^+ extrusion and the maintenance of cell pH homeostasis, reason why it is known as the "housekeeping" Na^+/H^+ exchanger. This exchanger has a high sensitivity to amiloride, a diuretic drug that was initially known to block Na^+ channels in tight epithelia. NHE2 and NHE3 are mostly apical transporters, present in epithelia such as renal tubules and intestinal mucosa, responsible mostly for transepithelial H^+ secretion and Na^+ reabsorption; these transporters have a low sensitivity for amiloride and its analogs such as hexamethylene-amiloride and ethyl-isopropyl-amiloride (Kleyman & Cragoe, 1988). In rabbit ileal brush-border membrane, both NHE2 and NHE3 were detected, and their activity evaluated by treatment with HOE 694, another amiloride analog, which inhibits only NHE3 at low doses. In control conditions, both isoforms are responsible for approximately 50% of Na^+/H^+ exchange, but only NHE3 is stimulated approximately 4 x by glucocorticoids. In this preparation, the exchangers are responsible for electroneutral NaCl absorption by working in parallel with the Cl^-/HCO_3^- exchanger (Wormmeester et al., 1998).

Several studies have shown how this exchanger is regulated. An important mechanism determining the rate of exchange is the pH inside the cell

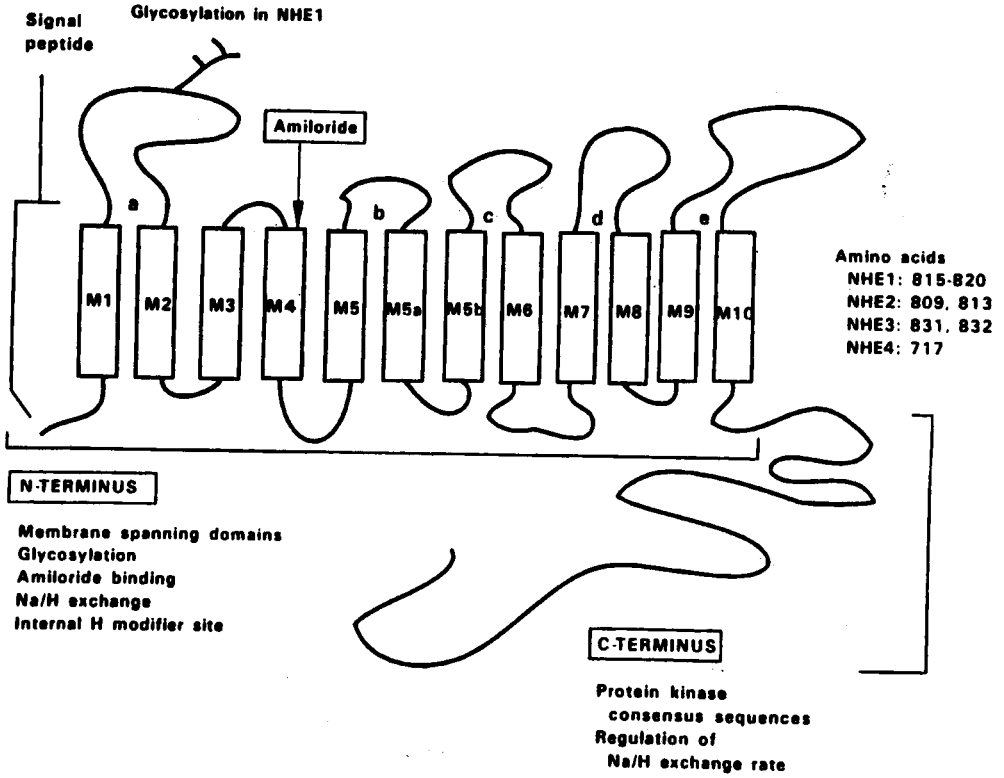

Figure 8. Schematic drawing of secondary structure of Na^+/H^+ exchanger with 10 hydrophobic membrane spanning domains (M1 to M10)and regulatory cytoplasmic C-terminus.

(Aronson, et al., 1982). It was shown that internal H^+ activates Na^+/H^+ exchange not by acting on the internal transport site, but by interacting with an allosteric site at the cytoplasmic surface of the membrane. The C-terminal, cytoplasmic end of the molecule has several phosphorylation sites, able to interact with Protein kinase A (PKA), an important inactivator of NHE, and with PKC, an activator of this transporter. This terminal is also the site of interaction with Ca^{++}/ calmodulin, also able to activate or to inactivate the molecule, depending on its site of action (Wakabayashi et al., 1994; Wakabayashi et al., 1997).

These exchangers are regulated by a number of hormones. Parathyroid hormone (PTH) is a known inhibitor, acting, e.g. in renal proximal tubule, via basolateral membrane receptors that activate adenylate cyclase, which liberates cAMP into the cytoplasm, causing PKA activation, which in turn phosphorylates inhibitory sites at the cytoplasmic C-terminal of the exchanger . Angiotensin, on the other hand, stimulates the exchanger in renal tubule and MDCK cells in culture, probably via an increase of cytosolic Ca^{++} and PKC activation (Nascimento-Gomes et al., 1995; Barreto-Chaves & Mello-Aires, 1996). Angiotensin was shown to increase cytosolic Ca^{++} levels, an effect that was opposed by atrial natriuretic peptide, which prevents this increase in Ca^{++} levels. It was also shown that arginine vasopressin (AVP) has a similar effect when applied to the luminal surface, acting via V1 receptors (Barreto-Chaves & De Mello-Aires, 1997), while basolateral AVP acts on V2 receptors that liberate cAMP and inhibit Na^+/H^+ exchange. Recently, it was shown that PKA acts on the exchanger via a regulating protein, NHE-RF, that inhibits basal rabbit renal brush-border (BBM) Na/H exchange activity. The amino acid sequences in the polypeptide containing only the NH2-terminal of NHE-RF have no intrinsic activity as an inhibitor but appear to be required for the full-length NHE-RF to express its full inhibitory effect on the BBM Na/H exchanger. One or more of the serine residues of the C-terminal segment represent the critical PKA phosphorylation site(s) on the NHE-RF protein that mediates the physiologic effect of cAMP on the renal BBM Na/H exchanger (Weinman et al., 1998; Zizak et al., 1999).

The role of the Na^+/H^+ exchanger has been evidenced by the study of "knock-out" mice. When the gene for the expression of NHE3 is deleted, in mutant homozygous knock-out mice a slight metabolic acidosis is observed, together with moderate diarrhea and hypotension. In these mice proximal bicarbonate reabsorption is markedly reduced, as well as reabsorption of intestinal fluids, which depend to a large part on the apical NHE3 exchanger. In spite of these alterations, the animals survive with only minor general alterations. This has been shown to be due to compensatory mechanisms, particularly due to increased expression of other transporters such as the Cl^-/HCO_3^- exchanger, the H^+-K^+ ATPase and Na^+ channels of distal nephron and colon (Schultheis et al., 1998).

V-type H^+-ATPase: A widely distributed H^+-ion transporter is the vacuolar (or vesicular) H^+-ATPase, found in a large number of subcellular organelles, including lysosomes, coated vesicles and endo/exocytotic vesicles, where its function is the acidification of the vesicular interior, which causes hydrolysis of proteins included in these vesicles. These ATPases (V-ATPases) are part of the general family of ATPases, composed of three sub-families: besides the V-ATPases, the P-type-ATPases, including the Na^+/K^+-ATPase and the Ca^{++} ATPases, which form a phosphorylated intermediate during their catalytic cycle, and use ATP for the transfer of ions across membranes, and the F-type-ATPases, from mitochondrial, chloroplast and bacterial membranes, which have the function of ATP synthesis. As opposed to P and F-type ATPases, V-ATPases are insensitive to vanadate and oligomycin, and inhibited by n-ethylmaleimide, bafilomycin and concanamycin. (Forgac, 1989; Myers & Forgac, 1993).

