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MINI-REVIEW

Passive H^+/OH^- Permeability in Epithelial Brush Border Membranes

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Abstract

Passive H^+/OH^- permeability across epithelial cell membranes is rapid and leads to partial dissipation of H^+/OH^- gradients produced by H^+ pumps and ion gradient-coupled H^+/OH^- transporters. A heterogeneous set of H^+/OH^- transport mechanisms exist in biological membranes: lipid solubility/diffusion, protein-mediated transport by specific proteins or by slippage through ion-coupled H^+/OH^- transporters, and transport at the protein/lipid interface or through protein-dependent defects in the lipid structure. A variety of methods are available to study protein transport mechanisms accurately in cells and biomembrane vesicles including pH electrode recordings, pH-sensitive fluorescent and magnetic resonance probes, and potentiometric probes. In brush border vesicles from the renal proximal tubule, the characteristics of passive H^+/OH^- permeability are quite similar to those reported for passive H^+/OH^- permeability through pure lipid bilayers; slippage of protons through the brush border Na^+/H^+ antiporter or through brush border water channels is minimal. In contrast, passive H^+/OH^- permeability in brush border vesicles from human placenta is mediated in part by a stilbene-sensitive membrane protein. To demonstrate the physiological significance of passive renal brush border H^+/OH^- transport, proximal tubule acidification and cell pH regulation mechanisms are modeled mathematically for states of normal and altered H^+/OH^- permeabilities.

Key Words: Proton permeability; brush border membrane; fluorescence; proximal tubule; placenta.

Introduction

A diverse ensemble of active and secondary active proton transport mechanisms have been identified in epithelial cell membranes including ATP-dependent

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H^+ uniport, neutral Na^+/H^+, K^+/H^+, and Cl^-/OH^- countertransport, electrogenic $Na^+/3HCO_3^-$ cotransport, and neutral $Na^+/Cl^-/HCO_3^-$ coupled transport (Alpern, 1985; Chen *et al.*, 1987; Reenstra *et al.*, 1981). Transport of H^+ equivalents against the H^+ electrochemical potential is energized either directly by ATP hydrolysis, or secondarily, by coupling to gradients of Na^+, K^+, and/or Cl^-. Precise regulation of epithelial H^+/OH^- transport is required for maintenance of cell pH and ion concentration in a narrow range and for transepithelial acid secretion in some tissues including the stomach and the renal proximal tubule.

Passive H^+/OH^- permeability, or alternatively the "proton leak" or "passive proton conductance," is a counterproductive H^+/OH^- transport process in biological membranes because it causes the collapse of electrochemical H^+/OH^- gradients arising from the above active or secondary active processes. As defined here, the proton leak is a poorly regulated H^+/OH^- conductive mechanism which is not coupled to gradients of other ions. While the proton leak may be wasteful in terms of cellular energetics, it has a potential role in providing an H^+/OH^- pathway which is coupled to ion movements by induced diffusion potentials (parallel conductances) and in the prevention of large transmembrane pH gradients.

These observations raise a compelling question: Why do biological membranes possess a proton leak pathway? Is the leak pathway a concomitant of all membranes containing the bilayer phospholipid structure? Is the leak pathway a "slippage" route across active or secondary active H^+/OH^- transport systems, or across membrane water channels? To examine these issues, we review here a series of measurements of passive H^+/OH^- transport in brush border epithelial membranes derived from the renal proximal tubule luminal membrane and the brush border membrane of the human placental syncytiotrophoblast cell. The data presented support the existence of both phospholipid and protein-mediated H^+/OH^- conductances across brush border membranes which are the result of specialized membrane structures capable of rapid ion transport and a wide array of enzymatic functions.

H^+/OH^- Permeability Measurements in Biological Cell Membranes

H^+/OH^- fluxes are most directly measured from the rate of dissipation of preformed pH gradients. A number of techniques are available to measure H^+/OH^- permeability which are particularly suited to closed structures such as cells and isolated membrane vesicles. Because proton flux rates are determined in each method, both passive H^+/OH^- permeability and H^+/OH^- transport coupled to ion gradients can be measured.

