# Lipid Interactions With The Cardiac $Na^+$-$H^+$ Exchanger

By

Danny Paul Goel

A Thesis
Submitted to the Faculty of Graduate Studies
In Partial Fulfillment of the Requirements
For the Degree of

**MASTER OF SCIENCE**

Department of Physiology
Faculty of Medicine
University of Manitoba
and the Division of Stroke and Vascular Disease
St. Boniface General Hospital Research Centre
Winnipeg, Manitoba

**THE UNIVERSITY OF MANITOBA**

**FACULTY OF GRADUATE STUDIES**

*****

**COPYRIGHT PERMISSION PAGE**

**Lipid Interactions with the Cardiac $Na^+$-$H^+$ Exchanger**

**BY**

**Danny Paul Goel**

**A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of**

**Master of Science**

# ABSTRACT

Cardiac sarcolemmal $Na^+$-$H^+$ exchange is critical for the regulation of intracellular pH and its activity contributes to ischemia / reperfusion injury. It also plays an important role during ischemia / reperfusion injury in diabetics. The $Na^+$-$H^+$ exchanger is altered during insulin-dependent diabetes mellitus, however, the mechanism for this inhibition has yet to be determined. Coincidentally, the cardiac sarcolemmal membrane phospholipids are also altered during insulin-dependent diabetes mellitus. It has been suggested that the membrane phospholipid environment does not modulate $Na^+$-$H^+$ exchange. The present study was carried out to determine the effects on $Na^+$-$H^+$ exchange of modifying the endogenous membrane phospholipids through the addition of exogenous phospholipase $A_2$, phospholipase C, phospholipase D, specific phospholipids and saturated and unsaturated fatty acids. Phospholipase D selectively hydrolyses phosphatidylcholine into phosphatidic acid and choline. Incubation of 0.825 U phospholipase D with 1 mg of porcine cardiac sarcolemmal vesicles hydrolyzed 34 ± 2% of the sarcolemmal phosphatidylcholine and increased the phosphatidic acid 10.2 ± 0.5 fold, as determined by thin layer chromatography. Treatment of cardiac sarcolemmal vesicles with phospholipase D resulted in a 46 ± 2% inhibition of $Na^+$-$H^+$ exchange. $Na^+$-$H^+$ exchange was measured as a function of reaction time, $pH_o$ and extravesicular $Na^+$. All of these parameters of $Na^+$-$H^+$ exchange were inhibited following phospholipase D treatment when compared to untreated controls. Passive efflux of $Na^+$ following phospholipase D treatment was unaffected. Phosphatidylcholine content was depleted

approximately 50% following phospholipase C treatment. However, treatment of sarcolemmal vesicles with phospholipase C had no effect on $Na^+$-$H^+$ exchange. Phospholipase $A_2$ generated only non-specific sarcolemmal effects.

The effects on $Na^+$-$H^+$ exchange of a direct addition of specific lipids to the sarcolemmal membrane was investigated. Lysophosphatidylcholine, lysoplasmenlycholine, lysophosphatidylserine, lysophosphatidylinositol and lysophosphatidylethanolamine had no effect. The fatty acids linoleic acid and linolenic acid also had no effect on cardiac $Na^+$-$H^+$ exchange. However, eicosapentanoic acid and docosahexanoic acid both had an inhibitory effect on the $Na^+$-$H^+$ exchanger. These cardiac SL vesicles were subsequently exposed to 10-100μM eicosapentanoic acid (EPA) or docosahexanoic acid (DHA). $^{22}Na$ was utilized as a tracer to determine the amount of $H^+$-dependent $Na^+$ uptake. $H^+$-dependent $Na^+$ uptake was inhibited by up to 50% following ≥ 50μM EPA or ≥25μM DHA treatment. This inhibition was observed as a function of reaction time and in the presence of varying transsarcolemmal $H^+$ gradients. These fatty acids inhibited $Na^+$-$H^+$ exchange without altering passive ion permeability. This study demonstrates that specific alterations in the cardiac sarcolemmal membrane phospholipids influence the activity of the $Na^+$-$H^+$ exchanger.

# ACKNOWLEDGEMENTS

# LIST OF FIGURES

# LIST OF TABLES

# A. REVIEW OF LITERATURE

## I. The Cell Membrane

### *1. Fluid Mosaic model*

Many have established the importance of cellular membranes both physiologically and pathophysiologically. The cell membrane maintains cellular isolation from its outer environment. It is also involved in cell signaling and provides a selective permeable barrier (1). The membrane is arranged in two layers classically known as a phospholipid bilayer. The functional role of lipids contained within biological membranes was initially proposed by Gorter and Grendell in 1925 (2). This was established through analysis of an erythrocyte membrane phospholipid monolayer on an air-water interface. Decades later, these preliminary experiments lead to the discovery of vertical "flip flop" movements of phospholipids and lateral motions of proteins embedded within and traversing the bilayer. Singer and Nicholson (460) further clarified this in 1976 and coined the term "Fluid Mosaic Model" **(Figure 1)**. Each of the numerous components within the lipid bilayer has a specific function and plays an identifying role. The major components of this cellular membrane include phospholipids, fatty acids, proteins and cholesterol.

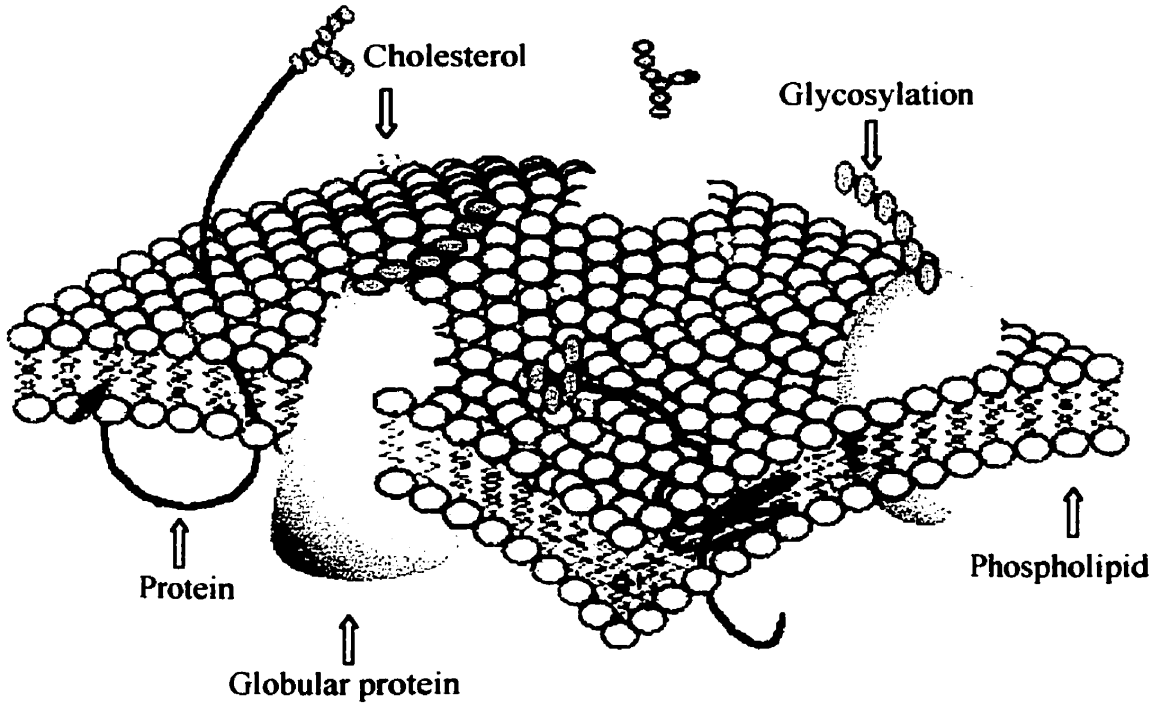


**Figure 1.** Singer and Nicholson's "Fluid Mosaic Model"

● = polar head groups

≋ = fatty acids

### *2. Phospholipids*

Within biological membranes exist two types of lipids, glycerophospholipids (GPL) and sphingolipids (SPL). Structurally, GPL's consist of a glycerol backbone to which two fatty acids are attached via an ester bond at positions sn-1 and sn-2. At sn-3, a phosphate group is attached to a highly polar head group **(Figure 2a)**. A plasmalogen is a GPL or an SPL where a vinyl ether bond attaches the fatty acid to the sn-1 position. SPL's have only one fatty acid attached to a sphingosine backbone as opposed to the glycerol backbone found in GPL's. For simplicity, both GPL's and SPL's will be referred to as phospholipids (1).