Structure of H^+-ATPase: Figure 9 gives a schematic view of the H^+-ATPase molecule. These molecules are composed of two functional domains, a catalytic, cytoplasmic and nucleotide binding domain (V_1) and an integral domain (V_o), which crosses the lipid bilayer membrane into which the transporter is inserted. The V_1 domain, in turn, is composed of 3 A (73 kD each) subunits and 3 B (58 kD) subunits. The A subunits are the moieties which interact with ATP, liberating the energy for transport, and the B subunits have also nucleotide interaction sites. This domain

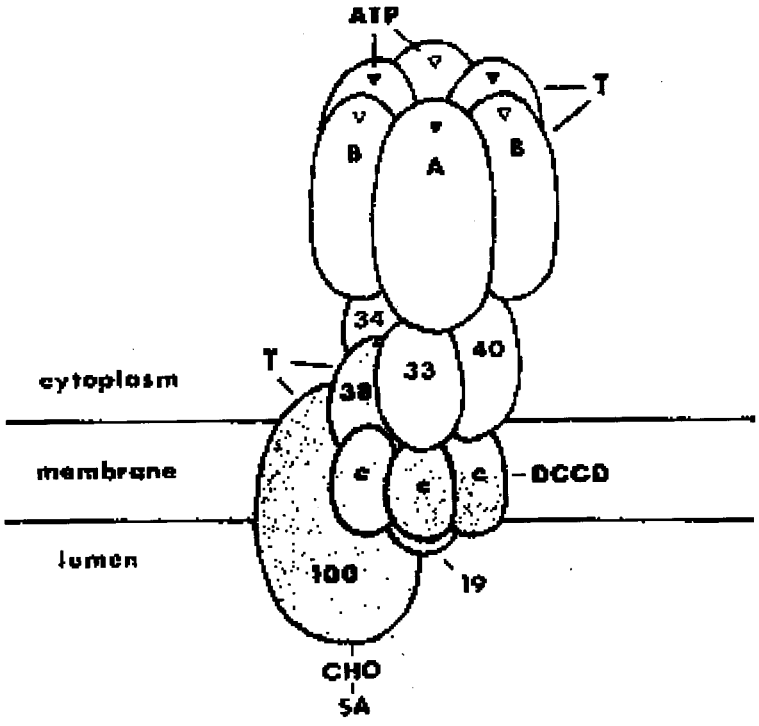

Figure 9. Schematic representation of V-type H^+-ATPase structure with a catalytic, cytoplasmic domain (V1) and a membrane integral domain (Vo) and their respective subunits. From Forgac (1989).

contains also a "stalk" sector, transition to the V_0 domain, composed of smaller subunits (see Fig. 8). The V_0, membrane-integral domain, is composed of six C subunits (of 17 kD) , and a number of other subunits of MW of 100, 38, and 19 kD. The C subunits are the site of binding of DCCD (dicyclo-hexyl-carbodiimide), a potent inhibitor of this ATPase. H^+ ions are thought to be "energized" in the catalytic portion of the molecule, probably reaching elevated free energy in a region within V_1, from there flowing through the V_0 channel across the lipid membrane. The structure of the vacuolar ATPase from bovine brain clathrin-coated vesicles has been determined by electron microscopy of negatively stained, detergent-solubilized enzyme molecules. The two major domains, V_1 and V_0, have overall dimensions of 28 x 14 x 14 nm. The V_1 domain is a more or less spherical molecule with a central cavity. The V_0 domain has the shape of a flattened sphere or doughnut with a radius of about 100 Å. The V_1 and V_0 domains are joined by a 60-Å long and 40-Å wide central stalk, consisting of several individual protein subunits. A large trans- membrane mass, probably the C-terminal domain of the 100-kDa subunit, is seen at the periphery of the c subunit ring in some projections. This large mass has both a lumenal and a cytosolic domain, and it is the cytosolic domain that interacts with the central stalk. Overall, the structure of the V-ATPase is similar to the structure of the related F(1)F(0)-ATP synthase, confirming their common origin (Wilkens et al., 1999). V-ATPases have also been identified to rod-shaped particles e.g. in apical cell membranes of the H^+ secreting, alpha- intercalated cells of turtle urinary bladder. These particles are characterized as "studs", cytoplasmic domains of V- ATPases on thin-section transmission electron microscopy and by intramembrane particles occurring as rod-shaped particles on freeze-fracture electron microscopy (Kohn et al., 1997).

It has been proposed that V-ATPase biosynthesis occurs within the endoplasmic reticulum. Assembly of the V_1 domain begins 5 – 10 min after the starting stimulus, and after 15-20 min this domain attaches to the membrane-bound V_o domain. In general, there is a large number of V_o domains inserted into the endoplasmic reticulum membrane, and a soluble cytosolic pool of assembled V_1 domains is available for connection to the V_o domains yielding the complete ATPase. This scheme involves the notion that the V_o domain alone is not functional as a proton channel, as is the case for the F_o of the mitochondrial membrane, but that only the complete molecule has a catalytic unit plus proton channel function (Myers & Forgac, 1993). The V-ATPase has the function of providing intracellular membrane compartments with an acidic lumen. This function increases along these compartments from the earliest endosomal compartments, where a pH of 7.0 is found, through late endosomes to lysosomes, where a pH of less than 5.0 can be found. A similar progression is found along the pathway constituted by the trans-Golgi to the

Golgi apparatus to lysosomes. The luminal acidification is essential for a number of intracellular processes such as nutrient uptake and secretion of protein molecules. Receptor-mediated endocytosis is one of the processes that may be regulated by vesicle pH. Several protein molecules may be taken up into the cell by surface membrane receptors in clathrin-coated pits, which then form clathrin-coated vesicles. These have H^+-ATPase in their membrane, although the transporter is activated only after loss of the clathrin coat. The lowest pH is found in the last station of the cycle, in lysosomes, with pH 4.5 – 5. The proteins that were taken up (insulin, transferrin, epidermal growth factor and others) are recycled or degraded in this process, including protein hydrolysis, which needs a low pH to be activated (Forgac, 1989).

Besides their presence in subcellular vesicles, in the endocytotic / exocytotic pathway, V-ATPases are found in apical membranes of epithelial tissues such as the renal tubule. In brush border membranes of mammalian proximal renal tubule, these ATPases are responsible for 20-30% of the reabsorption of bicarbonate ions; this has been verified by the inhibition of this moiety of bicarbonate reabsorption by bafilomycin, a specific inhibitor of the V-ATPase (Ulate et al., 1993). In cortical collecting duct, they are found both in a and in b intercalated cells, in the former at the apical membrane, where they constitute the most important distal acidifying mechanism of the urine, and in the latter, where they are inserted at the basolateral membrane transferring H^+ ions from cell to the interstitium, working as part of the mechanism of luminal bicarbonate secretion, in series with the luminal Cl^-/HCO_3^- exchanger. Here, this ATPase is able to produce large pH gradients, with a urine pH of 5.0 or less. This is a much greater value than that produced by the Na^+/H^+ exchanger of proximal tubule, which depends on the Na^+ gradient of the apical cell membrane (of the order of 140 mM, luminal, to 30 mM, cytoplasmic), corresponding to a ratio of 4.7 or log 4.7 = 0.67, that is, to a pH difference of only 0.67 across this membrane. The distal H^+-ATPase, on the other hand, being a primary active mechanism, and using metabolic ATP, is able to produce much larger pH differences.

Regulation of the H^+-ATPase: There are three main mechanisms for the regulation of V-ATPase. One of them involves modification of the activity of the pump by molecular modification. The optimum pH for the function of V-ATPase is of the order of 6.3, that, is, sensibly more acid than normal cell pH, showing that the reduction of cell pH is a factor that activates this molecule. The absence of a certain subunit may modify this activity; it has been shown that the large, 100 kD, transmembrane subunit may be added or deleted. Switching between different isoforms or subunits may also affect activity. In addition, the assembly of V_1 and V_0 may regulate the number of complete molecules available; there may be a number

of V_0 domains inserted into the membrane, which are then activated by the addition of V_1 domains which had been stored in the cytoplasm (Gluck, 1993).

