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Structure/function studies of mammalian Na–H exchangers – an update

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Four mammalian Na^+/H^+ exchangers have recently been cloned. Despite the structural similarity, these Na^+/H^+ exchanger isoforms differ in kinetic characteristics and their response to external stimuli. The present review deals with the recent developments in their functional characterization and their short-term regulation.

Mammalian Na–H exchangers are plasma membrane proteins which move Na^+ into cells in exchange for intracellular H^+. They are inhibitable by the diuretic amiloride and function in control of intracellular pH and volume, participate in cell division, and carry out Na^+ absorption and NH_4^+ absorption in epithelia. That a gene family of mammalian Na–H exchangers existed was predictable on the basis of different functions for Na–H exchangers in different cells and different amiloride sensitivities, not only in different cells but on different plasma membrane domains in the same epithelial cell.

Na–H exchanger gene family

Molecular cloning of mammalian Na–H exchanger

The first mammalian Na–H exchanger was cloned by Sardet *et al.* (1989) who used genetic complementation with fibroblast cell lines that they had selected to lack all endogenous Na–H exchangers. The cloned Na–H exchanger was named NHE, standing for *Na–H Exchanger*. The existence of multiple isoforms of mammalian Na–H exchangers had long been predicted largely on the basis of widely different sensitivity to inhibition by amiloride and differential regulation by protein kinases depending on cell type and cellular location (for instance apical *vs.* basolateral in polarized epithelial cells) (Clark & Limbird 1991; Tse *et al.* 1993*c*). Since the cloning of NHE1 by Sardet *et al.* (1989), three additional members of the mammalian gene family have been identified by our group (Tse *et al.* 1994*a*; Tse *et al.* 1993*b*; Tse *et al.* 1991) and by Orlowski and Shull (Orlowski *et al.* 1992; Wang *et al.* 1993). We named these clones in the order of their molecular identification as NHE1, NHE2, NHE3, NHE4, etc.

NHE1

NHE1 is the Na–H exchanger isoform initially cloned by Sardet *et al.* (1989) from human. NHE1 has also been cloned from rabbit (Tse *et al.* 1991), rat (Orlowski *et al.* 1992), pig (Reilly *et al.* 1991) and Chinese hamster. The size of NHE1 ranges from 815 to 820 amino acids. By Northern analysis and ribonuclease protection assays, NHE1 mRNA is present in nearly all mammalian cells (Tse *et al.* 1991). The only mammalian cells studied in which NHE1 mRNA was not identified are the OK cells (opossum renal proximal tubule cell line) and rat proximal tubule cortical segments S_1 and S_2 (Krapf & Solioz, 1991). All of these cells are known to lack functional basolateral membrane Na–H exchangers.

Immunohistochemical studies using antibody against NHE1 have shown that NHE1 is located on the basolateral surface of several epithelia. In the rabbit ileum, NHE1 is restricted to the basolateral membrane of both the villus and the crypt epithelial cells (Tse *et al.* 1991). It is also restricted to the basolateral membranes of the human colon cancer Cl^- secretory cell line, CaCo-2, under certain growth conditions (Watson *et al.* 1991) and the porcine renal epithelial cell line, LLC-PK_1 (Reilly *et al.* 1991). In rabbit kidney, NHE1 is on the basolateral membrane of proximal tubule cells, distal convoluted tubules, thick ascending limb of Henle's loop, and the collecting duct (Biemesderfer *et al.* 1992).

The diuretic amiloride and its 5′-amino substituted analogues are potent inhibitors of the Na–H exchangers and block the exchanger by competing with Na for the external Na binding site (Benos, 1988). Functional expression of NHE1 in the PS120 fibroblast cell line which lacks all endogenous Na–H exchange has shown that NHE1 is sensitive to inhibition by amiloride and its analogue. K_is for amiloride and ethylisopropyl amiloride (EIPA) are 1–3 μM and 20 nM, respectively.

NHE2

NHE2 has been identified in rabbit (Tse *et al.* 1993*b*), rat (Wang *et al.* 1993) and recently in human. NHE2 contains 809 amino acids in rabbit and 813 amino acids in rat.