Both GPL's and SPL's are considered amphipathic (i.e. hydrophobic and hydrophilic portions). The hydrophilic segment consists of the polar head group, whereas the hydrophobic portion is the fatty acid species bound to the glycerol backbone.

The nomenclature of GPL's depends on their specific components. Glycerophospholipids are named according to the head groups attached to the glycerol backbone. For example, phosphatidylcholine has a choline head group attached to the glycerol backbone. It is referred to as a GPL species, but within these species exist many different classes depending on the combination of fatty acids attached to the glycerol backbone. Some examples of fatty acids are linoleic, oleic, lauric, myristic, palmitic, stearic, linolenic and arachidonic acid. These fatty acids can either be saturated (contain no double bonds) or be unsaturated (contain double bonds). More information on fatty acids is found later in this treatise.

There are many phospholipids within the cellular membrane. Phosphatidylcholine (PC) is the most abundant phospholipid found within mammalian membranes. As much

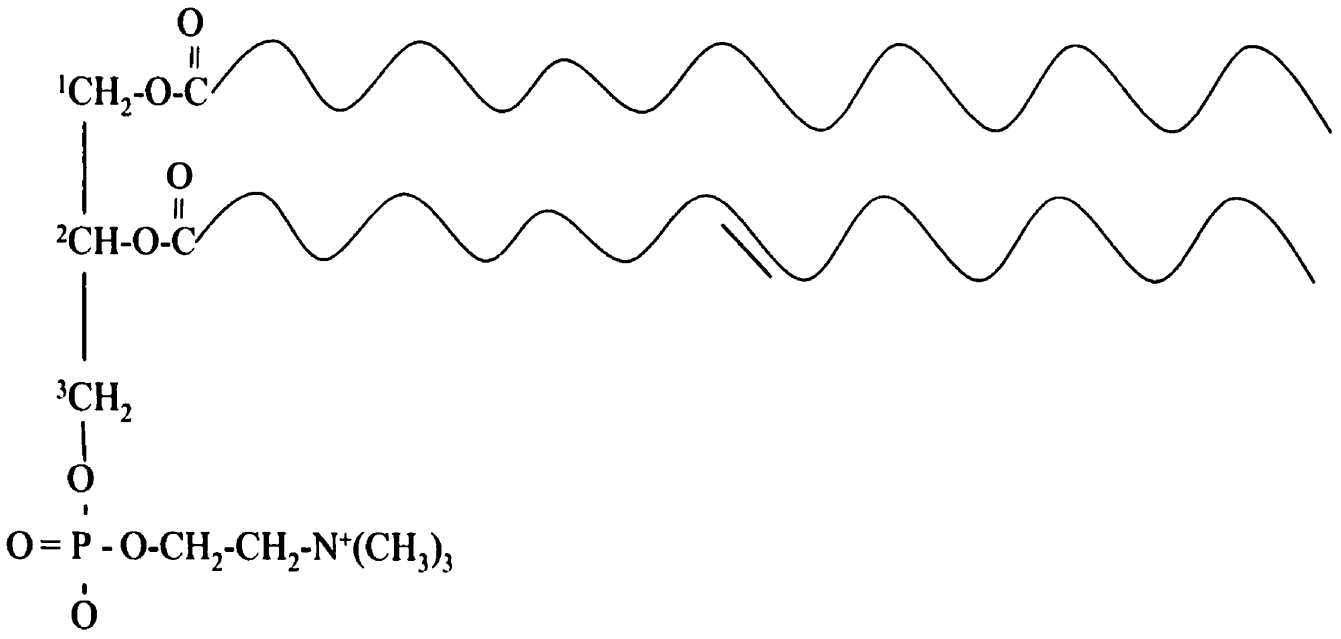


**Figure 2a. Phospholipid structure containing saturated and unsaturated fatty acids.**
Phospholipids include a head group (eg. Choline, ethanolamine etc), a phosphate group attached to a glycerol backbone and fatty acids (unsaturated or saturated) attached to the glycerol moiety.

as 50% of total membrane phospholipids consist of PC [3]. Others phospholipids include phosphatidylethanolamine (PE), sphingomyelin (SPH), phosphatidylinositol (PI), phosphatidylserine (PS), cardiolipin (CDL), phosphatidylglycerol (PG), lysophosphatidylcholine (LPC), and phosphatidic acid (PA). These phospholipids distribute themselves asymmetrically across the membrane. Certain phospholipids are found on the inner portion of the bilayer, and others on the outer layer [4] **(Figure 3)**. Phospholipids such as PC and SPH exist predominately in the outer membrane, while PI and PS occupy the inner membrane **(Figure 3)**.

The function of certain ion exchangers depends upon the membrane phospholipid composition. Philipson was the first to examine the influence of phospholipids on $Na^+$-$Ca^{2+}$ exchange [5,6]. Phosphatidylcholine enriched reconstituted proteoliposomes from dog heart sarcolemma showed no change in $Na^+$-$Ca^{2+}$ exchange (NCX). However, PC:PE reconstituted vesicles (90:10, weight:weight) stimulated $Na^+$-$Ca^{2+}$ exchange activity [7]. Vemuri and Philipson [8] reported optimal $Na^+$-$Ca^{2+}$ exchange occurs in the presence of PS, CDL and PA. NCX is depressed in the presence of PI and phosphatidylglycerol (PG) [8]. The stimulatory effect of acidic phospholipids on NCX has also been reported [8-10]. Other ion transporters such as the $Ca^{2+}$-ATPase [11-13] and $Na^+$-$K^+$ ATPase [14] are also dependent upon the phospholipid environment which surrounds them.

Lysophospholipids, such as lysophosphatidylcholine (LPC), lysophosphatidylethanolamine (LPE), lysophosphatidylinositol (LPI) and