Another regulatory mechanism is the dependence of H^+ secretion on the potential difference (PD) across the membrane into which the V-ATPase is inserted. Since its transport mechanism is electrogenic, H^+ transport into the vesicle lumen will turn its interior more positive, creating an electrochemical potential gradient unfavorable for further secretion. This gradient is normally dissipated by the presence of chloride channels in the same membrane, allowing for a shunt current of Cl^- to dissipate the PD established by the ATPase. It has been shown by immunocytochemistry that one form of these channels (ClC5) colocalizes with H^+-ATPase in renal cells (Sakamoto et al., 1999). ClC5 channels are widely distributed, but appear to be an important component of subcellular vesicles. The role of Cl^- channels in H^+-ATPase activity may, however, be different from a simple electrical shunting mechanism. It has been shown that acidification of microsomes from renal medulla depends on the presence of Cl^- channels, but when the PD across their membrane is dissipated by the presence of potassium in the medium plus valinomycin, a potassium ionophore, microsomal acidification was still dependent on the presence of Cl^- ions. The authors suggested that a more specific mechanism, denominated symport of H^+ and Cl^-, by the H^+-ATPase, may be responsible for these properties (Kaunitz et al., 1985). Since H^+-ATPases also are found in apical membranes of epithelial cells, particularly in the kidney, the role of Cl^- channels in renal tubule acidification was investigated. It was observed that the inhibition of Cl^- channels by blockers such as NPPB (5-nitro-2-(3-phenylpropylamino)-benzoic acid) markedly reduced distal tubule acidification, while there also occurred a small but significant alteration in transepithelial PD (Fernandez et al., 1997). In Madin-Darby Canine Kidney (MDCK) cells, regulation of cell pH depends mostly on Na^+/H^+ exchange, but a significant fraction is mediated by a V-ATPase; this fraction is impaired when Cl^- is deleted from the incubation solution or when NPPB is added, showing again the importance of the presence of Cl^- channels for the activity of the ATPase (Fernández & Malnic, 1998).

An important regulatory mechanism for H^+-ion secretion by epithelia such as the renal tubule and turtle bladder is the fusion of apical vesicles containing V-ATPases in their membrane with the apical membrane of the epithelial cell. This mechanism involves the exocytotic pathway, starting at the Golgi apparatus and following this path to the apical membrane when a stimulus for acidification such as CO_2 is applied. At the apical membrane the vesicles fuse, being now a part of the cell's plasma membrane, in this way incorporating the V-ATPases into the apical membrane and increasing the number of V-ATPases available for H^+ secretion. This

mechanism of regulation has been described for the stimulation of epithelial acidification by CO_2 in turtle bladder (Stetson & Steinmetz, 1983) and renal cortical collecting duct (Schwartz & AlAwqati, 1986). It was also observed in renal tubule cells that were acidified by incubation in sodium-free solutions, a situation that blocks the Na^+/H^+ exchanger (Malnic and Geibel, 2000). Figure 10 shows a segment of rat straight proximal tubule (S3 segment) subjected to this treatment, where the movement of vesicles from the perinuclear region to the subapical region is clearly shown.

H^+-K^+ ATPase: In the early and mid seventies Forte et al and Sachs et al demonstrated that the basic mechanism of gastric acid secretion was a P-type ATPase able to exchange K^+ for H^+ (Ganser et al., 1974; Sachs et al., 1976). However, more

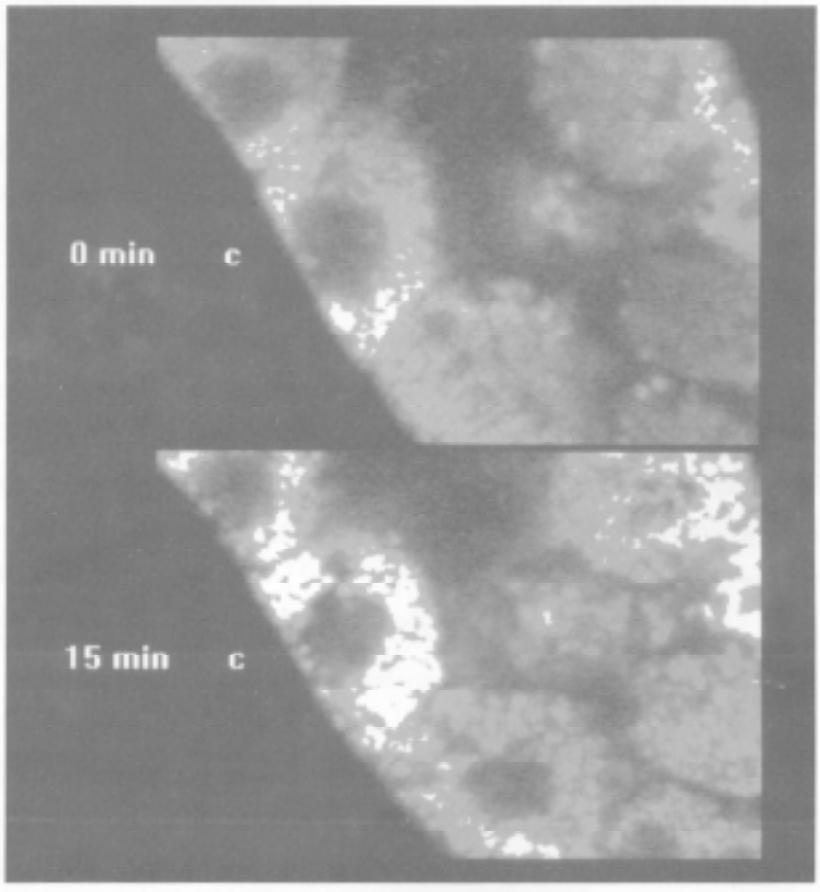

Figure 10. Proximal tubule (S3 segment) of mouse kidney with acridine orange-colored acid vesicles. Image by fluorescence confocal microscopy. Cell acidification by low-Na^+ Ringer superfusion causes vesicle transfer toward the apical membrane (see cell c) in a period of 15 min. G. Malnic & J. Geibel (2000).

recently it was found that H^+-K^+ ATPases were also present in colon and in the kidney. This ATPase is K^+ dependent, but Na^+ independent, and responsible for non-electrogenic K^+/H^+ exchange. In the stomach, it is able to reduce luminal pH to values as low as 1. In kidney and colon, its participation in urine and colon pH is not defined, but certainly minor. Nevertheless, the inhibition of this ATPase has significantly greater effects on urinary bicarbonate reabsorption than the inhibition (by bafilomycin), of the vacuolar H^+-ATPase. In addition, it appears to have considerable importance in the reabsorption of potassium, particularly in conditions of potassium depletion (Wingo & Smolka, 1995).

The H^+-K^+ ATPase, being a P ATPase like Na^+-K^+ ATPase, has a phosphorylated intermediate in its cycle; it is sensitive to vanadate, and has more specific sensitivities to the Schering compound (Sch 28080, an imidazopyridine) and to ouabain, depending on its different isoforms. Different isoforms of this ATPase have been cloned, and its primary and secondary structures are reasonably well known. It has two subunits, *a*, the catalytic, cytoplasmic subunit, with 8 to 10 hydrophobic transmembrane domains, and *b*, a subunit that has one or two transmembrane segments. Therefore, both subunits have membrane-bound components (Wingo & Smolka, 1995). The *a* subunit has several isoforms, one of them gastric and the other colonic. This means that the former has an extensive homology with the isoform initially isolated from stomach, and the other, with that isolated from colon. These isoforms were termed HKa_1 or HK1 (gastric) and HKa_2 or HK2 (colonic) by Wingo & Smolka (1995) and Jaisser & Beggah (1999), respectively. According to Doucet (1997), on the other hand, these isoforms are designated type I (gastric) and III (colonic). A type II, HK2 or HKa_3 isoform was also described, and has been isolated from toad bladder (*Bufo marinus)* and found also in the kidney, in proximal tubule and thick ascending limb. The following properties of these subunits have been described.

