The Na^+/H^+ Exchanger: A Target for Cardiac Therapeutic Intervention

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Abstract: The Na^+/H^+ exchanger (NHE) is a ubiquitous protein present in mammalian cells. In higher eukaryotes this integral membrane protein removes one intracellular H^+ for one extracellular Na^+ protecting cells from intracellular acidification. NHE is of essential importance in the myocardium. It prevents intracellular acidosis that inhibits contractility. NHE also plays a key role in damage to the mammalian myocardium that occurs during ischemia and reperfusion and is involved in hypertrophy of the myocardium. NHE is composed of a membrane bound domain of approximately 500 amino acids plus a hydrophilic regulatory cytoplasmic domain of approximately 315 amino acids. The NHE1 isoform is the only significant plasma membrane isoform present in the myocardium. The activity of NHE1 is elevated in animal models of myocardial infarcts and in left ventricular hypertrophy. During ischemia and reperfusion of the myocardium, NHE activity catalyzes increased uptake of intracellular sodium. This in turn is exchanged for extracellular calcium by the Na^+/Ca^{2+} exchanger resulting in calcium overload and damage to the myocardium. Numerous inhibitors of NHE have been developed to attempt to break this cycle of calcium overload. In animal models excellent success has been obtained in this regard. However in humans, clinical trials have resulted in only modest success and recently, significant detrimental side effects were note of one NHE inhibitor. The mechanisms by which these inhibitors affect NHE activity are presently being investigated and regions of the protein important in NHE activity and inhibitor efficacy are related but not identical. Future studies may develop superior inhibitors that may circumvent recently reported side effects. Recently, NHE inhibition has been shown to be remarkably effective in preventing hypertrophy in some animal models. Whether this proves to be a practical treatment for hypertrophy in humans has yet to be determined.

A. INTRODUCTION

The Na^+/H^+ exchanger (NHE) is an integral membrane protein ubiquitously expressed in mammalian cells. It functions in the electroneutral exchange of one intracellular H^+ for one extracellular Na^+ and therefore is an important protein in protecting the cells from damage due to intracellular acidification. The NHE is also involved in maintenance of cell volume as well as regulation of sodium fluxes [1]. To date there have been 9 isoforms of the exchanger identified [1] that differ in their tissue expression, cellular distribution, and inhibitor sensitivity.

In 1989 Sardet *et al.* cloned the first isoform of the NHE family, NHE1, and identified it as being an amiloride sensitive transporter involved in pH_i regulation [2]. It was later determined that this NHE was ubiquitously expressed and targeted to the plasma membrane where it existed as an integral membrane protein. Since that initial discovery there has been the identification of 8 other isoforms. The amino acid sequence identity of the various isoforms ranges from 25% to 70% although they are all predicted to share a similar secondary structure [3].

Although NHE1 is ubiquitously expressed and is considered the "housekeeping" isoform, after its initial discovery it was also identified as the predominant isoform expressed in the myocardium [4] and therefore is considered the cardiac specific isoform. NHE2-NHE5 are also localized to the plasma membrane of cells, but they show restricted tissue expression unlike NHE1. NHE2 and NHE3 show high expression in the kidney and intestine where they are localized to the apical membrane of epithelia and are thought to be involved in Na^+ and water reabsorption [5]. NHE4 like NHE1 is highly expressed in basolateral epithelial kidney cells, but has a reduced affinity for H^+ [6], suggesting that NHE4 is involved in a physiological role different from that of NHE1. NHE5 expression is restricted to the brain, where it is suggested to be involved in regulation of pH_i in neurons [7]. NHE6-NHE9 are not localized to the plasma membrane unlike the previously identified isoforms, but rather are present in the membranes of various organelles of the cell. NHE6 is localized to early recycling endosomes [8] while NHE7 is localized to the *trans*-Golgi network [9] and both were suggested to play a role in maintaining pH_i within the organelle. More recently NHE8 [10, 11] and NHE9 [11] have been identified and localized to the mid- to trans-Golgi and the late recycling endosomes respectively [11]. The intracellular localization of isoforms NHE6-NHE9 suggests a role for the NHE in organelle ion homeostasis as well as a mechanism for maintenance of the organelle specific pH values of the early recycling endosomes to trans-Golgi network system [11]. NHE1 is the only ubiquitously expressed isoform and the only plasma membrane isoform expressed in the myocardium. It is the isoform that is most thoroughly studied and is the topic of this review.

Structure and Domains of the Na^+/H^+ Exchanger

NHE1 is an 815 amino acid protein with a predicted molecular mass of 85kDa and an apparent molecular mass closer to 110kDa due to N- and O-linked glycosylation on extracellular loop 1 (EL1) [12], however the carbohydrate

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moieties are not essential to the ion transport action of the NHE1. In cells, NHE1 exists as a homodimer but the individual subunits do function independently [13, 14]. In 2000 Wakabayashi *et al.* generated a topological model of NHE1 using substituted cysteine accessibility analysis [15] that is now widely accepted (Fig. **1**). The topological model describes a protein that can be separated into an N-terminal membrane associated domain and a long C-terminal tail, with the N- and C- terminal domains being cytoplasmic. The N-terminal membrane associated domain was shown to consist of ~500 amino acids with 12 transmembrane spanning regions (12 TM) and 3 membrane associated loops: intracellular loop 2 (IL2) between TM3 and TM4, IL4 between TM8 and TM9, and EL5 between TM9 and TM10 [15]. Wakabayashi's work differs from the previous models generated from hydrophobicity analysis in that it demonstrated the presence of re-entrant loops, it assigned the region previously thought to be TM10 as EL5 (membrane associated region), and TM10 and TM11 were determined to be made up of different amino acid regions than previously identified [15]. It is this membrane domain of NHE1 that is responsible for homodimerization in cells [13, 14].

Unchanged from previous topology models was the region of NHE1 that makes up the C-terminal tail. The C-terminal tail consists of the last ~315 amino acids of NHE1 and exists exclusively in the cytoplasm. Analysis of the last 300 amino acids of the protein by circular dichroism spectroscopy revealed that the NHE1 cytoplasmic tail is 35% α-helix, 17% β-turn, and 48% random coil [16]. Further analysis of the circular dichroism data suggested a structure that was more compact at regions proximal to the membrane domain and regions distal to the membrane domain were more flexible in nature [16]. Circular dichroism analysis using only the C-terminal 182 amino acids of NHE1 further showed that the structure observed in the tail displayed conformational changes dependent on calcium (Ca^{2+}) [14] but how these conformational changes in the C-terminal tail affect the protein as a whole is yet to be determined.