Distribution of NHE2 mRNA is restricted to stomach, uterus, kidney, intestine, adrenal gland and much less in trachea and skeletal muscle (Tse *et al.* 1993*b*; Wang *et al.* 1993). In kidney, NHE2 mRNA is most expressed in the medulla, exceeding that in the cortex. In gastrointestinal tract, the ascending colon has most mRNA followed by descending colon, jejunum, ileum and duodenum. We have recently observed that by Western analysis with an anti-NHE2 polyclonal antibody that NHE2 is expressed in brush-border membranes isolated from rat jejunum, rabbit

kidney cortex, human jejunum and rabbit jejunum, but not in the basolateral membrane (Hoogerwerf *et al.* 1994). Szpirer *et al.* (1994) have recently shown that human NHE2 and rat NHE2 are mapped to human chromosome 2 and rat chromosome 9, respectively.

NHE2 is sensitive to amiloride but resistant to 5′-amino substituted amiloride analogues. K_i of amiloride of NHE2 is similar to that of NHE1 (1–3 μM). However, K_i of EIPA is 1 μM, which is 50-fold less sensitive than NHE1.

NHE3

NHE3 has been cloned from rabbit (Tse *et al.* 1994*a*), rat (Orlowski *et al.* 1992) and human (Brant *et al.* 1993). In rabbit, NHE3 mRNA is found exclusively in kidney, intestine and stomach (Tse *et al.* 1994*a*). Most mRNA is present in the kidney cortex, exceeding the medulla. The area of second most abundance is rabbit ascending colon which is approximately equal to ileum, followed by jejunum. NHE3 mRNA is not present in the duodenum or descending colon of rabbit. Thus the presence of NHE3 mRNA in rabbit gastrointestinal tract co-localizes with the expression of the neutral NaCl absorptive process. In rat, NHE3 mRNA is also found in descending colon, in addition to kidney, jejunum, ileum and ascending colon (Orlowski *et al.* 1992). The presence of NHE3 transcript in descending colon of rat but not of rabbit may be due to species difference. Rat descending colon also contains the neutral NaCl absorptive process.

We initially suggested that NHE3 was the Na–H exchanger isoform responsible for the neutral NaCl absorptive process based on our demonstration that an increase in Na–H exchange activity in rabbit ileum caused by glucocorticoids is accompanied by an increase in NHE3 mRNA, but not message of NHE1 or NHE2 (Yun *et al.* 1993*a*). Using an anti-NHE3 antibody, Biemesderfer *et al.* (1993) showed that NHE3 is expressed in the brush-border membrane of renal proximal tubule cells. Our group has recently shown by immunohistochemistry that NHE3 is present in brush border of villus epithelial cells and not goblet cells in human jejunum, ileum, ascending and descending colon and rectum (Hoogerwerf *et al.* 1994). It is also present in the crypt epithelial cell brush border in small amounts. In rabbit jejunum and ileum, NHE3 was present in the brush border of epithelial cells.

K_is of amiloride and EIPA of NHE3 expressed in PS120 fibroblast are 39 and 8 μM, respectively (Tse *et al.* 1993*a*). Therefore, NHE3 is about 39- and 400-fold more resistant to amiloride and EIPA, respectively, than NHE1.

NHE4

NHE4 has been identified only in rat. NHE4 is a unique Na–H exchanger isoform which exhibits no amiloride-sensitive Na^+ uptake under basal or acid-loaded isoosmotic conditions, but is activated only under hyperosmotic conditions based on a preliminary report (Bookstein *et al.* 1993). Again based on preliminary studies, immunohistochemistry using polyclonal anti-NHE4 antibody, it is found to be localized to the basolateral membranes of inner medullary tubules (Depaloi *et al.* 1993).