HK1 / HKa_1 or Type 1 (gastric): this was the first isoform, described in stomach and kidney, in outer medullary collecting duct (OMCD). This isoform is found in normal potassium balance, and not increased in potassium depletion. It has been located by immunocytochemistry to the intercalated cells, both α and β, of cortical collecting duct, where it colocalized with H^+-ATPase (Bastani, 1995). In inner medullary collecting duct, it has been found also in principal cells (Silver & Soleimani, 1999). Its primary structure shows a sequence of 1033 amino-acids, with approximately 114 kD. Hydropathy analysis has shown the following sequence starting at the NH_2 terminus: a short cytoplasmic segment is followed by four hydrophobic transmembrane α helices, in turn followed by a longer cytoplasmatic hydrophilic loop that contains phosphorylation sites. This loop is followed by four to six additional transmembrane hydrophobic α helices, ending with a hydrophilic cytosolic COOH

terminus. This isoform is stimulated by chronic and acute (10% CO_2) acidosis, is insensitive to ouabain and blocked by Sch 28080 and by omeprazole, a drug used for ulcer therapy. Besides gastric mucosa, this molecule has been detected in renal initial, cortical and outer medullary collecting duct.

HK2, HKa_2 or Type III (colonic): this subunit was originally described in the colon, but is also found in renal thick ascending limb and in initial, cortical and medullary collecting duct. Its activity is practically absent in normal conditions, and markedly increased in potassium depletion in collecting duct, but not in ascending limb. This increase in activity depends on increased expression of the molecule in collecting duct and rat distal colon, as demonstrated by the increase in mRNA for this molecule (Sangan et al., 1997). The HK2 derived from colon was shown to be sensitive to ouabain, but not to Sch 28080. In renal tubules, while HK1 is downregulated in potassium depletion, HK2 is upregulated and acquires Sch 28080 sensitivity (Buffin-Meyer et al., 1997). There are, thus, differences in inhibitor sensitivity depending on physiologic conditions of the studied animals, and also between "in vivo" and "in vitro" conditions.

HK3, HKa_3, Type II: originally detected in toad bladder, is also found in proximal tubule and thick ascending limb of Henle's loop. Its molecular structure has the same general distribution between cytoplasmic and transmembrane domains, and the difference is accounted for by changes in amino-acid residues. This subunit is sensitive to ouabain and also to Sch 28080, although its sensitivity to the latter is markedly lower than that of the gastric isoform.

Additional HK isoforms have been described, but we will not discuss their properties, since the most important isoforms are those described above. All described isoforms, HK1, 2 and 3, have extensive homology. They all belong to the P-ATPase group (like the Na^+-K^+ ATPase), and have highly conserved domains, such as the ATP-binding domain and the FITC binding site. All have 10 transmembrane domains, with their NH_2 and COOH terminus located within the cytoplasm.

The b Subunits: It is generally accepted that the H^+-K^+ ATPase is composed of 2 *a* and 2 *b* subunits (Jaisser & Beggah, 1999). However, it is not clear yet which *b* subunit is connected to which of the different *a* subunits described above. The *b* subunit of gastric H^+-K^+ ATPase has been the only such subunit well characterized up to this time. However, it appears that several other *b* subunits that were initially described as part of the Na^+-K^+ ATPase system may bind to non-gastric *a* subunits. Several *b* subunits of Na^+-K^+ ATPase have been described: *b*1 and *b*2, from glia, and *b*3, from amphibian tissue, but that later was also found in human and rat. Other subunits of this type were also described. It has been recently reported that the

colonic *a* subunit of H^+-K^+ ATPase may associate with the above described *b*1 subunit in kidney and in distal colon (Chow & Forte, 1993; Chow & Forte, 1995).

The Cl^-/HCO_3^- Exchanger: In tissues that secrete H^+ and thereby reabsorb bicarbonate, the following reaction generates these ions within the cell:

$$H_2O + CO_2 \Leftrightarrow H_2CO_3 \Leftrightarrow H^+ + HCO_3^-$$

As H^+ is secreted, HCO_3^- accumulates within the cell, and must be transferred to the interstitium. This may be performed by Cl^-/HCO_3^- exchange or by Na^+/HCO_3^- cotransport at the basolateral cell membrane. This process is widely distributed in renal tubule, where the exchanger is present in the basolateral membrane of proximal S3 segment and in α intercalated cells, while an opposite polarity is found in bicarbonate secreting β intercalated cell. A β cell polarity is also found in bicarbonate secreting epithelia, which are commonly found in intestinal mucosa.

The first Cl^-/HCO_3^- exchanger that was discovered was the Band-3 protein of the red blood cell (RBC), which is essential for CO_2 transport between tissues and lung. This exchanger has been cloned, and several isoforms have been detected in different tissues. The isoform of red cells is called AE1. This isoform is also found in the basolateral membrane of the α intercalated cell of the renal collecting duct. The renal isoform results from alternative splicing of the gene for AE1, resulting in a mRNA truncated at the 5' terminus. Presently, a new model for the topology of AE1 has been proposed, which indicates that the membrane domain has 12 transmembrane spans rather than the 14 of earlier models. The amino-terminal of the molecule is highly polar, intracellular, bound to the cytoskeleton by the protein ankyrin, which binds the AE1 molecule to the membrane as well as to haemoglobin. This part of the molecule is composed of about 400 amino-acids, the site of the lowest homology between isoforms (64 – 69%). On the other hand, the C-terminal is also bound to the membrane, and this part as well as the membrane spanning portion has about 500 amino-acids, and a high homology between isoforms (of the order of 80%). This part is responsible for Cl^-/HCO_3^- exchange. AE1 is found in a large number of tissues besides RBC. The human Band-3 gene has been completely sequenced, and this has facilitated the study of natural Band-3 mutations and their involvement in disease. Such diseases, resulting from AE1 mutations, are, e.g. spherocytosis, ovalocytosis, that is, morphological alterations of red blood cells; and familial distal renal tubule acidosis. Mice with AE-1 knockout present severe anaemia, but may nevertheless survive. The RBC of these animals contained a normally-assembled cytoskeleton, but are unstable, indicating that the normal red cell membrane is

stabilized by AE1 interaction with membrane lipids rather than with the spectrin skeleton (Tanner, 1997).

There are approximately 10^6 molecules of Band-3 in one erythrocyte. This isoform is very sensitive to di-isothiocyanate-stilbene-sulfonic acid (DIDS) (Kopito, 1990). It is responsible for CO_2 transport by RBC, more specifically for the Hamburger or chloride shift, by which bicarbonate, formed with catalysis by carbonic anhydrase inside the RBC, is transferred to blood plasma in exchange for Cl^-. It is also essential for renal bicarbonate reabsorption and bicarbonate secretion by the gut, transferring bicarbonate from cell to interstitium (reabsorption) or from cell to lumen (secretion).

Other isoforms that have been described are: AE2, found in the basolateral membrane of parietal cells of gastric mucosa, in the kidney and in brain, particularly in the choroid plexus. This isoform is probably located more in subcellular organelles than in plasma membrane. The isoform AE3 was found in brain cells, but is also present in kidney. AE1, AE2 and AE3 are produced by different genes, which constitute a presently well defined gene family (Kopito, 1990).