In addition to the N-and C-terminal domains of NHE1 being separated into structural domains they can also be separated into functional domains. The N-terminal membrane domain is associated with amiloride-sensitive Na^+/H^+ ion transport across the plasma membrane, while the C-terminal cytoplasmic tail is associated with regulation of exchanger activity [17]. The N-terminal membrane domain exchanges one intracellular H^+ for one extracellular Na^+ [18]. This transport is sensitive to both amiloride inhibition and competitive-inhibition by lithium (Li^+) [19]. NHE1 exhibits

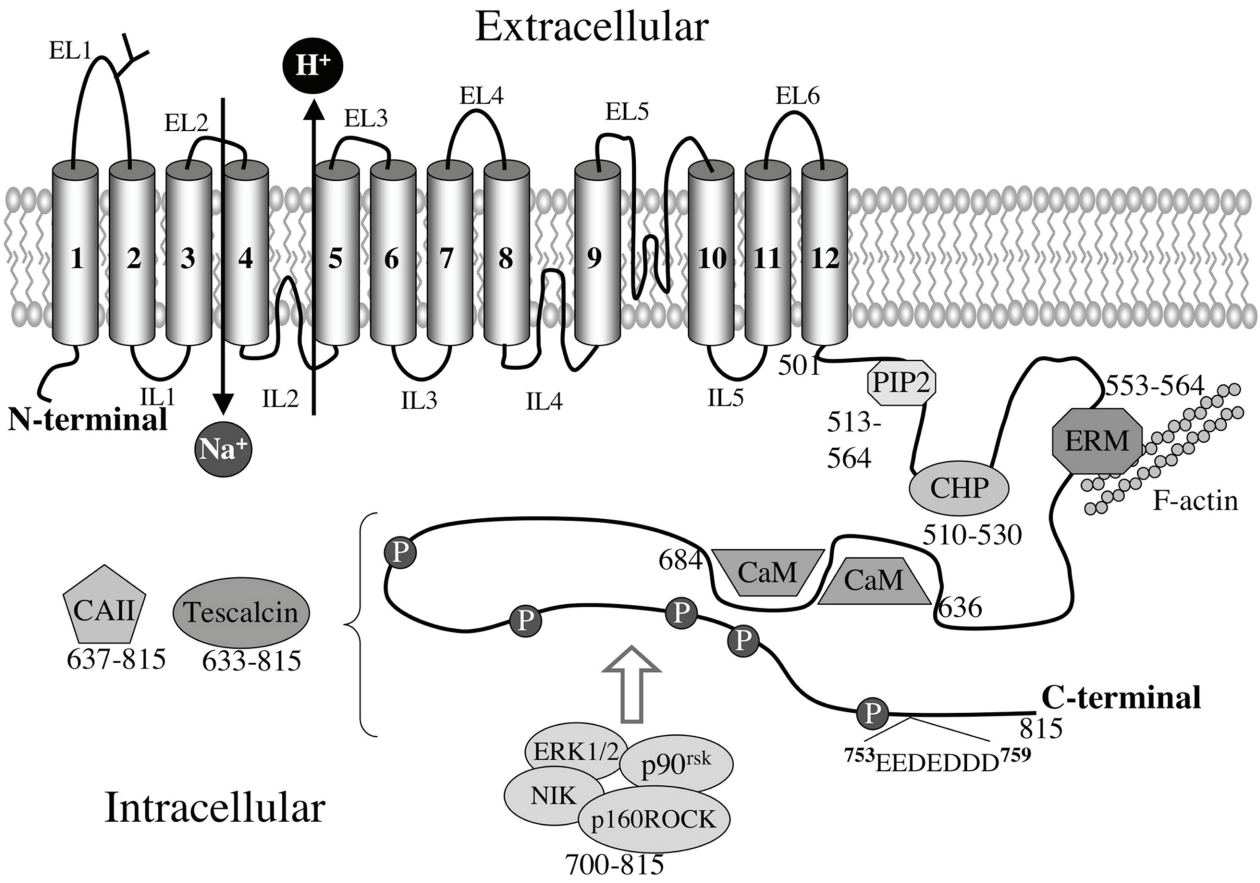

Fig. (1). Topology model of Na^+/H^+ exchanger isoform 1 [15] and regulatory elements. EL1-EL6 Extracellular loop 1-6; IL1-IL5 Intracellular loop 1-5; PIP2 Phosphatidylinositol bisphosphate; CHP Calcineurin homologous protein; ERM Ezrin/Radixin/Moesin proteins; CaM Calmodulin; CAII Carbonic anhydrase isoform 2; ERK1/2, p90rsk, NIK, p160ROCK Protein kinases; EEDEDDD Acidic region containing amino acids glutamic (E) and aspartic (D) acid.

maximal exchange activity at acidic pH (>6.5) but this dependence can be shifted to a more alkaline pH by the action of growth factors and hormones such as thrombin, endothelin, and angiotensin [20-23] as wells as by osmotic stress [24].

Regulation of NHE1 Activity

Regulation of NHE1 transport activity, as stated above, is a product of the cytoplasmic tail. The cytoplasmic tail of NHE1 can be split into a number of sub-domains that are individually involved in various systems employed to regulate NHE1 exchange, including protein-protein interaction, allosteric regulation, and covalent modification (Fig. **1**). The first sub-domain consists of overlapping binding sites for three different sets of factors and is most proximal to the membrane domain. The first factor is PIP2. The amino acids 513-564 contains two putative PIP2 binding sites and is involved in the ATP-dependent regulation of NHE1 [25]. Although NHE1 does not directly use ATP, when ATP is depleted there is a marked decrease in transport activity, and a mutant form of NHE1 lacking the two putative PIP2 binding sites shows a reduction in transport rate, suggesting that PIP2 binding is essential for optimal NHE1 activity [25]. The second factor is the ezrin, radixin, and moesin (ERM) family of proteins. Amino acids 553-564 contain the binding site for the ERM proteins [26]. Through a direct interaction with the ERM proteins and an indirect interaction with actin filaments *via* the ERM, NHE1 is involved in maintaining the structural integrity of the cell as well as in cell migration [18, 24, 27]. It has also been suggested [28] that NHE1 could act as a scaffold, binding the ERM proteins and mediating the formation of signaling complexes that would allow for coordinated regulation of NHE1 and restrict its targeting to specialized regions of the plasma membrane. A third part of this first sub-domain is the calcineurin B homologous protein (CHP). Amino acids 515-530 contain the binding site for CHP1 [29]. It was previously thought that CHP1 associated with NHE1 in a region critical for growth factor stimulation of the exchanger and over-expression of the protein resulted in negative regulation ion transport [30]. However, over-expression of CHP is not physiologically representative of the CHP and NHE1 association. In 2001 Pang *et al.* characterized the role of endogenous CHP in regulation of the exchanger and found it to be an essential cofactor for NHE1 [29]. In addition to CHP1 there exists another isoform, CHP2. CHP2 is expressed at high levels in tumor cells, and its association with NHE1 is proposed to contribute to the maintenance of alkaline pH_i and resistance to serum-deprivation induced death of malignant tumor cells [31].