Primary and secondary structures

All the mammalian Na–H exchanger isoforms are similar in primary structure (NHE1: 815–820 a.a.; NHE2: 809–813 a.a.; NHE3: 831–832 a.a.; NHE4: 717 a.a.) and the predicted secondary structure. Using the algorithms of Kite and Doolittle, and Engleman, all the Na–H exchangers are predicted to have two structurally and functionally distinct domains: a N-terminal domain consisting of 10–12 putative transmembrane helices, and a long cytoplasmic C-terminal domain. Although the presence of 12 putative transmembrane helices have been predicted in several epithelial exchangers and co-transporters, including the Cl^-–HCO_3^- exchanger, the Na^+-dependent and -independent glucose transporters, and the Na^+–Ca^{2+} exchanger, no homologous features have been identified with the NHE isoforms. Only limited information so far is available on the topology of the Na–H exchangers. Antibody studies confirm the topology that the C-termini for NHE1, NHE2 and NHE3 are intracellular, based on the requirement for membrane permeabilization for labelling by antibody. The N-terminus and the first transmembrane helix are predicted to be a signal peptide which is cleaved off in the intact proteins. Although a single putative N-linked glycosylation site is present in extracytoplasmic loop D in all isoforms, this site does not appear to be glycosylated.

Conservation of amino acid residues among the membrane-associated N-terminal domains is much higher than that among the cytoplasmic domains (50–60 *vs.* 20–23%). The hydropathy profiles and sequence comparisons have led to a proposal that the membrane-bound N-terminal domain of the Na–H exchanger contains 12 transmembrane α-helical spans. Of these, M5A and M5B are most conserved, suggesting that this region may be of importance in either binding or transport of extracellular or intracellular H^+. Replacement of a conserved amino acid E262 in putative M5B of NHE1 resulted in a non-functional Na–H exchanger without affecting expression of the protein (Fafournoux *et al.* 1994). Functional significance of these putative transmembrane helices is not yet known, with the exception of M4 which has been identified as a binding site for amiloride and its 5′-amino substituted analogues (Counillon *et al.* 1993).

NHE1 is a N-linked glycoprotein. Treatment of NHE1 protein expressed in fibroblast with N-glycosidase F (endoglycosidase F) reduces the molecular mass from 110 to 90 kD (Sardet *et al.* 1990). Insensitivity of NHE1 to endo-glycosidase H indicates that the glycosylation of NHE1 is of the high mannose complex type. NHE1 contains three consensus sequences for N-linked glycosylation at Asn75, -370 and -410. Site-specific mutation of all three potential N-glycosylation sites showed that only the first site (Asn75) is utilized. In addition, treatment of NHE1 with

N-glycosidase F followed by neuraminidase and O-glycanase resulted in a further shift in the molecular mass to 82 kD, indicating that NHE1 also contains O-linked glycosylation (Counillon *et al.* 1994). Unlike NHE1, NHE2 contains one potential N-linked glycosylation site at Asn350 which is conserved among NHE1–4. However, NHE2 is not N-glycosylated, since it is resistant to PNGase F and endoglycosidase H (Tse *et al.* 1994*c*). In contrast, neuraminidase treatment followed by O-glycanase shifted the molecular mass of NHE2 from 85 to 75 kD, indicating that NHE2 is an O-linked glycoprotein. On the other hand, NHE3 appears neither N- nor O- glycosylated based on its insensitivity to N-glycosidase F, neuraminidase and O-glycanase. State of glycosylation of NHE4 is not currently known. To date, no functional consequence of glycosylation of the cloned Na–H exchangers has been determined.

Deletion studies of the cytoplasmic domain of NHE1 (Wakabayashi *et al.* 1992) and NHE3 (Levine *et al.* 1994) showed that the N-terminal membrane-spanning domain alone is sufficient to carry out amiloride-sensitive Na–H exchange, although at a much slower rate than intact proteins, and is the site for the H^+-modifier site since the allosteric nature of the exchanger is preserved. Furthermore, the complete deletion of the cytoplasmic domain abolished growth factor/protein kinase-induced regulation of the exchanger and caused a marked shift in the pK value for cytosolic H^+ to the acidic range. Thus these studies indicate the Na–H exchangers can be divided into two structurally and functionally distinct domains.