An interesting aspect of the AE family, that has given rise to considerable controversy, is the nature of the exchanger located at the apical membrane of the β intercalated cell from collecting duct. As indicated above, this cell secretes bicarbonate, its prevalence is increased in metabolic alkalosis, and the exchanger is located at the luminal (apical) membrane, while the V-type H^+-ATPase is located basolaterally. However, antibodies against AE1 do not detect the presence of this isoform at the apical membrane of these cells; in addition, this exchanger has only a low sensitivity to DIDS, as opposed to the basolateral AE1 form. These properties have led to the suggestion that the apical AE could be a different form, AE2 or AE3, or even a still unknown molecule. No evidence for these suggestions has been, however, obtained. Figure 11 shows how it is possible to localize specific transporters by immunocytochemistry using antibodies bound to fluorescent molecules such as FITC. Figure 11A shows a cryosection of rat collecting duct immunologically labelled by a fluoresceine-bound monoclonal antibody against H^+-ATPase. The figure shows several cells having such binding at the apical membrane (*a* intercalated cells) and others having the V-ATPases at the basolateral membrane (*b* intercalated cells). Figure 11B shows cells labelled by a polyclonal antibody against AE1. It is noted that *a* intercalated cells are labelled basolaterally, while *b* intercalated cells show no label (Sabolic et al., 1997). Al-Awqati et al. (Al-Awqati et al., 1994) have shown that when the apical AE is isolated by biochemical methods it gains immunochemical properties of AE1, suggesting that the apical AE is actually AE1, but does not label immunocytochemically to AE1 antibodies and has low DIDS sensitivity due to a

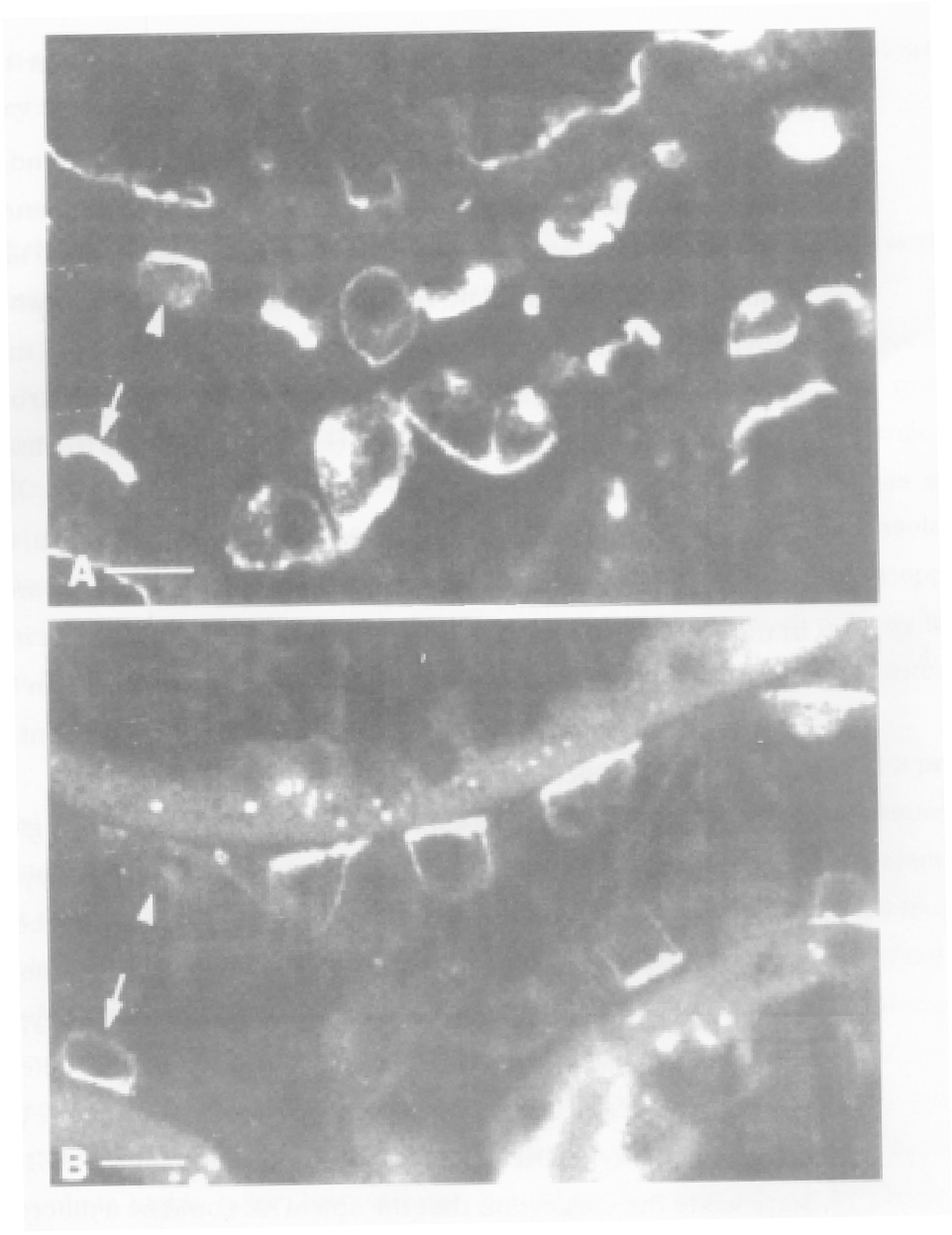

Figure 11. A: Cryosection of rat cortical collecting duct marked with fluorescein-bound monoclonal antibody against V-type H^+-ATPase. Arrow shows a intercalated cell with apical H^+-ATPase, and arrowhead shows basolateral ATPase in b intercalated cell. B: Cells marked with fluorescent polyclonal antibody against AE1 Cl^-/HCO_3^- exchanger. Arrow shows a intercalated cell with basolateral AE1. b intercalated cells do not react to this antibody (arrowhead). From Sabolic et al., 1997).

different lipid milieu at the apical membrane, which is richer in sphingomyelin and gangliosides than the basolateral membrane. These authors showed that "in vitro" these lipid components reduced the sensitivity of AE to DIDS. The different properties of the apical AE have also been attributed to an increased density of the subapical cytoskeleton network (Al-Awqati et al., 1994).

It has been shown that in acidosis the frequency of α intercalated cells is increased, while in alkalosis the density of β intercalated cells is increased. The cause of this property of the collecting duct has been discussed, and the possibility of interconversion between these cell types has been proposed. However, no evidence for this suggestion has been obtained. On the other hand, it has been proposed that a high density of intercalated cells in culture is responsible for the predominance of *a* intercalated cells, while cultivation at low density leads to predominance of *b*

cells. It was shown that under high density conditions the cells secrete toward the basolateral surface an extracellular matrix protein designated *hensin*, which would define the polarity of the intercalated cells toward the *a* type (Adelsberg et al., 1994).

The Na^+/HCO_3^- Cotransporter: The presence of Na^+ dependent HCO_3^- transport in basolateral cell membranes from proximal renal tubule was first shown by Froemter et al (Yoshitomi & Froemter, 1984; Yoshitomi et al., 1985). They showed that basolateral membrane PD presented a large depolarizing transient when bicarbonate was removed from capillary perfusate, which first was taken to represent a passive bicarbonate conductance, but was soon shown to be mediated by Na^+/HCO_3^- cotransport, since both electrical evidence for bicarbonate exit from the cell and its reflection on cell pH were impaired in sodium-free media, and since this mechanism was inhibited by SITS and DIDS, well-known anion exchanger inhibitors. This cotransporter is responsible for transfer of bicarbonate, generated inside cells during the process of H^+ ion generation from H_2O and CO_2, from cell to interstitium and to peritubular capillaries. As indicated above, this process is electrogenic, and therefore the cotransport is not 1:1. In renal cells this transport mechanism was shown to couple 3 HCO_3^- ions to 1 Na^+, being thus driven by the electrochemical gradient for bicarbonate (Boron & Boulpaep, 1983; Yoshitomi et al., 1985). On the other hand, in colon epithelium, bicarbonate secretion has been detected in colon crypts (Rajendran & Binder, 1999) and in cultured colon cells such as HT29 (Koettgen et al., 1994), IEC-6 (Wenzl et al., 1989) and T84 (Ramirez et al., 2000). Several of these cells have the Na^+/HCO_3^- cotransport in their basolateral membrane, loading the cells for apical secretion via Cl^-/HCO_3^- exchange. In these cells, where bicarbonate transport is in the opposite direction as in renal proximal tubule, the coupling ratio is 2 HCO_3^- to 1 Na^+ (Planelles et al., 1993).