The second sub-domain spans amino acids 636-684. This sub-domain contains two calmodulin (CaM) binding sites, a high affinity (amino acids 636-656) and a low affinity site (amino acids 657-684) [32]. The high affinity binding site functions as an "auto-inhibitory domain" that binds Ca^{2+}-bound CaM and allows activation of the exchanger. Removal of the CaM high-affinity binding site results in a constitutively active NHE1 [33]. In addition mutation of an acidic sequence of amino acids (753-759) in the C-terminal tail >100 amino acids downstream of the CaM binding site disrupts CaM binding, suggesting that the CaM binding site is sensitive to the over all conformation of the tail or interacts with other distal parts of the tail [34].

The third sub-domain of the C-terminal tail of the NHE1 is a region encompassing amino acids ~700-815 that contains a number of serine and threonine residues that are phosphorylated by protein kinases in response to hormone and growth factor stimulation as well as by sustained acidosis [22, 23, 35]. The pathways begin at the cell surface receptors and lead to the final product of NHE1 phosphorylation. They are complex and involve a number of signaling molecules, second messengers, binding proteins, and protein kinases. The pathways utilized can differ depending on the cell type [36]. Since the initial discovery by Sardet *et al.* in 1990 that NHE1 was phosphorylated in response to growth factors a lot of work has been done to further elucidate the nature of this event and to determine the players involved. The mitogen-activated protein kinases pathway is a major factor in regulation of NHE1 in the myocardium with ERK1/2 directly phosphorylating the C-terminal tail of the exchanger [37]. ERK1/2 can also activate $p90^{rsk}$, another NHE1 kinase [38]. Other protein kinases directly acting on the exchanger include p160ROCK [39], NIK [40], and p38 [21] (Fig. **1**), but they differ in that p160ROCK and NIK, like the mitogen-activated protein kinases activate exchange activity, while p38 is thought to inhibit NHE1 activity in response to angiotensin II [21]. It is of note that p38 may also stimulate NHE1 and induce intracellular alkalinization in an apoptotic pathway [41]. Protein kinase C and protein kinase D are also able to influence NHE1 activity in response to growth factor and hormone stimulation but they are not believed to directly phosphorylate the exchanger [42, 43].

The last 182 amino acids of the C-terminal tail of NHE also contain binding sites for two other proteins: carbonic anhydrase II [44] and tescalcin [45]. Carbonic anhydrase II has a stimulatory effect on NHE1 activity and its binding is believed to be dependent on NHE1 phosphorylation [44], while tescalcin shows Ca^{2+}-induced enhancement of binding and has an inhibitory effect on NHE1 activity [14].

How the various proteins interacting with the C-terminal tail are involved in regulation of NHE1 activity is not clear, but it is thought that their interaction with the tail influences how the tail modulates the N-terminal membrane domain altering the affinity for H^+ at an allosteric regulatory region distinct from the ion transport region of NHE1. This H^+ binding region is thought to "sense" the pH_i and act as an allosteric activator of the exchanger [46]. In IL5 and TM11 several amino acids influence the pH_i sensitivity of NHE1. Mutation of Arg^{440} (IL5) decreases the pH_i sensitivity, while mutation of Gly^{455}/Gly^{456} (TM11) increases the pH_i sensitivity of NHE1 [47]. How the pH sensitivity of these amino acids is modified by growth factor stimulation is not clear at this time.

The Many Physiological Functions of NHE1

The Na^+/H^+ exchanger's ability to regulate pH_i, cell volume, and ion flux implicates it in a number of physiological processes. NHE1 plays an important role in timing of the G2/M transition of the cell cycle. Cells expressing an ion

translocation deficient mutant fail to progress through the cell cycle and do not proliferate, implicating a role for NHE1 in cell proliferation [48]. NHE1 is also involved in cell growth and differentiation. Treatment of cells with an NHE1 inhibitor prevents retinoic acid-induced differentiation, and cells deficient in NHE1 expression have a reduced growth rate [49]. In fact, when the NHE1 gene is knocked out in mice the rate of postnatal growth is reduced, and the animals are ataxic and experience epileptic-like seizures [50]. Anchoring of NHE1 to the cytoskeleton *via* interactions with the ERM family of actin binding proteins also links NHE1 to a role in maintenance of cytoskeletal structure, focal adhesion, and cell migration [27, 51]. NHE1 is also thought to be involved in apoptosis, although its role seems to differ depending on the cells tested. Two groups show that NHE1 promotes cell survival in opposition of apoptotic signals [52, 53], while another group using pro-β-cells shows that NHE1 contribute to the apoptotic process [41].

B. ROLE OF NHE1 IN MEDIATING MYOCARDIAL ISCHEMIC AND REPERFUSION INJURY, ANIMAL-PRECLINICAL DATA

Research over the past many years has clearly demonstrated the ability of NHE1 inhibitors to protect the ischemic and reperfused myocardium in many different animal models. Indeed, this has led to the relatively rapid establishment of various clinical trials in patients with coronary artery disease. As will be discussed below, the outcomes of these trials have been much less than completely positive although, at least with respect to the most recent trial, the proof of principle of NHE1 inhibition as a cardioprotective strategy has been firmly established.

Mechanism of NHE1 Involvement in Myocardial Ischemic and Reperfusion Injury

The underlying basis for the protective effects of NHE1 inhibition reflects a close interaction between ion-regulatory processes found in the cardiac cell especially NHE1, Na^+-Ca^{2+} exchange and the Na^+-K^+ ATPase. Na^+-K^+ ATPase is inhibited in the ischemic cell and hence Na^+ entering *via* NHE cannot be effectively extruded. The increased intracellular Na^+ then results in either reduced removal of Ca^{2+} *via* Na^+-Ca^{2+} exchange or reverse mode Na^+-Ca^{2+} exchange activity resulting in elevations in intracellular Ca^{2+} levels and cell injury [reviewed in [54, 55]]. In addition, NHE1 is further activated by various hormonal, autocrine, or paracrine factors as well as metabolites produced either extracellularly or intracellularly during myocardial ischemia including hydrogen peroxide and lysophosphatidylcholine. Thus, the net result is a multifactorial stimulation of NHE under pathological conditions, not only due to increased intracellular acidosis but also due to activation by external factors. Such marked NHE1 stimulation increases an elevation in intracellular sodium levels that in turn increases intracellular calcium levels *via* Na^+-Ca^{2+} exchange resulting in cell injury. Previous evidence suggests that the Na^+-Ca^{2+} exchanger may actively contribute to calcium overload *via* reverse-mode calcium entry since transgenic mice over-expressing this exchanger show an increased sensitivity to ischemic injury, which would not be expected if elevated calcium would occur primarily *via* reduced efflux [56].