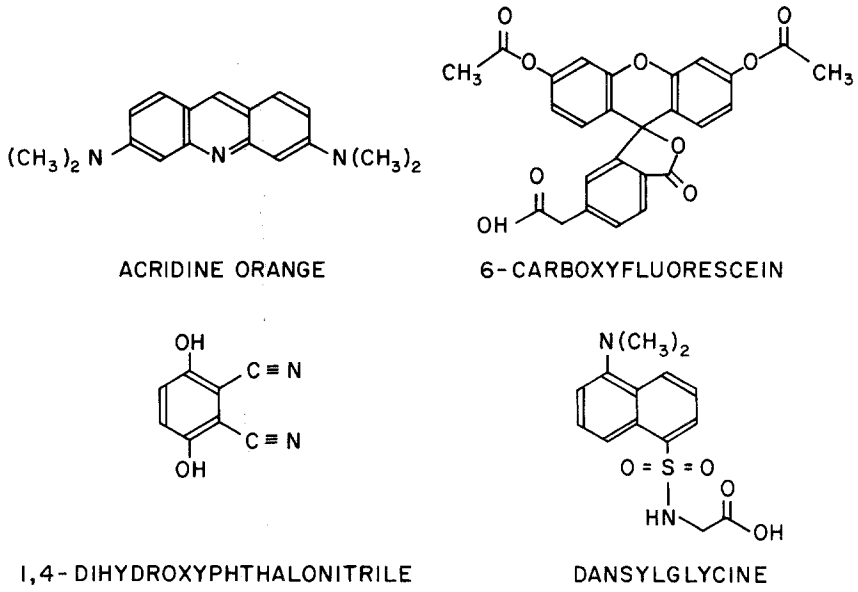


Fig. 1. Structures of some pH-sensitive fluorescent indicators.

Fluorescence methods are the most useful because of their simplicity and accuracy. Examples of pH gradient-sensitive and absolute pH-sensitive fluorescent probes are given in Fig. 1. Acridine orange and 9-aminoacridine are weak bases which partition into acidic intravesicular spaces where they undergo fluorescence quenching (Class I probes). The fluorescence intensity of these probes is therefore a measure of the transmembrane pH gradient. Fluoresceins (e.g., 6-carboxyfluorescein) are pH-sensitive probes that can be loaded into biological vesicles rapidly as nonfluorescent acetoxymethyl derivatives (Class II probes). The acetoxymethyl group is cleaved by intracellular or intravesicular esterases to give a charged, relatively impermeant fluorescent moiety. The fluorescein excitation spectrum is pH dependent with an isosbestic point at 450 nm, allowing a ratiometric normalization for absolute pH determination. A variety of fluorescein derivitives with varying pK's are available (e.g., dichloro-6-carboxyfluorescein, pK 5.5) with differing useful pH ranges. In addition, there exist a number of fluorescent probes with pH-dependent emission spectra (e.g., 1, 4-dihydroxyphthalonitrile) having emission isosbestic wavelengths. Each of the above probes resides in the aqueous intravesicular space. Recently, dansyl glycine has been used as a lipophilic membrane-bound pH-sensitive probe in phospholipid vesicles, where the degree of protonation of the surface glycine alters the dansyl emission spectrum (Class III probes).

Other approaches have been used to measure H^+/OH^- transport. Direct membrane impalement by double-barreled pH-sensitive microelectrodes is applicable to cells but not to small vesicles. Extracellular pH-sensitive macroelectrodes can be used noninvasively to measure H^+/OH^- transport using a very low buffer capacity extracellular solution (Ives *et al.*, 1986). Relative H^+ : ion conductances have been estimated by the biionic potential method using potentiometric cyanine probes (Wright *et al.*, 1984); however, effects of pH and ionic strength on cyanine fluorescence confound the interpretation of results. Phosphorus magnetic resonance and probes with pH-sensitive magnetic resonance spectra have also been used to measure cell pH.