The Na^+/HCO_3^- cotransporter has been cloned. Several isoforms have been described, including the renal proximal tubule isoform from the salamander *Ambystoma tigrinum*, which was expressed in *Xenopus* oocytes (Boron et al., 1997; Romero et al., 1997), and other isoforms that have been detected in brain, stomach, small and large intestine, heart and prostate. These isoforms are designated as NBC, and have 30 to 35% homology with the 3 AE molecules, therefore belonging to a bicarbonate transporter superfamily (Romero & Boron, 1999). They may be designated as NBC1, 2 and 3, etc, or akNBC (Ambystoma kidney), rkNBC (rat kindney), hhNBC (human heart), hkNBC (human kidney). NBC3 has been detected in brain, muscle and renal medulla; in the latter tissue it was detected in the apical membrane of α intercalated cells, and at the basolateral membrane of β intercalated cells,

showing that this cotransporter should have an important role in bicarbonate transport of intercalated cells. It was not detected in collecting duct principal cells, and colocalized with the H^+-ATPase in intercalated cells. An additional interesting property is the insensitivity of NBC3 to DIDS and its sensitivity to amiloride analogs (Pushkin et al., 1999; Kwon et al., 2000).

The foregoing considerations show that the regulation of cell pH is a very complex process, involving or not transepithelial H^+ or HCO_3^- transport. The knowledge about these processes has grown tremendously in the last years, allowing for the cloning and description of the molecular structure of most of the transporters that have been known from functional studies. In addition, functional determination of the role of these transporters by means of their expression in *Xenopus* oocytes, and fluorometric or electrode pH determinations in individual cells or tissues has given a quite detailed picture of their participation in a number of physiological and pathophysiological mechanisms.

References

Adelsberg JV, Edwards JC, Takito J, Kiss B, Al-Awqati Q. An induced extracellular matrix protein reverses the polarity of Band 3 in intercalated epithelial cells. Cell 1994;76:1053-61.

Al-Awqati Q, Mueller A, Steinmetz PR. Transport of H+ against electrochemical gradients in turtle urinary bladder. Am J Physiol Renal Fluid & Electrolyte Physiol. 1977;233:F502-F508.

Al-Awqati Q, Van Adelsberg J, Takito J. Plasticity in Epithelial Polarity. Current Topics in Membranes & Transport 1994;41:109-22.

Aronson PS, Nee J, Suhm MA. Modifier role of internal H+ in activating the Na+-H+ exchanger in renal microvillus membrane vesicles. Nature 1982;299:161-3.

Barreto-Chaves ML, Mello-Aires M. Luminal arginine vasopressin stimulates Na^+-H^+ exchange and H^+-ATPase in cortical distal tubule via V1 receptor. Kidney Int 1997;52(4):1035-41.

Barreto-Chaves MLM, Mello-Aires M. Effect of luminal angiotensin II and ANP on early and late cortical distal tubule HCO_3^- reabsorption. Am J Physiol Renal Fluid Electrolyte Physiol 1996;271(5):F977-F984.

Bastani B. Colocalization of H-ATPase and H,K-ATPase immunoreactivity in the rat kidney. J Am Soc Nephrol 1995;5:1476-82.

Bastani B, Gluck SL. New insights into the pathogenesis of distal renal tubular acidosis. Mineral Electrolyte Metab 1996;22(5-6):396-409.

Bidet M, Tauc M, Gastineau M, Poujeol P. Effect of calcitonin on the regulation of intracellular pH in primary cultures of rabbit early distal tubule. Pflügers Arch 1992;421:523-9.

Borensztein P, Juvin P, Vernimmen C, Poggioli J, Paillard M, Bichara M. cAMP-dependent control of Na^+/H^+ antiport by AVP, PTH, and PGE_2 in rat medullary thick ascending limb cells. Am J Physiol Renal Fluid Electrolyte Physiol 1993;264:F354-F364.

Boron WF, Boulpaep EL. Intracellular pH regulation in the renal proximal tubule of the salamander. Basolateral HCO3- transport. J Gen Physiol 1983;81:53-94.

Boron WF, Hediger MA, Boulpaep EL, Romero MF. The renal electrogenic $Na^+:HCO_3^-$ cotransporter. J Exp Biol 1997;200(2):263-8.

Boron WF, Roos A. Comparison of microelectrode, DMO, and methylamine methods for measuring intracellular pH. Am J Physiol 1976;231:799-809.

Brechue WF, Kinne-Saffran E, Kinne RKH, Maren TH. Localization and activity of renal carbonic anhydrase (CA) in CA-II deficient mice. Biochim Biophys Acta Bio-Membr 1991;1066:201-7.

Bright GR, Fisher GW, Rogowska J, Taylor DL. Fluorescence ratio imaging microscopy: temporal and spatial measurements of cytoplasmic pH. J Cell Biol 1987;104(4):1019-33.

Bright GR, Fisher GW, Rogowska J, Taylor DL. Fluorescence ratio imaging microscopy. [Review]. Methods in Cell Biology 1989;30:157-92.

Brion LP, Cammer W, Satlin LM, Suarez C, Zavilowitz BJ, Schuster VL. Expression of carbonic anhydrase IV in carbonic anhydrase II-deficient mice. Am J Physiol Renal Physiol 1997;273(2):F234-F245.

Brown D, Breton S. Mitochondria-rich, proton-secreting epithelial cells [Review]. J Exp Biol 1996;199(11):2345-58.

Buffin-Meyer B, Younes-Ibrahim M, Barlet-Bas C, Cheval L, Marsy S, Doucet A. K depletion modifies the properties of Sch-28080-sensitive K- ATPase in rat collecting duct. Am J Physiol Renal Physiol 1997;272(1):F124-F131.

Burg M, Green N. Bicarbonate transport by isolated perfused rabbit proximal convoluted tubules. Am J Physiol Renal Fluid & Electrolyte Physiol 1977;233(4):F307-F314.

Cano A. Characterization of the rat NHE3 promoter. Am J Physiol Renal Fluid & Electrolyte Physiol 1996;40(3):F629-F636.

Chow D-C, Forte JG. Characterization of the b-subunit of the H^+-K^+ -ATPase using an inhibitory monoclonal antibody. Am J Physiol Cell Physiol 1993;265:C1562-C1570.

Chow DC, Forte JG. Functional significance of the beta-subunit for heterodimeric P-type ATPases. J Exp Biol 1995;198(Pt 1):1-17.

Damasco MC, Ansaldo M, Malnic G. Effects of adrenalectomy and acute replacement by corticosteroids on distal acidification. Can J Physiol Pharmacol 1989;67:607-14.

Debellis L, Iacovelli C, Frömter E, Curci S. Model of bicarbonate secretion by resting frog stomach fundus mucosa. II. Role of the oxyntopeptic cells. Pflügers Arch 1994;428:655-63.

Doucet A. H^+,K^+-ATPase in the kidney: Localisation and function in the nephron. Exp Nephrol 1997;5(4):271-6.

DuBose Jr. TD, Caflisch CR. Validation of the difference in urine and blood carbon dioxide tension during bicarbonate loading as an index of distal nephron acidification in experimental models of distal renal tubular acidosis. J Clin Invest 1985;75:1116-23.

Fan LZ, Wiederkehr MR, Collazo R, Wang HM, Crowder LA, Moe OW. Dual mechanisms of regulation of Na/H exchanger NHE-3 by parathyroid hormone in rat kidney. J Biol Chem 1999;274(16):11289-95.

Fernandez R, Bosqueiro JR, Cassola AC, Malnic G. Role of Cl^- in electrogenic H^+ secretion by cortical distal tubule. J Membrane Biol 1997;157(2):193-201.

Fernandez R, Lopes MJ, Lira RF, Dantas WFG, Cragoe Jr. EJ, Malnic G. Mechanism of acidification along cortical distal tubule of the rat. Am J Physiol Renal Fluid & Electrolyte Physiol 1994;266:F218-F226.

Fernandez R, Malnic G. H^+ ATPase and Cl^- interaction in regulation of MDCK cell pH. J Membrane Biol 1998;163(2):137-45.

Forgac M. Structure and function of vacuolar class of ATP-driven proton pumps. Physiol Rev 1989;69:765-96.