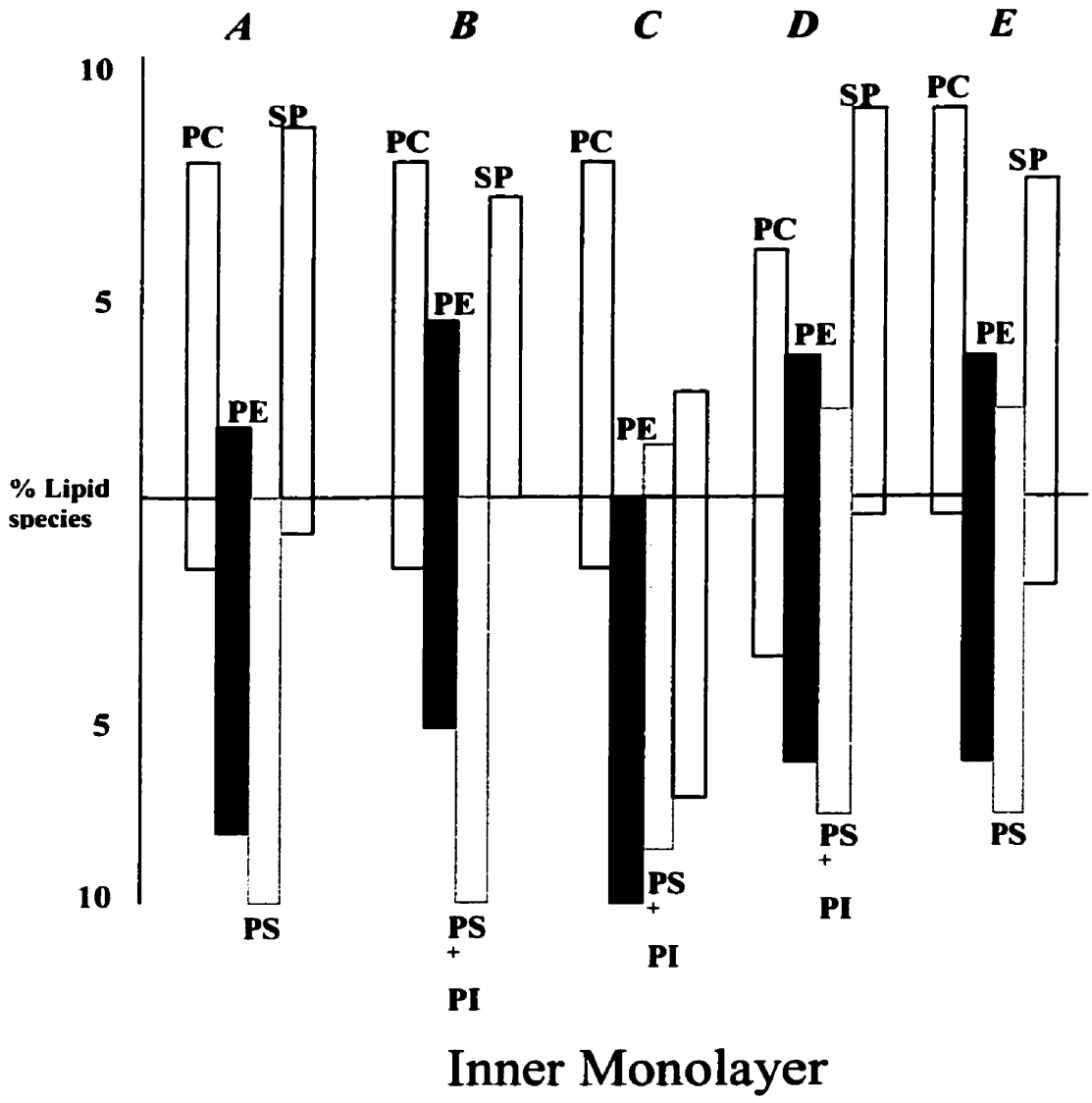


**Figure 3. Phospholipid asymmetry in plasma membranes.** (A). Human erythrocyte membrane, (B) rat liver blood sinusoidal plasma membrane, (C) rat liver continuous plasma membrane, (D) pig platelet plasma membrane, (E) VSV envelope derived from hamster kidney BHK-21 cells (adapted from [15])

lysophosphatidyserine (LPS) have been shown to modulate the activity of ion transporters. Lysolipids are phospholipids with one fatty acid group removed. Voltage dependent $Na^+$-channels [16], $Na^+$-$K^+$ ATPase [17], inward rectifier $K^+$ channels [18] and K-ATP channels [19] are affected by certain lysophospholipids. Lundback et al reported the effect of lysophospholipids on gramicidin channel function [20]. Yamaguchi et al have shown LPC inhibits the $Na^+$-$HCO_3^-$ [21]. LPC also affects cardiac $Na^+$ channels [16,22,23], $K^+$ channels [18,24] and inhibits the $Na^+$-$K^+$ ATPase [25,26]. The $Na^+$-$H^+$ exchanger has been reported to be unaffected by the phospholipid environment [21,27]. However, Hoque et al reported a stimulated $Na^+$-$H^+$ exchanger in rat cardiomyocytes following LPC addition [28]. The effects are thought to occur via alterations in the mechanical properties of the membranes [20].

Lysophospholipids have important biological actions outside of their effect on ion transport proteins. For example, lysophosphatidylcholine induces the expression of growth factors in human endothelial cells [29]. Lysophosphatidic acid induces vascular smooth muscle cell proliferation [30]. Lysoplasmenylcholine stimulates myocardial protein kinase (PKA) [31].

LPC is normally present in concentrations of 140 -150μM [32,33]. Certain pathological situations cause alterations in membrane phospholipids. Ischemia is one example. Phosphatidylethanolamine shifts from the inner membrane, where it is normally located, to the outer membrane following 60 minutes of ischemia in neonatal rat cardiomyocytes [34]. Phosphoinositide (phosphatidylinositol, phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate) breakdown is increased in

reperfused rat hearts subjected to 30 minutes of ischemia [35]. An elevation of LPC following ischemia has been reported by many labs [36-41]. Lysophosphatidylcholine is a mediator of ischemia-induced arrhythmias [42-45].

LPC can increase in the hearts of animals in a variety of diseases. In genetically determined myopathic hamster hearts, the levels of PC, PE, diphosphatidylglycerol (DPG) and PA were decreased while PI and LPC were elevated [46]. LPC is also increased in diabetic patients where it has been suggested to inhibit the activity of the $Na^+$-$K^+$ ATPase [47].

### *3. Cholesterol*

The integral role of cholesterol in stabilizing the phospholipid bilayer has been well established [48,49]. Interestingly, many authors have determined the preferential affinity for cholesterol to attach to certain phospholipids [50-52]. Sphingomyelin and other membrane stabilizing phospholipids attract cholesterol. Although this association for phospholipids is weak, the ratio of cholesterol to phospholipid contributes to overall membrane fluidity.

The quantity of cholesterol within membranes varies among the different subcellular membranes. This variation was first established in 1971 by Colbeau *et al* in rat liver subcellular membranes [53]. The inner mitochondrial membrane contains less cholesterol than the outer mitochondrial membrane. Similarly the smooth endoplasmic reticulum contains less cholesterol than the rough endoplasmic reticulum. The greatest quantity of cholesterol is found in the plasma membrane.

Cholesterol interacts with membrane-bound proteins (54-56). Kutryk *et al* (57) examined the effect of increasing membrane cholesterol on the $Na^+$-$Ca^{2+}$ exchanger. Cholesterol enrichment of sarcolemmal vesicles leads to a stimulation of $Na^+$-$Ca^{2+}$ exchange while inhibiting $Ca^{2+}$-$Mg^{2+}$ ATPase and $Na^+$-$K^+$ ATPase (57). This was confirmed by others (8). Oxidized cholesterol had the opposite effect on $Na^+$-$Ca^{2+}$ exchange, indicating the importance that cholesterol charge and structure has on membrane proteins and their function (461).

***4. Fatty Acids***

Fatty acids are carboxyl acids. They are attached to hydrocarbon chains which are 4-36 carbons long. They may be saturated (no double bonds) or unsaturated (double bonds). Single and multiple double bonds within fatty acids are referred to as monounsaturated (MUFA) and polyunsaturated fatty acids (PUFA), respectively. The double bonds within unsaturated fatty acids cause a "kink" to develop within the hydrocarbon chain. This prevents side-by-side alignment of fatty acids resulting in liquid fat at room temperature (eg. vegetable oil). Saturated fatty acids are solids or gels at room temperature (eg. margarine). They may also be branched or unbranched. Some fatty acids contain hydroxyl groups or three carbon ringed structures. Combined, these factors make up the specific physical properties of each individual fatty acid, including melting temperature and solubility. The length of the hydrocarbon chain determines the solubility of fatty acids. As the length of the hydrocarbon chain increases, solubility decreases (1).