There are several important technical consideration in the measurement of passive H^+/OH^- permeability in biological membranes. The specific considerations are:

1. *Choice of pH probe:* The class I fluorescent probes are easy to use for semiquantitative measurements of H^+/OH^- flux. They give large fluorescence signals and do not require membrane pretreatment. However, they cannot be used to measure very fast H^+/OH^- transport rates (because transmembrane partitioning is required), small pH gradients (< 0.5 pH units), and outwardly directed H^+ gradients. It is difficult to measure absolute H^+/OH^- fluxes because a fluorescence vs. ΔpH calibration is required for every probe and vesicle concentration, initial vesicle pH, temperature, and buffer composition. Class II probes are excellent for measurement of cell or vesicle pH and $d(\text{pH})/dt$. They response to pH changes in $< 1\ \mu s$ and the fluorescence vs pH calibration is relatively insensitive to buffer composition. They require cell or vesicle loading and washing of extracellular probe before the flux measurement. Class III probes have the advantages of class II probes and do not require the loading/washing procedures.

2. *Adequate voltage clamp:* It is essential that measured H^+/OH^- fluxes are not rate-limited by transmembrane movements of other ions required to maintain electroneutrality. To eliminate H^+/OH^- diffusion potentials, membranes are "voltage-clamped" with adequate ion/ionophore (e.g., K^+/valinomycin) concentrations. Adequacy of the voltage clamp must be demonstrated by the lack of effect of increasing further both the ion and ionophore concentrations on measured H^+/OH^- fluxes under experimental conditions in which H^+/OH^- flux is maximal.

3. *Eliminate coupled H^+/OH^- transport:* The buffer composition must be chosen to avoid the presence of coupled H^+/OH^- transport systems which would dissipate pH gradients by a mechanism in parallel with passive H^+/OH^- permeability. It should be recognized that even nominal HCO_3^- concentrations in air-equilibrated buffers could provide a parallel pathway for dissipation of pH gradients.

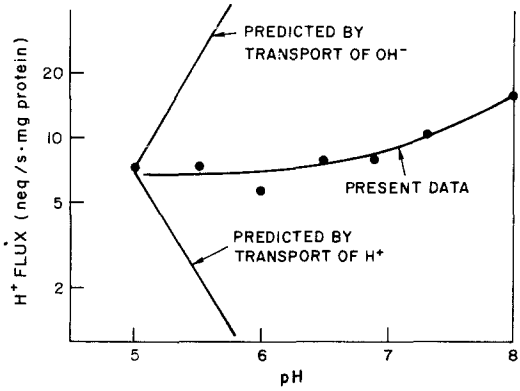
In general, passive H^+/OH^- permeability should be examined using a relatively uniform membrane population, homogeneous particularly in membrane composition and, if possible, in membrane surface-to-volume ratio. Membranes should be prepared freshly under conditions which minimize phospholipid oxidation and proteolysis which might result in formation of protonophoric lipid or protein products (Gutknecht, 1984). It is also important to evaluate whether the probe used to measure vesicle pH alters membrane transport properties or carries H^+ equivalents.

Passive H^+/OH^- Transport in Renal Brush Border Vesicles

The mechanism of passive H^+/OH^- transport was characterized in brush border membrane vesicles (BBMV) isolated from rabbit renal cortex. BBMV were prepared from homogenized renal cortex by a Mg aggregation and differential centrifugation procedure which yields a relatively pure population of small vesicles ($\sim 0.1\,\mu$ diameter) comprised of the apical (luminal) membrane of the proximal tubule (Booth and Kenny, 1974). As reported for other epithelial membranes, the renal brush border membrane is rich in cholesterol (~ 40 mol.%) and is heavily invested with transmembrane proteins. Major transporters of the brush border membrane include Na^+/H^+ and Cl^-/OH^- countertransport (Reenstra *et al.*, 1981; Chen *et al.*, 1987), Na^+/glucose and Na^+/amino acid cotransport (Hopfer and Gropeclose, 1980), K conductance, and a water channel (Meyer and Verkman, 1987).