Myocardial Protection by NHE1 inhibitors

Numerous studies using NHE inhibitors, especially those selective against the NHE1 isoform, have shown protection with respect to a large number of parameters of cardiac function including enhanced contractility, reduced contracture and a decrease in the incidence of arrhythmias. In addition, improvements in biochemical and ultrastructural indices have been extensively demonstrated with NHE inhibition [54, 55]. The extensive documentation demonstrating cardioprotective effects of NHE inhibitors has strongly supported the concept of the Na^+/H^+ exchanger's involvement in cardiac injury, especially under conditions of ischemia and reperfusion. Earlier studies utilized amiloride or amiloride analogues to demonstrate cardioprotective properties, however more recent data utilizing drugs targeted for clinical development reported excellent and consistent protection in a wide variety of experimental models and animal species. This success in treatment is likely unmatched in the cardioprotection literature. A particularly striking feature of NHE1 inhibitors is their ability to protect against various forms of dysfunctions including reduced mortality, limitation of infarct size, improvement of functional recovery after reperfusion, reduction of arrhythmias, attenuation of calcium and sodium miss-regulation, reduction of apoptosis as well as preservation of metabolic status such as attenuation of high energy phosphate depletion. Of clinical relevance, protection of the ischemic and reperfused myocardium with NHE1 inhibitors is also manifested in hypertrophied hearts [57].

Supporting the observation that NHE1 activity is critical in ischemic and reperfusion damage to the myocardium is the observation that hearts from mice in which NHE1 was genetically ablated exhibit enhanced resistance to ischemic and reperfusion injury [58]. This observation suggests that the beneficial effects of pharmacological agents are not due to nonspecific actions.

Many of the newer drugs have been tested for their ability to protect the myocardium when administered only at reperfusion, a property that would be important in terms of treatment of patients who present with acute myocardial infarction. Most agents do indeed demonstrate protective effects when administered at this period although it should be stated that, in general, such protection is less than that seen with pre-ischemia drug administration. From a mechanistic perspective, these findings are not surprising since NHE1 activation during ischemia contributes substantially to the sodium and calcium overloading conditions and resultant cell injury with further NHE1 activation occurring immediately upon reperfusion. As such, *optimum* protective effects of NHE1 inhibitors are likely realized when treatment can be maintained during both ischemia and reperfusion.

It is interesting to note that, at least from a theoretical perspective, the potential for toxicity or untoward side effects of NHE1 inhibitors is relatively small in view of the specificity of newer agents. Moreover, it is important to point out that drugs targeted at NHE1 inhibition have limited potential for disruption of normal cell homeostasis since NHE1 activity is generally restricted under normal conditions, thus these drugs should have the potential for selectivity inhibiting a process associated primarily with pathology.

Although most clinical studies with cariporide or eniporide revealed excellent tolerance, the exception is the recent EXPEDITION study (see below) that revealed a surprisingly higher increase in cerebrovascular events in high-risk patients undergoing coronary artery bypass surgery.

Does NHE1 Inhibition Represent a Superior Cardioprotective Strategy?

A critical consideration in designing novel cardioprotective strategies is to assess whether such new approaches surpass currently available modes of protection. Extensive head-to-head comparisons between NHE1 inhibitors and other strategies have not been reported although some of the earlier evidence with cariporide demonstrated reduction of arrhythmias in the infarcted myocardium that were generally refractory to classical antiarrhythmic agents [59]. Indeed, despite the lack of direct electrophysiological effects of NHE1 inhibitors, these agents have been shown to exert excellent antiarrhythmic properties against a wide array of arrhythmias [reviewed in [60]]. Overall, as cardioprotective strategies NHE1 inhibitors are unique in the sense that virtually no contradictory data have been reported and, the approach appears to be effective against various forms of injury and in a variety of experimental models. These properties are not seen with other cardioprotective approaches suggesting that these drugs represent a superior cardioprotective strategy [61].

It is likely that the currently most widely studied cardioprotective strategy is ischemic preconditioning, a phenomenon in which multiple episodes of brief ischemia confers protection against subsequent prolonged ischemic insult [62]. The mechanism of ischemic preconditioning is not completely understood but is most likely distinct from NHE1 inhibition, indeed NHE1 inhibitors offer added protection when administered to preconditioned hearts [63]. Protection by ischemic preconditioning is indeed impressive and it has generally been considered for many years as the most effective known cardioprotective strategy. This has been challenged, however, in a study that demonstrated comparable protection by NHE1 inhibition and in terms of infarct size reduction in dogs subjected to coronary artery ligation for 60 minutes. However, when the period of occlusion was extended to 90 minutes, preconditioning failed to exert salutary effects although NHE1 inhibition reduced infarct size by approximately 70% indicating that NHE1 inhibition confers superior protection compared to ischemic preconditioning, at least in this model [64].

C. CLINICAL EVALUATION OF NHE1 INHIBITORS IN PATIENTS WITH CORONARY ARTERY DISEASE

The GUARDIAN Study

Clinical development and testing of NHE1 inhibitors in cardiac disease states has been relatively rapid, possibly reflecting the consistent and excellent protection with these agents demonstrated in animal studies. The first such study, termed the GUARDIAN (Guard During Ischemia Against Necrosis) was an ambitious combined Phase II/Phase III double-blind randomized placebo-controlled study of more than 11,590 patients to assess different doses of cariporide in individuals with acute coronary syndromes with outcomes evaluated at 36 days [65]. The population of patients included those 1) with unstable angina/non Q wave myocardial infarction, 2) undergoing high-risk percutaneous transluminal coronary angioplasty and 3) undergoing high-risk coronary artery bypass graft surgery. The GUARDIAN study revealed that cariporide is well tolerated although it failed to demonstrate an overall significant attenuation (10%) of the two primary events, mortality and incidence of myocardial infarction. However, favorable effects among the 3 major subgroups were observed especially a significant reduction in event rate in CABG patients receiving the highest dose (120 mg IV every 8 hours) of the drug. This dose appeared to be effective in reducing the overall incidence of Q wave myocardial infarction by about 40%. In view of the fact that complete details of GUARDIAN results have yet to be published, an in-depth assessment of the study is difficult to provide at the present time. However, it should be noted that the GUARDIAN study carried some inherent risks in view of the heterogeneity of patients recruited, some of which were apparently not subjected to reperfusion protocols thus precluding any potential myocardial salvaging benefit of cariporide. Moreover, as a dose finding study and the observation that only the highest dose exerted any benefit, it is likely that optimal dosing and plasma therapeutic levels were not achieved in this study.

