REVIEW ARTICLE

The Na^+/H^+ exchanger: an update on structure, regulation and cardiac physiology

Larry FLIEGEL*‡ and Otto FRÖHLICH†

*Departments of Biochemistry and Pediatrics, 417 Heritage Medical Research Building, University of Alberta, Edmonton, Alberta, Canada T6G 2S2, and †Department of Physiology, Emory University School of Medicine, Atlanta, GA 30322, U.S.A.

INTRODUCTION

Most mammalian cells maintain a physiological cytoplasmic pH of approximately 7.2 despite the prediction of a more acidic internal pH on the basis of thermodynamic and metabolic considerations. If the transmembrane gradient of hydrogen ions were determined solely by a hydrogen ion leak pathway in the presence of a membrane potential of −60 mV, then the cytoplasmic pH would be about 6.2, that is one pH unit more acidic than the extracellular pH. Besides membrane potential, metabolic conditions can contribute an intracellular acid load. This includes production of CO_2 in the cell and subsequent conversion to carbonic acid, and metabolic reactions producing other acids. Since there are many conditions, physiological and pathological, which tend to shift the metabolic acid balance, regulatory mechanisms need to be in place to maintain intracellular pH in the face of acidic or alkaline challenges. It is also necessary to change intracellular pH to support changes in the growth or functional state of the cell [1–3]. This is achieved by the interplay of several different mechanisms, including bicarbonate-transporting carriers and Na^+/H^+ exchange, the relative contributions of which vary among the different cell types [4]. The Na^+/H^+ exchanger is a universal pathway employed by essentially all eukaryotic cells to regulate intracellular pH [5]. Na^+/H^+ exchange activity is widely expressed in the animal and plant kingdoms in virtually all cell types. As implied by the name, the exchanger transports Na^+ and H^+ ions in opposite directions across the bilayer membrane. The direction of exchange is governed solely by the two ions' gradients and requires no additional metabolic energy. In higher organisms such as mammals, the free energy in the inward Na^+ gradient is greater and therefore powers the H^+ movement out of the cell against its own gradient. In mammalian cells the stoichiometry of Na^+/H^+ exchange is 1:1 [6]. In contrast, bacterial exchange is electrogenic [7] with a stoichiometry of 1:2 [8], reflecting the fact that the Na^+/H^+ exchangers in the different systems appear to be essentially unrelated to each other.

The best inhibitor of the Na^+/H^+ exchanger is amiloride and its analogues. Amiloride inhibits the mammalian Na^+/H^+ exchanger with a K_i of 1–100 μM, depending on the cell type. The more specific amiloride analogues also inhibit this activity with greater efficacy [9]. The exchanger is normally nearly quiescent when the cytoplasmic pH is at the physiological level. Activation occurs by various stimuli including hormones (insulin, vasopressin), growth factors (platelet-derived growth factor, epidermal growth factor), and other stimuli such as chemotactic factors and fertilization of eggs [5,6]. In higher organisms, Na^+/H^+ exchange fulfils different functions, depending on the cell type. The most common and important role of Na^+/H^+ exchange is to protect the cell from intracellular acidification, which is evident from mutant cell lines devoid of Na^+/H^+ exchange [10]. Na^+/H^+ exchange also participates in cell volume regulation after osmotic shrinkage [11].

Both pharmacological and kinetic criteria indicate that there are different isoforms of the Na^+/H^+ exchanger. Amiloride and its analogues distinguish two forms (or classes of isoforms): an epithelial luminal transporter with a K_i for amiloride near 0.1 mM, and the higher-affinity non-epithelial transporter (K_i = 1–10 μM). The latter form is now referred to as the ubiquitous or 'housekeeping' form, or NHE-1 (for Na^+/H^+ exchanger type 1). It appears to exist in the plasma membrane of most cells, including the basolateral membrane of epithelial cells [12]. Thus pharmacological criteria have established that different isoforms of antiporter can exist in the same cell albeit partitioned into different domains of the plasma membrane [12–14].

Some aspects of the Na^+/H^+ exchanger have recently been reviewed, such as its regulation by phosphorylation [15]. In this review we examine several newer and very interesting areas of research related to the Na^+/H^+ exchanger. We focus on three areas: (1) homology and diversity between the different members of the Na^+/H^+ exchanger family, (2) regulation of expression of the protein in response to external stimuli, and (3) the role of the Na^+/H^+ exchanger in the myocardium. Several different NHE isoforms have been isolated and their similarities and differences will be discussed. We will also survey studies on the regulation of the Na^+/H^+ exchanger gene which demonstrate how the level of the antiporter responds to chronic acidosis and other stimuli. Finally, we will examine the Na^+/H^+ exchanger in the myocardium. In this tissue in particular, pH regulation and the consequences of this regulation may play an important role in health and disease.

THE Na^+/H^+ EXCHANGER AND ITS ISOFORMS

Several different isoforms of the Na^+/H^+ exchanger have been identified and the number of species from which exchanger clones have been isolated is growing (Table 1). Sardet et al. [16,17] isolated the first known Na^+/H^+ exchanger cDNA clone through a series of elegant experiments involving complementation of exchanger-deficient cell lines. The cDNA coded for the human housekeeping (amiloride-sensitive) isoform that is now referred to as NHE-1 [16,17]. Portions of the cDNA of NHE-1 have been used for screening libraries for other isoforms and from other animals. To date, four isoforms from a number of species are known from mammals: NHE-1 to NHE-4 (from man, sheep, rabbit, rat, hamster and pig; Table 1) [16–28a]. In addition, cDNA clones have been isolated from trout [29], turtle [35] and the nematode, *Caenorhabditis elegans* [30]. As expected

Abbreviations used: TM segment, membrane-spanning segment; CaM kinase II, calmodulin-dependent protein kinase II; PKA, cyclic AMP-dependent protein kinase; PKC, protein kinase C.

‡ To whom correspondence and reprint requests should be addressed.

Table 1 Cloned Na^+/H^+ exchangers

NA, not applicable.

Species	Isoform	Tissue	Accession nos.	Reference
Mammalian				
Human	NHE-1	*	M81768, J03163	16
Human	NHE-1	Kidney, placenta, breast carcinoma	M81768, J03163	18
Human	NHE-1	Heart	M96066, M96067	19
Hamster	NHE-1	CCL39	CGNHE1	20
Pig	NHE-1	LLC-PK_1 (Kidney)	M89631, S71135	21
Rabbit	NHE-1	Heart	X56536	22
Rabbit	NHE-1	Ileal villus	X59935, S40845	23
Rabbit	NHE-2	Ileal villus	L13733	28a
Rabbit	NHE-3	Kidney (cortex)	M87007, S99441	24
Rabbit	NHE-1	Kidney	X61504, S73423	25
Rat	NHE-1	Heart	M85299	26
Rat	NHE-2	Intestine	L11004	27
Rat	NHE-2	Stomach	L11236	28
Rat	NHE-3	Kidney	M85300	26
Rat	NHE-4	Stomach	M85301	26
Non-mammalian eukaryotes				
Trout	β-NHE	Cephalic kidney	M94581	29
Caenorhabditis elegans		NA	M23064	30
Schizosaccharomyces pombe	sod2	NA	Z11736	31
Prokaryotes				
Bacillus firmus	nhaC	NA	M73530	32
Enterococcus hirae	napA	NA	M81961	33
Escherichia coli	nhaA	NA	J03879	7
Escherichia coli	nhaB	NA	M83655	34

* This cDNA clone was isolated by complementation of an exchanger-deficient cell line.

from kinetic and pharmacological studies of Na^+/H^+ exchange in different tissues, the various isoforms differ in their tissue distribution and in their kinetic and pharmacological properties. In mammals, NHE-1 appears to exist essentially in every tissue examined with a message size of 5.0–5.4 kb [16,17]. NHE-2 and NHE-3 are found in intestinal and renal epithelial tissues. NHE-4 occurs mainly in the stomach, which also contains large amounts of NHE-1 and some NHE-3 mRNA [26]. The two epithelial isoforms, NHE-2 and NHE-3, are believed to reside in the apical membrane [28a]. They participate in trans-epithelial NaCl transport. Expression of NHE-2 in polarized human epithelial cells results in a functional transporter in the apical membrane [28a]. NHE-1 resides in the basolateral membrane of epithelia [36], as well as in the plasma membrane of non-polarized cells, where it participates in pH regulation. It should be noted that in at least one epithelial tissue (the human placenta), NHE-1 resides in the apical membrane instead of the basolateral membrane [37].