Two approaches were used to examine the mechanisms of BBMV H^+/OH^- permeability: measurement of the nonequilibrium thermodynamic force–flow relations (Verkman and Ives, 1986), and studies of the effects of protein/lipid perturbing agents on H^+/OH^- flux (Ives and Verkman, 1985).

As discussed elsewhere in this volume (Nagle, 1987), measurement of the dependences of H^+/OH^- flux on pH and the driving forces ΔpH and membrane potential aids in distinguishing models of H^+/OH^- transport. BBMV H^+/OH^- flux in neq/s/mg vesicle protein was calculated from the initial rate of collapse of a performed pH gradient in the absence of Na and Cl using 6-carboxyfluorescein as an entrapped intravesicular pH probe. For small pH gradient (0.4 units), H^+/OH^- flux was nearly independent of pH between 5 and 8 (Fig. 2, top). Such a dependence is similar to findings in pure phospholipid bilayers (Cafiso and Hubbell, 1983; Deamer and Nichols, 1983; Gutknecht, 1984; Nichols and Deamer, 1980) and is not compatible with simple diffusion of H^+ or OH^-, or carrier-mediated transport mechanisms involving a single mobile carrier. At pH 7, the proton permeability coefficient ($P_H = J_H/\Delta[H^+]$), calculated from the data in Fig. 2, the BBMV surface-to-volume ratio $2 \times 10^5\,cm^{-1}$, and glucose space $1\,\mu$l/mg protein is 0.2 cm/s,


PREDICTED BY
TRANSPORT OF OH⁻
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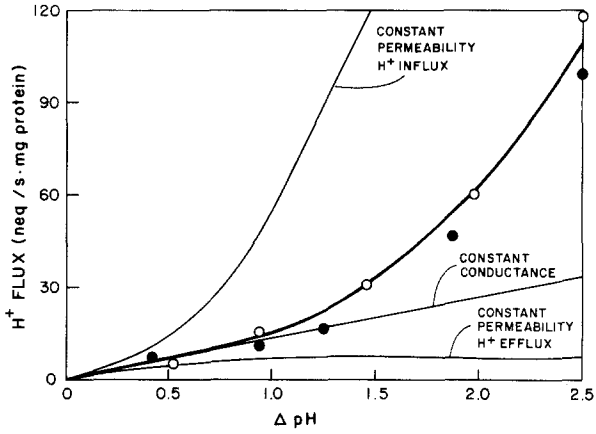
CONSTANT
PERMEABILITY
H⁺ INFLUX
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H⁺ EFFLUX
H⁺ FLUX (neq/s·mg protein)
120
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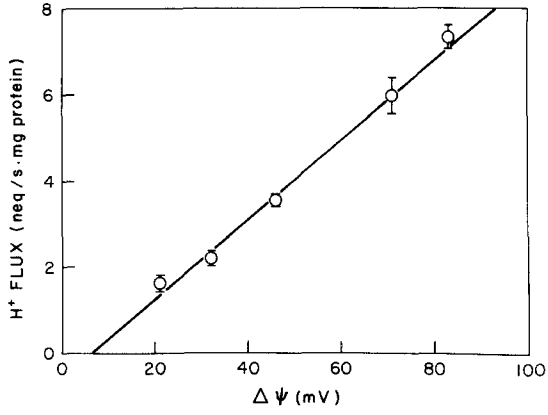
1.0
1.5
2.0
2.5
Δ pH


