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Topical Review

Mechanisms of Regulation of the Na^+/H^+ Exchanger

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Introduction

Exchange of Na^+ for H^+ was first proposed as a mechanism for renal acidification almost 40 years ago (Pitts, Ayer & Schiess, 1949). However, it was not until 1976 that the existence of such an exchange system was directly demonstrated in vesicles prepared from brush border membranes of kidney tubules (Murer, Hopfer & Kinne, 1976). From studies in renal systems, it has become clear that the exchanger plays an important role in acid secretion and in transepithelial salt and water transport.

More recent studies have demonstrated that the Na^+/H^+ exchanger (antiporter) is not unique to epithelial tissues. It appears to be present in most, if not all animal cells, where it has been implicated in a variety of functions, including the regulation of the cytoplasmic pH and Na^+ concentration, the control of cell volume and the initiation of growth and proliferation. Given the general interest in these phenomena, the rapidly expanding interest in Na^+/H^+ exchange is not surprising. Many aspects of its properties and functions have been the subject of recent symposia and review articles (Krulwich, 1983; Ives & Warnock, 1984; Aronson, 1985; Mahnensmith & Aronson, 1985).

In many unstimulated nonepithelial cell types the antiport appears to be nearly quiescent when the cytoplasmic pH (pH_i) is in the physiological range. However, it can be activated by a wide variety of stimuli including hormones, growth factors, tumor promoters and hypertonic shrinking. An emerging pattern of information reveals striking similarities between the modes of activation of several stimuli and suggests that they may share a common mechanism. It is the intent of this brief and speculative review to summarize the evidence concerning the nature of the regulation and to consider the possible molecular mechanisms that underlie it.

Key Words antiport · pH regulation · volume regulation · amiloride · growth factors

Characteristics of the Na/H Exchanger

The operational properties of the exchanger have been described in detail elsewhere (Aronson, 1985; Mahnensmith & Aronson, 1985) and will only be summarized briefly here, except for those aspects which are of particular importance to the regulation of the system. The antiporter has been studied in a variety of cell types and it is clear that the operating characteristics are generally quite similar in all cases. Without exception, the stoichiometry of the Na^+/H^+ exchange process is one for one (Cala, 1980; Boron & Boulpaep, 1983; Grinstein, Cohen & Rothstein, 1984*a*). Consistent with this transport ratio, the antiport is electrically neutral (Aickin & Thomas, 1977; Cala, 1980; Grinstein et al., 1984*a*) and insensitive to maneuvers that alter the transmembrane potential (Kinsella & Aronson, 1980). When the transmembrane concentration gradient for Na^+ is thermodynamically balanced by an equivalent but opposite gradient for H^+ (i.e., when $[Na^+]_o/[Na^+]_i = [H^+]_o/[H^+]_i$), the exchanger is at equilibrium and mediates no net flux of these ions (Kinsella & Aronson, 1982; Cala, 1983). These findings confirm that the stoichiometry is one to one and that exchange is electroneutral. In addition, they suggest that the sole driving force is the combined chemical Na^+ plus H^+ gradient, without direct intervention of energy from metabolic reactions. Since under physiological circumstances $[Na^+]_o \gg [Na^+]_i$ and $[H^+]_o \leq [H^+]_i$, the antiport will normally operate in the Na_o^+/H_i^+ exchanging mode. However, under certain conditions the direction of exchange can be reversed by inverting the direction of the gradient, e.g., by removal of extracellular Na^+ (Moolenaar et al., 1983; Cassel, Zhuang & Glaser, 1984).