A great deal of effort is currently focused on comparing the transport properties of the different isoforms in transfected cells with those found previously in the different tissues. Such expression experiments are performed in cells which have been mutated and selected for the absence of intrinsic Na^+/H^+ exchange activity [16]. After expression in host cells, NHE-1, NHE-2 and NHE-3 can be activated by growth factors [38,39], but they differ in their response to phorbol esters in that NHE-1 and NHE-2 are activated while NHE-3 is inhibited [39]. These phorbol ester responses agree with findings with the endogenous exchangers, where basolateral Na^+/H^+ exchange is stimulated whereas apical Na^+/H^+ exchange is inhibited by phorbol esters and agents that activate protein kinase [40]. In an analogous manner, the trout Na^+/H^+ exchanger can be activated by isoprenaline after expression in a fibroblast cell line [29]. The amiloride sensitivity of the expressed isoforms is also close to that expected from previous studies: NHE-1 exhibits the same high affinity for amiloride and its higher-potency analogues while NHE-2 and NHE-3 have a lesser affinity for these drugs. When NHE-2 is expressed in exchanger-deficient cell lines, its sensitivity to amiloride is the same as that of NHE-1, but it is 25-fold more resistant to ethyl isopropyl amiloride than is NHE-1 [28a]. NHE-3 is the most resistant, consistent with the observed low inhibitory affinity of amiloride for apical epithelial transport [41]. The first identification of a site that influenced amiloride binding was made by Counillon et al. [42]. They identified Leu-167 as the amino acid that was altered in an amiloride-resistant mutant and whose mutation into a phenylalanine residue conferred the same resistance phenotype to NHE-1. Preliminary studies also examined the kinetic properties of the expressed proteins, in particular examining the activation of transport by intracellular hydrogen ions and the extent of apparent co-operativity in the activation curve. In this respect, however, the correlation with the *in situ* studies is not as clear: all reconstituted isoforms exhibited an apparent Hill coefficient of greater than 2 [39], whereas in the originating tissues the coefficient ranged from 1.3 in epithelia to about 2 in fibroblasts and to 3 and above in muscle cells. It remains to be seen to what extent the cellular environment might influence the activation state and protein modifier site of the Na^+/H^+ exchanger, and thus determine what appears to be an isoform-specific phenotype. This is particularly important since a recent observation shows that the same NHE-1 clone, transfected into different cell lines, is differentially regulated by different stimuli [43].

Table 2 Putative phosphorylation and glycosylation sites on Na^+/H^+ exchanger isoforms

Species	Isoform	Tissue	Phosphorylation sites XRXXSX	XRRXSX	XPX(ST)P	Glycosylation sites NX(ST)	References
Human	NHE-1	†	56,324,648,703,796		723,726	75,370,410	16
Hamster	NHE-1	CCL39	57,328,652,707,803		727,730,780	76,374,414	20
Pig	NHE-1	LLC-PK_1	56,324,648,703,799		723,726,774	75,370,410	21
Rabbit	NHE-1	Ileal villus	11, 56,648,703,797		723,726,772	75,370,410	23
Rat	NHE-1	Heart	57,328,652,801		727,730,776	41,76,374,414	26
Rat	NHE-2	Intestine	503,554,665	528*,546,683		235,508,577	27
Rat	NHE-2	Stomach	47,619,670,781	644*,662,799	52	351,624,693	28
Rabbit	NHE-2	Ileal villus	618,669,777,806	643*,661,795	51	350,623,685	28a
Rat	NHE-3	Kidney	560,661,691	552,605,690	791,825	323,588,689,705,805	26
Rabbit	NHE-3	Kidney (cortex)	515,562,663,694	554,607*,693	797	325,692,811	24
Rat	NHE-4	Stomach	10,400,609,660			31,297,342	26

† This cDNA clone was isolated by complementation of an exchanger-deficient cell line.

* This site also contains an R residue, two amino acids after the serine, forming consensus sequence XRXXSXRX. One example of each species was used for each isoform except for NHE-2, which varied between the tissues. Only full-length sequences were used for analysis.

Although Na^+/H^+ exchange has also been observed in bacteria and yeast, and in organelles such as mitochondria (Table 1 and [44]), it appears that these antiporters belong to different gene families. Several of these exchangers have been cloned. However, none show significant homology to NHE-1 or other members of the NHE family. The different Na^+/H^+ exchanger proteins may have diverged from each other very long ago, or they may have evolved independently from each other and are not related at all. The stoichiometry and physiological missions of these exchangers differ markedly from those of the NHE family members.

Are there yet other isoforms to be discovered? Despite the fact that four different forms have already been identified (not counting trout and *C. elegans*), this is still a possibility. Several studies have shown the presence of other possible isoforms or have provided evidence for the possible differential splicing of some messages. Northern analysis revealed a message in testis which is smaller than the NHE-1 message in other tissues [26]. Dyck et al. [45] recently reported a smaller (3.8 kb) message in ischaemic hearts. This message hybridized with different NHE-1 cDNA probes derived from the open reading frame but not from the untranslated regions. Finally, there are reports of an Na^+/H^+ exchange function that is completely resistant to amiloride [46–48]. Since the currently expressed isoforms only exhibit reduced affinities as opposed to no binding at all, the underlying transporter could be considered as a candidate Na^+/H^+ exchanger isoform as well.

STRUCTURE OF THE Na^+/H^+ EXCHANGER

The members of the NHE family have very closely related structures. The different Na^+/H^+ exchanger isoforms range between 717 and 835 amino acids in length. Human NHE-1, for example, consists of 815 residues. It may occur in the cell membrane as a dimer with an apparent molecular mass of over 200 kDa [49]. The monomeric protein has a higher apparent mass (110 kDa) than expected from the amino acid sequence because it is glycosylated [17] on at least two glycosylation sites [50]. The human NHE-1 isoform has three consensus sites for glycosylation, and NHE-3 and NHE-4 have between three and five depending on the species (Table 2). All isoforms share a hydropathy profile which clearly delineates two different domains for the protein. The first 500–510 amino acids form alternating hydrophilic/hydrophobic stretches which suggest membrane-spanning (TM) segments. The remainder of the molecule, the C-terminal domain, is on average quite polar so that one is compelled to assign it to an aqueous environment (cytoplasm). In order to detect the exchanger protein immunologically with an antibody against the C-terminal region, one needs to permeabilize the cell, which provides evidence for the intracellular location of this region [17].

The structures of the hydropathy plots of the N-terminal membrane-intrinsic domains are nearly identical for the different isoforms [16,23,26]. These plots suggest that the peptide crosses the membrane up to 12 times. However, this suggestion of 12 TM segments can at best be considered tentative since different algorithms can lead to differing arrangements with a lower or higher TM number [51]. The near identity of the hydropathy profiles is based on the close sequence similarity among the different isoforms in the membrane-resident domain. In the hydrophilic domain, the hydropathy profile varies much more, suggesting that much of this domain confers isoform-specific properties.

In general, the NHE proteins are very well conserved within a given isoform and among different species, and even among different isoforms of the same species. On the peptide level, the different NHE-1 representatives from human, pig, hamster, rabbit and rat are 93–96% identical among themselves. The NHE-3 forms from rat and rabbit are also 87% identical in their peptide sequence. The catecholamine-responsive trout isoform, β-NHE, is 64% identical with human NHE-1 over its entire sequence length. When compared from the beginning of the second until the end of the twelfth TM segment (corresponding to residues 100–550 of human NHE-1), the trout sequence is 80% identical with human NHE-1. Even the exchanger of *C. elegans* is 44% identical with human NHE-1 over that membrane-intrinsic stretch, and 38% identical when all 699 residues of the partial-length clone are compared. Good conservation also exists among the different isoforms within one species, in particular for the stretch from TM-2 to TM-12. Comparing three published rat sequences (NHE-1, NHE-3 and NHE-4), for example, one finds for this stretch identity values of 47–53%.