H⁺ FLUX (neq/s·mg protein)
8
6
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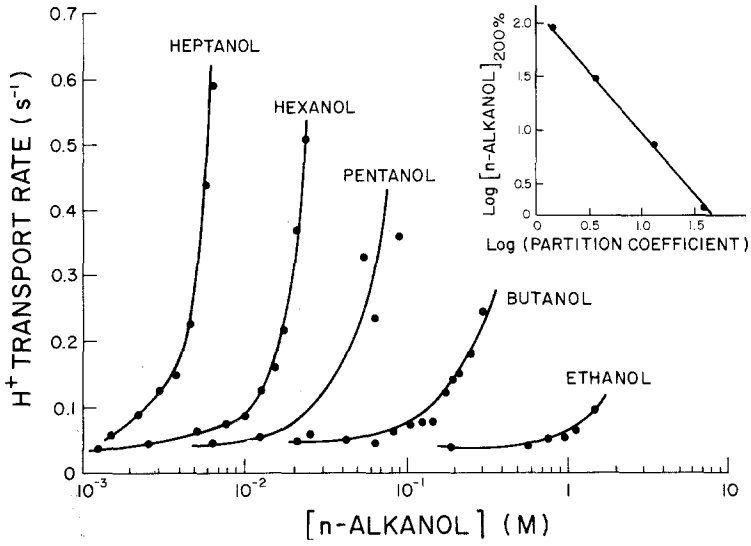

Fig. 3. Effect on *n*-alkanols on BBMV proton permeability. H^+ flux, expressed as the reciprocal exponential time constant for pH equilibration, was determined by the acridine orange method for a 1.5 pH unit outwardly directed H^+ gradient. BBMV were incubated with specified concentrations of the *n*-alkanols. Inset: The *n*-alkanol concentration required to double basal BBMV H^+ flux is plotted against the estimated *n*-alkanol partition coefficient. Fitted line has slope 1.1 ± 0.1. With permission from Ives and Verkman (1985).

many orders of magnitude higher than the permeabilities of other monovalent ions and even higher than P_H in pure lipid bilayers.

The dependence of H^+/OH^- flux on ΔpH for constant initial vesicle pH is curvilinear (Fig. 2, middle) but does not fit the predicted dependences for a constant H^+ permeability or for a constant H^+/OH^- conductance. Interestingly, the dependence of J_H on ΔpH was independent of initial intravesicular pH and the direction of the H^+ gradient. In agreement with the observations made by Nagle (1987), the shape of the J_H vs ΔpH plot is quite dependent on the assumed form of the H^+/OH^- driving force; although the measured J_H vs ΔpH relation is not compatible with simple diffusive/conductive models, it would certainly be consistent with other

Fig. 2. Dependences of BBMV proton flux on pH, ΔpH, and membrane potential. Top: H^+ flux is plotted against initial vesicle pH for a 0.4 pH unit outwardly directed H^+ gradient. The predicted dependences of H^+ flux on pH for diffusive transport of either H^+ or OH^- are plotted. Middle: H^+ flux is plotted against ΔpH for inwardly (open circles) or outwardly (closed circles) directed H^+ gradients. The predicted dependences of H^+ flux on ΔpH for constant H^+ permeability and constant H^+ conductance are shown. Bottom: H^+ was measured in response to K^+/valinomycin diffusion potentials at initial equal vesicle and solution pH values of 7.0. With permission from Verkman and Ives (1986).

more complex models. The dependence of J_H on K/valinomycin induced diffusion potentials is linear within the range of potentials (< 85 mV) which can be examined reliably in membrane vesicles (Fig. 3, bottom). Taken together, these results are similar to those reported in lipid bilayers and are consistent with a primary lipid-mediated pathway in BBMV. Of the mechanisms for lipid-mediated transport, the data are most compatible with models involving transient formations of hydrogen-bonded water chains.

If passive H^+/OH^- permeability in BBMV is a lipid-mediated process, then J_H should be dependent strongly on the physical state of the membrane phospholipid. Figure 3 top, shows that J_H is increased when the membrane is perturbed by *n*-alkanols which increase membrane fluidity. The increase in J_H is directly correlated with the efficacy of the *n*-alkanols to decrease the steady-state fluorescence anisotropy of diphenylhexatriene (DPH), a lipophilic probe of membrane fluidity. A similar correlation between increased J_H and decreased DPH anisotropy was found for a series of non-alkanol fluidizing agents (Ives and Verkman, 1985). In addition, the activation energy (E_a) for BBMV H^+/OH^- permeability is 21.6 kcal/mol, similar to that measured for H^+/OH^- permeability in pure lipid bilayers (Rossignol *et al.*, 1982).