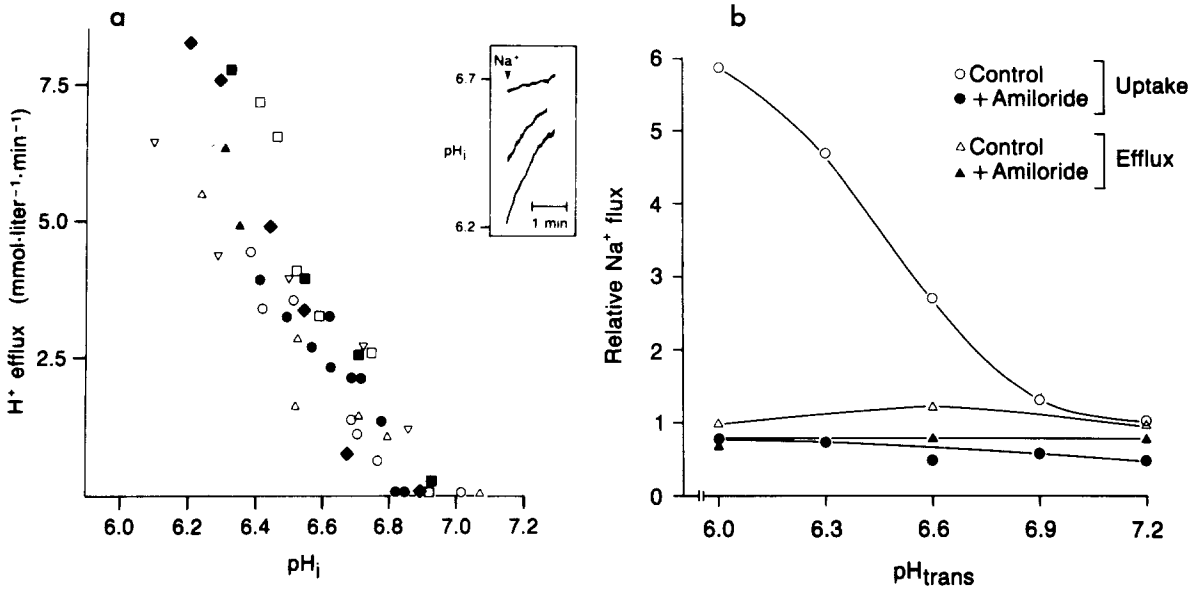

Fig. 1. (*a*) Internal pH (pH_i) dependence of the rate of Na^+-induced acid extrusion from rat thymic lymphocytes. Acid extrusion was calculated from the rate of ΔpH_i measured fluorimetrically. The pH_i was brought to the desired value using nigericin. [Reproduced from *J. Gen. Physiol.* (1984) Vol. 83, pp. 341–369, by copyright permission of the Rockefeller University Press]; (*b*) Dependence of the rate of unidirectional Na^+ flux on the pH on the *trans* side of the membrane. The fluxes were normalized to facilitate comparison; unity is the control flux at $pH_{trans} = 7.2$. [Reproduced from *J. Gen. Physiol.* (1984), Vol. 84, pp. 585–600, by copyright permission of the Rockefeller University Press]

In addition to Na^+ and H^+, the exchanger can transport Li^+ and probably NH_4^+, but not K^+, Rb^+ or organic cations such as choline$^+$ or *N*-methyl-D-glucamine$^+$ (Aronson, 1985). Na^+/H^+ exchange can be inhibited competitively by other transported cations and also by amiloride, a K^+-sparing diuretic, and its analogues (Benos, 1982; Aronson, 1985). With few exceptions, the inhibitory effects of amiloride have been found to be competitive with respect to Na_o^+, presumably resulting from binding of the inhibitor to the externally exposed transport sites. Forward (Na_o^+/H_i^+) exchange can also be inhibited by elevating the extracellular H^+ concentration (Aronson, Suhm & Nee, 1983). Competition with Na_o^+ for the transport site is probably the predominant factor in the inhibition (Kinsella & Aronson, 1981; Paris & Pouyssegur, 1983; Grinstein et al., 1984*a*), but mixed type and even noncompetitive kinetics have been reported in some systems.

Whereas the interaction of external Na^+ with the antiport follows Michaelis-Menten kinetics, consistent with one single binding site, the dependence of transport on $[H^+]_i$ is considerably steeper (Aronson, Nee & Suhm, 1982; *see also* Fig. 1). Such behavior is not predicted for a simple one-for-one exchanger that obeys Michaelian kinetics and suggests that additional effects must exist. This was first realized by Aronson and coworkers, who postulated the existence of a second cytoplasmic H^+ binding site that allosterically activates the antiport. Support for this allosteric "modifier" site comes from observations of stimulation of Na_i^+ efflux by increasing $[H^+]_i$ (Aronson et al., 1982), an effect opposite to the expected competition for the internally facing transport site. The allosteric effects of increasing $[H^+]$ are highly asymmetric: contrary to the marked effect of pH_i on Na_o^+ uptake, extracellular acidification fails to significantly increase Na^+ efflux (Fig. 1*b*). Therefore, the modifier site appears to be confined to the cytoplasmic side of the antiport, unlike the transport site(s), which must have access to both faces of the membrane. These features are summarized in the model of Fig. 2.

An important consequence of the operation of the modifier site is apparent in the relationship between pH_i and the rate of Na^+/H^+ exchange (Fig. 1). The antiporter becomes virtually quiescent above a certain pH_i. This activity threshold or "set point" happens to coincide with the normal physiological pH_i of the cells, consistent with a central role of the Na^+/H^+ exchanger in pH_i homeostasis. At lower values of pH_i, protonation of the modifier greatly enhances the rate of Na^+/H^+ countertransport. On the other hand, by curtailing the activity of the exchanger above the "set point," the modifier kinetically protects the cell from approaching the very alkaline pH_i at which the antiport would otherwise stop after attaining thermodynamic equi-

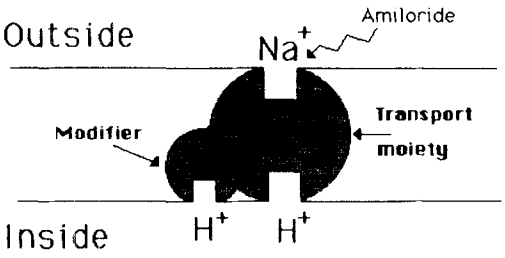

Fig. 2. Diagrammatic representation of the Na^+/H^+ exchanger. The modifier and substrate binding site(s) are indicated. Two separate transport sites are shown for didactic purposes. However, a single site alternating across the membrane is also compatible with the available data

librium (pH_i = 8.3, for pH_o = 7.3 and a $[Na^+]_o/[Na^+]_i$ ratio of 10). These observations suggest the deprotonation of the modifier results in inactivation of the exchanger, in spite of the prevailing combined Na^+ plus H^+ gradient.

In addition to H_i^+, other ions can potentially interact with the modifier site. In dog red blood cells, Li^+ has been found to activate amiloride-sensitive Na^+ uptake at normal pH_i and cell volume (J. C. Parker, *personal communication*). In lymphocytes, activation of the antiport has been observed when the internal $[Na^+]$ is lowered (Grinstein, Goetz & Rothstein, 1984*b*), suggesting that Na_i^+ might exert an inhibitory effect on the modifier. These interactions could be important in the regulation of the cytoplasmic concentration of alkali cations by the antiport.