The different isoforms do differ completely, however, in their

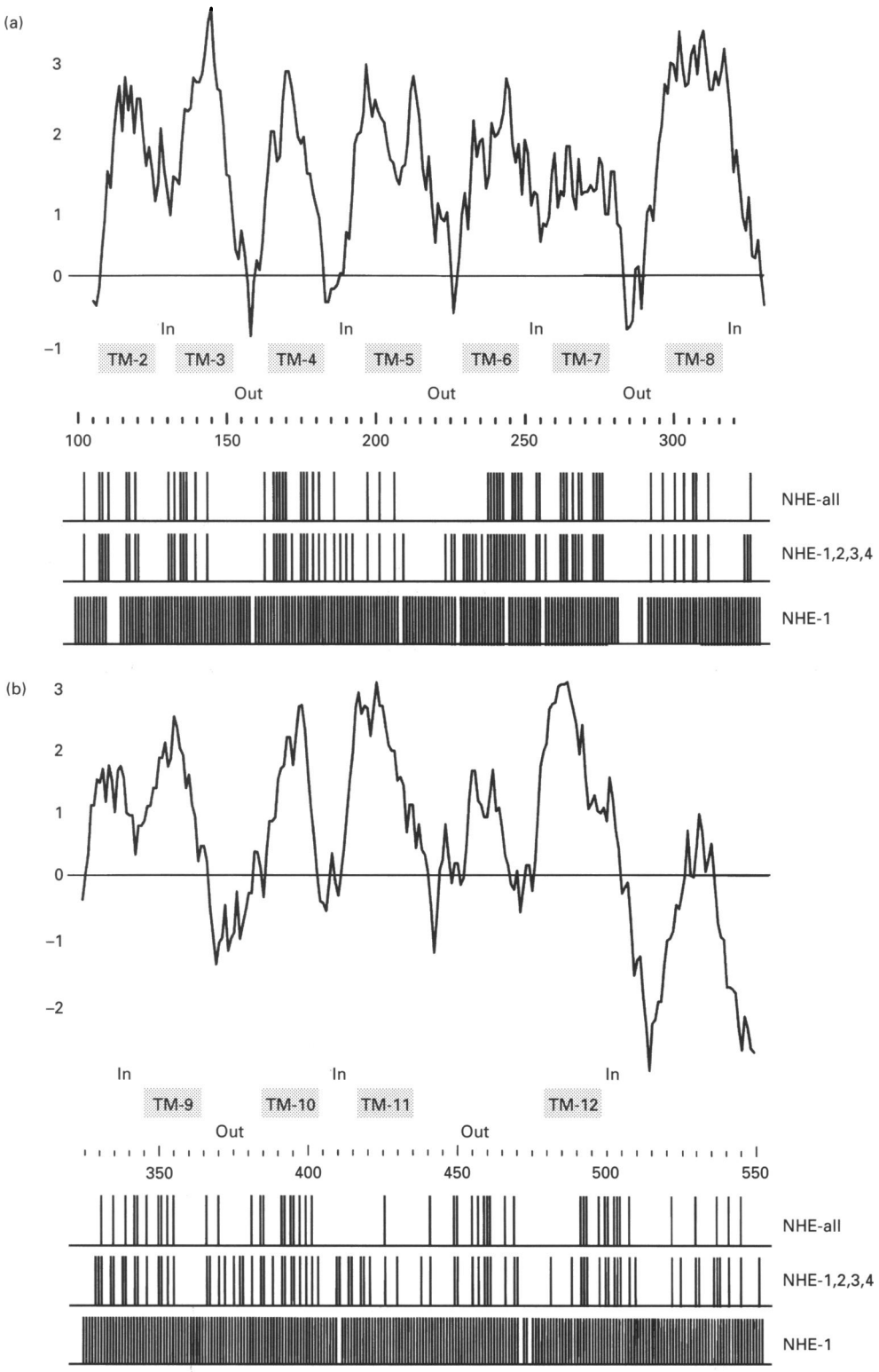

Figure 1 Analysis of the hydropathy profile and sequence identity of different NHE isoforms

The *x*-axis of the graph represents the amino acid chain of the exchanger protein, with the numbering corresponding to that of human NHE-1. Only the portion of the sequence in which there is identity between the different NHE isoforms is shown. The top line shows the hydropathy profile of human NHE-1, calculated as a moving average of the hydrophobicity values of Kyte and Doolittle [138] with a moving window size of 13. The boxes below indicate where one could expect the peptide to cross the membrane (TM-2–TM-12). TM-1 is not shown because it lies in a region where no identity exists among the different isoforms. The three bar graphs at the bottom give a measure of the identity among the different NHE members: among all published sequences (top), among NHE-1, NHE-2, NHE-3 and NHE-4 from rat (middle), and among all published mammalian NHE-1 members (bottom). The presence of a bar indicates identity at this position.

first putative TM segment (TM-1) and the following first extracellular loop. The strong identity begins only shortly before the peptide presumably enters the membrane for the second time. This curious phenomenon becomes more plausible in light of the suggestion that the first TM segment may comprise a leader sequence that is cleaved off during post-translational processing [15]. If this is the case, then the first TM segment would be lost after processing, leaving behind mature proteins that contain 11 TM segments of very similar structure. Hence, the only significant remaining isoform-specific difference within the membrane-resident domain would then be the first 20–60 (depending on the isoform) hydrophilic residues of the mature protein whose N-terminus would reside in the extracellular space. Perhaps the first transmembrane stretch contains the information that guides the protein into the proper region of the cell, such as the apical membrane in the case of the epithelial isoforms NHE-2 and NHE-3. However, this information could also reside in the C-terminal domain, which also varies between isoforms. Future studies are necessary to show whether this first transmembrane region can target the various exchangers to their proper location.

Figure 1 is a graphical summary of an analysis of the identity among the transmembrane regions of different transporter forms beginning after the first transmembrane passage. It shows vertical bars at the (equivalent) position of the human NHE-1 peptide sequence where the amino acid is conserved. The different NHE-1 representatives (human, pig, sheep, rabbit, rat and hamster) are closely related and most of the amino acids are conserved so that a bar is found in almost all positions (bottom row). In contrast, there is much less identity between NHE-1, NHE-2, NHE-3 and NHE-4 (from rat). When all sequences are included and compared (top row), the conservation is reduced further. It is apparent that most of the selection for the conserved sites on the exchanger protein has already occurred among the different isoforms within a species. Even including evolutionarily more distant representatives (trout and *C. elegans*) eliminated only about 30% of the identical sites among NHE-1, NHE-2, NHE-3 and NHE-4. However, it should be noted that this analysis deals with strict identity; if conservative replacements are included, the degree of similarity is considerably higher.

The strictly conserved residues belong almost exclusively to the membrane-associated domain. One can argue that these residues are somehow essential for basic transport function, or at least for structural integrity. Unfortunately, the number of conserved sites is fairly high (109 residues in the stretch corresponding to positions 100 and 550 of human NHE-1), and it is not possible to tell with any certainty which sites are crucial for transport function. This is aggravated by the lack of experimental verification of the putative topology of the TM segments. However, within the framework of the 12-TM model, there are several conserved charged sites in positions which one might suspect to reside in a TM segment, and which are therefore the best candidates for cation-binding sites. McDaniel et al. [52] have tentatively assigned a 'charge-relay' mechanism to the combination of Lys-116, His-120 and Glu-131 in TM-2, based on a molecular model which places them in close proximity to each other. Only one of these three sites is strictly conserved among all NHE members: in *C. elegans*, His-120 is replaced by Asn and Glu-131 is replaced by Asp. Since one could consider these substitutions to be conservative, this proposed mechanism is probably still a viable hypothesis. It is interesting to note that the most highly conserved region of the protein, corresponding to the stretch that includes putative segments TM-6 and TM-7, contains eight conserved negatively charged amino acids. These residues could be part of a cation-binding or transport site, or they could be essential for the proper local environment around an access channel to the binding site. They would provide a high negative charge density which in turn would result in a higher surface concentration of cations (protons?) and a higher apparent affinity for these cations. There are several other positions along the NHE sequence which one could consider as potential candidates for essential transport sites. However, further structural information is necessary before their analysis.