Ganser AL, Tanisawa AS, Forte JG. The K^+ stimulated ATPase system of microsomal membranes from gastric oxyntic cells. Ann NY Acad Sci 1974;242:255-67.

Gillis JM, Gailly P. Measurements of $[Ca^{2+}]_i$ with the diffusible Fura-2 AM: Can some potential pitfalls be evaluated. Biophys J 1994;67:476-7.

Gluck SL. The vacuolar H+ -ATPases: Versatile proton pumps participating in constitutive and specialized functions of eukaryotic cells. International Review of Cytology 1993;137C:105-37.

Good DW, Knepper MA, Burg MB. Ammonia and bicarbonate transport by thick ascending limb of rat kidney. Am J Physiol Renal Fluid & Electrolyte Physiol 1984;247:F35-F44.

Gottschalk CW, Lassiter WE, Mylle M. Localization of urine acidification in the mammalian kidney. Am J Physiol 1960;198:581-5.

Green HH, Steinmetz PR, Frazier HS. Evidence for proton transport by turtle bladder in presence of ambient bicarbonate. Am J Physiol 1970;218:845-50.

Helmle-Kolb C, Montrose MH, Murer H. Parathyroid hormone regulation of Na+/H+ exchange in opossum kidney cells: polarity and mechanisms. Pfluegers Arch 1990;416:615-23.

Henry RP. Multiple roles of carbonic anhydrase in cellular transport and metabolism. Annu Rev Physiol 1996;58:523-38.

Hersey SJ, Sachs G. Gastric acid secretion. Physiol Rev 1995;75:155-90.

Hüser J, Lipp P, Niggli E. Confocal microscopic detection of potential-sensitive dyes used to reveal loss of voltage control during patch-clamp experiments. Pflugers Arch 1996;433(1-2):194-9.

Igarashi P, Reilly RF, Hildebrandt F, Biemesderfer D, Rebouças NA, Slayman CW, Aronson PS. Molecular biology of renal Na^+-H^+ exchangers. Kidney Int 1991;40(suppl 33):S84-S89.

Jaisser F, Beggah AT. The nongastric H^+-K^+-ATPases: molecular and functional properties. Am J Physiol Renal Physiol 1999;276(6):F812-F824.

Kaunitz JD, Gunther RD, Sachs G. Characterization of an electrogenic ATP and chloride-dependent proton translocating pump from Rat Renal Medulla. J Biol Chemistry 1985;260(21):11567-73.

Kinsella JL, Aronson PS. Properties of the Na-H exchanger in renal microvillus membrane vesicles. Am J Physiol 1980;238:F461-F469.

Kinsella JL, Aronson PS. Determination of the coupling ratio for Na+-H+ exchange in renal microvillus membrane vesicles. Biochim Biophys Acta 1982;689:161-4.

Kleyman TR, Cragoe EJ. Amiloride and its analogs as tools in the study of ion transport. J Membrane Biol 1988;105:1-22.

Koettgen M, Leipziger J, Fischer KG, Nitschke R, Greger R. pH regulation in HT29 colon carcinoma cells. Pfluegers Arch 1994;428:179-85.

Kohn OF, Hand AR, Mitchell PP, Steinmetz PR. Intra- and submembrane particle densities during CO_2 stimulation of H^+ secretion in turtle bladder. Am J Physiol Renal Physiol 1997;272(4):F491-F497.

Kopito RR. Molecular biology of the anion exchanger gene family. Int Rev Cytology 1990;123:177-99.

Kwon TH, Pushkin A, Abuladze N, Nielsen S, Kurtz I. Immunoelectron microscopic localization of NBC3 sodium-bicarbonate cotransporter in rat kidney [In Process Citation]. Am J Physiol Renal Fluid Electrolyte Physiol 2000;278(2):F327-F336.

Levine DZ, Iacovitti M, Buckman S, Harrison V. In vivo modulation of rat distal tubule net HCO_3 flux by VIP, isoproterenol, angiotensin II, and ADH. Am J Physiol Renal Fluid Electrolyte Physiol 1994;266:F878-F883.

Loennerholm G, Ridderstrale Y. Intracellular distribution of carbonic anhydrase in the rat kidney. Kidney Int 1980;17:162-74.

Lopes AG, Mello-Aires M, Malnic G. Behavior of the antimony microelectrode in different buffer solutions. Brazilian J Med Biol Res 1981;14:29-36.

Malnic G. Role of the kidney in controlling acid-base balance. Child Nephrol Urol 1989;9:241-52.

Malnic G, Geibel JP. Regulation of cell pH and H^+ secretion by S3 segment of mammalian kidney. J Membrane Biol 2000;178:115-25.

Malnic G, Mello-Aires M. Kinetic study of bicarbonate reabsorption in proximal tubule of the rat. Am J Physiol 1971;220:1759-67.

Maren TH. Carbonic anhydrase: chemistry, physiology and inhibition. Physiol Rev 1967;47:595-781.

Meech RW, Thomas RC. Voltage-dependent intracellular pH in helix aspersa neurones. J Physiol (London) 1987;390:433-52.

Meneton P, Ichikawa I, Inagami T, Schnermann J. Renal physiology of the mouse. Am J Physiol Renal Physiol 2000;278(3):F339-F351.

Miller DS, Letcher S, Barnes DM. Fluorescence imaging study of organic anion

transport from renal proximal tubule cell to lumen. Am J Physiol Renal Fluid Electrolyte Physiol 1996;271(3):F508-F520.

Murer H, Hopfer U, Kinne R. Sodium-proton antiport in brush-border membrane vesicles isolated from rat small intestine and kidney. Biochem J 1976;154:597-614.

Myers M, Forgac M. Mechanism and function of vacuolar acidification. NIPS 1993;8:24-9.

Nakamura S, Amlal H, Schultheis PJ, Galla JH, Shull GE, Soleimani M. HCO-3 reabsorption in renal collecting duct of NHE-3-deficient mouse: a compensatory response [In Process Citation]. Am J Physiol 1999;276(6 Pt 2):F914-F921.

Nascimento-Gomes G, Souza MO, Vecchia MG, Oshiro MEM, Ferreira AT, Mello-Aires M. Atrial natriuretic peptide modulates the effect of angiotensin II on the concentration of free calcium in the cytosol of Madin-Darby canine kidney cells. Braz J Med Biol Res 1995;28:609-13.

Noel J, Roux D, Pouysségur J. Differential localization of Na^+/H^+ exchanger isoforms (NHE1 and NHE3) in polarized epithelial cell lines. J Cell Sci 1996;109(5):929-39.

Orlowski J, Grinstein S. Na^+/H^+ exchangers of mammalian cells. J Biol Chem 1997;272(36):22373-6.

Pitts RF. Renal excretion of acid. Federation Proc 1948;7:418-26.

Planelles G, Thomas S, Anagnostopoulos T. Change of apparent stoichiometry of proximal tubule Na+-HCO3- cotransport upon experimental reversal of its orientation. Proc Natl Acad Sci USA 1993;90:7406-10.

Pushkin A, Yip KP, Clark I, Abuladze N, Kwon TH, Tsuruoka S, Schwartz GJ, Nielsen S, Kurtz I. NBC3 expression in rabbit collecting duct: colocalization with vacuolar H(+)-ATPase. Am J Physiol 1999;277(6 Pt 2):F974-F981.

Rajendran VM, Binder HJ. Distribution and regulation of apical Cl/anion exchanges in surface and crypt cells of rat distal colon. Am J Physiol Gastrointest Liver Physiol 1999;276(1):G132-G137.

Ramirez M, Fernandez R, Malnic G. Permeation of NH_3/NH_4^+ and cell pH in colonic crypts of the rat. Pflugers Arch 1999;438(4):508-15.

Ramirez M, Toriano R, Parisi M, Malnic G. Control of cell pH in the T84 colon cell line. J Membrane Biol 2000;177:149-57.

Reboucas NA, Malnic G. Regulation of rat proximal tubule Na/H exchange by protein kinase C. Renal Physiol Biochem 1996;19(2):87-93.