Modulation of the Rate of Na^+/H^+ Exchange

Over the past few years, a large number of diverse stimuli have been reported to modulate the activity of the Na^+/H^+ exchanger in a variety of cells. These include a series of growth factors and hormones, mitogenic lectins, fertilization of eggs, diacylglycerol and related tumor promoters, vanadate, intracellular Ca^{2+} and osmotic shrinking. A detailed listing is given in Table 1. Included are cells of invertebrates, fish, amphibia and various mammalian species. Regulation has been observed in various tissues, as well as in cultures of primary cells or established lines. All of the effectors listed in the Table activate the exchanger, but it should be noted that two agents, parathyroid hormone and cyclic AMP diminish the activity (Pollock, Strewler & Warnock, 1984; Kahn et al., 1985; Reuss & Peterson, 1985). It is clear that the Na^+/H^+ exchanger is widely (perhaps universally) distributed and that its regulation is a widespread event.

The nature of the modulation can be tentatively placed in two categories: one in which the effects are almost immediate (seconds to minutes) and another in which activation is apparent only after many hours or even days. The slow type of activation has been reported mainly in kidney proximal tubular cells as a result of long term exposure to acidosis (Cohn, Klahr & Hammerman, 1983; Kinsella, Cujdik & Sacktor, 1984*a*,*b*; Tsai et al., 1984), renal insufficiency (Cohn et al., 1982; Harris, Seifter & Brenner, 1984; Nord et al., 1985), thyroxine (Kinsella & Sacktor, 1985), parathyroidectomy (Cohn et al., 1983) and glucocorticoids (Freiberg, Kinsella & Sacktor, 1982; Kinsella et al., 1984*b*; Kinsella, Freiberg & Sacktor, 1985). The precise mechanisms involved are not clear but, where available, kinetic evidence indicates an increase in the maximal transport velocity. This is consistent with either increased turnover of individual sites, or with an elevated number of antiporters. The latter hypothesis is attractive in view of the time required for the appearance of the response, which is consistent with *de novo* synthesis of exchangers. The slow effectors will not be further considered here, but they are reviewed by Kinsella and Sacktor (1984). The remainder of this review addresses the mechanism of action of the more rapid modulators.

Rapid Activation of Na/H Exchange

Although a detailed assessment has not been made in each case, it appears that the rapid activation of the antiport by the agents listed in Table 1 occurs without prior acidification of the cytoplasm. Therefore, increased exchange cannot be attributed to the "normal" pH_i-dependent control exercised by the modifier site, as described in the previous sections. Furthermore, in a substantial number of cases it has been reported that the activation leads to an alkalinization of the cytoplasm above the normal "set point" of the modifier. It is evident that the model in Fig. 2 cannot account for these observations and that additional mechanisms must intervene.

An indication of the possible mechanism involved has been obtained by comparing the pH_i dependence of transport in resting and activated cells. This was accomplished by manipulating pH_i with the NH_4^+-preloading method (Moolenaar et al., 1983), using weak organic acids (Grinstein & Furuya, 1984) or by means of ionophores (Grinstein et al., 1985*a*; Grinstein, Rothstein & Cohen, 1985*d*) as described for Fig. 1. Typical results obtained for cells activated with growth factors, phorbol esters and osmotic shrinking are illustrated in Fig. 3. In all

Table 1. Factors that activate Na^+/H^+ exchange in various cell types

A) Rapid responses (seconds to minutes)		
1) Hormones and growth factors		
Serum	Mouse neuroblastoma	Moolenaar et al., 1981
	Human fibroblasts	Villereal, 1981; Moolenaar et al., 1982, 1983; Muldoon et al., 1985; Owen & Villereal, 1982*a,b*, 1983
	Swiss 3T3 fibroblasts	Frelin et al., 1983, 1985; Burns & Rozengurt, 1983
	3T3 Variant, NR6	Cassel et al., 1983
	Human epidermal carcinoma (A431)	Cassel et al., 1985
	C-6 glioma	Sapirstein & Benos, 1984
	Rat smooth muscle	Owen, 1984
	Rat myoblasts	Vigne, Frelin & Lazdunski, 1985
Epidermal growth factor	Human epidermal carcinoma (A431)	Whiteley et al., 1984; Cassel et al., 1985; Rothenberg et al., 1983*a,b*
	Human fibroblasts	Villereal, 1981; Moolenaar et al., 1982, 1983; Muldoon et al., 1985
	C-6 glioma	Sapirstein & Benos, 1984; Benos & Sapirstein, 1983
	Neuroblastoma	Benos & Sapirstein, 1983
Platelet-derived growth factor	Swiss 3T3 fibroblasts	Schuldiner & Rozengurt, 1982; Burns & Rozengurt, 1983
	3T3 Variant, NR6	Cassel et al., 1983
	Rat smooth muscle	Owen, 1984
Insulin	Frog skeletal muscle	Moore, 1979, 1981; Putnam, 1985
	Cultured myocytes	Rosic, Standaert & Pollet, 1985
	Hepatocytes	Fehlman & Freychet, 1981
	Swiss 3T3 fibroblasts	Schuldiner & Rozengurt, 1982
	Hamster lung fibroblasts	Paris & Pouyssegur, 1983; L'Allemain et al., 1984
	Human fibroblasts	Muldoon et al., 1985
Catecholamines (isoprenaline)	Trout red cells	Baroin et al., 1984
Vasopressin	Swiss 3T3 fibroblasts	Schuldiner & Rozengurt, 1982; Burns & Rozengurt, 1983
	Human fibroblasts	Muldoon et al., 1985; Villereal & Owen, 1984
Bradykinin	Human fibroblasts	Muldoon et al., 1985; Owen & Villereal, 1983; Villereal & Owen, 1984
	C-6 glioma	Sapirstein & Benos, 1984
Angiotensin	Rat smooth muscle	Smith & Brock, 1983
2) Phorbol esters and diacylglycerol:		
TPA and/or oleoylacetylglycerol	Swiss 3T3 fibroblasts	Burns & Rozengurt, 1983
	Sea urchin eggs	Swann & Whitaker, 1985
	Human epidermal carcinoma (A431)	Whiteley et al., 1984
	Human leukemic (HL-60) cells	Besterman et al., 1985; Besterman & Cuatrecasas, 1984
	C-6 glioma	Sapirstein & Benos, 1984
	Pre B lymphoblast (70Z/3)	Rosoff, Stein & Cantley, 1984
	Human fibroblast	Moolenaar et al., 1984
	HeLa	Moolenaar et al., 1984
	Neuroblastoma	Moolenaar et al., 1984
	Porcine neutrophils	Grinstein et al., 1985*b*
	Rat thymocytes	Grinstein et al., 1985*a*
	Rat myoblasts	Vigne et al., 1985
3) Other agents		
Ca^{++}-ionophore	Human platelets	Siffert, Fox, Michenkoff & Scheld, 1984
	Human fibroblasts	Villereal, 1981; Owen & Villereal, 1982*a*; Villereal & Owen, 1984
Vanadate	Human epidermal carcinoma (A431)	Whiteley et al., 1984; Cassell et al., 1984
Chemotactic factors	Rabbit neutrophils	Molski et al., 1980
	Human neutrophils	Grinstein & Furuya, 1984
Thrombin	Human platelets	Siffert et al., 1984; Greenberg-Seperski & Simons, 1984; Horne et al., 1981; Sternberg et al., 1984
	Hamster lung fibroblasts	Paris & Pouyssegur, 1983; L'Allemain et al., 1984
Caerulein	Guinea pig pancreatic acini	Dufresne et al., 1985
Melittin (lipase activator)	Human fibrobasts	Villereal & Owen, 1984
Lipopolysaccharide	Pre B-lymphoid line	Rosoff & Cantley, 1983