GLYCOSYLATION AND PHOSPHORYLATION SITES

A comparison of the glycosylation sites of the various isoforms is shown in Table 2. In most species, including man, NHE-1 possesses three potential sites of glycosylation, located in the first and fifth exoplasmic loops, and near the cytoplasmic entry of TM-11 (all in terms of the 12-TM hypothetical model); an additional site can be found still closer to the beginning of the protein in the rat exchanger. According to this 12-TM model, the exchanger would only be glycosylated at the first two sites. Although NHE-1 is known to be glycosylated [17] and the glycosylation is of the complex biantennary type [50], the exact glycosylation pattern is not yet clear. For example, there is evidence from partial glycolytic digestion experiments that the human placental transporter contains at least two and possibly three glycosylation sites [50]. The glycosylation sites are well conserved throughout the different NHE-1 representatives, but the carbohydrates do not appear to be essential for transport function [50,53]. Perhaps the carbohydrates' role lies in providing post-translational processing or targeting information. Only the second site is conserved among the different isoforms. NHE-3 and NHE-4 (from rat) contain additional potential glycosylation sites without NHE-1 counterparts; however, with NHE-3 they are mainly in the cytoplasmic domain or otherwise in locations that the 12-TM model would consider intracellular and therefore not glycosylated. NHE-2 is similar to NHE-3 in this regard. Of the three glycosylation sites, two are probably within the cytoplasmic domain [27].

The greatest differences among the different isoforms are found in the C-terminal domain. NHE-3 and the Na^+/H^+ exchanger of *C. elegans* exchanger lose their resemblance to NHE-1 beyond positions 540–550 of NHE-1. NHE-4 [26] and β-NHE of trout [29] retain significant identity with NHE-1 for approximately another 100 residues, beyond which only small patches of potential identity are observed. One major functional difference among the different isoforms lies in the way they are hormonally regulated. One might suspect that the structural correlate of this difference is found in the cytoplasmic domain since it comprises the interface between cytoplasmic second-messenger signalling and the exchanger function. However, the list of physiological roles of the cytoplasmic domain quite likely does not end here. For example, this portion of the protein could also contain binding sites for cytoskeletal elements [15,38]. Of greatest current interest, however, is to test the hypothesis that the C-terminal domain contains the phosphorylation site(s) that are utilized during hormonal regulation of the exchanger's activity. This hypothesis has been confirmed with C-terminally truncated exchanger protein. When the cytoplasmic domain was removed, the protein could no longer be stimulated by growth factors and the cytoplasm did not become more alkaline [38]. Interestingly, the truncated protein also exhibited a strongly acid-shifted pH_i dependence, as if the C-terminal domain also contained elements that controlled the hydrogen ion affinity of the presumed regulatory modifier site. To examine the functional role of the C-terminal domain in fibroblasts, Winkel et al. [54] microinjected polyclonal antibodies raised against the C-terminal

157 amino acids of NHE-1. The antibodies blocked activation by endothelin and α-thrombin, but did not block activation by phorbol esters, cell acidity and osmotic shrinkage. This suggests that the exchanger can be activated by phosphorylation or another form of regulation at different loci, with each locus specific for a different mechanism.

Both the C- and N-terminal domains of the exchanger proteins contain a number of putative sites of phosphorylation. Table 2 provides a summary of consensus phosphorylation sequences in the different isoforms of the Na^+/H^+ exchanger. These include the ideal recognition motifs for multifunctional calmodulin-dependent protein kinase II (CaM kinase II) (XRXXSX), for cyclic AMP-dependent protein kinase (PKA) (XRRXSX), and for protein kinase C (PKC) (XRXXSXRX) [55]. The presence of the sequence XPX(ST)P is also examined in Table 2. This represents the optimal consensus sequence for most of the isoforms of the mitogen-activated protein (MAP) kinase family and for $p34^{cdc2}$, the cyclin-dependent protein kinase [56]. The analysis of Table 2 shows that mammalian NHE-1 does not contain the consensus sequences XRRXSX and XRXXSXRX. The human, hamster and porcine NHE-1 isoforms all contain five conserved XRXXSX sites: two in the N-terminal and three in the C-terminal domain. One of the N-terminal and two of the C-terminal sites are also conserved in rabbit and rat NHE-1, and one even in rat NHE-4. NHE-2, NHE-3 and NHE-4 (rat) also contain additional sites for potential phosphorylation by CaM kinase II. Of the exchanger isoforms, only NHE-2, NHE-3 and β-NHE contain XRRXSX(RX) sites, which means that direct phosphorylation of the protein by PKA and PKC would be possible.

Mitogen-activated protein kinases (MAP kinases) and cyclin-dependent protein kinases have recently been implicated in the control of many cellular events. In particular, they are suggested to be involved in the cell cycle and meiosis [56]. For most isoforms of MAP kinase and for $p34^{cdc2}$ the consensus sequence XPX(ST)P defines the preferred site of phosphorylation [56]. In most species the NHE-1 isoform of the Na^+/H^+ antiporter contains three such sites (Table 2). Two of these are interesting in that they overlap, while a third site further downstream occurs in all species, except in human NHE-1. NHE-3 contains one or two such sites depending on the species and the rat NHE-4 contains none. In all representatives of NHE-3 and NHE-4, these consensus sequences are located in the C-terminal domain of the proteins. The Na^+/H^+ exchanger is known to be important in regulation of cell proliferation and in control of the cell cycle [57,58]; it remains to be seen whether these sites are involved in these processes and in the hormonal stimulation of the Na^+/H^+ exchanger in general.

Specifically which sites on the C-terminal domain are phosphorylated *in vivo* is not yet known since they possess many serine and threonine residues. It is known that serine residues are phosphorylated [17] but their exact location is not known. The situation is rather complex in that most NHE-1 members do not possess a good consensus sequence for PKC even though this kinase is involved in many signalling chains following receptor activation [17]. The same set of serine residues appears to be phosphorylated in response to different agonists, whether these activate a tyrosine kinase or phosphoinositide hydrolysis [59]. Therefore one or more additional, yet to be identified, kinases must be involved. Also, dephosphorylation by ATP depletion and rephosphorylation by ATP repletion phosphorylates what appears to be another class of sites (probably serine residues as well [17,60]). Finally, under at least one condition, namely in response to cell shrinkage, transport activation can also occur without detectable phosphorylation [61,62]. It was suggested that dual control of NHE-1 may occur by phosphorylation-dependent and phosphorylation-independent mechanisms [62].

There are some sites that would appear to be reasonable candidates for regulation through other signalling pathways. The NHE-1 sequence contains several consensus sites for CaM kinase II. Several of these sites from the C-terminal region have been successfully phosphorylated *in vitro* by CaM kinase II but not by PKA or PKC [63]. Also, β-NHE has two consensus sequences for phosphorylation by PKA which are probably the sites through which the trout red cell exchanger is stimulated by β-adrenergic pathways [29,64]. However, the existence of a consensus sequence does not necessarily mean that this site is phosphorylated. Despite indications that the cytoplasmic domain between residues 566 and 635 is required for mitogen activation of transport, mutating each of the eight serine residues in this stretch did not alter this activation [15]. The same negative result was obtained when Ser-648, a promising candidate in a consensus context for phosphorylation, was mutated [15]. This could mean that more than one residue needs to be phosphorylated for transport activation, and/or that one has to look elsewhere for additional phosphorylation sites, possibly even on the membrane-resident N-terminal domain. An alternative explanation is that phosphorylation of this domain is degenerate. With the cystic fibrosis transmembrane regulator it was found that although four sites were phosphorylated *in vivo*, no one specific site was necessary for responsiveness to cyclic AMP. One site alone was sufficient for regulation [65]. Future studies will be necessary to determine if this is the case for NHE-1.

It should be noted that while phosphorylation of NHE-1 is usually considered to be stimulatory, for the apical exchanger this is not necessarily the case. Stimulation of either PKC or CaM kinase II can inhibit the ileal brush border membrane exchanger [66,67]. Both NHE-2 and NHE-3 are expressed in this tissue [24,27,28a] and both contain consensus sequences for phosphorylation by either kinase. Which sites are used for phosphorylation is not yet known at this time.