The ESCAMI study

The ESCAMI (Evaluation of the Safety and Cardioprotective effects of Eniporide in Acute Myocardial Infarction) was Phase II trial in patients undergoing thrombolysis or angioplasty for acute myocardial infarction in which eniporide was administered before the start of reperfusion [66]. Efficacy of treatment was measured by enzymatic determination of infarct size. No beneficial effects were observed, although interestingly, a significant reduction in the incidence of heart failure was found in those patients undergoing late reperfusion (>4 h).

Although treatments in the ESCAMI study failed to limit infarct size, the results could have been anticipated in view of the fact eniporide was administered during reperfusion only, thus potentially limiting the drug's salutary effect when compared to coverage during both ischemia and reperfusion as in cardiac surgery scenarios (see EXPEDITION study below). This comment notwithstanding, a small (100 patients) study revealed that cariporide reduced infarct size and improved left ventricular function in post-infarction patients undergoing angioplasty [67].

The EXPEDITION Study

Beneficial Effects

The EXPEDITION (Na^+/H^+ Exchange Inhibition to Prevent Coronary Events in Acute Cardiac Con ditions) is the most recent study aimed at determining the effect of cariporide on mortality and the incidence of myocardial infarction in high-risk patients undergoing coronary artery bypass graft surgery. The results have been published in abstract form [67]. More than 5700 patients were randomized to either intravenous cariporide or placebo with primary endpoints assessed at day 5 and up to 6 months. The results

were promising with death and MI at day 5 being reduced from 20.3% in the placebo group to 16.6% with cariporide (P=0.0002) which was maintained for the 6 month follow up period. The primary beneficial effect of cariporide was due to a dramatic reduction in myocardial infarction 18.9% in the placebo group to 14.4% with cariporide (P=0.000005) at day 5 and 18.5% (placebo) *vs*. 13.8% (cariporide) at 6 months (P=0.000001).

Adverse Effects

Unfortunately, a significant increase in mortality was observed at day 5 (1.5% in placebo versus to 2.2% with cariporide, P=0.028) although no significant differences were observed at 6 months. Cariporide use was associated with an increase in the overall rate of cerebrovascular events (2.7% in placebo to 4.8% with cariporide, P<0.001). The increased cerebrovascular risk was surprising and certainly unexpected particularly in view of evidence that NHE inhibition exerts cerebrovascular protective effects. The mechanism for the increased cerebrovascular risk in EXPEDITION is not known but it should be noted that knockout of NHE1 has a pro-epileptic effect [50]. On the positive side, EXPEDITION [68] represents the first large scale drug-intervention study in cardiac surgery patients to date which demonstrates the feasibility of cardioprotection with pharmacological approaches and confirms the excellent cardioprotective properties associated with NHE1 inhibition. Regretfully, however, the study also showed an unfavorable safety profile for cariporide although whether this reflects NHE1 inhibition or a specific effect of cariporide per se needs to be determined with further research.

D. ROLE OF NHE1 IN CARDIAC HYPERTROPHY AND HEART FAILURE

The Need for New Therapeutic Strategies for Treating Heart Failure

The past number of years have seen substantial improvement in the therapeutic approaches for the treatment of heart failure [reviewed in [69]]. The introduction of ACE (angiotensin-converting enzyme) inhibitors and increased use of α adrenoreceptor blockers have added to the armamentarium treating this complex syndrome. The incidence of heart failure is expanding rapidly due primarily to an aging population and also to increased survival rates after myocardial infarction. Indeed, in up to 70% of patients with heart failure the causative factors can be related to myocardial infarction. In the United States alone, more than 500,000 new cases of heart failure are diagnosed yearly. Yet despite improved therapeutic strategies, mortality rates in patients with heart failure continues to be high. Understanding the fundamental underlying mechanisms for the development of heart failure, particularly in the chronic maladaptive responses or remodeling, likely holds the key for potentially effective heart failure management. As discussed below, NHE1 may represent one such key target.

Theoretical Considerations for NHE1 Involvement in Heart Failure

Myocardial hypertrophy, remodeling and heart failure represent many complex events but in general involve initiating factors such as increase in mechanical load and upregulation of a large number of hormonal, paracrine and autocrine factors which contribute to the process through receptor-mediated changes in intracellular signaling [69]. One of the major reasons for considering NHE1 as a potentially important contributor to the heart failure process is based on the fact that, as already alluded to above, the antiporter represents a key downstream factor activated by many such factors including α_1 adrenoreceptor agonists [70-72], angiotensin II [73-75] and endothelin-1 [76-78]. Indeed, in cardiac cells, NHE inhibitors block hypertrophic responses to various stimuli. Stretch-induced stimulation in protein synthesis in neonatal cardiac myocytes, as well as stretch-induced alkalinization in feline papillary muscles, can be blocked by NHE inhibitors [79, 80]. In addition, norepinephrine-induced protein synthesis in cultured rat cardiomyocytes can be blocked by NHE1 inhibition [81]. These studies reveal a commonality in terms of responses to a wide array of hypertrophic factors. They support the suggestion that NHE1 activation represents a common response to mechanical stretch and a key player in the hypertrophic process [82]. Thus, cellular deformation under pathological conditions could lead to a cascade of events resulting in cell hypertrophy and eventual myocardial remodeling. It has been proposed that this occurs as a consequence of the activation of both angiotensin II AT_1 receptors as well as the endothelin-1 ET_A receptor which then activates intracellular signal transduction pathways leading to increased NHE1 activity and cell growth [82, 83]. This may occur in an autocrine or paracrine fashion in which stretch stimulates the local release of these peptides which then act on their respective receptor. It is interesting to note that NHE1 upregulation in the hypertrophied myocardium can be inhibited by anti-hypertrophic factors which do not directly target the exchanger such as activators of the mitochondrial ATP-sensitive potassium channel [84].

Experimental Evidence for NHE Involvement in Cardiomyocyte Hypertrophy and Heart Failure

Although experimental studies are still in their relative infancy, initial findings using NHE1 inhibitors have been encouraging and support the concept that inhibition of NHE1 is conducive to attenuation of the remodeling process and heart failure. Such findings have been observed in both *in vitro* and *in vivo* models of hypertrophy and heart failure. As already noted above, NHE inhibitors block norepinephrine induced protein synthesis in cultured neonatal rat ventricular myocytes, although in that particular study, the nonspecific inhibitor amiloride was used to inhibit the antiporter [81]. However, similar effects have been reported with HOE 694, a much more selective NHE1 inhibitor [85].