Does passive H^+/OH^- permeability in BBMV represent slippage through the Na^+/H^+ antiporter or passage of protons through the BBMV osmotic water transport pathway? Several lines of evidence suggest that both answers are no. Na^+/H^+ exchange in BBMV is amiloride inhibitable, inactivated by heating to 60°C for 1 min and is unaffected by *n*-alkanols. Passive H^+/OH^- permeability is amiloride and temperature insensitive, and strongly increased by *n*-alkanols. Osmotic water permeability in BBMV is in part mediated by an aqueous pore or channel with mercurial sensitivity, osmotic-to-diffusion permeability coefficient ratio $\sim$ 3.5, and low E_a (2.5 kcal/mol, < 33°C) which increases (8 kcal/mol) with mercurial inhibition (Meyer and Verkman, 1987). BBMV osmotic water permeability is not altered at *n*-alkanol concentrations which increase H^+/OH^- permeability fivefold, and has E_a very much lower than E_a measured for H^+/OH^- permeability. These results suggest that the primary route for passive H^+/OH^- transport in BBMV is through membrane phospholipid; it is unclear whether protein-induced changes in the phospholipid structure can account for the high BBMV P_H compared to values measured in pure lipid bilayers.

H^+/OH^- Transport in Placental Microvillus Vesicles

The entrapped 6-carboxyfluorescein method was also used to examine the mechanism of H^+/OH^- permeability in microvillus vesicles (MVV)

isolated from human placenta (Cabrini *et al.*, 1986). The microvillus and basolateral membranes of the placental syncytiotrophoblast cell form the interface between the maternal and fetal circulations, and thus serve important transepithelial transport functions. MVV are isolated by stirring placental fragments in isotonic buffer to shear off microvilli, followed by Mg aggregation, differential centrifugation, and sucrose density gradient centrifugation (Illsley and Verkman, 1987). There were several interesting differences in the H^+/OH^- transport characteristics in the renal and placental microvillus membranes.

In voltage-clamped MVV (K/valinomycin) in the absence of the transportable ions Na^+ and Cl^-, the H^+/OH^- flux induced by a 1.4 pH unit proton gradient was inhibited 20% by the stilbene dihydro-4,4′-diisothiocyano-2,2′-disulfonic stilbene (H_2DIDS) with a K_i of 8 μM; BBMV H^+/OH^- transport was not inhibited under similar conditions. E_a for passive H^+/OH^- permeability in MVV was 9.1 kcal/mol, much lower than the value of 21.6 kcal/mol in BBMV. These results raised the possibility that part of the MVV H^+/OH^- flux involved a membrane transport protein.

Another unexpected result was the ability of the anions Cl^-, Br^-, and I^- (but not SO_4^{-2} or PO_4^{-3}) to enhance H^+/OH^- flux. For a 1.5 pH unit inwardly directed proton gradient, H^+/OH^- was enhanced $\sim$30% by the presence of Cl^- in the external solution. There was no effect of internal Cl^- on H^+/OH^- flux both in the presence and absence of external Cl^-. These results suggested that Cl^- was acting at an external modifier site rather than participating as a transportable ion. Evidence for this interpretation was supported by the ability of external Cl^- to enhance the H^+/OH^- flux induced in response to a 1.5 pH unit outwardly directed proton gradient. The Cl^- effect was blocked by H_2DIDS. In similar experiments performed using renal BBMV (Chen *et al.*, 1987), there was no effect of Cl^- on passive H^+/OH^- permeability.