REGULATION OF THE Na^+/H^+ EXCHANGER

Na^+/H^+ exchange is subject to regulation on several different levels: (1) through modification of exchanger turnover rate and (2) through mechanisms which modulate the number of exchanger units available for transport. On the first level, the enzymic activity can be modified by an intrinsic H^+ modifier site giving rise to the steep activation of exchange at a lowered intracellular pH. This gives the protein the means to adjust its activity sensitively to changes in intracellular pH. Extrinsically, hormones and mitogens modulate the exchanger's activity through phosphorylation, which can further depend on the cellular milieu in which the Na^+/H^+ exchanger is expressed [43]. These two aspects of regulation of activity have been reviewed most recently by Wakabayashi et al. [38] and will not be described here. The other important level of regulation of the Na^+/H^+ exchanger deals with the numbers of exchanger units in the plasma membrane. Regulation may occur during transcription of the Na^+/H^+ exchanger gene into message and during translation from the message to protein. In addition, recruitment of Na^+/H^+ exchanger from intracellular stores to the plasma membrane may occur. We focus on transcriptional control of Na^+/H^+ exchanger numbers and to a lesser extent on translational control and recruitment. We will concentrate on the NHE-1 isoform (or generic Na^+/H^+ exchange), since little information is available in this area on the other isoforms.

Although the Na^+/H^+ exchanger exists in all tissues, it is generally present in only small amounts and is not easily

detectable by immunological methods [49]. One possible explanation for the low protein levels would be an intrinsically low rate of translation of the message into protein. Translational control of the exchanger has not yet been studied in detail, but at least for NHE-1 there are suggestions that the message contains elements that serve to keep translational activity low. Wakabayashi et al. [38] showed that the 5′ untranslated region of the NHE-1 message inhibits translation of NHE-1. Removal of most of the 5′ untranslated region of the cDNA resulted in higher cellular Na^+/H^+ exchange transport activity after transfection and expression in an exchanger-deficient cell line. Depending on the species, the 5′ untranslated region of NHE-1 contains up to three minicistrons. These are short, open reading frames which contain an initiation codon, code for only a few amino acids and end in a termination codon. In NHE-1 these minicistrons are all in-frame with each other, but are out-of-frame with the initiating ATG of the main open reading frame. Such out-of-frame minicistrons can be inhibitory to translational efficiency. They cause the translational machinery to fall off the mRNA before reaching the major open reading frame or can cause a failure to recognize the relevant major ATG codon [68]. Whether or not this is the reason for the effects noted earlier on translational efficiency [38] is not yet known. Interestingly, rat NHE-3 possesses one in-frame minicistron a few codons upstream of the major open reading frame. On the other hand, rat NHE-4 possesses several minicistrons in all three reading frames, with one bracketing the main initiation site. There are no studies yet that have tested the potential role(s) of these minicistrons in more detail. In addition, it is not known whether these hypothetical minicistron effects are constitutive or are subject to additional control by modulation of the translational machinery.

Probably a greater regulatory influence lies in the transcription step from Na^+/H^+ exchanger gene to mRNA. All other factors being equal, increased mRNA levels by transcriptional upregulation will result in increased levels of the gene product. That the Na^+/H^+ exchanger may be transcriptionally regulated has been suspected for some time. A number of external environmental stimuli have been shown to affect the maximal rate of Na^+/H^+ exchanger activity, suggesting a possible increase in Na^+/H^+ exchanger message and protein levels. Earlier studies examined the maximal rate of Na^+/H^+ antiport activity, since specific antibodies or probes for mRNA were not yet available. For example, brush border membrane vesicles from rat proximal tubules showed increased Na^+/H^+ exchange after treatment of the animals with glucocorticoids [69]. The activation was specific in that glucose uptake was not affected and Na^+ gradient-dependent phosphate uptake was decreased. Also, the mineralcorticoid aldosterone caused no such increase [69]. A similar effect was observed in isolated proximal cells from kidney where glucocorticoid treatment increased the $V_{max.}$ of Na^+/H^+ exchange. The stimulation was blocked by actinomycin D or cycloheximide, suggesting that both RNA and protein synthesis were required [70]. The mechanism by which glucocorticoids activate the exchanger is not known but is generally thought to be by classical steroid hormone action [71]. A recent study suggests that, at least under some circumstances, it is the NHE-3 isoform of the exchanger that is regulated by glucocorticoids and not the NHE-1 or NHE-2 isoforms [72]. Glucocorticoids were shown to elevate the ileal brush border exchanger NHE-3 message levels and the activity of the protein in rabbits treated with methylprednisolone [72]. Thyroid hormone also affects the rate of Na^+/H^+ exchange in kidney brush border membranes from rats. Hypothyroid rats show decreased Na^+/H^+ exchange and hyperthyroid rats show increased maximal exchange rates [71,73,74]. Mineralocorticoids such as aldosterone are not thought to affect the protein levels; however, in some special cell types they may act similarly to the glucocorticoids [75]. These findings, combined with the other studies, show that glucocorticoids and other hormones regulate the level of Na^+/H^+ exchange activity and that the variations occur in a physiologically adaptive way.

Regulatory effects have also been observed in different renal models. In renal hypertrophy, increased binding of the amiloride analogue, [^{3}H]ethyl isopropyl amiloride, also supports the theory that Na^+/H^+ exchanger numbers can increase in response to external stimuli [69]. Unilateral nephrectomy causes an increase in the Na^+/H^+ exchange activity of renal cortical brush border membrane vesicles. The increase in Na^+/H^+ exchange after 48 h is abolished by prior administration of actinomycin D, indicating that protein synthesis is involved [76]. Several other conditions such as renal disease and diabetic nephropathy may also affect Na^+/H^+ exchange activity, possibly acting through other mechanisms such as systemic acidosis (reviewed in Fine et al. [77]).

Perhaps the most interesting physiological response is the increase in level of the Na^+/H^+ exchanger activity in response to long-term acidosis. There appears to be a physiologically adaptive mechanism by which some eukaryotic cell types are able to upregulate this acid-removing transporter in response to chronic acid load. In an early study, chronic metabolic acidosis, induced by addition of NH_4Cl to drinking water, increased the $V_{max.}$ of Na^+/H^+ exchange in rat renal cortical brush border membrane vesicles [73]. Similarly, chronic acid feeding increased the $V_{max.}$ of apical Na^+/H^+ exchange in the rat proximal tubule [78]. At that time, little was known about the different isoforms of the exchanger and it is likely that these workers were not examining the activity of the NHE-1 isoform in the apical membranes. Probably their results originated from a more amiloride-resistant isoform of the Na^+/H^+ antiporter [12–14], possibly the NHE-3 isoform [26]. However, Akiba et al. [79] found similar effects in both brush border and basolateral membranes of rabbit renal cortex. Others [80,81] have also shown that chronic metabolic acidosis induced by NH_4Cl feeding results in increased renal cortical mRNA levels (NHE-1). Similar results were obtained with whole renal proximal tubule cells incubated in acidic media. This treatment resulted in increased Na^+/H^+ antiporter activity, and this increase depended on protein synthesis. There was no stimulation in fibroblasts treated in the same way [82], suggesting that the effects of acidosis on the Na^+/H^+ antiporter are tissue-specific. Similar effects occur in mouse renal cortical tubule cells and in opossum kidney cells [80]. Incubation in acid media increases Na^+/H^+ exchanger mRNA by up to 90% as detected by an NHE-1 probe. The same treatment decreased the mRNA abundance of the Na^+/H^+ exchanger of 3T3 fibroblasts, again demonstrating the tissue specificity of the effect [80]. LLC-PK_1 renal epithelial cells also show increased expression and activity after 48 h of treatment at pH 6.9 [83]. When rat hearts are subjected to relatively short periods of ischaemia, intracellular acidosis occurs. This results in a small increase in NHE-1 mRNA levels and a larger increase in a related isoform (3.8 kb) of the message [45]. Primary cultures of isolated myocytes also increase Na^+/H^+ exchanger activity in response to exposure to external media of low pH (L. Fliegel, unpublished work). The stimulatory effects of acidosis are not unique to the Na^+/H^+ exchanger. Chronic metabolic acidosis also causes increases in activities of the basolateral membrane Na^+/HCO_3^- cotransporter and phosphoenolpyruvate carboxykinase [78,79,84].