All isoforms appear to consist of a single subunit based on complementation of Na–H exchange activity in an exchanger-deficient cell (Sardet *et al.* 1989; Tse *et al.* 1991; Tse *et al.* 1993*a*; Orlowski, 1993; Tse *et al.* 1993*b*; Wang *et al.* 1993). A number of findings, including kinetic studies and radiation inactivation analysis, suggest Na–H exchangers exist as oligomers. In addition, Fafournoux *et al.* (1994) has recently demonstrated that NHE1 and NHE3 form homodimers *in vivo* in fibroblasts, and that the N-terminal membrane domain is sufficient for dimerization. It should be noted that NHE1 and NHE3 do not form heterodimers, indicating that dimerization requires sequences intrinsic to each isoform. Whether this absence of heterodimerization is universal among all Na–H exchangers is not known. It will be of interest to find out whether NHE2 and NHE3, both which are present on the apical membrane in some cells, form a heterodimer. Despite the existence of oligomeric forms, it remains to be determined whether the functional unit of the mammalian Na–H exchanger is a monomer or an oligomer.

Kinetics

The kinetic nature of Na–H exchangers in cells and tissues has previously been described, and we will focus here on the kinetics of the cloned mammalian Na–H exchangers. The cloned Na–H exchangers have been studied in Na–H exchanger-deficient cells such as Chinese hamster lung fibroblast cells (PS120), Chinese hamster ovary cells (AP-1) or mouse fibroblast cells (LAP). Expression of a Na–H exchanger in procaryotes or non-mammalian eucaryotes has so far shown limited success. We largely utilize PS120 fibroblast cells and the human colonic carcinoma cell line, CaCo-2, for functional studies of stably transfected NHEs.

The cloned exchangers show similar Na-dependent pH recovery following an acid load. The kinetics for external Na^+ follow a classical Michaelis–Menten model with a Hill coefficient of 1, suggesting there is a single binding site for external Na^+. All isoforms of rabbit NHEs (NHE1–3) expressed in PS120 show similar K_m (Na^+) (15–18 mM). On the other hand, rat NHEs expressed in AP-1 cells exhibit differences between isoforms. NHE1 in these cells had a 2-fold lower K_m (Na^+) (4·6 mM) than that of NHE3 (10 mM). The reason for this discrepancy in K_m (Na^+) is not known, although it may be due to different measurement techniques (i.e. spectrofluorometry *vs.* $^{22}Na^+$ uptake), the variation in host cell system or species variation in NHEs.

The presence of extracellular cations including H^+, Li^+ and NH_4^+ has been shown to inhibit H_i^+-dependent Na^+ influx. In AP-1 cells, external cations such as Li^+ and H^+ compete with external Na^+ for the single binding site to inhibit NHE1 and NHE3. However, external K^+ shows a differential effect on NHE1 and NHE3; external K^+ inhibits H_i^+-dependent Na^+ influx by NHE1 but it has no effect on NHE3, suggesting a structural difference between the external cation binding site for NHE 1 and that of NHE3.

The kinetics for H^+ are also similar for the three cloned exchangers with an asymmetric nature of intracellular *versus* extracellular H^+. One important kinetic feature of Na–H exchangers is that while external H^+ follows a Michaelis–Menten model with a single binding site, the kinetics of internal H^+ deviates from the classical Michaelis–Menten kinetics with a Hill coefficient of 2–3. The data describing the effect of intracellular H^+ best fit an allosteric model with at least two independent binding sites. It is thought that in addition to the H^+ transport site, there is an internal H^+-modifier site at which binding of H^+ turns on the operation of Na–H exchange.

The deletion studies of the cytoplasmic domain of NHE1 (Wakabayashi *et al.* 1992) and NHE3 (Levine *et al.* 1994) showed that the cation binding sites are located within the N-terminal membrane-spanning domain. Complete removal of the cytoplasmic domain of the exchanger maintained the allosteric nature of the exchanger, suggesting that the proton modifier site is also located within the N-terminal domain.