These results in placental MVV indicate that the mechanisms of passive H^+/OH^- permeability in MVV are more complex than simple diffusion through membrane phospholipid. The apparent allosteric regulation of H^+/OH^- transport by external Cl^-, the inhibition by H_2DIDS, and the low E_a suggest that one or more membrane proteins mediate directly, or by an indirect mechanism, regulate H^+/OH^- transport. The physiological role for a regulated or protein-mediated passive H^+/OH^- permeability remains unknown.

Role of Passive H^+/OH^- Permeability in Proximal Tubule Function

To understand the biological implications of proton leak pathways in a well-defined epithelium, effects of passive H^+/OH^- permeability on renal

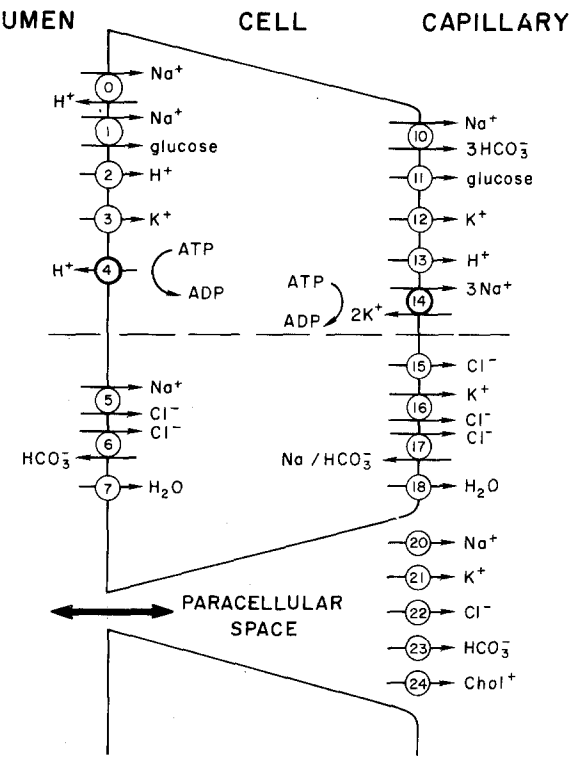

Fig. 4. Schematic of proximal tubule cell membrane transporters.

proximal tubule function are examined. The proximal tubule consists of a single layer of polarized epithelial cells arranged in a cylinder. The outside of the cylinder is bathed in capillary blood and the inside of the cylinder contains the bloodless glomerular ultrafiltrate which is to become urine. A major function of the proximal tubule is to reabsorb the majority of Na^+, Cl^-, HCO_3^-, glucose, amino acids, and water filtered by the glomerulus. The major transport systems in the proximal tubule are given in Fig. 4 based on a mathematical model of proximal tubule acidification reported recently (Verkman and Alpern, 1987).

Transport of base equivalents from lumen to capillary (or equivalently, luminal acidification) is energized by the basolateral membrane Na/K ATPase and the luminal membrane H ATPase. Decreased cell Na activates the Na^+/H^+ antiporter which pumps H^+ equivalents from cell to lumen. The -70 mV cell membrane potential, produced primarily from K^+ gradients, provides the electrochemical driving force to cause transprt of HCO_3 from