Table 1—Continued

Lectins	Murine T lymphocytes	Hesketh et al., 1985
Caffeine	Frog skeletal muscle	Connett, Hays & Gilda, 1984
Osmotic shrinking	*Amphiuma* erythrocytes	Cala, 1980, 1983
	Dog erythrocytes	Parker, 1983; Parker & Castranova, 1984
	Rat thymocytes	Grinstein et al., 1985*c,d*
	Frog skeletal muscle	Abercrombie & Roos, 1983
	Human epidermal carcinoma (A431)	Whiteley et al., 1984; Cassel, 1985
	Necturus gall bladder	Fisher & Spring, 1984
	Rabbit proximal tubular primary cultures	Fine et al., 1985
	Human lymphocytes	Grinstein et al., 1983
Fertilization	Sea urchin eggs	Cuthburt & Cuthburt, 1978; Johnson, Epel & Paul, 1976; Payan, Gerrard & Ciapa, 1983; Whitaker & Steinhardt, 1982; Busa & Nuccitelli, 1984
Activation	Sea urchin sperm	Lee, Johnson & Epel, 1983; Wong, Lee & Tsang, 1981; Christen, Schackmann & Shapiro, 1982
B) Slow Responses (hours to days)[a]		
1) Acidosis	Dog	Cohn et al., 1983
	Rabbit	Tsai et al., 1984
	Rat	Kinsella et al., 1984*a,b*
2) Parathyroidectomy	Dog	Cohn et al., 1983
3) Renal insufficiency (reduction in mass)	Rabbit	Nord et al., 1985
	Rat	Harris, et al., 1984
	Dog	Cohn et al., 1982
4) Glucocorticoids (dexamethasone)	Rat	Freiberg et al., 1982; Kinsella et al., 1984*b*, 1985
5) Thyroxine	Rat	Kinsella & Sacktor, 1985
6) Chronic K^+ depletion	Rat	Seifter & Harris, 1984

[a] Most experiments involve *in vivo* exposure of kidney to stated conditions. Evaluation of Na^+/H^+ exchange rates are done with brush border vesicle preparations of renal proximal tubular cells. In one study (Kahn et al., 1985), however, a proximal tubular suspension was exposed *in vitro* to modulating factors, followed by assessment of the exchange in brush border vesicles.