The PUFA's have a special clinical importance in a variety of disease states. For example, mortality by coronary heart disease is reduced as a consequence of dietary long chain PUFA administration [58-61]. The long chain PUFA's prevent arrhythmias induced by ischemia. This was shown in animals [62-65] and is thought to function in a similar manner in humans [59,66-68]. There is also a role for PUFA's in diabetes mellitus. Dietary addition of docosahexanoic acid (DHA) improved metabolic control in diabetic patients [69]. Interestingly, the basal levels of DHA, EPA and arachidonic acid (AA) are similar to control non-diabetic levels [70].

Fatty acids have also been reported to affect the activity of certain ion exchangers. The PUFA's linoleic acid and linolenic acid stimulate the $Na^+$-$Ca^{2+}$ exchanger [71]. Eicosapentanoic acid, a PUFA, inhibits the $I_{Na}$ channel in human embryonic kidney cells (HEK293t) [72]. Other fatty acids such as palmitoleic acid caused a large stimulation of the $Na^+$-$Ca^{2+}$ exchanger [73]. The unsaturated fatty acids oleic, linolenic, linoleic and linoelaidic acid had similar effects [73]. These fatty acids also stimulate the $Ca^{2+}$-ATPase in erythrocyte membranes [74]. However, the opposite effects have been reported by others. Animals restricted to an unsaturated n-3 fatty acid diet containing docosahexanoic acid (C22:6) and eicosapentanoic acid (C20:5) show a decrease in rat cardiac sarcoplasmic reticulum $Ca^{2+}$-ATPase activity [75]. These fatty acids (DHA and EPA) also prevent ouabain toxicity [76]. This prevention is due to a decrease in $Ca^{2+}$ influx following ouabain addition [77]. The $Na^+$-$Li^+$ antiporter is inhibited in DHA fed diabetic patients [69].

As indicated in the preceeding pages, PUFA's also prevent ventricular fibrillation observed in rats following coronary ligation (62) and in monkeys (63). Intravenous infusion of DHA and EPA into canine myocardium prevented ventricular fibrillation following ischemia (65,78). This anti-arrhythmic effect of PUFA's may function via $Ca^{2+}$ (79) or $K^+$ channel inhibition (80). The effect of DHA and EPA on the $Na^+$-$H^+$ exchanger remains undetermined. However, $Na^+$-$H^+$ exchange activity in linoleic or linolenic enriched cells remains unaltered (81).

***5. Phospholipases***

Phospholipases are enzymes which hydrolyse phospholipids. This breakdown is necessary for constant phospholipid turnover. Several phospholipases exist within biological membranes. Phospholipase $A_2$, C and D are the most common. Each phospholipase has a specific phospholipid cleavage point. Each phospholipase will yield specific, structurally different by-products.

***i. Phospholipase A2***

There are three types of intracellular phospholipase$A_2$ ($PLA_2$) species found within mammalian tissue. The first $PLA_2$ is a 14-15 kDa protein which is known as low-molecular weight $PLA_2$. This low-molecular weight $PLA_2$ is localized in the membrane of cardiomyocytes (82). The second type is the 85 kDa high-molecular weight $PLA_2$ (83,84). Both of these phospholipases are $Ca^{2+}$ dependent. The $Ca^{2+}$ permits the translocation of the phospholipases to the plasma membrane from the cytoplasm (84). The third type is a $Ca^{2+}$-independent, 40 kDa phospholipase. This phospholipase has

been identified in canine myocardium [85] and is specific for *sn*-2-arachidonoyl plasmalogens [86].

The function of phospholipase $A_2$ is to cleave the ester linkage at the sn-2 position of phosphatidylcholine. This cleavage gives rise to a free fatty acid (usually arachidonic acid as it is most abundant in choline and ethanolamine containing phospholipids) and lysophosphatidylcholine **(Figure 2b)**. The $Ca^{2+}$-independent $PLA_2$ yields lysoplasmenylcholine as it is plasmalogen specific [86]. Removal of the choline from the LPC yields lysophosphatidic acid (LPA).

Gross and colleagues report an increase in $PLA_2$ activation following 15 minutes of ischemia [87]. This increase in PLA2 activity has also been reported following a 2-minute ischemic period [88]. The levels of plasmalogen specific $PLA_2$ also increase following 15 minutes of ischemia in rabbit myocardium [89]. This $PLA_2$ is also stimulated in response to interleukin-1 and thrombin [90,91].

The effect of $PLA_2$ on membrane ion exchangers has also been examined. Its effects are both direct and indirect. For example, although the $Ca^{2+}$-ATPase remains unaffected following the addition of $PLA_2$ [92], the by-products of $PLA_2$, lysophosphatidylcholine and arachidonic acid do affect ion transport [16,18,21-26,28,93]. Angiotensin II dependent $Na^+$-$K^+$ ATPase inhibition is thought to occur via a $PLA_2$ signaling pathway [94]. Inhibition of $PLA_2$ by mepacrine prevents angiotensin II induced inhibition of the $Na^+$-$K^+$ ATPase [94].

### *ii. Phospholipase C*

There are ten isozymes of phosphoinositide specific-phospholipase C (PI-PLC). These have been localized in many types of organisms including bacteria, plants and mammals [95-99]. These isozymes can be classified as either β (4), α (2) or δ (4) according to their structure [100,101]. Of the three types of subfamilies, the δ group is the smallest, approximately 85 kDa. In addition, the structure of the δ PLC isozyme is simple as it is contained in eukaryotes such as yeast and slime molds [100,102,103]. The β and γ isozymes are between 140 kDa – 150 kDa.

The isozymes share some similar features, while differ in others [103-106]. The activity of PI-PLC is dependent on the presence of $Ca^{2+}$. The major substrates are the phosphoinositides (phosphatidylinositol (PtdIns), phosphatidylinositol-4-phosphate (PtdIns 4-P) and phosphatidylinositol-4,5-bisphosphate (PtdIns-4,5-$P_2$) [107]. However, the discovery of two $Ca^{2+}$-independent non-phosphoinositide utilizing PI-PLC's have been reported [108,109].

The location of the isozymes varies. The $\delta_1$ and $\gamma_1$ isoforms are located in ventricular myocytes [110,111]. Little, however, is known about the localization of the PLC isozymes within cardiac cells.

The function of phospholipase C is to hydrolyze the phosphoinositides. This generates 1,4,5-triphosphate ($IP_3$) and *sn* 1,2-diacylglycerol (DAG) [112] **(Figure 2b)**. $IP_3$ and DAG are potent second messengers. $IP_3$ is known to cause $Ca^{2+}$ release from the sarcoplasmic reticulum [113,114], while DAG activates protein kinase C which activates a number of regulating factors downstream [115,116].