In vivo studies using clinically relevant animal models have further advanced the concept of NHE1 mediated hypertrophy and heart failure and have provided very strong evidence for the exchanger's involvement in these processes. For example, early studies have shown that orally administered amiloride, a nonspecific NHE inhibitor, reduces fiber diameter in rat coronary ligation [86] and murine dilated cardiomyopathy models [87]. Dietary administration of the NHE1 specific inhibitor cariporide completely abrogates the increased length of surviving myocytes after one week after coronary artery occlusion and ameliorates contractile

dysfunction in the absence of afterload reduction, thereby implicating a direct effect of the drug on myocardial remodeling [88]. Furthermore, more severe hypertrophy and heart failure observed at three months post-infarction follow-up are reduced by approximately 50% in animals treated with the NHE1 inhibitor cariporide [89]. As noted, these effects of cariporide were observed in the absence of afterload reduction or reduction in infarct size, although others have reported a moderate infarct size reduction in cariporide-treated rats subjected to coronary ligation [90].

The evidence for a direct anti-remodeling influence of NHE1 inhibition is further reinforced by the findings that heart failure not associated with myocardial ischemia is also attenuated by NHE1 inhibition. One such model involves the acute administration of monocrotaline to rats which produces pulmonary neointimal thickening and compensatory right ventricular hypertrophy. As noted previously, we have recently reported that cariporide can effectively attenuate the right ventricular hypertrophic response as well as the accompanying hemodynamic defects whereas the pulmonary artery responses were unaffected [91]. Further, such evidence stems from the spontaneously hypertensive rat which develops left ventricular hypertrophy. It has recently been reported that cariporide causes a regression of left ventricular hypertrophy similarly to that seen with the vasodilators enalapril and nifedipine, but with no decrease in blood pressure [92]. Cariporide has also been shown to inhibit interstitial fibrosis, hypertrophy and heart failure in transgenic mice that overexpress the cardiac α_1 adrenergic receptor [93]. NHE1 inhibition can also attenuate cardiac hypertrophy produced by isoproterenol administration in rats [94]. Another NHE1 specific inhibitor BIIB722 has recently been shown to improve ventricular function in rabbits in which heart failure was produced by 3 weeks of rapid left ventricular pacing [95]. Thus, it appears that NHE1 inhibition may have the potential to favorably influence heart failure related to multiple initiating factors.

A number of recent studies have also implicated a role for NHE1 in mediating the deleterious effects of mineral-corticoids on the heart. For example, aldosterone was found to directly produce hypertrophy in cultured neonatal rat ventricular myocytes which was associated with increased gene and protein expression of NHE1 and which was blocked by NHE1 inhibition [96]. Moreover, cardiac hypertrophy and fibrosis produced by desoxycorticosterone in uninephrectomized rats was inhibited by cariporide [97].

We have recently found that EMD87580, a specific and potent NHE1 inhibitor, not only prevents heart failure and hypertrophy but can also reverse these processes when treatment is delayed by up to 4 weeks after coronary artery ligation in rats, a time when the remodeling process has already been established [98]. Moreover, these effects occurred in the absence of afterload reduction or in diminution of infarct size thus strongly implicating a direct salutary effect of the drug on the myocardial remodeling process. The ability to reverse remodeling and heart failure by NHE1 inhibitors is a particularly intriguing finding as it offers the prospect of potential reversal of the disease process in patients with established heart failure.

Potential Mechanisms

Despite the emerging strong evidence for NHE1 involvement in the heart failure process and the prospect for novel therapeutic interventions, the mechanisms underlying the role of NHE1 is presently unknown. Although NHE1 dependent intracellular pH changes may be proposed in view of the role of pH in protein synthesis, this is unlikely since it would be expected that under long term NHE1 blockade other intracellular pH regulatory processes would likely compensate to ensure maintenance of physiological pH levels. It has been suggested that NHE1 activation results in the influx of sodium ions which then in turn activate various protein kinase C isozymes which then alter gene expression and protein synthesis [99]. Recent studies in rabbits subjected to a model of combined pressure and volume overload showed that 3 months of cariporide feeding abolished heart failure and prevented cellular remodeling among which were marked inhibition of cytosolic calcium and sodium contents with an NHE1 dependent elevation in intracellular sodium preceding other parameters associated with cellular remodeling [100, 101].

Another potential mechanism by which NHE1 inhibition could exert beneficial effects in heart failure is through improved energy metabolism. In this regard, Javadov and coworkers have recently shown that the NHE1 specific inhibitor EMD87580 prevented mitochondrial dysfunction associated with chronic artery occlusion in rats [102]. These effects were demonstrated by inhibition of mitochondrial permeability transition pore opening, improved respiration and attenuation of mitochondrial vulnerability to exogenous calcium.

Although these represent potential contributory factors to salutary effects of NHE1 inhibition in the heart failure process, a precise cause and effect relationship has not been established and the precise mechanism for NHE1 involvement in myocardial remodeling and hypertrophy requires extensive further elucidation.

Conclusion, NHE1 and Heart Disease

NHE1 plays a unique role in mediating the cardiac responses to both acute ischemic and reperfusion injury as well as chronic post infarction remodeling. The cellular bases for these effects are presented in Figure **2** and demonstrate a potential role for intracellular ion dysregulation in mediating both acute and chronic responses. In terms of ischemic and reperfusion injury the salutary effect of NHE inhibition as a cardioprotective strategy has been more convincingly demonstrated than for any pharmacological agent. The increased cerebrovascular risk in patients receiving cariporide was unpredicted and further research is required to determine whether this presents a drug class effect or a property of cariporide per se. In addition to its role in acute injury, extensive evidence over the past few years has supported the notion that NHE1 contributes to chronic remodeling thereby suggesting that inhibitors of the exchanger could offer therapeutic effects in heart failure. This would have various advantages over current treatments including the absence of afterload reduction. Although animal studies have been encouraging, further studies are