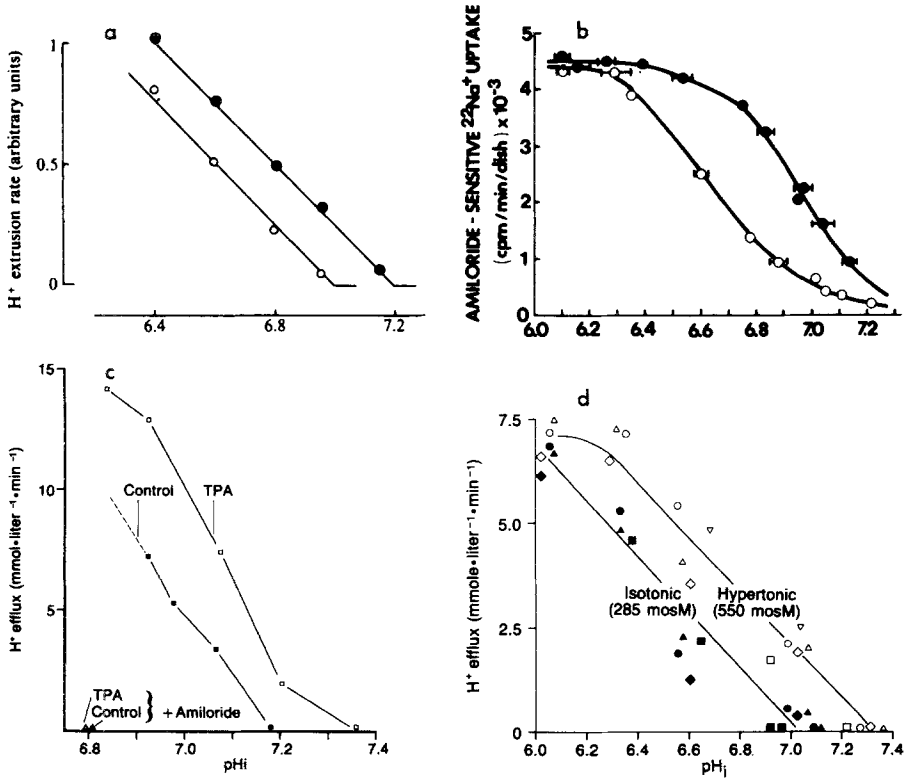

Fig. 3. Cytoplasmic pH (pH_i) dependence of Na^+/H^+ exchange in resting and activated cells. (*a*) Effect of fetal calf serum (solid symbols) on human foreskin fibroblasts. Control: open symbols. [Reprinted by permission from *Nature*, Vol. 304, pp. 645–648, copyright 1983, Macmillan Journals, Ltd.] (*b*) Effect of growth factors on lung fibroblasts. Control: open symbols; plus growth factors: solid symbols. [From Paris and Pouyssegur (1984)]. (*c*) Effect of 12-O-tetradecanoylphorbol 13,acetate (TPA) on rat thymic lymphocytes. Experimental: open symbols; control: solid symbols. [From Grinstein et al. (1985*a*)]. (*d*) Effect of osmotic shrinking on rat thymic lymphocytes. Hypertonic: open symbols; isotonic control: solid symbols. [Reproduced from *J. Gen. Physiol.* (1985), Vol. 85, pp. 765–787, by copyright permission of the Rockefeller University Press]

Table 2. Protein kinase stimulation by agents that activate Na^+/H^+ exchange

Agent	Primary response	Kinase stimulated	References
Epidermal growth factor	Activation kinase	Tyrosine kinase	Cohen et al., 1982; Heldin & Westermark, 1984
Platelet-derived growth factor	Activation kinase	Tyrosine kinase	Ek et al., 1982; Heldin & Westermark, 1984
Insulin	Activation kinase	Tyrosine kinase	Roth & Cassell, 1983; Heldin & Westermark, 1984
Thrombin	Activation phospholipase C	Protein kinase C	Bell & Majerus, 1980; Chambard & Pouyssegur, 1983
Caerulein	Activation phospholipase C	Protein kinase C	Dixon & Hokin, 1984
Angiotensin	Activation phospholipase C	Protein kinase C	Garrison et al., 1984; Berridge & Irvine, 1984
Vasopressin	Activation phospholipase C	Protein kinase C	Vicentini & Villereal, 1984; Garrison et al., 1984
Bradykinin	Activation phospholipase C	Protein kinase C	Vicentini & Villereal, 1984; Berridge & Irvine, 1984
Chemotactic factor	Activation phospholipase C	Protein kinase C	Dougherty et al., 1984; Korchak et al., 1984
β-Phorbol diesters	Activation kinase	Protein kinase C	Berridge, 1984; Nishizuka, 1984
Diacylglycerol	Activation kinase	Protein kinase C	Berridge, 1984; Nishizuka, 1984
Serum	a) Activation kinase b) Activation phospholipase C	a) Tyrosine kinase b) Protein kinase C	Chambard et al., 1983 Vicentini & Villereal, 1984
Lectins	a) Activation phospholipase C ?	a) Protein kinase C b) Tyrosine kinase	Taylor et al., 1984 Hirata et al., 1984
B lymphocyte mitogens	a) Activation phospholipase C ?	a) Protein kinase C b) Tyrosine kinase	Coggeshall & Cambier, 1984 Nel et al., 1984
Vanadate	?	Tyrosine kinase	Tamura et al., 1984

instances the pH_i dependence of the untreated cells resembles that depicted in Fig. 1, with near quiescence at $pH_i \approx 7.2$. More importantly, all the stimuli induced an alkaline shift of the pH_i-dependence curve of approximately 0.2 to 0.3 units. This shift in the curve presumably reflects an altered behavior of the modifier site, inasmuch as this site largely determines the pH_i sensitivity of the exchanger (*see* above). According to this model, the "set point" of the modifier is adjusted upward. As a result the nearly quiescent exchanger is activated, but the activation persists only until pH_i attains a value of 7.4–7.5, the new "set point." It is noteworthy that even at this elevated pH_i the exchanger is not at thermodynamic equilibrium (*see* above), indicating that the allosteric control of transport has not been lost but merely shifted to a more alkaline setting.

Molecular Mechanism of the Rapid Activation of the Antiport

Even though the kinetic properties of the activation of the Na^+/H^+ exchanger by certain agents listed in Table 1 have been described in some detail (*see* above), little is known about the specific molecular mechanisms involved. The relatively slow time course of the activation (seconds to minutes), compared to the virtually immediate effect of acid loading, is suggestive of intervening biochemical reactions, involving intermediate steps. This possibility is further suggested by the reported ATP dependence of the activation: while acid loading-induced exchange appears to proceed in the nominal absence of ATP (as suggested by experiments in isolated membranes), the stimulations induced by phorbol esters (Grinstein et al., 1985*a*,*c*) and by shrinking (Parker & Hoffman, 1965; Grinstein et al., 1985*c*) are largely eliminated by ATP depletion.