Regulation

Growth factor-induced regulation

The differential response to growth factors by the cloned Na–H exchanger isoforms expressed in fibroblasts has previously been described in detail (Levine *et al.* 1993; Tse *et al.* 1993; Tse *et al.* 1994*b*) and, therefore, will not be

reviewed in detail. However, the biochemical nature of the growth factor-dependent regulation of the Na–H exchanger is just being uncovered, largely due to the works by Pouyssegur and his co-workers on NHE1. It has been shown that the stimulation of NHE1 by growth factors/proteins kinases (thrombin, EGF, PMA and okadaic acid) is partially through an increase in phosphorylation (Sardet *et al.* 1990; Sardet *et al.* 1991). Phosphorylation of NHE1 on serine residues but not on threonine or tyrosine residues by these growth factors and the increase in phosphorylation of a single phosphopeptide suggests convergence of the signal transduction pathways on a common activating kinase. Involvement of MAP kinase (mitogen-activated protein kinase) (Sardet *et al.* 1990) or a kinase associated with the exchanger (NHE1 kinase) (Sardet *et al.* 1991), perhaps similar to the regulation of the β-adrenergic receptor by a receptor-related kinase, has been suggested.

Deletion studies on NHE1 by Pouyssegur and his co-workers have shown that the cytoplasmic C-terminal domain is essential for the growth factor-induced regulation of NHE1 (Wakabayashi *et al.* 1992, 1994). In addition, it was recently described that the chimeric Na–H exchanger made between the N-terminal domain of cyclic AMP-insensitive human NHE1 and the C-terminal domain of cyclic AMP-activatable βNHE isoform from trout red blood cells became cyclic AMP activatable. This showed that the cytoplasmic portion of Na–H exchanger determines the nature of regulation (Borgese *et al.* 1994). In the case of NHE1, growth factor-induced phosphorylation sites were found in the region of a.a. 636–815. However, deletion of this major phosphorylation site failed to abolish the growth factor-induced activation of NHE1, but reduced the magnitude of the regulation by 50%. In contrast, further deletion of a region between a.a. 567 and 635 completely obliterated high pH_i sensitivity and the activation of NHE1 in response to serum, thrombin, platelet-derived growth factor, okadaic acid (OA) and phorbol ester, although the increase in phosphorylation in response to these mitogenic signals was preserved. Therefore, growth factor-induced activation of NHE1 occurs at least in part by a mechanism that does not involve direct phosphorylation of the exchanger. It is postulated that the phosphorylation independent activation of NHE1 is mediated through an interaction of the region of a.a. 567–635 (known as the regulatory domain) with an 'accessory or regulatory' protein which then interacts with the H^+-sensor located within the N-terminal membrane domain (Wakabayashi *et al.* 1994). The presence of such a accessory protein has been suggested in cyclic AMP-dependent inhibition of the native renal brush border Na–H exchanger (Weinman & Shenolikar, 1986; Weinman *et al.* 1993). In addition, NHE1 is co-localized in fibroblasts with adhesion plaques necessary for PKC-dependent activation making cytoskeletal elements potential candidates as accessory proteins that can mediate extracellular signals in Na–H exchanger regulation (Schwartz & Lechene, 1992).

The model of a single regulatory domain for NHE1, however, has not been confirmed in all reports. A study by Winkel *et al.* (1993) using microinjection of antibody directed at the region of a.a. 658–815 of NHE1 found that this antibody blocked regulation by endothelin-1 and α-thrombin, but that protein kinase C and osmotic-induced activation, as well as pH sensing, were mediated via a separate, more proximal region of the cytoplasmic tail. In addition, while βNHE with the putative PKA sites deleted is no longer activated by cyclic AMP and its analogues or catecholamines, α-thrombin and phorbol esters stimulated both the mutated form and the wild-type βNHEs, suggesting that there are separate regions in this exchanger, with at least one region for cyclic AMP and one for PKC (Borgese *et al.* 1992).

