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Na^+/H^+ ANTIPORT: MODULATION BY ATP AND ROLE IN CELL VOLUME REGULATION

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Summary

Na^+/H^+ antiport is a major determinant of intracellular pH (pHi) and also plays an important role in the maintenance of cellular volume. Na^+/H^+ exchange through NHE-1, the ubiquitous isoform of the antiporter, is accelerated by cytosolic acidification and also by osmotically induced cell shrinking, thereby promoting recovery of the physiological pHi and volume, respectively. Although hydrolysis of ATP is not required for transport of ions through the antiporter, metabolic depletion exerts a marked inhibitory effect. Depletion of ATP also prevents osmotic activation and volume regulation. Contrary to earlier suggestions, however, changes in the phosphorylation state of the antiporter itself are not involved in the effects of either metabolic depletion or osmotic stimulation. Nevertheless, the cytosolic carboxy-terminal segment of the antiporter, which contains the major phosphorylation sites, is essential for the ATP dependence as well as for osmotic activation. It is conceivable that this domain interacts with ancillary phosphorylated or nucleotide-binding proteins, with the cytoskeleton and/or with specific phospholipids, which modulate the rate of transport.

Nucleotide dependence and osmotic sensitivity have been compared in three different isoforms of the antiporter, heterologously expressed in fibroblastic cells. Like NHE-1, NHE-2 and NHE-3 were severely inhibited by depletion of ATP. In contrast, whereas NHE-2 was stimulated by osmotic shrinkage, NHE-3 was inhibited. The possible physiological significance of the ATP-dependence and osmotic responsiveness of the antiporter isoforms is discussed.

Introduction

In animal cells, acid equivalents are continuously generated as a byproduct of a number of metabolic pathways. Consequently, the cytosol tends to become progressively acidified. This tendency is further compounded by the passive uptake of extracellular acid equivalents, driven by the membrane potential, which is negative inside the cells. Protons must therefore be actively extruded from the cells in order to maintain the cytosolic pH (pHi) within the physiological range. This task is performed in part by the Na^+/H^+ antiporter (NHE), also called exchanger, that catalyzes the exchange of extracellular Na^+ for intracellular H^+.

Key words: antiporter, Na^+/H^+ exchange, pH regulation, volume regulation, ATP dependence, phosphorylation.

Under physiological conditions, the antiporter facilitates the uptake of extracellular Na^+ with concomitant extrusion of cytosolic H^+, a process that is effectively blocked by amiloride and its 5-N-alkylated derivatives (for reviews, see Grinstein *et al.* 1989; Wakabayashi *et al.* 1992*b*). Despite the large inward Na^+ gradient established by the Na^+/K^+-ATPase, the rate of Na^+/H^+ exchange is dictated primarily by the intracellular pH. This pronounced sensitivity of the antiporter to pHi has been attributed to the existence of an allosteric modifier site that controls the rate of countertransport (Aronson *et al.* 1982; Aronson, 1985). When protonated, the modifier site activates cation exchange, protecting the cytosol against excessive acidification. As pHi approaches neutrality, deprotonation of the allosteric site curtails the activity of the antiporter, preventing alkalization of the cytosol beyond the physiological set point. Thus, the intracellular H^+ concentration is the primary determinant of the rate of Na^+/H^+ exchange, a finding consistent with a major role for the antiporter in pHi homeostasis. However, Na^+/H^+ antiporters have additionally been implicated in other cellular functions. Because they are stimulated by a variety of growth promoters, a role in the initiation and/or control of mitogenesis has been proposed (reviewed by Moolenaar, 1986; Grinstein *et al.* 1989). In addition, Na^+/H^+ exchange is believed to be central to the maintenance and regulation of cell volume.

The predominant antiporter of non-epithelial cells, designated NHE-1 because it was the first one to be identified, is an integral membrane phosphoglycoprotein of approximately 110 kDa (Sardet *et al.* 1989, 1990). Hydropathy analysis of its primary structure suggests that the antiporter spans the membrane 10–12 times and has, in addition, a sizable hydrophilic tail, which is thought to be cytosolic, on the basis of immunochemical data (Sardet *et al.* 1989, 1990). The transmembrane region is concentrated in the amino-terminal domain of the protein, whereas the carboxy terminus constitutes the cytosolic tail. Mutational analysis has shown that the transport site of NHE-1 resides, as anticipated, in the amino-terminal transmembrane domain of the protein (Wakabayashi *et al.* 1992*a*,*b*). This portion of the protein similarly retains the amiloride-binding site as well as the intracellular modifier site, as deduced from the steep pHi-dependence of truncated constructs lacking the last carboxy-terminal 300 amino acids (Wakabayashi *et al.* 1992*a*,*b*).