What is the nature of the ATP-dependent reaction(s) involved in the stimulation of the exchanger? Perusal of the list of acute activators in Table 1 discloses a common property: the biological effects of most of these agents are believed to be mediated by activation of protein kinases. A partial summary of the available evidence is presented in Table 2. Therefore, it seems reasonable to hypothesize that agonist-induced protein phosphorylation is required for the shift in pH_i dependence that underlies the activation of Na^+/H^+ exchange. The antiport itself,

or protein(s) capable of regulating its activity could be the targets of the kinases. It follows that stimulation of countertransport could be obtained not only by increasing the rate of phosphorylation, but also by reducing the rate of dephosphorylation, which would also lead to increased levels of protein-phosphate. This mechanism has been proposed to account for the observed stimulatory effects of vanadate, a known phosphatase inhibitor (Cassel et al., 1984).[1]

In A431 cells, a good correlation appears to exist between the protein kinase activity of the epidermal growth factor (EGF) receptor and the magnitude of the EGF-induced activation of Na^+/H^+ countertransport. The tyrosine kinase activity associated with receptor occupancy by EGF is known to be inhibited by pre-treatment of the cells with 12-O-tetradecanoylphorbol 13,acetate (TPA; Cochet et al., 1984). Treatment with the phorbol ester, which by itself has only a small activating effect in these cells, substantially inhibits the response of the antiport to EGF (Whiteley et al., 1984).

The kinetic observations of a shift in the pH_i-dependence of the antiport and the possible involvement of protein kinases can be consolidated into a unifying hypothesis, if it is assumed that phosphorylation at or near the modifier site is responsible for the shift in the "set point." This could result from a conformational change of the protein or simply from the increased lcoal $[H^+]$ created by the negative charges of the phosphate group.

From the data summarized in Table 2, it is evident that the agents capable of stimulating the antiport fall into two distinct groups: those that activate tyrosine-specific kinases and those that activate protein kinase C (whether directly or through diacylglycerol, following stimulation of phosphoinositide phosphodiesterases). The latter type of kinase is known to phosphorylate serine and threonine, but not tyrosine residues of the target proteins (Nishizuka, 1984). Because the stimulation of the antiport is ostensibly similar in both instances, two converging but distinct mechanisms must be involved. Three possibilities can be envisaged at present:

i) The simplest hypothesis is that phosphorylation at two separate sites on the same target protein may have identical functional consequences. As discussed, the target need not be the antiporter itself but could be a regulatory protein analogous to phospholamban. When phosphorylated, this protein stimulates the activity of the Ca^{2+} ATPase of the cardiac sarcoplasmic reticulum. Two different control sites on a transport protein with similar biological consequences are known to exist in the case of the plasma membrane Ca^{2+} ATPase (Michell, 1982). Moreover, there is precedent for the phosphorylation of a single substrate protein by multiple kinases (Blenis, Spivack & Erikson, 1984; Welch, 1985). Indeed, in these studies tryptic mapping indicated that in all cases the phosphorylated residue was in the same peptide.

ii) A single activating mechanism might exist if the effects of phorbol esters, and by extension those of diacylglycerol, were mediated by a tyrosine kinase. Although their acknowledged primary target is protein kinase C (Berridge, 1984; Nishizuka, 1984), addition of phorbol esters could result in stimulation of a tyrosine kinase either directly or indirectly. Increased tyrosine phosphorylation has been reported following addition of TPA and other biologically active phorbol esters to cell extracts (Grunberger et al., 1984) or isolated membranes (Moon, Palfrey & King, 1984). At least in one case (Moon et al., 1984), activation of protein kinase C did not seem to be a prerequisite for tyrosine phosphorylation, suggesting that the phorbol esters directly stimulated a tyrosine-specific kinase.

Alternatively, activation of protein kinase C could indirectly stimulate tyrosine kinase, through phosphorylation of the latter. Phosphorylation of growth factor receptors by phorbol esters has been reported, though this is usually associated with a decrease in their tyrosine kinase activity (e.g. Cochet et al., 1984).

iii) A single mechanism would explain the observations if the effects of tyrosine kinases on Na^+/H^+ exchange were mediated by their stimulation of protein kinase C. Though, to our knowledge, no direct modulation of protein kinase C by tyrosine kinases has been reported, indirect stimulation is possible through the phosphoinositide cycle. Indeed, epidermal and platelet-derived growth factors as well as insulin have been shown to stimulate phosphoinositide metabolism (Machicao & Wieland, 1984; Macara, 1985). *In vitro,* several tyrosine kinases have been demonstrated to phosphorylate phosphoinositides, including phosphatidylinositol and phosphatidylinositol 4,phosphate (Hunter & Cooper, 1985; Macara, 1985). In the cell, this would be expected to increase the levels of phosphatidylinositol 4,5-bisphosphate, the primary target of the receptor-activated phospholipase C. It must be borne in mind, however, that increased availability of the substrate will not necessarily result in increased degradation. In this regard, Taylor et al. (1984) have reported increased synthesis of polyphosphatides without a concomitant change in hydrolysis, as measured by the release of inositol trisphosphate.

[1] Direct stimulation of kinase activity by vanadate has also been reported.