The importance of phosphorylation in the growth factor-induced regulation of NHE1 remains unclear. Although a 50% reduction in the extent of growth factor-induced activation of NHE1 upon deletion of the major phosphorylation area suggests the presence of a phosphorylation-dependent regulatory pathway, its direct role in the activation awaits further studies including identification of the specific phosphorylation sites. The direct requirement of phosphorylation in regulation has been shown for cyclic AMP-activatable βNHE isoform (Borgese *et al.* 1994). It was shown that site-directed mutation of two putative protein kinase A (PKA) consensus sites of the cytoplasmic domain reduced the extent of cyclic AMP-induced activation by 72%. We have recently determined that NHE3 is a phosphoprotein, although the effect of phosphorylation in its regulation is not known. Whether other mammalian Na–H exchanger isoforms, NHE2 and NHE4, are phosphoproteins and whether their regulation is phosphorylation dependent has not been addressed.

We have recently generated a series of C-terminal deletion mutants of NHE3 to identify specific regions of the C-terminus responsible for growth factor-induced regulation (Levine *et al.* 1994). These studies demonstrated that there are two separate domains within the cytoplasmic tail of NHE3, one involved in stimulation and one in inhibition. The domain between a.a. 455 and 585 is responsible for the stimulation by fibroblast growth factor (FGF), OA and serum, whereas the domain between a.a. 586 and 832 is necessary for inhibition by PMA and calmodulin. Moreover, within the stimulatory and inhibitory domain, there are discrete subdomains for each of these growth factors and kinases. The presence of discrete subdomains for individual growth factors and protein kinases is a major difference from NHE1 which is postulated to contain a single regulatory domain and a phosphorylation domain (Wakabayashi *et al.* 1994).

The nature of the interaction of the cytoplasmic tail of NHE3 with the N-terminal membrane domain is unknown, although it was previously shown for NHE3 that growth factor-induced regulation does not affect

affinity for H_i^+, but alters only V_{max} of the exchanger (Levine *et al.* 1993). The V_{max} effect is due to either insertion or removal of pre-existing exchangers from the membrane or change in turnover number as a result of interaction of the cytoplasmic tail with effector sites on the membrane-spanning domain.

Regulation by cell shrinkage

Na–H exchange is thought to play a role in the process of Na^+ uptake by osmotically shrunken cells (Grinstein *et al.* 1985). In some cells, osmotic shrinkage stimulates Na–H exchange (Grinstein *et al.* 1985; Grinstein *et al.* 1986). NHE1 expressed in fibroblasts is activated by hyperosmolarity (Grinstein *et al.* 1992). Although this activation is ATP dependent, the phosphorylation state of NHE1 was not affected during this activation.

We have recently determined that hyperosmolarity inhibits NHE2 and NHE3 expressed in PS120 fibroblasts (Nath *et al.* 1994). The inhibition of NHE3 by cell shrinkage has also been reported in cultured renal proximal tubule cells, LLC-PK_1 and OK (Soleimani *et al.* 1994), Chinese hamster ovary cells (AP-1) transfected with rat NHE3 (Kapus *et al.* 1994), and the medullary thick ascending limb (Watts & Good, 1994). Mechanistically, hyperosmolarity significantly reduced V_{max} of NHE2 and NHE3 but did not alter the K_m for intracellular H^+ and the hyperosmolarity-induced inhibition is reversible. It is noteworthy that studies using truncated mutants have shown that the hyperosmolar effects on the truncated NHE3s were similar to that of the wild type NHE3, suggesting that elements necessary for the hyperosmolarity-induced inhibition of NHE3 is located within the N-terminal membrane-spanning domain (Nath *et al.* 1994). The molecular mechanism and the signalling pathway of the hyperosmolar effects on Na–H exchange is not known. Current postulated mechanisms for the hyperosmolar effect are by inducing change in the physicochemical property of the cell membrane, concentration of intracellular solutes or cytosolic macromolecules or G proteins (Garner & Burg, 1994). Involvement of protein kinase C (Grinstein *et al.* 1986), Ca^{2+}/CaM-dependent kinase (Dascalu *et al.* 1992; Chinet, 1993), and phosphorylation-independent mechanisms (Grinstein *et al.* 1992) have been suggested. However, we have found that protein kinase C, tyrosine kinase, elevation of Ca^{2+}, cyclic AMP, and cytoskeletal elements including inhibition of microtubules and actin did not affect the hyperosmolarity-induced NHE3 inhibition (Nath *et al.* 1994).