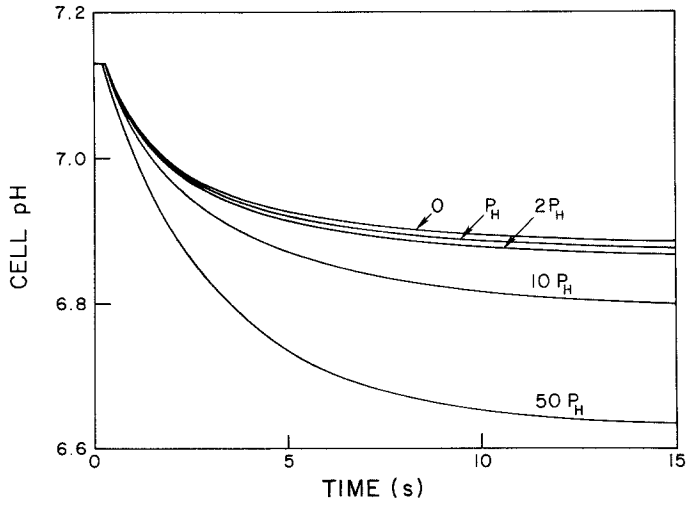

Fig. 5. Effect of passive H^+/OH^- permeability on defense of cell pH in response to perturbations in extracellular pH. The time course of cell pH was calculated using the model of Verkman and Alpern (1987) in response to a change in capillary pH from 7.32 to 6.63. Curves plotted for several values of basolateral membrane H^+/OH^- permeabilities. P_H refers to the physiological basolateral membrane passive H^+/OH^- permeability.

cell to capillary via the electrogenic $Na^+/3HCO_3^-$ cotransporter. Because intracellular carbonic anhydrase causes rapid CO_2–HCO_3^- equilibration and because of the high membrane CO_2 permeability, transport of H^+ into the lumen is equivalent to transport of OH^- or HCO_3^- into the capillary.

The luminal and basolateral (capillary) membrane passive H^+/OH^- transporters (transporters 2 and 13, Fig. 4) cause a small lumen-to-cell and capillary-to-cell proton leak driven by the negative cell potential. Because the luminal pH is less than the capillary pH and because of the high paracellular electrical conductance (transepithelial potential difference 1 mV), passive H^+/OH^- permeability causes a small lumen-to-capillary proton "backleak." The effective transcellular permeability coefficient for proton leak has been estimated in the intact perfused proximal tubule to be 0.3–0.5 cm/s (Hamm *et al.*, 1984; Schwartz, 1981) at pH ~ 7. This value is in agreement with the high BBMV P_H calculated above. It has been estimated that the transcellular proton backleak is equal to 5–20% (early-to-late proximal tubule) of the net transepithelial capillary-to-lumen proton flux (Alpern *et al.*, 1986). Although there are no clinically recognized disorders of an abnormally large H^+/OH^- permeability in the proximal tubule, there is a well-defined distal tubule acidification defect in patients receiving high dose amphotericin for systemic fungal infections (McCurdy *et al.*, 1968). Amphotericin forms pores in the

distal tubule cell membranes resulting in high H^+/OH^- permeability and inability to sustain a transepithelial pH gradient necessary to excrete an acid urine.

In addition to impairing tubule acidification, an abnormally high passive H^+/OH^- permeability would alter proximal cell pH regulation in response to perturbations in extracellular pH. Quantitative predictions given in Fig. 5 were calculated from the model of Verkman and Alpern (1987). Using the magnitude of pasive H^+/OH^- permeability measured in the proximal tubule, cell pH decreases from a steady-state value of 7.13 to 6.86 with a half-time of 2–3 s in response to a sudden decrease in capillary pH from 7.32 to 6.63. As the passive H^+/OH^- permeability coefficient of the basolateral membrane is increased many times over the normal physiological value, there is notably impaired pH regulation. Similarly, a very high passive H^+/OH^- permeability impairs regulation of cell pH and Na^+ concentration in response to external perturbations in luminal or capillary pH or Na^+ concentration.

Summary and Conclusion

As stressed by all of the papers in this issue, the mechanisms of passive H^+/OH^- transport across a homogeneous bilayer lipid membrane are poorly understood at present. The validity of mobile carrier and hydrogen-bonded water models remain unproven. Definition of passive H^+/OH^- permeability mechanisms in biological membranes is yet more difficult because of phospholipid heterogeneity and domain structure, and the presence of transmembrane protein pathways. This membrane heterogeneity allows for a variety of pathways and mechanisms for H^+/OH^- transport, some of which have been studied in the renal and placental epithelial membranes. Passive H^+/OH^- transport does not appear to play an important physiological role, but may be a necessary concomitant of specialized epithelial cell membranes capable of multiple rapid transport and enzymatic processes.

Acknowledgments

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