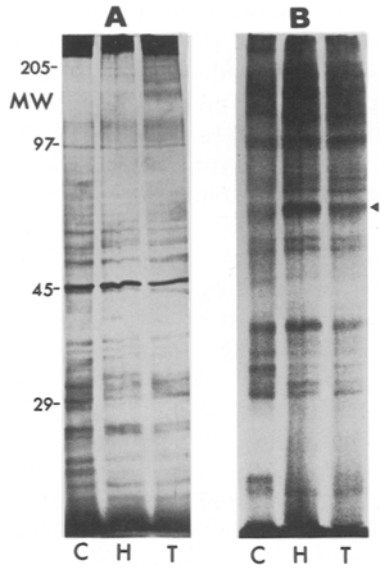

Fig. 4. Phosphorylation of membrane proteins by TPA and osmotic shrinking in thymic lymphocytes. Thymocytes were preloaded with ^{32}P to label the nucleotide pool. The cells were then incubated for 5 min in isotonic medium in the absence (*C*) or presence of TPA (*T*), or in hypertonic (*H*) medium (550 mosM). The reaction was stopped by homogenizing the cells in ice-cold medium, followed by separation of a plasma membrane-rich fraction by differential centrifugation. The membranes were then analyzed by polyacrylamide gel electrophoresis in SDS followed by autoradiography. (*A*) Silver staining; (*B*) autoradiogram. The molecular weights of standards (in kilodaltons) are indicated. The arrowhead shows a polypeptide of $M_r \approx 60{,}000$ that displayed increased labeling following treatment with hypertonic media or with TPA

Mechanism of Activation during Volume Changes

In several cell types, a normally quiescent Na^+/H^+ exchanger can be turned on by osmotically induced shrinking (Cala, 1980, 1983; Parker, 1983; Parker & Castranova, 1984; Grinstein et al., 1985*d*). In some of these cells the exchanger is also activated by factors that stimulate tyrosine kinase or protein kinase C (Grinstein et al., 1985*a*; Cassel et al., 1985). In lymphocytes, where the mechanism has been studied in some detail, the pharmacological and kinetic properties of the osmotic- and phorbol ester-induced activations are remarkably similar: in both cases the activation can be explained by a shift in the pH_i dependence of the modifier site; moreover, both processes are ATP dependent and can be inhibited by comparable concentrations of trifluoperazine (Grinstein et al., 1985*c*). These similarities, and the finding that the TPA and osmotic responses are not additive, are suggestive of a common underlying mechanism, namely, protein phosphorylation.

This hypothesis was tested by measuring the incorporation of ^{32}P into the membrane proteins of osmotically shrunken lymphocytes. For comparison, parallel samples were treated with and without TPA in isotonic solution. As shown in Fig. 4, increased phosphorylation of specific polypeptides was obtained not only with phorbol ester, but also with the osmotic treatment. Of particular interest are polypeptides of molecular weights 50–55 and ≈60 kilodaltons, which were phosphorylated by both procedures. Phosphoamino acid analysis by two-dimensional high-voltage electrophoresis further showed that, with both stimuli, the labeled residues in the ≈60 kilodalton polypeptide (the only one analyzed) were serine and threonine.

Concomitantly with the increase in protein phosphorylation, osmotically-treated lymphocytes also displayed an increased turnover of phosphoinositides, measured by ^{32}P incorporation (Grinstein et al., 1986). This is consistent with increased degradation of phosphatidylinositol 4,5 bisphosphate, which prompted the hypothesis that shrinking might activate phospholipase C. However, measurements of release of inositol phosphates failed to confirm this prediction (*unpublished observations*). Moreover, migration of cytoplasmic C kinase to the membrane, which can be induced by TPA and presumably by diacylglycerol, was not observed in osmotically shrunken cells. Therefore, the increased ^{32}P turnover in phospholipids is probably due to activation of a phosphoinositide kinase. Moreover, the osmotic activation of protein phosphorylation is not likely due to activation of phospholipase C followed by stimulation of C kinase by diacylglycerol. In summary, osmotic shrinking leads to increased protein and phospholipid phosphorylation. One or both of these phenomena could underlie the concomitant stimulation of Na^+/H^+ exchange.

Is Phospholipase A Involved in the Activation Process?

In contrast to the findings with phospholipase C, there is direct evidence of stimulation of phospholipase A in osmotically shrunken cells (Dise, Goodman & Rasmussen, 1980). Moreover, other agents that activate the antiport, such as thrombin and the chemotactic factor f-met-leu-phe, are also known to activate phospholipase A (Bormann et al., 1984). Thus, it is possible that one of the products of phospholipid hydrolysis by this enzyme plays a role in activation of Na^+/H^+ countertransport.

Participation of phospholipases in the stimulation was first suggested by Villereal and coworkers (Vicentini, Miller & Villereal, 1984), based on the

following observations: (i) amiloride-sensitive Na^+ uptake in fibroblasts was found to be stimulated by melittin, a component of bee venom that is known to activate phospholipase A_2; (ii) the stimulation induced by melittin or by serum growth factors was inhibited by mepacrine, a phospholipase inhibitor and (iii) the stimulation was also reduced by long preincubations with dexamethasone, a drug known to induce the synthesis of lipomodulin, an intrinsic phospholipase inhibitor.

Free fatty acids, which are one of the products of phospholipid hydrolysis by phospholipase A_2, are active in cellular signalling. They can also be produced by the combined action of phospholipase C, which releases diacylglycerol, and diglyceride lipase. Fatty acids, particularly arachidonate, could mediate activation of the antiport by two pathways: (i) by direct stimulation of protein kinase C (McPhail, Clayton & Snyderman, 1984) or (ii) indirectly via the synthesis of prostaglandins and leukotrienes. The possible role of eicosanoids in the regulation of Na^+/H^+ exchange remains to be elucidated.