The existence of additional isoforms of the antiporter, distinct from NHE-1, was initially postulated on the basis of the differential substrate and inhibitor affinities of basolateral and apical antiporters of epithelial cells (Haggerty *et al.* 1988). This prediction was recently borne out by the cloning of three new members of a gene family, termed *NHE-2*, *NHE-3* and *NHE-4*, which are structurally related to *NHE-1* (Orlowski *et al.* 1992; Tse *et al.* 1992, 1993*c*; Orlowski, 1993). Heterologous expression in antiporter-deficient mutants has confirmed the ability of the NHE-2 and NHE-3 gene products to catalyze Na^+/H^+ exchange, but similar evidence for NHE-4 is still lacking. The approximate size and transmembrane topology of NHE-2, NHE-3 and NHE-4 closely resemble those of NHE-1 (Fliegel and Frolich, 1993; Tse *et al.* 1993*a*). Nevertheless, the isoforms differ significantly in their primary structure, particularly near their cytosolic carboxy termini, in their tissue distribution and in their pharmacological profile (Fliegel and Frolich, 1993; Tse *et al.* 1993*a*).

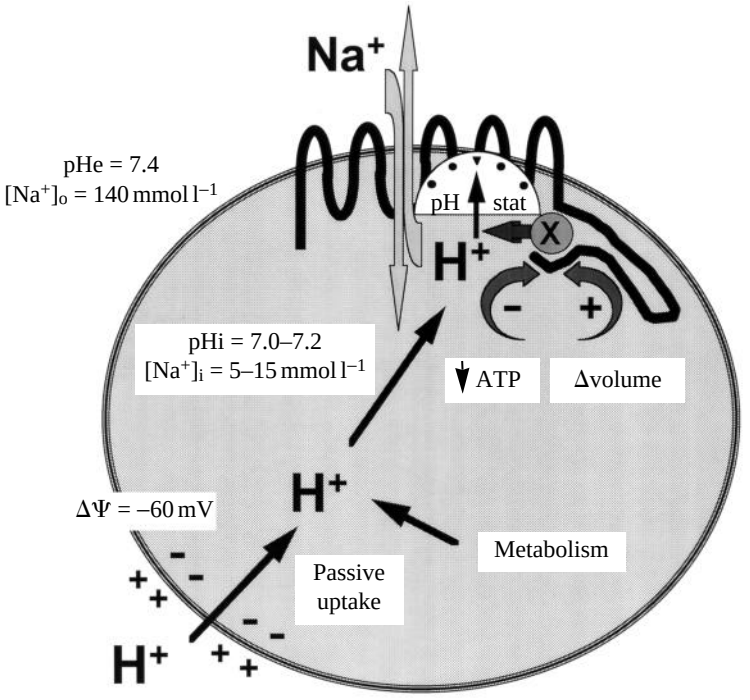

Fig. 1. Regulation of Na^+/H^+ exchange by ATP and by cell volume. The scheme illustrates the role and regulation of Na^+/H^+ exchange. The cytosol of animal cells tends to become progressively acidified as a result of both metabolic acid generation and passive H^+ uptake across the plasma membrane, down the electrochemical gradient. This acid can be extruded by the Na^+/H^+ antiporter, which catalyzes the equimolar exchange of extracellular Na^+ for intracellular H^+. The activity of the antiporter is under the control of an allosteric H^+ binding site, the 'pH stat', which activates cation exchange only when the intracellular pH (pHi) falls below the physiological set point. The effects of ATP depletion, which inhibits exchange activity, and of a decrease in cell volume, which stimulates exchange activity, are both through changes in the set point of the pH stat. These effects are not meditated by phosphorylation of the antiporter itself, but require the presence of the cytosolic carboxy-terminal segment of the antiporter. We postulate the existence of an additional component X, whose nature remains to be identified, that interacts with the cytoplasmic tail and modulates the rate of transport. pHe, extracellular pH.

The existence of multiple isoforms of the antiporter, combined with their functional versatility, precludes the compilation of a comprehensive review of NHE structure and function in the limited space available here. We have instead chosen to concentrate on two aspects of the function and regulation of the antiporter, namely its paradoxical ATP-dependence and its activation by osmotically induced volume changes. Updated overviews of these topics are presented separately below. The findings are summarized in Fig. 1.