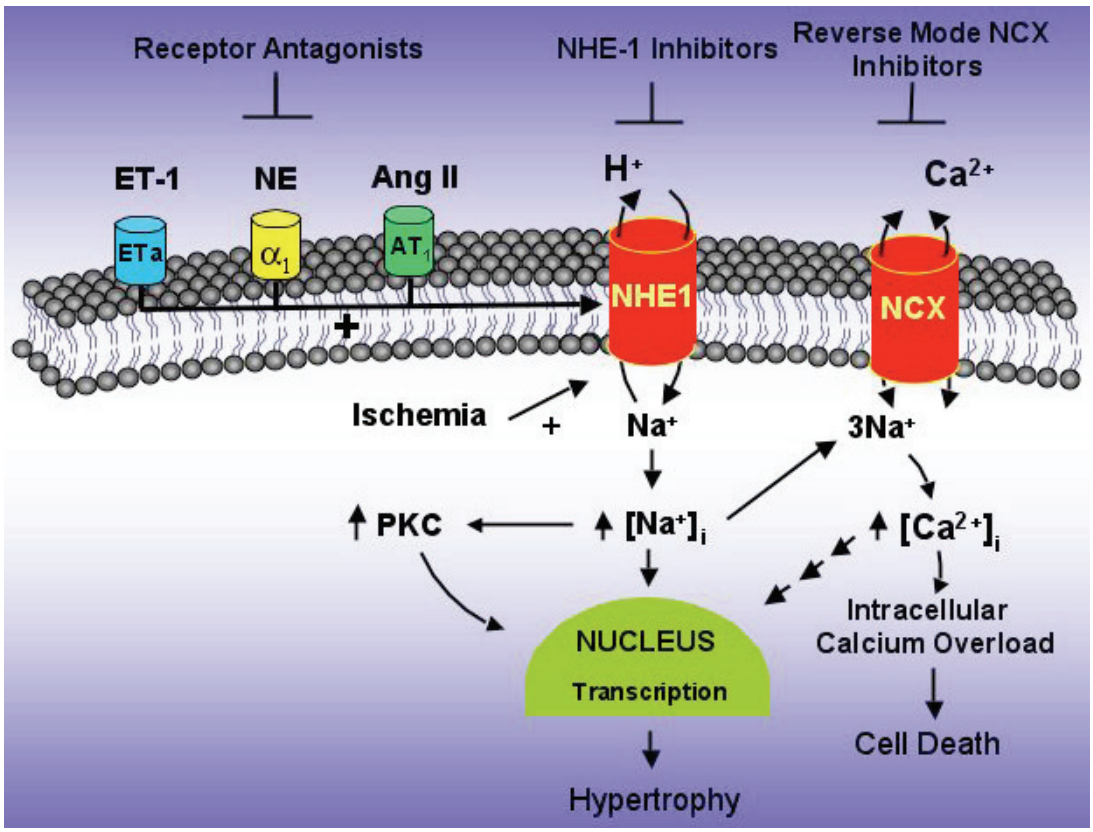

Fig. (2). Central role of NHE-1 in mediating acute and chronic responses to myocardial ischemia and reperfusion. Ischemia activates NHE-1 secondary to intracellular acidosis resulting in an elevation in intracellular Na^+ concentrations. The increased in intracellular Na^+ levels results in a subsequent increase in intracellular Ca^{2+} levels as result of diminished efflux of Ca^{2+} *via* the $3Na^+$-Ca^{2+} exchanger (NCX) or due to reverse mode NCX activity. In acute responses, intracellular Ca^{2+} overloading would result in cell death. Under chronic conditions, such as post-infarction remodeling, the increased Ca^{2+} and Na^+ levels would produce transcriptional changes *via* multiple intermediates such as for Ca^{2+}, or direct, or PKC-dependent mechanisms for Na^+ resulting in hypertrophy. The activation of NHE-1 is further enhanced by upregulation of various paracrine, autocrine or hormonal factors such as endothelin-1 (ET-1), angiotensin II (Ang II) and norepinephrine (NE) which exert their effects through their receptors. Although antagonists for these receptor or reverse mode NCX inhibitors could inhibit some of the deleterious effects of these processes, NHE-1 inhibitors would be expected to produce more effective results due to the pivotal and central role of the antiporter in mediating the actions of a variety of factors which contribute to both acute and chronic pathological responses.

required to better elucidate this phenomenon before the use of NHE1 inhibitors for the treatment of heart failure is realized.

E. MECHANISMS OF INHIBITION OF THE NA^+/H^+ EXCHANGER

While it is clear that NHE1 inhibition has tremendous potential clinical benefit, it is not yet certain that the present day inhibitors are the most potent and specific ideal compounds. The structural and functional attributes of the inhibitors merit revisiting, especially in the light of the detrimental side effects found in the EXPEDITION study. The classical inhibitor of NHE is amiloride or 3,5 diamino-6-chloro-N-(diaminomethylene)pyrazine-carboxamide. It contains a guanidine group and has a relatively poor specificity for NHE affecting several other proteins including the Na^+/Ca^{2+} exchanger, Na^+/K^+ ATPase, Na^+ channels and other proteins. More specific inhibitors were initially made based on amiloride including 5 –(N-ethyl-N-isopropyl) aminoloride and 5-(N,N-hexamethylene) amiloride that have potencies of 380 nM and 160 nM respectively *vs*. 84 μM for amiloride [103]. While being relatively potent inhibitors of NHE and useful for studying the protein *in vitro*, these compounds were not suitable for use in humans due to a number of side effects [104]. For this reason, a whole new class of inhibitors was developed. The first of these developed were Hoe 694 and Hoe 642 (cariporide). These benzoylguanidines inhibit NHE1 with an IC50 of < 1 μM [105] and have high potency and selectivity for NHE1 and excellent solubility properties and resorbtion and bioavailability [106]. The initial development of these compounds has spawned the offshoot of a variety of related derivatives. This includes eniporide, EMD 87580, KB-R9032, BIIB 513, KB-R9032, SM-20220 and others [106].

Mechanisms of NHE inhibition and activity

The mechanism by which NHE inhibitors function is important in designing new improved inhibitors and understanding NHE function. In this regard it has been

suggested that in aqueous medium the Na^+ ion is surrounded by 3 molecules of water and that the guanidinium ion was similar in structure, and thereby could inhibit NHE by binding to the extracellular Na^+ binding site [106]. However the question of the identity of the NHE inhibitors binding site is complex and the inhibitor binding site is not necessarily at the Na^+ binding site. A number of approaches have been used to elucidate the identity of the inhibitor binding site. One study used chimeric NHE's to examine TM segments that confer sensitivity to pharmacological antagonists [107]. Using hybrid NHE's of the sensitive NHE1 and resistant NHE3, transmembrane segment (TM) IX was shown to be important in mediating sensitivity to the antagonists.

Studies mutating particular amino acids or isolating mutants that are resistant to NHE inhibitors have also given novel insights into potential sites of NHE inhibitor action. One group mutated cells and isolated NHE inhibitor resistant mutants by successive exposure to NHE inhibitors during acid loading. The amino acid leucine 167 (leucine number 163 in the human sequence) was mutated to phenylalanine and this NHE mutant was extremely resistant to inhibition by amiloride analogs. Figure three shows a crude model of transmembrane segment IV of NHE1, that contains leucine 167. Aside from this residue a number of other amino acids have been mutated in this and other transmembrane segments. Table **1** summarizes the effects of these mutations. Briefly, for TM IV the sequence in humans is ^{155}FLQSDV FFLFLLPPII LDAGYFLP^{178}. Within this sequence the underlined residues affect either the Na^+ affinity or the inhibitor resistance of human or other mammalian Na^+/H^+ exchangers (Table **1**) [108-110]. In deciding whether the amiloride and Na^+ binding site are identical it was informative that some mutations gave differential effects on Na^+ affinity and affinity of inhibitors while others did not. For example, the F162S mutation greatly increased resistance to cariporide, but also decreased the Na^+ affinity. In contrast, the double mutant F162S/I169S and the mutant L163F/G174S both increased inhibitor resistance greatly but had no or only relatively small effects on Na^+ affinity. This evidence as a sum, suggests that there may be some overlap between the Na^+ binding site and the inhibitor binding site, however they do not appear to be identical.