Activation by Cytoplasmic Ca^{2+}

In fibroblasts, elevation of the cytoplasmic free Ca^{2+} concentration with ionophore A23187 markedly stimulated Na^+/H^+ exchange (Villereal, 1981; Owen & Villereal, 1982*a*,*b*). Even though this does not appear to be a general phenomenon (*see* Moolenaar, Tertoolen & DeLaat, 1984; Frelin, Vigne & Lazdunski, 1983, for different findings), several possible mechanisms can be envisaged: The effects on cation exchange could be mediated by activation of phospholipase C or phospholipase A_2, both of which are known to be stimulated by increased $[Ca^{2-}]$. The products of phospholipid hydrolysis would in turn stimulate protein kinase C. In addition, this kinase or a calmodulin-dependent kinase could be directly stimulated by Ca^{2+}. Alternatively, the stimulation of Na^+/H^+ exchange could be indirectly due to changes in cellular volume. Addition of Ca^{2+} ionophores to lymphocytes and Ehrlich ascites cells is known to induce cellular shrinking, as a consequence of increased K^+ and Cl^- permeability (Grinstein, DuPre & Rothstein, 1982; Hoffmann, 1985). Therefore, activation of Na^+/H^+ exchange could be secondary to the decrease in cell volume.

Membrane Recycling and the Stimulation of Na^+/H^+ Exchange

In recent years it has become apparent that stimulation of a number of transport systems involves activation of latent transport sites. In some instances, these sites are initially located in internal vesicles and become inserted into the plamsa membrane by an exocytotic process. This mechanism has been suggested to account for the induction of acid extrusion in the turtle bladder and for the effects of insulin on hexose transport in fat cells.

Whether a similar mechanism applies to the activation of Na^+/H^+ exchange will only become clear when the number and subcellular distribution of antiporters in resting and activated cells is defined. However, available evidence suggests that, at least in some cell types, insertion of transporters from internal membranes is not the primary mechanism. The most convincing argument can be made for the osmotic stimulation of Na^+/H^+ exchange in dog red blood cells (Parker, 1983; Parker & Castranova, 1984), which, unlike nucleated cells, lack an internal membrane system. In neutrophils, a phorbol ester-induced activation can be obtained not only in intact cells, but also in cytoplasts (Grinstein, Elder & Furuya, 1985*b*). These are cellular fragments essentially devoid of nuclei and secretory vesicles. These observations argue against the involvement of the secretory vesicles, but the participation of lighter microsomal elements cannot be ruled out.

Inhibition by Cyclic AMP

In two reported instances, the rate of Na^+/H^+ exchange was reduced by elevating the cytoplasmic levels of cAMP (Kahn et al., 1985; Reuss & Petersen, 1985). Little is known about the mechanism involved, but the effect of the nucleotide on transport parallels the inhibitory action of cAMP on receptor-activated stimulation of phospholipase C and on mitogen-induced phosphorylation (Taylor et al., 1984). If, as proposed above, the degree of phosphorylation determines the rate of Na^+/H^+ exchange, it is possible that inhibition by cAMP is due to a reduced activity of the relevant kinases. Another agent reported to inhibit the Na^+/H^+ antiport, parathyroid hormone (Pollock et al., 1984; Kahn et al., 1985), presumably acts by stimulating adenyl cyclase and increasing the levels of cAMP.

Summary and Concluding Remarks

It is becoming increasingly clear that a Na^+/H^+ antiport is present in the plasma membrane of virtually all vertebrate cells and in at least some invertebrates. It appears to be important in the regulation of the cytoplasmic pH and Na^+ concentration, in transepithelial ion transport, in the control of cell

volume, and may also participate in the initiation of proliferation. The rate of activity of the antiport can be modulated by a variety of agents and conditions, such as growth factors, hormones and osmotic challenge. The ubiquity of the antiport, together with the wide range of agents that regulate its rate, suggest a central role of Na^+/H^+ exchange in cellular homeostasis.

The activation of the antiport can lead to three immediate consequences: an alkalinization of the cytoplasmic pH, an increased intracellular Na^+ concentration and the uptake of osmotically obliged water. In cells that have a Na^+/Ca^{2+} exchanger, the changes in $[Na^+]_i$ can in turn lead to an increased $[Ca^{2+}]_i$. A hyperpolarization resulting from a secondary acceleration of the electrogenic Na^+-K^+ pump is also possible. Each one of these parameters can have important consequences on cell function. Thus, the cytoplasmic alkalinization has been claimed to be permissive for cellular proliferation (L'Allemain, Paris & Pouyssegur, 1984), the elevated Na^+ content is thought to signal differentiation in lymphoid cells (Rosoff & Cantley, 1983) and the osmotic gain has been shown to underlie the regulatory volume increase observed in shrunken cells (Cala, 1980; Grinstein, Clarke & Rothstein, 1983).

The biological actions of most of the agents that activate the antiport are known to be mediated by protein kinases (Table 2). Therefore it is suggested that the stimulation of countertransport, which results from a shift in the pH_i dependence of exchange, could also be mediated by phosphorylation of the antiporter or of a regulatory protein. Though attractive, this hypothesis has not been directly tested, partly because the molecular identity of the Na^+/H^+ exchanger has not been established. This obstacle may soon be overcome by current studies of antiport-enriched and deficient mutants, and by the development of high affinity radiolabeled inhibitors, such as the 5,N-substituted analogs of amiloride. These advances, coupled to the development of simplified cell-free systems where the responses can be elicited *in vitro*, may soon disclose the nature of the regulation of the Na^+/H^+ exchanger.

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