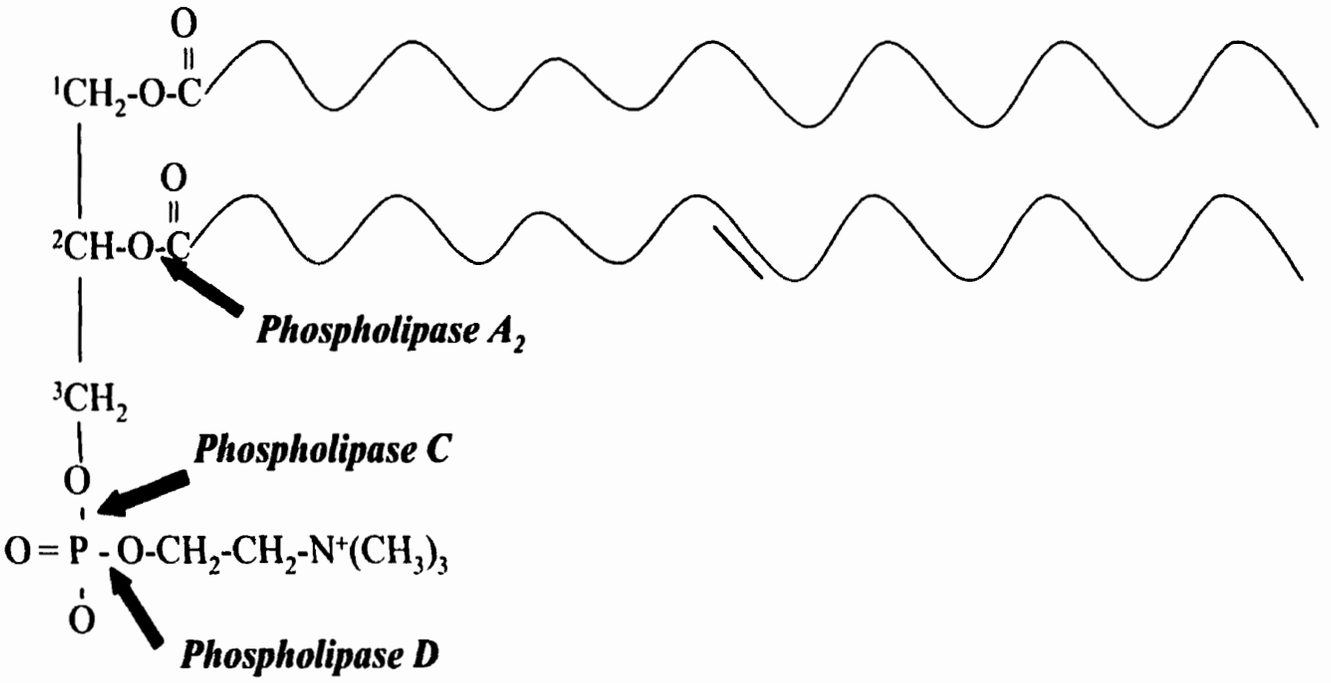


**Figure 2b. Phospholipase action and their specific by-products.** Each phospholipase cleavage point is site specific, thereby generating specific by-products.

The addition of phospholipase C modulates the activity of many ion exchangers. Phospholipase C addition to vesicular lipids causes $Ca^{2+}$ uptake to decrease (117). Phospholipase C also decreases the interaction of ATP with the membranes (117). A decrease in $PIP_2$ causes depression in NCX and $Ca^{2+}$ pump (118). $Na^+$-$K^+$ ATPase, SR $Ca^{2+}$, $Mg^{2+}$ ATPase and gastric $K^+$-$H^+$ ATPase have all been modulated by phospholipase C (14,119,120). Furthermore, $Na^+$-$Ca^{2+}$ exchange activity is enhanced following the addition of phospholipase C (6). This stimulation occurs irrespective of the increase in $Ca^{2+}$ permeability observed (6). This stimulation of the $Na^+$-$Ca^{2+}$ exchanger was also observed by others using phosphatidylinositol-specific PLC (121).

***iii. Phospholipase D***

Phosphatidylcholine, phosphatidylethanolamine and phosphatidylinositol are the preferential substrates for phospholipase D. Cleavage of the phospholipid generates two products, a polar head group and phosphatidic acid (122) **(Figure 2b)**.

Kanfer *et al* were the first to isolate phospholipase D (PLD) from rat brain tissue (123). Phospholipase D was also isolated from rat lung (124). Phospholipase D exists in a variety of organelles and tissue types and is stimulated by many factors (125). The majority of the PLD within the myocardium has been localized to the sarcolemmal membrane (126). A significant portion also exists in the sarcoplasmic reticulum membrane (126). Others have confirmed this localization of phospholipase D within the myocardium (127,128).

The PLD's can be separated into two categories. The first category consists of membrane bound PLD's which are stimulated by phosphoinositides such as phosphatidylinositol –4,5-bis phosphate [129]. Within this primary subdivision are two types of phospholipase D sub-species, PLD1 and PLD2 [130,131]. Both PLD1 and PLD2 are stimulated by phosphatidylinositol 4,5 bisphosphate and phosphatidylinositol 3,4,5 triphosphate. However, only PLD1 is stimulated by fatty acids, such as oleate [132]. The activity of this PLD is dependent on the presence of sodium oleate [133,134]. The second subdivision is dependent upon G-protein ribosylation factor (ARF) [135,136]. The oleate and ARF regulated PLD's are membrane bound [137]. Other PLD isozymes present within the plasma membrane are dependent upon other factors such as guanosine 5'-(3-thio)-triphosphate (GTPγS) [138-140].

The second subdivision of PLD's is a cytosolic isozyme which was identified in a number of tissue types [141-143]. Calcium stimulates this form of PLD [141,143]. In addition, the cytosolic isoform of PLD cleaves phospholipids non-specifically. Unlike the membrane bound PLD isozymes, the cytosolic PLD differs in its preference for non-phosphatidylcholine substrates. Substrates of the cytosolic PLD include PE or PI [141-143].

The modulatory effect of phospholipase D on certain ion exchangers has been demonstrated. Philipson and colleagues report a stimulation of $Na^+$-$Ca^{2+}$ exchange following the addition of phospholipase D [144]. This stimulation occurred following a 10-fold increase in phosphatidic acid levels subsequent to phospholipase D addition [144]. Phosphatidic acid also affects ion transport. Phosphatidic acid increases

intracellular levels of $Ca^{2+}$ (145). This increase is thought to occur via sarcoplasmic reticulum $Ca^{2+}$ release. The addition of thapsigargin and ryanodine (sarcoplasmic reticulum $Ca^{2+}$ channel inhibitors) attenuated the phosphatidic acid induced increase in intracellular $Ca^{2+}$ (145). The increase in $Ca^{2+}$ release from the sarcoplasmic reticulum by phosphatidic acid was also reported under in vitro conditions (146).

Plasmalogen phosphatidic acid augments $Na^{+}$-$Ca^{2+}$ exchange activity (147). This experiment was carried out in proteoliposomes containing plasmenylcholine. These proteoliposomes were exposed to phospholipase D generating plasmalogenic phosphatidic acid. This resulted in an 8-fold stimulation of the $Na^{+}$-$Ca^{2+}$ exchanger (147). At present, no study has examined the influence of phospholipase D, or its by-products, on the $Na^{+}$-$H^{+}$ exchanger.

Laminin increases the production of collagenase IV. This increase in collagenase IV is thought to occur through a laminin-induced phospholipase D intermediate (148). Williger *et al* report the reliance of matrix metalloproteinase-9 secretion on the presence and subsequent production of phosphatidic acid by phospholipase D (149). A role for phospholipase D during meiosis has also been shown (150).

Phospholipase D activity is reduced in the sarcolemma, sarcoplasmic reticulum and mitochondrial membranes of diabetic myocardium (151). The reduction of PLD activity in diabetic cardiac sarcolemma is reversed by insulin administration. Insulin only partially restored phospholipase D activity in the sarcoplasmic reticulum and mitochondrial membranes (151). Ischemia-reperfusion insults stimulate PLD activation (152). This activation also occurs in pre-conditioned hearts (153,154).

## II. The $Na^+$-$H^+$ Exchanger

### *1. Historical perspective*

Cells acquire the raw materials from the extracellular environment that are necessary for energy production. The by-products of this cellular metabolism must be released back into the external environment. The mechanism most frequently used to release such products from the cell is simple diffusion. Simple diffusion occurs when there is an unequal solute concentration on either side of a permeable membrane. Through this process the solute will eventually equilibrate its concentration in accordance with the second law of thermodynamics. However, achieving maximum entropy and equilibrium in living organisms would ultimately lead to death. The cellular membrane in living organisms prevents this by allowing only certain solutes to traverse. This selective permeability is accomplished in part through the physical characteristics of the lipid bilayer. The hydrophobic core in the lipid bilayer prevents uncontrolled movement of all polar solutes and inorganic ions, with the exception of water. To regulate transport of substances such as sugars, amino acids and inorganic ions, certain proteins are present within the bilayer. These protein exchangers import solutes in some instances, and export solutes in others.