Rector FC, Carter NW, Seldin DW. The mechanism of bicarbonate reabsorption in the proximal and distal tubules of the kidney. J Clin Invest 1965;44:278-90.

Romero MF, Boron WF. Electrogenic Na^+/HCO_3^- cotransporters: Cloning and physiology. Annu Rev Physiol 1999;61:699-723.

Romero MF, Hediger MA, Boulpaep EL, Boron WF. Expression cloning and characterization of a renal electrogenic Na^+/HCO_3^- cotransporter. Nature 1997;387(6631):409-13.

Roos A, Boron WF. Intracellular pH. Physiol Rev 1981;61:296-434.

Rosenberg SO, Berkowitz PA, Li L, Schuster VL. Imaging of filter-grown epithelial cells: MDCK Na(+)-H+ exchanger is basolateral. Am J Physiol 1991;260:C868-76.

Sabolic I, Brown D, Gluck SL, Alper SL. Regulation of AE1 anion exchanger and H+-ATPase in rat cortex by acute metabolic acidosis and alkalosis. Kidney Int 1997;51(1):125-37.

Sachs G, Chang HH, Rabon E, Schackmann R, Lewin M, Saccomani G. A nonelectrogenic H^+ pump in plasma membranes of hog stomach. J Biol Chem 1976;251:7690-8.

Sakamoto H, Sado Y, Naito I, Kwon TH, Inoue S, Endo K, Kawasaki M, Uchida S, Nielsen S, Sasaki S, et al. Cellular and subcellular immunolocalization of ClC-5 channel in mouse kidney: colocalization with H(+)-ATPase. Am J Physiol 1999;277(6 Pt 2):F957-F965.

Sanders SS, Hayne VB, Rehm WS. Normal H rates in frog stomach in absence of exogenous CO_2 and a note on the pH-stat method. Am J Physiol 1973;225:1311-21.

Sangan PC, Rajendran VM, Mann AS, Kashgarian M, Binder HJ. Regulation of colonic H-K-ATPase in large intestine and kidney by dietary Na depletion and dietary K depletion. Am J Physiol Cell Physiol 1997;272(2):C685-C696.

Sasaki S, Marumo F. Mechanisms of inhibition of proximal acidification by PTH. Am J Physiol Renal Fluid Electrolyte Physiol 1991;260:F833-F838.

Schultheis PJ, Clarke LL, Meneton P, Miller ML, Soleimani M, Gawenis LR, Riddle TM, Duffy JJ, Doetschman T, Wang T, et al. Renal and intestinal absorptive defects in mice lacking the NHE3 Na^+/H^+ exchanger. Nature Genet 1998;19(3):282-5.

Schwartz GJ, Al-Awqati Q. Regulation of transepithelial H+ transport by exocytosis and endocytosis. Ann Rev Physiol 1986;48:153-161.

Schwartz GJ, Winkler CA, Zavilowitz BJ, Bargiello T. Carbonic anhydrase II mRNA is induced in rabbit kidney cortex during chronic metabolic acidosis. Am J Physiol Renal Fluid Electrolyte Physiol 1993;265:F764-F772.

Silver RB, Soleimani M. H^+-K^+-ATPases: regulation and role in pathophysiological states. Am J Physiol Renal Physiol 1999;276(6):F799-F811.

Smith HW, Finkelstein N, Aliminosa L, Crawford B, Graber M. The renal clearances of substituted hippuric acid derivatives and other aromatic acids in dog and man. J Clin Invest 1945;24:388-404.

Spicer Z, Miller ML, Andringa A, Riddle TM, Duffy JJ, Doetschman T, Shull GE. Stomachs of Mice Lacking the Gastric H,K-ATPase alpha-Subunit have Achlorhydria, Abnormal Parietal Cells, and Ciliated Metaplasia. J Biol Chem 2000;275:21555-65.

Steinmetz PR, Lawson LR. Effect of luminal pH on ion permeability and flows of Na and H in turtle bladder. Am J Physiol 1971;220:1573-80.

Stetson DL, Steinmetz PR. Role of membrane fusion in CO2 stimulation of proton secretion by turtle bladder. Am J Physiol 1983;245:C113-C120.

Stevens TH, Forgac M. Structure, function and regulation of the vacuolar (H^+)-ATPase. Annu Rev Cell Dev Biol 1997;13:779-808.

Tanner MJ. The structure and function of band 3 (AE1): recent developments (review). Mol Membr Biol 1997;14(4):155-65.

Thomas J, Buchsbaum R, Zimniak A, Racker E. Intracellular pH measurements in Ehrlich ascites tumor cells utilizing spectroscopic probes generated in situ. Biochemistry 1979;18(11):2210-8.

Tse CM, Brant SR, Walker MS, Pouysségur J, Donowitz M. Cloning and sequencing of a rabbit cDNA encoding an intestinal and kidney-specific Na+/H+ exchanger isoform (NHE-3). J Biol Chem 1992;267:9340-6.

Tsien RY. Fluorescence measurement and photochemical manipulation of cytosolic free calcium. Trends in Neurosciences 1988;11:419-24.

Ulate G, Fernandez R, Malnic G. Effect of bafilomycin on proximal bicarbonate absorption in the rat. Braz J Med Biol Res 1993;26:773-7.

Vieira FL, Malnic G. Hydrogen ion secretion by rat renal cortical tubules as studied by an antimony microelectrode. Am J Physiol 1968;214:710-8.

Vurek GG, Warnock DG, Corsey R. Measurement of picomole amounts of carbon dioxide by calorimetry. Anal Chem 1975;47:765-7.

Wakabayashi S, Bertrand B, Ikeda T, Pouysségur J, Shigekawa M. Mutation of calmodulin-binding site renders the Na^+/H^+ exchanger (NHE1) highly H^+-sensitive and Ca^{2+} regulation- defective. J Biol Chem 1994;269:13710-5.

Wakabayashi S, Shigekawa M, Pouyssegur J. Molecular physiology of vertebrate Na^+/H^+ exchangers. Physiol Rev 1997;77(1):51-74.

Weinman EJ, Steplock D, Tate K, Hall RA, Spurney RF, Shenolikar S. Structure-function of recombinant Na/H exchanger regulatory factor (NHE-RF). J Clin Invest 1998;101(10):2199-2206.

Wenzl E, Sjaastad MD, Weintraub WH, Machen T. Intracellular pH regulation in IEC-6 cells, a cryptlike intestinal cell line. AJP (Gastrointest Liver Physiol) 1989;257:G732-G740.

Wilkens S, Vasilyeva E, Forgac M. Structure of the vacuolar ATPase by electron microscopy. J Biol Chem 1999;274(45):31804-10.

Wingo CS, Smolka AJ. Function and structure of H-K-ATPase in the kidney. Am J Physiol Renal Fluid Electrolyte Physiol 1995;269:F1-F16.

Wistrand PJ, Knuuttila K-G. Renal membrane-bound carbonic anhydrase. Purification and properties. Kidney Int 1989;35:851-9.

Wormmeester L, De Medina FS, Kokke F, Tse CM, Khurana S, Bowser J, Cohen ME, Donowitz M. Quantitative contribution of NHE2 and NHE3 to rabbit ileal brush-border Na^+/H^+ exchange. Am J Physiol Cell Physiol 1998;274(5):C1261-C1272.

Yoshitomi K, Burckhardt B-C, Froemter E. Rheogenic sodium-bicarbonate cotransport in the peritubular cell membrane of rat renal proximal tubule. Pfluegers Arch 1985;405:360-6.

Yoshitomi K, Froemter E. Cell pH of rat renal proximal tubule in vivo and the conductive nature of peritubular HCO3- (OH-) exit. Pfluegers Arch 1984;402:300-5.

Zizak M, Lamprecht G, Steplock D, Tariq N, Shenolikar S, Donowitz M, Yun CHC, Weinman EJ. cAMP-induced phosphorylation and inhibition of Na^+/H^+ exchanger 3 (NHE3) are dependent on the presence but not the phosphorylation of NHE regulatory factor. J Biol Chem 1999;274(35):24753-8.