ATP-dependence of Na^+/H^+ exchange

Functional observations and structural correlates

Flux through the antiporter is electroneutral and is driven by the combined chemical gradients of Na^+ and H^+. Metabolic energy is not directly expended during the transport event. The notion that Na^+/H^+ exchange is an ATP-independent process was derived from early measurements in isolated plasma membrane vesicles. In these studies, epithelial brush-border vesicles, which had been thoroughly washed and were therefore presumably devoid of ATP, still demonstrated Na^+/H^+ exchange activity (Murer *et al.* 1976; Kinsella and Aronson, 1980). Thus, the cation transport process *per se* can occur in the nominal absence of ATP. However, in intact cells, ATP appears to be required for optimal Na^+/H^+ exchange, since the activity of the antiporter is greatly diminished by procedures that reduce the intracellular levels of the nucleotide. Following the initial report of this effect in rat thymic lymphocytes (Grinstein *et al.* 1985*a*), the inhibition of Na^+/H^+ exchange activity by ATP depletion has been consistently found in a variety of cellular systems, as well as in antiporter-deficient cells stably transfected with selected isoforms of the antiporter. A summary of these studies is presented in Table 1. It is noteworthy that in all cases the intracellular pH was measured in both control and ATP-depleted cells and was similar. Alternatively, an identical pHi was imposed and maintained throughout the experiment in the untreated as well as in the depleted cells. Thus, the effect of nucleotide depletion on the rate of transport was not mediated by a change in pHi. In at least some of the studies, care was also taken to minimize changes in the transmembrane Na^+ gradient. Therefore, alterations to the concentration of the substrate and/or regulatory ions cannot account for the observed changes in the rate of countertransport. Hence, a modification of the intrinsic properties of the antiporters must have occurred.

To elucidate the mechanism responsible for inhibition of exchange upon ATP depletion, several transport parameters have been compared in intact and depleted cells. The pHi-dependence of the rate of Na^+/H^+ exchange following metabolic inhibition has been analyzed in some detail in seven studies. In all cases, ATP depletion reduced the apparent affinity of the antiporter for intracellular H^+, shifting its H^+-sensitivity by approximately 0.5 pH units. In addition, in five studies metabolic depletion also reduced the maximal rate of exchange (V_{max}). However, V_{max} was unchanged in the remaining two studies. It must be noted that accurate extimates of the V_{max} are complicated by limitations in the range of $[H^+]$ that can be explored without inflicting cell damage. The effect of ATP depletion on the allosteric activation of the antiporter by protons is more controversial. Some authors have reported a decrease in cooperativity, whereas most other reports suggest increased cooperativity. These inconsistencies may stem from the use of different techniques for ATP depletion, pH-clamping and measurement of Na^+ or H^+ fluxes. Thus, it is at present difficult to define conclusively the effect of metabolic inhibition on the allosteric 'modifier' site. Nevertheless, the claim made by Levine *et al.* (1993) that ATP depletion reduces the number of H^+ binding sites and allows the functional differentiation of the H^+ substrate site from the H^+-modifier site is attractive and warrants further investigation.

In conclusion, ATP depletion appears to modify drastically the pHi-sensitivity of the

Table 1. *Summary of the effects of ATP depletion on NHE function*

Cell type	Isoform(s)	Technique	ATP depletion time (min)	Affinity for H^+	V_{max}	Reference
Thymic lymphocytes (rat)	?	$^{22}Na^+$ flux	20[a]	ND	ND	Grinstein *et al.* (1985*b*)
A431 carcinoma cells (human)	?	$^{22}Na^+$ flux	30[b]	Decreased	Unchanged	Cassel *et al.* (1986)
Primary aortic smooth muscle (adult rat)	?	$^{22}Na^+$ flux	30[c]	Decreased	Reduced	Little *et al.* (1988)
Primary cardiocytes (neonatal rat)	?	pHi measurement	30[d]	ND	ND	Weissberg *et al.* (1989)
LLC-PK1 renal epithelial cells (pig)	?	$^{22}Na^+$ flux	60[e]	Decreased	Reduced	Burns *et al.* (1991)
Transfected PS120 fibroblasts (hamster)	NHE-1 (human)	$^{22}Na^+$ flux	30[b]	Decreased	Unchanged (reduced 14%)	Wakabayashi *et al.* (1992*a*)
Transfected PS120 fibroblasts (hamster)	NHE-1, NHE-2 or NHE-3 (rabbit)	pHi measurement	30[b]	Decreased (all isoforms)	Reduced (all isoforms)	Levine *et al.* (1993)
Transfected PS120 fibroblasts (hamster)	NHE-1 or truncated mutants (human)	$^{22}Na^+$ flux	7[f]	Decreased (full-length clone)	Reduced (all clones)	Goss *et al.* (1994)
Transfected CHO cells (hamster)	NHE-1, NHE-2 or NHE-3 (rat)	$^{22}Na^+$ flux	10[f]	Decreased (NHE-1 and NHE-2)	Reduced (all isoforms)	Kapus *et al.* (1994)

[a]Antimycin; [b]DOG + oligomycin; [c]DOG; [d]DOG + ouabain; [e]DOG + rotenone; [f]DOG + antimycin.
ND, not determined; DOG, deoxyglucose.