The exact mechanism by which low external pH increases Na^+/H^+ exchanger mRNA levels and transport activity is still in question. Some of the increases in message observed *in vivo*

may be accounted for by acidosis-induced increases in circulating adrenal corticosteroids [71]. However, the effect has been shown in cultured cell lines, suggesting that it is intrinsic to the cells and does not depend on additional external factors. In the case of phosphoenolpyruvate carboxykinase, both an increased transcription rate [85] and increased mRNA stability may be responsible for some of the acidosis-induced increases in message [86]. A potential mediator of the acidosis effect is the transcription factor AP-1, the activity of which is increased during acid stimulation [87]. The human NHE-1 gene contains three AP-1 binding sites [88]. In comparison, the mouse NHE-1 promoter region contains only one AP-1-like site (L. Fliegel and J. Dyck, unpublished work), as does the rabbit gene [89]. However, it has not yet been conclusively demonstrated that AP-1 directly activates the antiporter gene or that the acid-induced increases in NHE-1 message are solely transcriptionally mediated. AP-1 could also activate other genes whose products activate the exchanger, or other protein kinase pathways could mediate the effect [87,90]. Experiments with reporter genes coupled to a string of six AP-1 sites demonstrated the involvement of cellular AP-1 protein in acidosis-induced upregulation in renal epithelial cells [87]. The next step will be to demonstrate the AP-1 effect with an NHE-1 promoter-coupled reporter gene, and to show that AP-1 actually binds to the expected sites on the promoter. In the case of phosphoenolpyruvate carboxykinase, the 5′ flanking region of the gene has been shown to mediate increased transcription of a reporter gene in response to pH [91]. In addition, other mechanisms such as changes in RNA stability could also be involved. It is, however, clear that a mechanism of compensation has evolved by which chronic increases in acid load in the cell result in a compensatory increase in the Na^+/H^+ exchanger. Figure 2 shows several hypothetical mechanisms that would result in increased NHE-1 expression. Future studies may confirm, disprove or replace these mechanisms.

In addition to transcription, recruitment of protein from intracellular stores may participate in the acidosis-mediated upregulation of Na^+/H^+ exchanger activity. Soleimani et al. [92] showed that acute treatment of rabbit renal proximal tubule cells with acidotic media can affect Na^+/H^+ exchanger activity. Treatment for 2 h with an acidic solution resulted in an increased $V_{max.}$ in both brush border and basolateral membrane vesicles from these cells. This upregulation might reflect activities of two different isoforms of the exchanger. The presence of cycloheximide did not block the increased activities, suggesting that protein biosynthesis was not required for this effect. In this case the increased activity could be due to changes in regulation of the protein or in recruitment from intracellular stores. Regulation of the protein by phosphorylation is a viable candidate since it is known that the exchanger can be phosphorylated [17]. In addition, the Na^+/H^+ exchanger is present in endosomal vesicles [93], and hormonally induced translocation has been reported previously in proximal tubules [94]. This short-term stimulation of Na^+/H^+ exchange appears different from that observed after longer periods of acidosis, which involves an increased message and a requirement for protein biosynthesis [80-82]. Thus acid-induced upregulation of Na^+/H^+ exchange appears to be the result of several different regulatory pathways, in parallel or sequentially, by which cells protect themselves from acute or chronic acidosis.

Several other factors have also been shown to affect or be involved in regulation of NHE-1 mRNA levels in a variety of cell types. In vascular smooth muscle, serum and platelet-derived growth factor increase Na^+/H^+ exchanger mRNA levels by up to 25-fold [95], but serum only causes a minor increase in the $V_{max.}$ of the protein [96]. The human NHE-1 gene [88] and the mouse gene (L. Fliegel et al., unpublished work) contain only portions of the serum response element, leaving the mechanism of the effect of serum in question [97]. Other mitogens such as phorbol esters, fibroblast growth factor and platelet-derived growth factor also cause 5–15-fold increases in mRNA levels, while angiotensin II has only marginal effects. In smooth muscle, the response can be characterized as an increase in Na^+/H^+ exchanger mRNA levels in response to mitogenic but not hypertrophic stimuli [96]. Rao et al. [98] examined the regulation of NHE-1 gene expression during monocytic differentiation of HL60 cells. During phorbol ester-induced differentiation, mRNA levels increased 50-fold and protein levels also increased 30-fold. Nuclear run-on assays showed that increased transcription accompanied the increased mRNA levels [98]. Activation of PKC alone with a synthetic diacylglycerol which does not induce differentiation did not cause the large increase in transcription. This study showed that increased transcription can, at least with some types of stimulation, be responsible for the increases in Na^+/H^+ exchanger mRNA levels. Similar results were observed with HL60 cells during differentiation to granulocytes induced by retinoic acid. In this case the level of the Na^+/H^+ exchanger message increased 7–18-fold. The increase was also due to an increased rate of gene transcription, and the level of protein also increased 7-fold [99].

To investigate further the role of PKC, Horie et al. [90] examined the effect of phorbol esters on exchanger levels in proximal tubule cells. Long-term stimulation of the cells with phorbol esters resulted in increased activity of the exchanger that could be blocked by cycloheximide and actinomycin D. However, Northern blot analysis showed only a 2-fold increase in the message levels upon treatment with phorbol ester. Preliminary experiments by Horie et al. [90] suggest that inhibition of PKC can prevent the increase in antiporter activity induced by acid media. Future experiments are necessary to confirm this suggestion. Overall, it is apparent that besides acid-induced increases in Na^+/H^+ exchanger message and activity, stimuli such as activation of PKC and differentiation of some cell types can have profound effects on Na^+/H^+ exchanger levels.

THE MYOCARDIAL Na^+/H^+ EXCHANGER IN HEALTH AND DISEASE

The regulation of internal myocardial pH is of especial importance to the function of the heart. The resting intracellular pH is typically near 7.2. It can drop dramatically during ischaemia, in the process depressing contractility of the myocardium. The negative inotropic effect of acidosis has been demonstrated in a variety of cardiac preparations ranging from cardiac muscle fibres [100] to the isolated perfused heart [101] and the rabbit heart *in vivo* [102]. The lowered pH_i depresses the contractility by affecting a number of steps of excitation–contraction coupling (reviewed in [103]). In order to protect the physiological role of the heart muscle as a pump, one would therefore expect that the heart muscle cells possess mechanisms to maintain the cellular pH_i within fairly narrow limits. One important mechanism is Na^+/H^+ exchange.

The Na^+/H^+ exchanger shares the physiological mission of regulating the myocardial pH with at least two other transporters: a Cl^-/HCO_3^- exchanger and a Na^+–HCO_3^- cotransporter [104,105]; in some cardiac tissues possibly also a Na^+-dependent Cl^-/HCO_3^- exchanger [106]. In this collective mission the different transporters appear to have specialized roles with relatively little overlap. The Cl^-/HCO_3^- exchanger, which at least in some cells is activated by a more alkaline pH_i [107,108], serves to acidify the cell when needed. The Na^+/H^+ exchanger, which is activated by acidic pH [109], helps the cell to recover

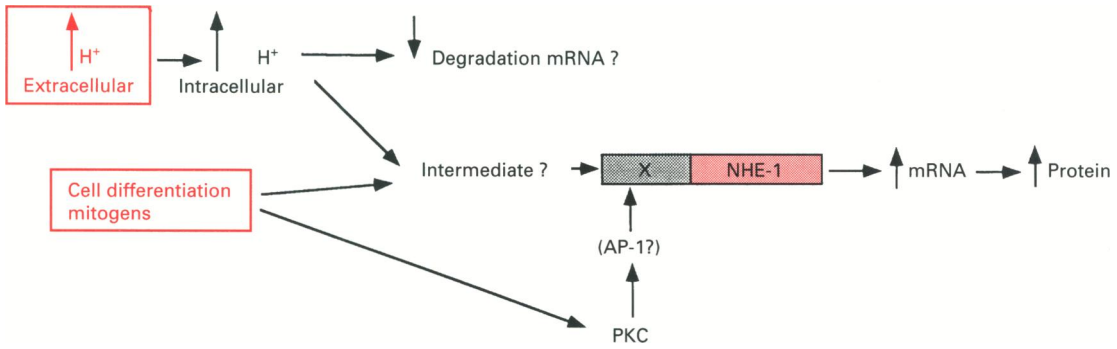

Figure 2 Possible mechanisms of induction of expression of the Na^+/H^+ exchanger

The temporal sequence is shown from left to right. A number of stimuli including acidosis and mitogens are known to stimulate Na^+/H^+ exchanger activity. In some cases increased transcription of the NHE-1 gene has been demonstrated. Whether all stimuli act through the same mechanisms, such as through AP-1 or another intermediate, is not yet known. Increased levels of message, protein and activity have been demonstrated as a result of some types of stimuli. For some other proteins, decreased mRNA degradation has been shown to be responsible for increased message. Whether this occurs with the Na^+/H^+ exchanger is not yet known. X represents the promoter–enhancer region of the gene.

from a strong acid challenge. The Na^+–HCO_3^- cotransporter also exports acid equivalents by importing bicarbonate together with Na^+, and supports the Na^+/H^+ exchanger during recovery from acidosis. However, its contribution relative to Na^+/H^+ exchange varies with the cardiac preparation between 20 and 40% [104,105]. Because of the steep activation curve of Na^+/H^+ exchange with lowered pH_i [109], it is reasonable to assume that at resting pH_i the Na^+/H^+ exchanger is only marginally active and that the job of regulation of resting pH is physiologically assigned mainly to Na^+–HCO_3^- cotransport.