This type of regulation is present in the myocardium. Cardiac cells are constantly producing protons because of their intense metabolism (155). The internal pH of myocytes is maintained between 7.3 –7.4. However, at an external pH of 7.4 and a membrane potential of –60mV, it would be expected that the intracellular pH would be approximately 6.4 with passive distribution according to the Donnan equilibrium.

Therefore, the internal pH in cardiac cells is maintained at one pH unit more alkaline than would be expected. This strongly suggested to early scientists in this area that a $H^+$ extruding mechanism must be present in the myocardium. In 1961, P. Mitchell suggested the existence of an antiporter which regulated fluctuations in the $[H^+]$ of the cell. The ability of cardiac cells to regulate their pH was first demonstrated by Ellis and Thomas (156). They examined $pH_i$ in Purkinje fibers from rat, guinea pig and ferret ventricle. Extracellular $CO_2$ was varied while $pH_i$ was measured. Variations in extracellular $CO_2$ initially increased pH. This was followed by pH restoration, however, at a new basal pH (156), suggesting the existence of a pH regulatory mechanism.

Knowledge concerning the regulation of pH in the myocardium was greatly advanced by the discovery of the $Na^+$-$H^+$ exchanger in the 60's. This was followed by discoveries of several $Na^+$-$H^+$ exchangers within other cell types and in bacterium *Streptococcus faecalis* (157,158). In 1976, the $Na^+$-$H^+$ exchanger was discovered in membrane of epithelial cells (159), rat liver mitochondria (160) and analyzed in the kidney (161). Several investigators have now identified the NHE as the major protein responsible for $H^+$ extrusion in cardiac cells (162,163). However, it wasn't until 1989 that the human $Na^+$-$H^+$ exchanger was cloned (164). The role of the NHE during certain pathological situations has stimulated the need for more information concerning the structural and functional characteristics of the $Na^+$-$H^+$ exchanger. **Table 1** chronologically illustrates the discoveries involving the NHE over the past 40 years.

**Table 1. Significant findings regarding the $Na^+$-$H^+$ exchanger in chronological order (165).**

| Date | Finding | Ref. |
|---|---|---|
| 1961 | First prediction of $Na^+$-$H^+$ antiport by P.Mitchell | (166) |
| 1967 | First experimental demonstration of $Na^+$-$H^+$ antiport in rat liver mitochondria | (167) |
| 1972 | Demonstration of NHE in bacterium S. faecalis. | (157) |
| 1976 | Electroneutral NHE is documented in the plasmalemmal of epithelial cells. | (161) |
| 1981 | Demonstration of inhibition of mammalian NHE antiporter by amiloride and analogs | (168) |
| 1982 | First evidence that the activity of the mammalian exchanger is allosterically activated by intracellular protons | (169) |
| 1981-1983 | Growth factor activation of NHE participates in mitogenesis | (170-173) |
| 1983 | First demonstration of NHE activity in heart | (462) |
| 1985 | First biochemical identification and characterization of NHE activity in myocardial sarcolemmal membranes | (174) |
| 1987 | Cloning of the major NHE antiporter gene of E. coli | (175) |
| 1989 | Cloning of the human NHE-1 isoform | (164) |
| 1990 | Elucidation that growth factors induce phosphorylation of the NHE-1 | (176) |
| 1991 | Purification and reconstitution of the functional NhaA, the electrogenic NHE anti-porter of E. coli | (177) |
| 1992 | Cloning of the plasmalemmal NHE of the yeast Schizosaccharaomyces pombe | (178) |
| 1989-1995 | Cloning of the distinct isoforms of NHE (NHE 1-5); determination of their expression in different tissues | (179-185) |
| 1993-1994 | Experimental evidence of oligomerization of mammalian NHE antiporters: NHE1 and NHE3 form stable homodimers in the | (186,187) |

| | | |
|---|---|---|
| | membrane | |
| 1994 | The cytoplasmic carboxyl terminal domain of the NHE-1 can bind calmodulin in a $Ca^{2+}$- dependent manner | (188,189) |
| 1995 | The C-terminal segment of NHE-1 interacts with mammalian heat-shock protein | (190) |
| 1998 | Identification of a novel mitochondrial NHE isoform, NHE-6 | (191) |
| 1999 | Clinical trials using NHE inhibitors during ischemia-reperfusion injury and balloon angioplasty | (192) |

### *2. Structure*

The NHE-1 is a 110-kDa glycoprotein containing 815 amino acids. The model initially proposed of the NHE-1 by Sardet *et al* consisted of 2 domains (164). The first was a hydrophobic N-terminal domain containing 500 amino acids forming 10-12 transmembrane helices. A second domain, a 315 amino acid hydrophilic portion referred to as the C-terminal cytoplasmic domain, served as the "sensor" for activation of the NHE-1. The topology of the NHE-1 is represented in **Figure 4**.

The eukaryotic NHE cytoplasmic domain is larger than that present in bacterium (193). Functionally, the cytoplasmic domain translates signals into either inhibitory or stimulatory modulation of the NHE. The cytoplasmic domain shares little similarity between isoforms. Stimulation of the NHE via phosphorylation occurs at the cytoplasmic domain. For example, activation of the NHE-1 proceeds via a phospholipase C-diacylglycerol-PKC induced phosphorylation of this cytoplasmic domain (164). In addition to the PKC dependent NHE phosphorylation, phorbol esters and other growth factors activate certain kinases to phosphorylate the NHE (176,194). NHE phosphorylation occurs at residue 635 of the cytoplasmic tail. Removal of this residue does not prevent NHE activation. This indicates that NHE stimulation must be occurring through some other mechanisms (195,196). To examine the location upon which these factors were stimulating NHE, the non-phosphorylatable residues, 567-635 were removed. This eliminated growth factor induced NHE stimulation, however NHE activation remained (195). For this reason, NHE stimulation through $Ca^{2+}$-calmodulin binding was suggested (197,198)