antiporter, shifting it to more acidic values. Because the pHi-dependence of the antiporter is dictated chiefly by its modifier site, the latter is presumably the prime target of metabolic inhibition. However, other modifications are also likely to occur, to the extent that V_{max} is seemingly also reduced. Because both the transport and modifier sites have been tentatively assigned to the transmembrane amino-terminal moiety of the protein, the effects of ATP depletion would be expected to persist in mutants with carboxy-terminal truncations. This simplistic prediction, however, was not fulfilled. The mutant lacking virtually the entire cytosolic 'tail' (Δ*515*) had functional properties similar to those of the wild-type antiporter measured in ATP-depleted cells: its pHi-sensitivity was shifted by 0.5 pH units towards more acidic values and its V_{max} was reduced (Wakabayashi *et al.* 1992*a*). The decreased V_{max} must be regarded with reservation, inasmuch as the transport rates could not be normalized per amount of functional plasmalemmal antiporter. Nevertheless, the finding that the pHi-sensitivity was altered by the truncation suggested that the cytoplasmic domain may be involved in the ATP-dependence of the antiporter. This notion was corroborated by a more recent study (Goss *et al.* 1994), where the ATP-dependence of Na^+/H^+ exchange was directly investigated in a series of mutants with progressively greater carboxy-terminal truncations. The pronounced inhibition of exchange observed following metabolic depletion in the wild-type NHE-1 was diminished in the Δ*635* mutant and was essentially insignificant in the Δ*566* mutant.

These experiments indicate that the cytosolic tail plays a role in mediating the effect of ATP on the rate of transport. However, it has not been possible to attribute this feature to a particular sequence within this carboxy-terminal region. In fact, similar effects of ATP depletion on transport have been reported in recent studies of cells transfected with *NHE-2* and *NHE-3* (Levine *et al.* 1993; Kapus *et al.* 1994). As summarized in Table 1, decreased H^+ affinity and V_{max} were described for both isoforms following metabolic inhibition. As mentioned earlier, the primary structure of these isoforms shows divergence from NHE-1, particularly in the cytosolic domain. Therefore, either ATP-dependence is conferred to all the isoforms by a short conserved motif or, more likely, no unique primary sequence is required for this effect.

Mechanism of ATP-dependence

What is the molecular mechanism underlying the ATP-dependence of the antiporter? As discussed earlier, studies performed in isolated membrane vesicles as well as thermodynamic considerations indicate that hydrolysis of ATP is not required to fuel the transport cycle. Direct modulation involving binding of ATP to the antiporter also seems unlikely, as consensus nucleotide-binding sites, such as the Walker motifs, are not identifiable in the sequences of the four NHE isoforms studied to date (Sardet *et al.* 1989; Orlowski *et al.* 1992; Wang *et al.* 1993). Therefore, alternative mechanisms must be postulated to explain the effects of metabolic inhibition.

A simple, original hypothesis postulated that the ATP effect involves changes in the phosphorylation of the antiporter. This hypothesis was supported by the following observations. (a) The state of phosphorylation dictates, or is at least associated with, changes in the Na^+/H^+ exchange activity of NHE-1 and of the equivalent trout isoform (Sardet *et al.* 1990, 1991; Wakabayashi *et al.* 1992*b*). Similar conclusions have been

inferred for NHE-2 and NHE-3, whose activity is also modulated by protein kinase agonists (Fliegel and Frolich, 1993; Tse *et al.* 1993*c*). (b) The antiporter has been shown to be constitutively phosphorylated (Sardet *et al.* 1990, 1991; Goss *et al.* 1994). Indeed, multiple phosphorylation sites have been identified by phosphopeptide mapping in NHE-1 isolated from unstimulated, serum-deprived cells (Sardet *et al.* 1991; Grinstein *et al.* 1992; Goss *et al.* 1994). It was therefore hypothesized that constitutive phosphate groups may be an important determinant of the activity of the antiporter (Wakabayashi *et al.* 1992*b*). Conceivably, such phosphate moieties may be lost upon depletion of ATP as a result of endogenous phosphatase activity, thereby providing a rationale for the inhibitory effect of metabolic depletion.

Recent findings, however, contradict this hypothesis, since (a) phosphorylation of NHE-1 was found to be similar in control and ATP-depleted fibroblasts, under conditions where a concomitant inhibition of exchange could be demonstrated (Goss *et al.* 1994); there were no detectable differences in the NHE-1-derived phosphopeptide maps obtained from control and depleted cells, ruling out dephosphorylation of one site with concomitant phosphorylation of another; (b) a deletion mutant of NHE-1 (Δ*635*), which was found to lack all the major phosphorylation sites, still displayed a normal pHi-dependence (Wakabayashi *et al.* 1994); and (c) phosphorylation-independent, yet ATP-dependent activation, of the antiporter has been reported (see below).