In other words, steady-state pH_i can be seen as the balance between acidifying mechanisms (residual Cl^-/HCO_3^- exchange, a background hydrogen ion leak through the membrane or cation channels, metabolic acid production, etc.) and the alkalinizing effects of residual Na^+/H^+ exchange and the Na^+–HCO_3^- cotransporter. The notion of a relatively minor role for Na^+/H^+ exchange near resting pH_i is supported by the observation that amiloride has only a small acidifying effect on steady-state pH in heart cells. One should keep in mind, however, that it takes only a small alkaline shift (by 0.1 to 0.2 pH units) in the pH-dependence curve of Na^+/H^+ exchange to raise the relative importance of this exchange and cause cytoplasmic alkalinization, such as is observed after exposure to α-adrenergic agonists [110,111], especially if this is accompanied by inhibition of Na^+–HCO_3^- cotransport [104,105].

Myocardial Na^+/H^+ exchange exhibits a transport kinetic feature which seems to distinguish it from Na^+/H^+ exchange in other cells, namely its dependence on the intracellular H^+ concentration. Intracellular hydrogen ions do not activate Na^+/H^+ exchange in a Michaelis–Menten-type manner. Rather, the pH_i-dependence exhibits positive co-operativity, as if more than one hydrogen ion is involved in the activation. When fitted to a Hill-type expression, Na^+/H^+ exchange in renal brush border vesicles could be characterized by a Hill coefficient of $h = 1.2$–1.5 [112,113]. This value of h appears to be typical for epithelial cells (and by implication probably for the epithelial isoforms), but it is in general higher (1.5–2.0) [5] in tissues where NHE-1 is expected to exist. In heart and skeletal muscle, however, the fitted Hill coefficient is still higher, with $h = 2.5$–3 [109,114,115].

Such a difference in transport kinetics would suggest that the heart expresses its own cardiac isoform. However, this notion is not borne out by experiments and the fact that NHE-1 is the predominant isoform in the heart. Screening of rabbit and rat heart cDNA libraries led only to clones of NHE-1 [22,26]. In addition, Northern blot analysis of heart muscle mRNA with NHE-1 probes reveals mainly the message size of 5 kb that is consistent with NHE-1 [26,45]. However, a smaller message of 3.8 kb also hybridizes to NHE-1 probes under low stringency, which becomes more pronounced after ischaemic exposure [45]. In conclusion, it is not clear whether the kinetic difference is due to the contribution by an additional, as yet unidentified, exchanger isoform or whether it represents a difference in the modulation state of the exchanger in different cell types.

The high value of the Hill coefficient observed in cardiac myocytes is a quantitative description of the fact that the Na^+/H^+ exchanger is activated by intracellular hydrogen ions over a quite narrow pH range. The Na^+/H^+ exchanger goes from being nearly inactive to nearly maximally activated within one pH unit [109]. This sensitive response to intracellular acidification is needed if the Na^+/H^+ exchanger's main role is to protect the cell from an acidic challenge. Na^+/H^+ exchange can handle even a strong acidification because of the large cellular transport capacity (of all Na^+/H^+ exchangers combined). At $V_{max.}$, Na^+/H^+ exchange has probably the highest transport capacity among the different carrier systems in the sarcolemma. Unfortunately, this also means that during recovery from strong acidosis there is a high influx rate of Na^+ ions in exchange for extruded H^+ ions. This rate is higher than the transport capacity of the Na^+/K^+-ATPase whose role is to maintain a low intracellular Na^+ concentration. As a consequence, the intracellular Na^+ concentration rises and with it, because of the tight coupling of the Ca^{2+} gradient to the Na^+ gradient, the intracellular free Ca^{2+} concentration rises. A high intracellular Ca^{2+} concentration in turn can cause cellular damage in many different ways: biochemically through uncoupling mitochondrial function, mechanically through local cellular hypercontraction, and by triggering arrhythmias that impede the pumping function of the heart and further aggravate the ischaemic cause (Figure 3).

Under normal physiological conditions this scenario does not occur, and even a mild acidosis is handled by the combination of Na^+/H^+ exchange and Na^+ extrusion. However, the advantage of a responsive, powerful acid extrusion mechanism becomes detrimental to the heart when the acid challenge is too large, such as during and after an ischaemic episode. This is not to say that the sequence of events described above is the only mechanism that contributes to reperfusion injury after ischaemia. However, it represents a new viable hypothesis for one process which has

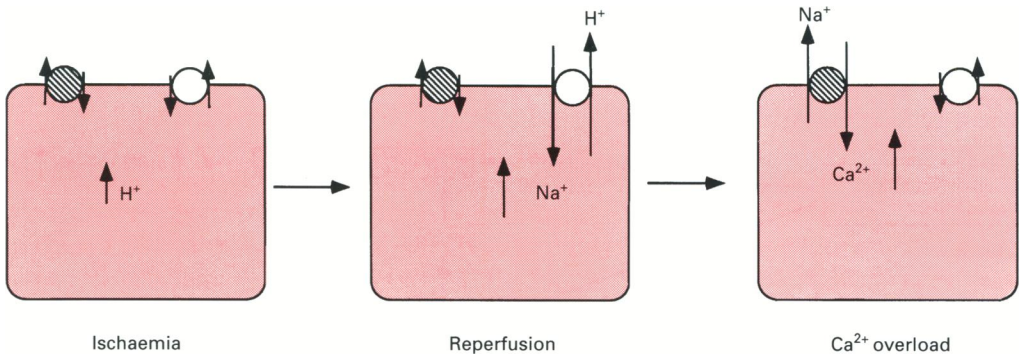

Figure 3 Putative series of events illustrating how intracellular acidosis can lead to Ca^{2+} overload

Excess intracellular protons as a result of ischaemia lead to decreased intracellular pH. During reperfusion the resulting acid load activates the Na^+/H^+ exchanger, resulting in increased intracellular Na^+. Subsequently, the increased intracellular Na^+ results in increased intracellular Ca^{2+} through the actions of the Na^+/Ca^{2+} exchanger. Excess intracellular Ca^{2+} is known to have a variety of detrimental effects resulting in cell damage and possibly in generation of arrhythmias.

recently received strong support from a number of different laboratories.

When hearts are rendered ischaemic for long periods of time, they do not completely recover their contractile strength after perfusion is resumed. Myocardial damage associated with these episodes occurs with reperfusion of the myocardium and not with the initial ischaemic episode [116,117]. Therefore it is not the low intracellular pH or the change in oxidation state of the cell that elicits injury, but an event that starts with reperfusion. This is very interesting in terms of the observation that Na^+/H^+ exchange is inhibited by a low extracellular pH [109]. During ischaemic acid build-up of mainly lactate, both intracellular and extracellular pH levels drop, but the low intracellular pH cannot stimulate Na^+/H^+ exchange since the low extracellular pH has shut down the exchanger. Once reperfusion is started, the first change is a return to normal extracellular pH, which removes the block on the Na^+/H^+ exchanger. The rising intracellular Na^+ concentration then activates Ca^{2+} influx via the Na^+/Ca^{2+} exchanger, resulting in Ca^{2+} overload and cell death or arrhythmias [118–120]. Figure 3 shows this proposed mechanism of damage to the myocardium.