Interestingly, rat NHE2 isoform expressed in AP-1 cell was activated by hyperosmolarity (Kapus *et al.* 1994). The reason for the discrepancy between rabbit NHE2 expressed in PS120 fibroblasts and rat NHE2 expressed in AP-1 ovary cells is not known. This difference is unlikely to be due to the variation between rabbit and rat isoforms since the degree of homology is 94%. However, it is conceivable that the host cell types determine the response by, at least, NHE2 to hyperosmotic stress.

Activation of NHE1 by cell shrinkage is thought to underlie volume regulation. Hyperosmolarity-induced inhibition of NHE3 has been postulated to be relevant in regulating renal acid–base handling. Relevant clinical examples of a putative role for hyperosmotic regulation of NHEs include response to pathological conditions in which serum hyperosmolarity occurs, such as high blood sugar, advanced renal failure, and toxicity from ingestion of methanol and polyethylene glycol (Soleimani *et al.* 1994). Inhibition of NHE3 may also have a relevant role in preventing cell volume increase or rise in intracellular Na^+ under circumstances of increased Na^+ coupled absorption of glucose/galactose and amino acids (MacLeod & Hamilton, 1990).

Effects of ATP depletion

Depletion of ATP drastically reduces Na–H exchange activity, although Na–H exchangers are not known to hydrolyse ATP. ATP depletion has qualitatively similar effects on NHE1–3 expressed in fibroblasts resulting in drastic decreases in V_{max} and in affinity for intracellular H^+, the effect being most profound in NHE3 (Levine *et al.* 1993). In addition, Hill coefficients for all three isoforms decreased from 2 to about 1, such that the kinetic profile with respect to intracellular H^+ then fits Michaelis–Menten kinetics. These observations were interpreted such that under ATP-depleted conditions, the H^+-modifier site can be separated from H^+ transport site, and the H^+-modifier site of the Na–H exchangers is likely to be phosphorylated or require an ATP-dependent process to function. It is noteworthy that Wakabayashi *et al.* (1992) also reported a decrease in H^+ affinity by ATP depletion in NHE1 but there was still co-operativity of intracellular H^+. Their observation that the H^+-modifier site does not require phosphorylation or an ATP-dependent process is in contradiction with our studies.

Recently, Kapus *et al.* (1994), studying rat Na–H exchanger isoforms expressed in AP-1 ovary cells, observed the inhibitory effects of ATP depletion on rat NHE1, NHE2 and NHE3. Although their results are qualitatively in agreement with ours, their interpretation is that the mechanism of inhibition differs among the isoforms. ATP depletion reduced the V_{max} of NHE1 and NHE2, and also decreased the affinity for intracellular H^+, as we had seen earlier. In contrast, inhibition of NHE3 was largely attributed to a decrease in V_{max}, with no observable change in H^+ affinity.

It is interesting that a shift in pH_i dependence in deletion mutants of NHE1 resembles the effect of ATP depletion. Moreover, the ATP depletion effect was absent in the truncated NHE1 mutant, suggesting that the ATP-sensitive motif is located in the cytoplasmic tail of NHE1 (Wakabayashi *et al.* 1992; Kapus *et al.* 1994). It is, therefore, thought that depletion of ATP alters the conformation of the cytoplasmic tail by reducing the phosphorylation state of NHE1 so as to decrease the

affinity for intracellular H^+ at the H^+-modifier site. However, Goss *et al.* (1994) recently reported that ATP depletion did not cause any noticeable change in phosphorylation in NHE1, ruling out dephosphorylation of the exchanger as being involved in changes of Na–H exchanger. Nonetheless, one cannot rule out dephosphorylation of an accessory protein(s) that is required for optimal activity of the exchanger, although such a protein is yet to be identified. Other potential causes of the inhibition by ATP depletion are a disturbance of phospholipid asymmetry and rearrangement of cytoskeletal elements including F-actin (Kapus *et al.* 1994).

Structure/function studies of cloned Na–H exchangers are just beginning. Applying this analysis to dissecting their involvement in normal physiological function and in pathobiology of disease promises an opportunity to move molecular transport to a new dimension.

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