Thus, it appears that some of the early observations that led to the phosphorylation hypothesis must be re-examined. Whereas early studies pointed to a close correlation between phosphorylation and activation by growth promoters (Sardet *et al.* 1990, 1991), recent analyses of deletion mutants (Wakabayashi *et al.* 1994) suggest that phosphorylation may not, after all, be an essential requirement for stimulation of transport. A construct lacking the cytoplasmic region between amino acids 567 and 635 displayed a normal pattern of phosphorylation yet showed no activation of Na^+/H^+ exchange following stimulation. More importantly, a second mutant (Δ*635*) lacking all the major phosphorylation sites responded to stimulation by growth factors with an amiloride-sensitive cytoplasmic alkalization. Although the response was smaller than that of wild-type transfectants, the data nevertheless imply the existence of a phosphorylation-independent mechanism of activation, resembling the reported phosphorylation-independent activation of NHE-1 by changes in cell volume (see below).

Both growth factors and osmotic challenge stimulate the antiporter by inducing a shift in the pHi-dependence of the antiporter towards more alkaline values (Sardet *et al.* 1990; Grinstein *et al.* 1992; see below for details of osmotic effect). Conversely, ATP depletion shifts the pHi-sensitivity of the antiporter in the acidic direction (Table 1). It is unclear whether three distinct states of the antiporter exist (basal, stimulated and depleted), or whether the experimental conditions chosen merely highlight three points of a functional continuum. Nevertheless, it is clear that the transitions between these stages can be attained without changes in the phosphorylation state of the antiporter itself.

The results summarized above suggest that another, as yet unidentified, component mediates the paradoxical ATP sensitivity of the antiporter and possibly also (part of) its activation by growth factors. Several mechanisms can be envisaged.

(a) A protein possessing a nucleotide-binding domain may be involved. Certain ion-

transport proteins can directly bind ATP through nucleotide-binding folds containing the Walker consensus motifs (Mimura *et al.* 1991). In one such protein, the cystic fibrosis transmembrane regulator, nucleotide binding regulates Cl^- fluxes through the channel moiety (Anderson *et al.* 1991; Welsh *et al.* 1992). However, ATP-binding domains are not identifiable in the primary structure of the Na^+/H^+ antiporter. Therefore, if binding of ATP is the determinant of NHE function, additional components containing nucleotide-binding sites must be invoked. Alternatively, a GTP-binding protein might be involved, as the levels of GTP probably decline in parallel with the levels of ATP during most metabolic depletion protocols, including those described in Table 1. Indeed, the involvement of G-proteins in the control of antiporter activity had been previously postulated, on the basis of the effects of exogenous guanine nucleotides and of toxins on the activity of the antiporter (Davis *et al.* 1992).

(b) An auxiliary phosphoprotein may be involved. In this instance, the effect of ATP depletion would reflect changes in the basal phosphorylation state of this ancillary component. At least part of the growth factor response might also depend on enhanced phosphorylation of this protein. This dependence might explain why activation of transport by growth promoters is preserved in the mutants lacking all the major phosphorylation sites.

(c) Intact cytoskeletal assembly may be required for normal antiporter function. According to this hypothesis, ATP depletion would inhibit the antiporter by inducing cytoskeletal rearrangement. In this context, it is noteworthy that ATP depletion has been shown to be accompanied by drastic redistribution of F-actin in several cell types (Wang, 1986; Glascott *et al.* 1987; Gabai *et al.* 1992). However, disruption of microfilaments with cytochalasin D does not mimic the effects of ATP depletion on NHE-1 function. Therefore, disaggregation of actin filaments is insufficient to inhibit the antiporter and more complex cytoskeletal changes must be postulated to control Na^+/H^+ exchange activity.

(d) A specific phospholipid distribution may be essential for the operation of the antiporter. Phospholipid 'flippases' create an asymmetric surface charge in the plasma membrane by preferentially distributing the negatively charged lipids to the cytoplasmic leaflet. This negative cytosolic surface charge, or a particular phospholipid composition of the inner or outer monolayers of the membrane, may be required to stabilize the antiporter molecule in its functional state. Inasmuch as the flippases require ATP to function, the effects of metabolic depletion on Na^+/H^+ exchange may be secondary to changes in lipid distribution.