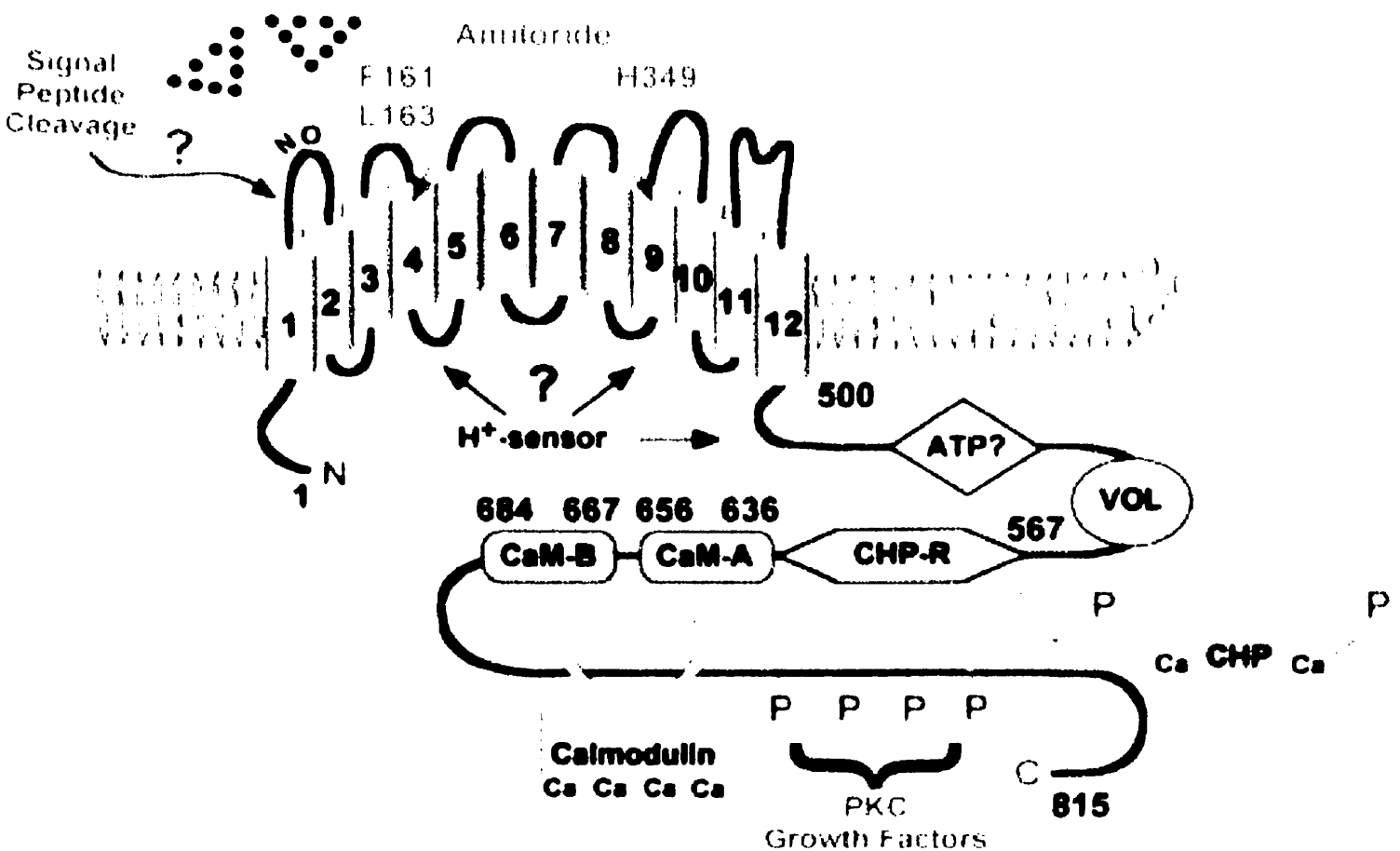


**Figure 4.** Topological representation of NHE-1 (199)

(203-205). Residues 637-656 of the cytoplasmic tail are responsible for the $Ca^{2+}$-calmodulin binding site on the NHE (188,206) **Figure 4**. Activation of protein kinase II via $Ca^{2+}$-calmodulin binding is thought to stimulate NHE (197,198,203-205). Other factors which bind and activate the NHE are hsp70 (189), which binds the carboxyl-terminal region of the NHE and a 24kDa protein which remains unidentified (207).

The transmembrane segments (TMS) are responsible for actual ion exchange (208,209). The number of transmembrane spanning regions varies between NHE isoforms. There is homology of amino acids within the transmembrane segments between NHE isoforms. The first TMS forms a sequence necessary for signaling (196), and shares the least homology amongst the various NHE isoforms (210). The different transmembrane segments (TMS) have special functions. Sites for glycosylation, amiloride binding and $H^+$ sensors are present.

The N and O linked glycosylated sites are restricted to the first N-terminal domain (211). This glycosylation adds 20 kDa to the molecular mass of the NHE-1 protein (176,212). The actual molecular mass of NHE-1 is 91 kDa. Glycosylation promotes proper packaging and transport of the NHE to the membrane (213,214). The exact function of glycosylation in $Na^+$-$H^+$ exchange has yet to be determined. Some tissues may contain different levels of glycosylation serving an unknown function (212). Removal of the glycosylated segments has no effect in some cell types (211,212,215), yet stifles ion exchange in others (216). Not all NHE isoforms, such as NHE-3, are glycosylated (211,217).

The TMS vary between isoforms in their sensitivity for inhibitors [218-220]. The fourth and ninth TMS are involved in amiloride sensitivity. For example, loss of sensitivity occurs following replacement of the Leu167 residue with Phe167 in the 4th TMS [221,222]. Mutagenesis of an analogous site in rabbit NHE-2 (Leu143 to Phe143) replicates the previous finding [223]. Alterations of the His349 residue in the ninth TMS to Tyr, Phe, Gly or Leu have minimal effects on amiloride sensitivity [224].

The stimulation of the NHE by $H^+$ occurs via allosteric activation [225]. This activation is thought to occur via protonation of an ionizable group such as a histidine residue on the cytoplasmic side. Protonation is thought to cause a conformational change and subsequent stimulation of the NHE. A histidine residue between the 7th and 8th TMS has been suggested to serve as the $H^+$ sensor in *Escherichia coli* NHE [226]. Although the bacterial NHE is not completely homologous with mammalian NHE, a similar histidine residue may serve an analogous role. Wakabayashi *et al* have established the N-terminal domain as the site for $H^+$-induced activation [195], whereas the cytoplasmic domain senses a certain $pH_i$ to which the NHE becomes active [195].

Little is known about the tertiary and quaternary structure of the NHE. Some suspect NHE functions as a monomer [164], while others suggest a homodimeric configuration [187,227]. Monomer interaction is thought to occur within the transmembrane region [187] via disulfide bonds [227], however, the exact location has yet to be determined.

### *3. Location and isoform*

Several different isoforms of the $Na^+$-$H^+$ exchanger have been identified throughout mammalian tissue. To date, six different isoforms of the $Na^+$-$H^+$ exchanger

exist and are all located in select areas of mammalian tissue. $Na^+$-$H^+$ exchange isoform-1 (NHE-1), is a ubiquitous glycoprotein that is the predominant isoform found in the myocardium. The NHE-1 isoform is also found in the human thyroid gland, adult brain, placenta and breast. Other isoforms such as NHE-2, NHE-3 and NHE-4 are located in areas of the stomach, intestine and kidney, respectively (181,184). The NHE-5 isoform of the $Na^+$-$H^+$ exchanger is localized in non-epithelial tissue, which is distinct from all other isoforms (180). Recently, a sixth isoform of NHE has been discovered in the mitochondrial inner membrane (191) **(Table 2)**. The NHE-6 isoform shares the least amino acid similarity to the other five, with a ≈20% homology. This is in contrast to the other isoforms, which share anywhere from a 34%-60% amino acid similarity. The function of NHE-6 is still under debate but is considered to regulate $Na^+$ and $H^+$ in the mitochondria (191).

It is apparent that not only is the function of the NHE-1 important, so is its tissue distribution. Petrecca *et al* have localized the distribution of the NHE-1 in adult rat atrium and ventricle (228). They characterized the exact location of the NHE-1 at the intercalated disks adjacent to connexin 43 and in the transverse tubules (228). This may be important as connexin 43 and NHE may function synergistically to regulate gap junction conductance. The relationship between $pH_i$ and gap junction conductance has been reported (229,230). Amiloride-induced NHE inhibition and subsequent intracellular acidosis inhibits gap

**Table 2. $Na^+$-$H^+$ isoforms and their specific locations within mammalian tissue.**

| Isoform | Location |
|---|---|
| NHE-1 | Ubiquitous; Fibroblasts, cardiac cell membrane, intestinal and renal epithelial cells, skeletal muscle, vascular smooth muscle, brain |
| NHE-2 | Stomach, kidney, intestine, adrenal gland and trachea and skeletal muscle . |
| NHE-3 | Intestine, kidney and stomach. |
| NHE-4 | Stomach, kidney. |
| NHE-5 | Brain, testes, spleen and skeletal muscle. |
| NHE-6 | Inner mitochondrial membrane. |

**References:** (164,179-181,183-185,191,231-234)

junction conductance (235). The subcellular location of the NHE differs from other ion-transporters. The $Na^+$-$Ca^{2+}$ exchanger has been localized to areas similar to the NHE, as it is present in the sarcolemma of rat and guinea pig myocytes (236,237).