The individual steps of the reaction sequence described above have been subject to experimental scrutiny by examining the effect of the inhibitor amiloride on reperfusion injury. First, in n.m.r. experiments to examine intracellular pH in rat hearts, it was shown that the recovery of myocardial pH from ischaemia is slowed down in the presence of amiloride [121]. Several studies also showed that intracellular Na^+ levels increase significantly during reperfusion and that inhibiting the antiporter with amiloride can reduce Na^+ accumulation during this period [119,122]. In the same manner, amiloride analogues prevent reperfusion-induced increases in Na^+ and Ca^{2+} concentrations [119,122–124] and lead to an improved return of mitochondrial function [125]. They also reverse lactate-induced depression in post-ischaemic ventricular recovery [126]. The amiloride analogues ethyl isopropyl amiloride, dimethyl amiloride and hexamethyl amiloride are much more potent inhibitors of the exchanger than is amiloride [9]. They are also more potent in preventing the detrimental effects of reperfusion. A similar mechanism may occur when amiloride and its analogues improve recovery from hypoxia and subsequent reoxygenation [127,128].

Amiloride and its analogues also improve the recovery of cardiac contractility and reduce the severity of the arrhythmias after ischaemia and reperfusion in isolated myocardial tissue preparations [118,119,122,123,129,130]. The beneficial effect of amiloride and ethyl isopropyl amiloride on reperfusion-triggered arrhythmias was also observed *in vivo* with rats whose hearts were made ischaemic by coronary artery ligation [131]. Again, this is consistent with the idea of a central role of the Na^+/H^+ exchanger in the mechanism of cellular damage and necrosis [131,132]. Significantly, amiloride needs to be present during the reperfusion phase and there is no noticeable improvement in the protection when amiloride is also present at the time perfusion is stopped and the ischaemic period begins [123]. This suggests that protons accumulate during ischaemia through the formation of metabolic acid, but their exchange with intracellular Na^+ through the antiporter occurs only during reperfusion of the myocardium. In support of this notion, when the heart is reperfused after an ischaemic period with media of a more acidic pH, it is protected relative to the extent of damage observed with reperfusion media of normal pH (Figure 3 and [22]).

These observations demonstrate the importance of Na^+/H^+ exchange under physiological and pathological conditions. A surprising and seemingly paradoxical conclusion is that inhibition of the normal function of this transporter actually improves the health of the myocardium in certain pathological disease states. A reasonable explanation would be that it is more beneficial for the heart muscle as a pump to respond efficiently to a minor acid challenge and rapidly restore its contractility than to be ready for a catastrophic event that usually occurs at a later stage in the life of the individual. As a logical extension, one could surmise that if the protein were overexpressed in certain pathological states *in vivo*, it could have an even more deleterious effect on the myocardium. Does chronic ischaemia or acidosis cause increased expression of the antiporter in the heart, similar to what is observed in some other tissues? Could increased expression of the antiporter further aggravate some pathological conditions? The answers to these question lie in future experiments and may lead to a better understanding of the mechanism of myocardial dysfunction in disease.

FUTURE PROSPECTS

The Na^+/H^+ exchanger with its several isoforms represents a major pathway for the removal of acid equivalents. It plays important roles in cellular homeostasis and development, as well as under certain pathological conditions. While a fundamental understanding of its functioning is emerging, many questions

remain unanswered about this family of transport proteins. For example, little is known about the mechanism by which the exchanger is regulated by extrinsic factors, a topic which is not addressed in detail here but which has received extensive coverage elsewhere [15,133). A number of other important questions remain. How many different phosphorylation sites (or classes of sites) are there? Does their phosphorylation lead to the same kinetic modulation of the exchanger? Which are the kinases that directly phosphorylate and regulate the exchangers? To what extent does the regulation involve interactions among different exchanger molecules in a multimeric complex? Or could it involve interactions between the membrane and the protein's membrane anchoring site, as could potentially happen during volume-dependent modulation?

Targeting and localization of the Na^+/H^+ exchanger is another largely unexplored issue. In epithelial tissues, the epithelial exchanger isoforms (NHE-2 and NHE-3, and probably also NHE-4) are presumed to be localized in the apical membrane and NHE-1 on the basolateral side, but in the human placenta the localization of the different isoforms is reversed [37]. How this differential localization is programmed and how the targeting to the correct membrane is executed is not yet known. It could be the result of specific signals present in the sequence of the protein itself that are recognized in a tissue-specific fashion. This question may receive an answer in the near future with the expression of the different isoforms and the development of isoform-specific antibodies.

The physical mechanism of transport through the Na^+/H^+ exchanger is a complete unknown and has previously only been accessible through transport-kinetic experiments. Now that the protein can be expressed in cells, as the wild type or containing specific mutations, its kinetics can be studied again with the goal of correlating structure with function. Recent studies have provided evidence for sites whose mutation drastically lowers the affinity for amiloride [134]. Other experiments suggest that the cytoplasmic domain contains elements of the internal H^+ modifier site [15]. However, a rational search for the structural correlates of the kinetic properties will only be possible once the topology of the protein is better understood.

Beyond the division of the exchanger protein into two domains, little is certain about the structure of the protein. An analysis of the hydrophobicity profile of the amino acid sequence suggests that the hydrophobic N-terminal domain can contain as many as 12 membrane-spanning segments. However, predicting the transmembrane orientation by hydropathy analysis is not overly reliable [51]. Other algorithms of predicting structural properties of the protein lead to differing estimates of how many TM segments one can expect (down to 10 or even less). For example, Orlowski et al. [26] proposed that TM-1 and TM-2 of the 12-TM model do not span the plasma membrane but constitute a cytoplasmic membrane-associated domain. Only specifically designed experiments will reveal details of the protein's topography. A first handle could be a careful analysis of the glycosylation pattern since extensive glycosylation is found essentially only on the exoplasmic face of the protein. NHE-1 is indeed glycosylated [17], and the placental form possesses at least two, perhaps even three, glycosylation trees [50]. If indeed three such sites were found by additional experimentation, this would challenge the 12-TM model that is currently favoured (see Figure 1), since this model would place the third site on the cytoplasmic face. A comparative study with the other NHE isoforms could be helpful since not all sites are conserved within the NHE family (Table 2). Further tests on cloned exchangers await the development of an expression system that provides sufficient quantities of fully functional protein. Initial attempts to overproduce the protein in insect cells using the baculovirus system have met with mixed success, with much of the protein apparently existing in a non-functional form [53]. We have noted that attempts to produce the protein *in vitro* have not been as successful as with other proteins (L. Fliegel and O. Fröhlich, unpublished work). It is not clear to what extent possible low-usage codons, secondary structures in the message or other mechanisms can account for these results.

A newly emerging and interesting aspect of the Na^+/H^+ exchanger is the regulation of the gene. As discussed above, cells possess mechanisms which respond to acid loads by increasing the levels of Na^+/H^+ exchanger mRNA and protein. It is up to future experiments to determine the details of how this response is mediated. Do similar mechanisms exist for the other isoforms? How widespread is this phenomenon among the different tissues? Apparently it is not observed in all cells [82].

In this context, future studies will in addition deal with the different transcription factors which may also be involved in regulation of the exchanger. There are consensus sequences for several DNA-binding proteins present on the human and mouse NHE-1 genes, including Sp-1, AP-1, AP-2 glucocorticoid response element, cyclic AMP response element and others ([88]; L. Fliegel, unpublished work). Finding how this gene is regulated will not only lead to the mechanism behind the response to chronic acid load, but it may also reveal information on the possible involvement of the exchanger in the cell cycle and in some pathological states.

There is at least one experimental pathological model in which the normally beneficial action of the Na^+/H^+ exchanger can trigger events that lead to tissue damage, namely during the reperfusion injury of the isolated animal heart after an ischaemic period. However, whether these findings are applicable in the clinical setting has yet to be determined. Of great value in demonstrating this role have been amiloride and its more potent and specific analogues such as methyl isobutyl amiloride and ethyl isopropyl amiloride. Unfortunately, the action of amiloride and these analogues is not confined to inhibiting the Na^+/H^+ exchanger [9,135]. These compounds are moderately membrane-permeant and can enter the tissue in significant quantities, and at the necessary dosage they affect other cellular functions in the cytosol [136], in addition to amiloride's action as a diuretic. It would be of great interest to develop either more specific amiloride analogues or other unrelated inhibitors of the exchanger. Such drugs would be valuable during the treatment of myocardial infarctions. They would also have tremendous potential in the treatment of cancer tissue, since they could promote cellular acidification of the transformed cells to the point of their death (reviewed in Harguindey [137]). The challenges therefore are great and may require multidisciplinary approaches for their solution.

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