A similar mechanism is believed to be responsible for the ATP-dependence of another ion transport system, the Na^+/Ca^{2+} antiporter (Hilgemann and Collins, 1992). Although the Na^+/H^+ antiporter and the Na^+/Ca^{2+} antiporter show no structural homology to each other or to any other protein, they share topological as well as functional features. (a) Both antiporters have 10–12 transmembrane regions and a large cytosolic domain. In the Na^+/Ca^+ antiporter, this domain is a loop located between transmembrane regions 5 and 6. (b) Both transport systems are activated allosterically by their intracellular substrate ion (H^+ or Ca^{2+}) and deletion of the cytoplasmic domain does not abolish this effect. (c) More importantly, as is the case with Na^+/H^+ exchange, ATP hydrolysis is not

required for Na^+/Ca^{2+} exchange, yet ATP depletion has a pronounced inhibitory effect. Since the ATP-sensitivity of Na^+/Ca^{2+} exchange has recently been reported to be mediated by a phospholipid flippase (Hilgemann and Collins, 1992), it is tempting to speculate that a similar mechanism applies in the case of Na^+/H^+ exchange.

Na^+/H^+ exchange and cell volume regulation

Even when suspended in iso-osmotic media, animal cells passively gain volume as a result of the accumulation of permeable inorganic ions that are driven into the cytosol by the presence of charged impermeant macromolecules. This tendency to swell is continuously counteracted by the active extrusion of monovalent ions, resulting in the steady-state maintenance of cellular volume. In many tissues, cell volume is regulated even in aniso-osmotic solutions; following rapid swelling in hypotonic solutions, cells regain nearly normal size by rapid loss of solutes and water (Grinstein and Foskett, 1990; Hoffmann and Simonsen, 1989). This response is known as regulatory volume decrease. Conversely, shrunken cells can reswell towards their original size by a process named regulatory volume increase (RVI).

Two principal pathways are activated when shrunken cells undergo RVI. Many cells display a tightly coupled electroneutral symport of $Na^+/K^+/2Cl^-$ which is inhibited by loop diuretics such as furosemide and bumetanide. Four osmolytes are translocated inwards during each turnover of the symporter, effectively altering the tonicity of the cells. The second system activated when cells shrink is the Na^+/H^+ antiporter. Though the antiporter catalyzes the strictly coupled exchange of one osmolyte for another, exchange of Na^+ for H^+ results in net osmotic gain for two reasons: while Na^+ moves inwards and H^+ outwards, the intracellular concentration (activity) of the latter is simultaneously compensated by dissociation of intracellular buffers. In addition, while the cellular buffers are being depleted, the pHi increases significantly and, as a consequence, the cytoplasmic concentration of HCO_3^- rises. This rise in turn drives the inward flow of external Cl^-, in exchange for intracellular HCO_3^-, through the anion exchange system. The latter is not only stimulated indirectly by the change in the concentration of its substrate ions, but also directly by the cytosolic alkalization. Olsnes and colleagues (Ludt *et al.* 1991) demonstrated that the anion antiporters of a variety of mammalian cells are exquisitely pHi-sensitive, being markedly stimulated as the cytosol becomes progressively alkaline. Together, the parallel stimulation of the cation and anion antiporters promotes uptake of NaCl, coupled to osmotic water uptake, thereby favoring cell swelling and RVI.

As discussed above, pHi is the primary determinant of antiporter activity, with stimulation occurring in the acidic range. Notably, direct measurements of pHi have shown that the activation of Na^+/H^+ exchange during RVI is *not* preceded by a detectable cytosolic acidification. However, measurements of plasma membrane potential have shown that activation of Na^+/H^+ exchange was not accompanied by changes in membrane potential. Therefore, stimulation of exchange is not due to alterations of the driving force or the concentration of allosteric activators (i.e. H^+) but is, instead, a consequence of changes in the properties of the antiporter itself. Accordingly, when the pHi-dependence

of the activity of NHE was explored in detail, a sizable shift in the alkaline direction was detected (Grinstein *et al.* 1985*b*). As a result, whereas the antiporter is nearly quiescent at normal pHi in isotonic media, significant transport is observed at a similar pHi when the cells shrink in response to hypertonicity. Inasmuch as the modifier site largely defines the pHi-dependence of the unstimulated antiporter, the alkaline shift induced by osmotic challenge can be attributed to an alteration in the behavior of this site.

As briefly mentioned earlier, mitogens, hormones and other chemical stimuli can also activate Na^+/H^+ exchange. As in the case of RVI, stimulation by such agents is associated with cytoplasmic alkalization due to an upward displacement of the pHi set point of the modifier site. The similarity of the two effects is further emphasized by the observation that they are not additive; maximal osmotic activation entirely precludes subsequent stimulation by growth promoters and other stimuli that bind to cellular surface receptors. Moreover, both forms of activation appear to depend on the availability of intracellular ATP, since metabolic depletion not only depresses the basal rate of transport (see above) but, in addition, obliterates hypertonicity- as well as receptor-induced activation. Such observations initially led us to suggest that phosphorylation plays an important role in the activation of the Na^+/H^+ antiporter. Early studies of the phosphoprotein content of control and osmotically activated cells revealed the appearance of at least one phosphorylated polypeptide in response to cell shrinking (Grinstein *et al.* 1986). These studies pre-dated the molecular identification of the antiporter, so the identity of the responsive phosphoproteins was not defined. Osmotically induced activation of protein phosphorylation has been found to be a rather widespread phenomenon (Sadoshima and Izumo, 1993).