Transmembrane segment IV clearly appears to play a key role in inhibitor binding and in Na^+ affinity. Studies have examined other structural and functional aspects of this part of the protein. Fig. **3** illustrates a model of TM IV. We [111] have recently shown that the structure of TM IV is unusual in that it is structured, but not as a classical alpha helix. Residues 159-162 contain a series of β-turns, residues 165-168 were extended in structure while residues 169-176 were more helical in structure. We demonstrated that Phe161 is a residue that appears to line the pore of the Na^+/H^+ exchanger. Analysis of the structure of TM IV showed that Asp159 may

Table 1. Summary of Effect of Mutations of Amino Acids on Inhibitory Constants and Na^+ Affinity of NHE1. The Change in IC50 with Inhibition by NHE Inhibitors is Indicated Relative to the Wild Type. For Reference [109] Numbering of Amino Acids was Changed to the Corresponding Residues in Human NHE1. NE, no Effect; NM, Not Measured; ^{M}, *N*^{5}-methyl-*N*^{5}-propylamiloride; ^{C}, Cariporide; h, Hoe694

AA Mutant	Δ IC50	Δ Na^+ Km	Reference
F162S	+1500X^c	+11X	[110]
I169S	+2X^c	NE	[110]
I170T	+5X^c	NE	[110]
F162S/ I169S	+2500X^c	NE	[110]
F162S/ I170T	+4600X^c	+1.3X	[110]
I169S/ I170T	+10.5^c	+3X	[110]
F162S/I169S/I170T	+3500^c	-0.7X	[110]
L163F	+30X^M	NM	[108]
F164Y	NE^M	NM	[108]
L163F/F164Y	+20X^M	NM	[108]
F161Y/F164Y	+20X^M	NE	[108]
L163A	+6^h	NM	[109]
L163R	+8^h	NM	[109]
G174A	NE^h	NM	[109]
G174D	+6^h	NM	[109]
L163F/G174S	111X^h	+2X	[109]
E346D	300^h	+4X	[115]

also be a pore lining residue. A critical part of the structure and function of TM IV appears to be proline residues 167 and 168. Proline residues are known to produce kinks in transmembrane segments of proteins [112]. We have demonstrated that the two prolines that occur in the middle of TM IV are essential for activity [113]. The structure of this region of the protein demonstrates that this region is in fact in an extended conformation [111]. With the presence of the prolines, the region is both more flexible and has free backbone carbonyls that can participate in cation coordination [113].

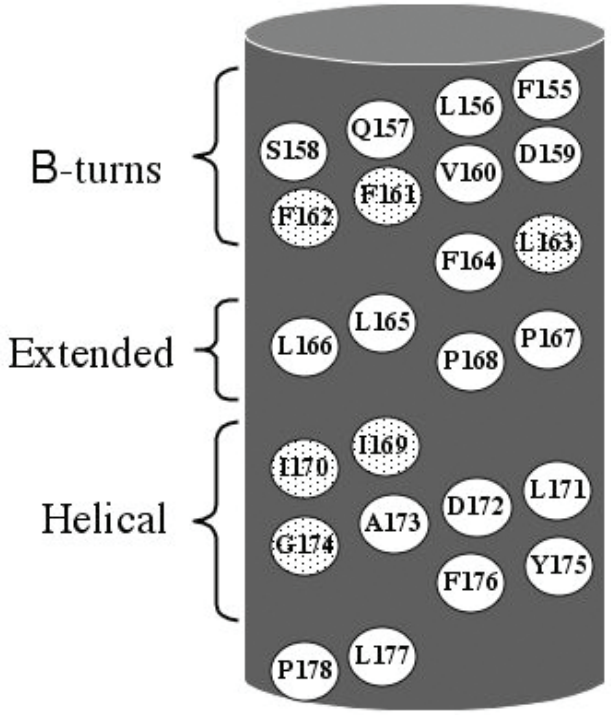

Fig. (3). Schematic diagram of transmembrane segment IV of the Na^+/H^+ exchanger. Stippled residues indicate which amino acids change the IC50 of inhibitory compounds when mutated. The results of the analysis of the structure of TM IV are indicated at left [111].

Some amino acid residues in other transmembrane segments and membrane associated parts of the protein, have also been shown to affect NHE inhibitor efficacy. Surprisingly, mutation of amino acid His349 with either Gly or Leu resulted in an amiloride resistant NHE [114]. This amino acid is within TM IX which supports the results shown by domain swapping that indicated that TM IX also plays a role in NHE inhibitor resistance [107]. Glu346 of TM IX has also been shown to play a key role in inhibitor resistance and Na^+ affinity [115]. We [116] also demonstrated that Glu262 and Asp267 of TM VII are critical for activity. Mutation to uncharged amino acids destroyed activity of NHE while conservative mutations had little effect on activity. The Glu262Asp mutant altered lithium, but not sodium affinity, suggesting that this residue and TM VII may be critical for cation coordination.

Summary, NHE1 inhibition and activity

Overall the results have given us a partial picture of the mechanisms of activity and inhibitor action on the Na^+/H^+ exchanger. It is clear that amino acids in TM's IV, VII and IX play an important role in inhibitor binding. Clearly, TM IV has a critical role in inhibitor binding and also in the activity of the protein. The unusual structure of TM IV and its sensitivity to mutagenesis are supportive of a critical role for this transmembrane segment.

F. FUTURE DIRECTIONS

There is tremendous promise in the application of inhibition of NHE to cardiovascular research. Of prime importance is the determination of whether the detrimental effects seen in the EXPEDITION are an effect of NHE1 inhibition or a specific side effect of cariporide. It is not known whether other NHE1 inhibitors such as eniporide, would have the same effect. An understanding of the exact mechanism of NHE1 inhibition and possibly, the development of more potent and specific NHE1 inhibitors may help in this regard. A greater understanding of the structure of NHE1 would be helpful. In addition, further confirmation of the mechanism by which NHE contributes to myocardial hypertrophy would also be helpful in designing treatments.

ABBREVIATIONS

ERM = Ezrin, radixin, moesin

CaM = Calmodulin

CHP = Calcineurin homologous protein

IL = Intracellular loop

EL = Extracellular loop

NHE = Na^+/H^+ exchanger,

PIP2 = Phosphatidylinositol 4, 5- bisphosphate

TM = Transmembrane

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