More direct means of analyzing the molecular basis of RVI became available following the identification of the NHE family of antiporters. To date, mechanistic studies have concentrated on NHE-1, which has been shown to mediate RVI in non-epithelial cells. This is suggested by experiments in which cells deficient in antiporter activity, isolated by the H^+-suicide method (Pouyssegur *et al.* 1984), were transfected with cDNA encoding for NHE-1. Whereas the antiporter-deficient cells failed to alkalize in response to hypertonic stress, an amiloride-sensitive alkalization was restored in the transfectants (Grinstein *et al.* 1992). Moreover, whereas antiporter-deficient Chinese hamster ovary (CHO) cells were found to remain shrunken after osmotic challenge, cells expressing NHE-1 reswell towards their original volume (Rotin and Grinstein, 1989).

As mentioned above, simultaneously with the activation of Na^+/H^+ exchange, growth promoters induce the phosphorylation of NHE-1 near its carboxy terminus (Wakabayashi *et al.* 1992*a*, 1994). Moreover, stimulation of transport and phosphorylation is also observed when cells are treated with serine/threonine-phosphatase antagonists such as okadaic acid (Bianchini *et al.* 1991; Sardet *et al.* 1991). On the basis of the kinetic similarities between the receptor-induced and osmotically induced responses, we hypothesized that stimulation by hypertonic shrinking was similarly linked to phosphorylation of NHE-1. To test this possibility, we metabolically labelled transfected fibroblasts with radioactive orthophosphate and immunoprecipitated NHE-1 before and after osmotic challenge. As reported earlier, basal phosphorylation was present in isotonic medium. Unexpectedly, under conditions where hypertonic stimulation of

Na^+/H^+ exchange was readily detectable, no additional phosphorylation was measurable (Grinstein *et al.* 1992). Phosphoamino acid analysis and peptide mapping of radiolabelled samples confirmed that the same residues were phosphorylated in control and stimulated cells, ruling out the possibility of phosphorylation of one site with concomitant dephosphorylation of another. It was concluded that osmotic activation of the antiporter can occur *without* phosphorylation of NHE-1. We therefore proposed the existence of dual control of Na^+/H^+ exchange by phosphorylation-dependent and phosphorylation-independent mechanisms (Grinstein *et al.* 1992).

A recent publication has revised the original hypothesis regarding the requirement for phosphorylation during growth-factor induced stimulation. Wakabayashi *et al.* (1994) found that the sites that become phosphorylated during activation are located very near the carboxy terminus of the protein. This region (residues 635–815 of the human NHE-1) had been shown to be dispensable, i.e. growth factor stimulation persisted in truncated mutants lacking this domain. It must therefore be concluded that phosphorylation is not absolutely required for the receptor-mediated activation of the antiporter. It is unclear why deletion of the upstream region inhibited the effect of growth promoters. This effect may result from a wholesale, deleterious conformational change or from deletion of a site that is essential for binding of a separate activator molecule (see below).

In view of these results, it would appear that the osmotically and growth-factor-induced responses are, after all, not dissimilar. This notion is reinforced by recent, unpublished analysis of the series of deletion mutants originally generated by Wakabayashi *et al.* (1992*a*). Dr L. Bianchini has found that, as in the case of growth factors, the osmotic stimulation persists after truncation at position 635, but disappears after truncation at position 566 (unpublished observations). Hence, though generated by different signals, both responses may converge and share a common effector mechanism.

Although NHE-1 itself appears not to be phosphorylated during osmotic activation, it is nevertheless possible that phosphorylation modulates the antiporter through ancillary regulatory proteins, which could themselves be substrates of protein kinases. To date, proteins that specifically associate with the NHE-1 isoform of the antiporter have not been described. A few phosphoproteins co-sediment with the antiporter during immunoprecipitation, but their yields are variable and the specificity of the interaction has not been established. More importantly, the amount or extent of phosphorylation of these co-sedimenting polypeptides does not appear to change when the cells are challenged hypertonically. Therefore, if present, ancillary phosphoproteins are loosely associated with NHE-1 and are not detectable by conventional immunoprecipitation protocols. It is also possible that the interaction with a phosphorylated regulator is indirect, with intervening adaptor proteins. One variant of this hypothesis is that the antiporter interacts with, and is regulated by, macromolecular complexes such as those forming the cytoskeleton. Not only are other transport proteins known to associate with the cytoskeleton (e.g. Nelson and Veshnock, 1987; Smith *et al.* 1991; Johnson and Byerly, 1993), but cytoskeletal-disrupting agents have been reported to impair volume regulation in some systems (Foskett and Spring, 1985; Cornet *et al.* 1993). To date, there is no compelling evidence that NHE-1 interacts directly with cytoskeletal elements. If present, such an interaction could conceivably modulate the activity of the antiporter. In this

event, a mechanism for transducing cell volume changes into varying rates of ion transport can be readily envisaged.

The responsiveness of the individual NHE isoforms to changes in cell volume was recently compared using heterologous expression of the rat antiporters, by stable transfection of the full-length cDNAs in CHO cells deficient in endogenous antiporters (Kapus *et al.* 1994). Not all isoforms respond equally to osmotic stress. As discussed above, NHE-1 is stimulated by hypertonicity. Under similar conditions, NHE-2 is also stimulated to a similar extent. Conversely, both isoforms are inhibited when the cells are suspended in hypotonic media. In contrast, NHE-3 was found to be markedly inhibited by hypertonic cell shrinking, yet was unaffected by hypotonicity. Osmotic inhibition of NHE-3 was rapid, reversible and detectable at the single-cell level. The pattern of responsiveness of the isoform to osmolarity parallels that observed with some protein kinase agonists. Phorbol esters that activate protein kinase C stimulate NHE-1 and NHE-2 yet inhibit NHE-3 (Fliegel and Frolich, 1993; Tse *et al.* 1994). In cells transfected with the rat constructs, protein kinase A agonists similarly inhibit NHE-3, while stimulating NHE-1 and NHE-2 (J. Orlowski, personal communication). This remarkable parallelism again suggests that the kinase- and volume-induced effects converge upstream of the antiporter.

At present, we can only speculate about the possible functional significance of the inhibition of NHE-3 by volume changes. This isoform has been localized to the apical membrane of epithelial cells of the renal and gastrointestinal tracts (Biemesdeifer *et al.* 1993; Bookstein *et al.* 1994). Inhibition of NHE-3 when cells are shrunk may contribute to natriuresis during glucosuria and during mannitol infusion. In the renal medulla, inhibition of apical NHE-3 could explain the observed reduction in bicarbonate reabsorption reported to occur during hypertonic exposure. The peculiar osmotic response of NHE-3 may also be a component of electrolyte or sorbitol-induced diarrhea, favoring loss of Na^+ and water. The validity of these assumptions and the mechanism underlying the osmotic inhibition of NHE-3 remain to be established in the future.

Concluding remarks

In this brief review we intended to compile the current information regarding the control of Na^+/H^+ exchange by ATP and by cell volume and to compare and contrast the mechanisms of these regulators. Functional studies indicate that ATP depletion induces a shift in the pHi-sensitivity of the antiporter to more acidic values. Conversely, stimulation by shrinking is mediated by a shift in the alkaline direction. Therefore, both effectors seem to target the modifier site, which is believed to be the prime determinant of the pHi-sensitivity of the antiporter.

The similarities between the ATP-dependent effects and volume-dependent effects extend further. In both instances, regulation requires an intact cytosolic, carboxy-terminal tail. This finding would superficially appear to contradict the notion that the modifier site is involved, since the latter has been postulated to be located in the amino-terminal segment of the protein. However, interaction between the tail and transmembrane domain very probably occurs and will be an important determinant of the set point of the modifier.

The shift in pHi-dependence observed following truncation of the tail is consistent with this hypothesis.

Both the basal and osmotically stimulated rates of transport are ATP-dependent. Nevertheless, changes in the state of phosphorylation of the antiporter itself do not appear to be involved. Other, as yet unidentified, components are seemingly involved. At present, it is not known whether the transport rate is influenced by exogenous factors such as ancillary phosphorylated or nucleotide-binding proteins, cytoskeletal elements or phospholipid flippases or kinases that can change the lipid composition of the membrane. These possibilities are currently under investigation.

Finally, it is worth stressing that, although remarkable analogies exist between the osmotic and nucleotide sensitivities of the antiporters, some divergence is also apparent. In particular, the differential responsiveness of the isoforms towards osmotic stress must be highlighted. This difference contrasts with the parallel behavior of NHE-1, NHE-2 and NHE-3 following ATP depletion. It is therefore unlikely that the alkaline and acidic pHi shifts observed following shrinking and ATP depletion, respectively, are simply opposite manifestations of a single mechanism.

In summary, substantial progress has been accomplished in our quest to understand the mode of modulation of Na^+/H^+ exchange by nucleotides and by changes in cell volume, yet many essential pieces of the puzzle remain unsolved. We hope that the present chapter provides a useful summary that will facilitate the pursuit of future research in this area.

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