### *4. Function of the $Na^+$-$H^+$ exchanger*

The regulation of pH is crucial. Whole body physiology is affected by changes in body fluid [$H^+$]. The intracellular pH is normally acidic compared to the extracellular environment. The [$H^+$] of body fluids averages 0.00004 mEq/liter. The changes observed during whole body acidosis result from alterations in intracellular pH. Both bicarbonate and hydrogen ions diffuse through the cellular membrane but may take several hours to equilibrate with the external environment. The only exception to this is red blood cells which can equilibrate rapidly.

The functional pH within the myocardium is ~ 7.0-7.3. (156,238). Alterations in pH cause changes in electrical-contraction coupling, contraction and cardiac action potential disturbances (239). Intracellular acidosis has been reported to potentiate the amount of atrial natriuretic peptide secreted during a given pressure overload (240). The pH in a cell also regulates protein synthesis (241), metabolic enzyme systems (242), contractile apparatus (243), ion conduction and cell division and proliferation (244-246). For example, the rate limiting enzyme in glycolysis, phosphofructokinase, increases its activity in response to a rise in intracellular pH (242,247). A decrease in pH negatively affects contractility in perfused hearts and in Purkinje fibers (248-251). A decrease in pH of 0.22 units led to a 50% decrease in contractility (250). Sodium and calcium currents

are also reduced by acidosis in a dose dependent manner (252-254). These alterations in ionic conductance are not only associated with changes in contractile tension in the heart but may also lead to arrhythmias. Intracellular pH can effectively regulate cell growth as well. Alterations in pH of only 0.3 units cause diminished mitogenic stimulation of cells in culture (245).

To maintain intracellular [pH], the NHE-1 extrudes a single $H^+$ in exchange for one $Na^+$ while maintaining electroneutrality (255,256). The NHE can also function in reverse. Reversing the $Na^+$ gradient leads to intracellular acidification and extrusion of $Na^+$ (257) (258-260). Approximately 60% of all $H^+$ ions removed from myocytes are accounted for by the NHE (261). The ability of the NHE to restore pH following acidosis has been shown in sarcolemmal vesicles (174), freshly isolated rat myocytes (262), chick myocytes (257) and sheep Purkinje fibers (156,263).

$Na^+$-$H^+$ exchange is highly regulated and can increase or decrease depending upon the ionic conditions. Although there are two ions exchanged, the $Na^+$-$H^+$ exchanger requires the presence of both gradients in order to function optimally. For example, the activity of the exchanger, when measured in cardiac sarcolemmal vesicles, is increased when the concentration of extravesicular $Na^+$ is increased (257). Therefore, NHE activity is influenced by the [$Na^+$], and the intracellular [$Na^+$] is influenced by NHE activity as well (257).

Ideally, optimal exchange occurs in the presence of a $Na^+$ gradient. However, because under basal conditions a large $Na^+$ gradient is normally present with142mEq/L $Na^+$ in the extracellular space and 10mEq/L intracellularly, it is the $H^+$ gradient which commonly fluctuates the most and stimulates $Na^+$-$H^+$ exchange activity. Therefore, the

activity of the $Na^+$-$H^+$ exchanger is primarily dependent upon the presence of a $H^+$ gradient. Vesicular $Na^+$ uptake is near zero when both internal and external pH are similar (257). The NHE has a differential sensitivity to intracellular and extracellular $H^+$. Intracellular $H^+$ will stimulate the exchanger more than $H^+$ in the extracellular space **(Figure 5)**. This differential sensitivity to intracellular $H^+$ is likely due to the structure of the NHE. The NHE possesses a cytoplasmic domain that is highly sensitive to $[H^+]$ (195). A co-operative relationship also exists between $H^+$ and NHE as the activity of the exchanger can increase substantially following small pHi fluctuations (257). The NHE is, however, particularly sensitive within a specific range of intracellular and extracellular $[H^+]$'s. At a $pH_o$ of 7.6, the NHE functions optimally when $pH_i$ is 6.8, but decreases activity and eventually ceases as $pH_i$ approaches 7.6 (257). It is controversial whether the NHE is inactive during basal conditions in the heart when intracellular pH is in the range of 7.2-7.4.

The NHE is also capable of substituting other ions for $Na^+$ during exchange. Intravesicular release of $H^+$ increases with the presence of extracellular $Na^+$, $K^+$, $Rb^+$ and $Li^+$ (264). This indicates a non-specific exchange mechanism for $Na^+$ (264). In addition, the uptake of $K^+$ and $Rb^+$ was diminished when $H^+$ was replaced with intracellular $Na^+$ and $Li^+$. Sodium uptake is decreased as intracellular $H^+$ was replaced with $Li^+$ or $Rb^+$ (264). Prior to this, Pierce and colleagues demonstrated the ability of $Mg^{2+}$ and $Li^+$ to inhibit $H^+$-dependent $Na^+$ uptake (265). This was consistent with competitive inhibition at the active transport site. They also reported an increase in $Na^+$ efflux with $Na^+$, $Ca^{2+}$, $Mg^{2+}$ and $Li^+$

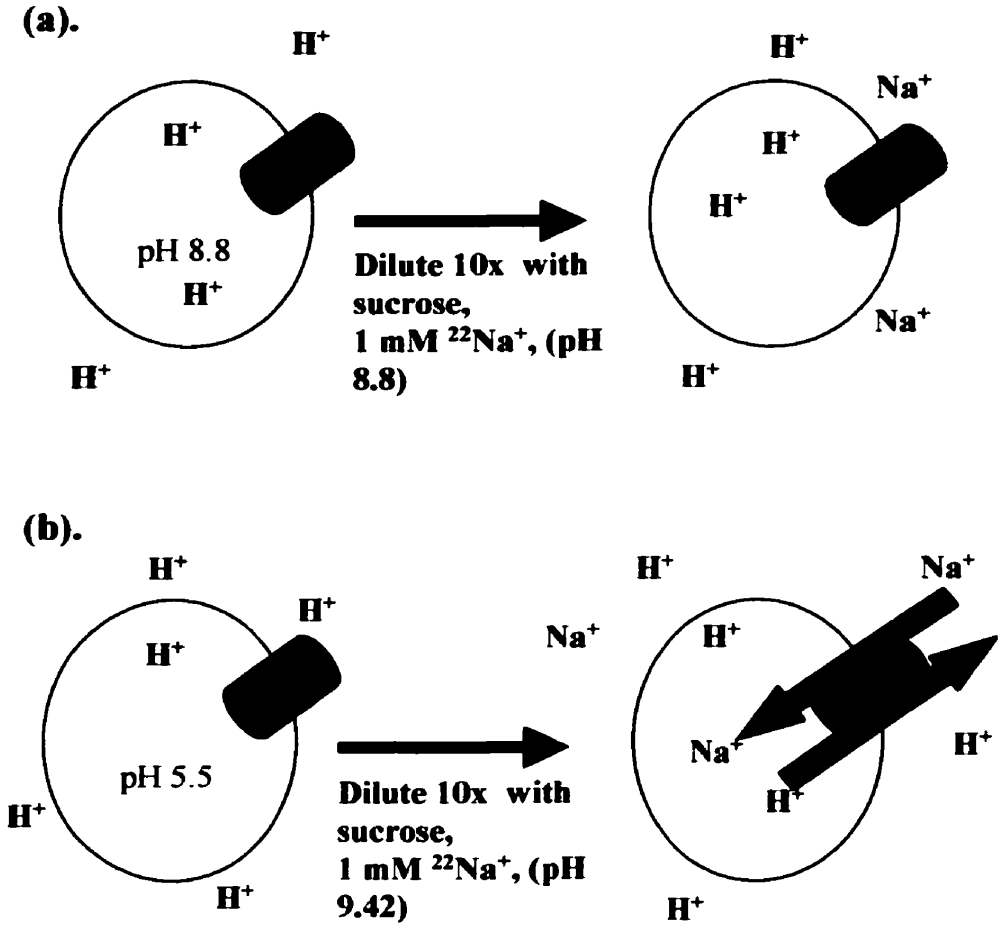


**Figure 5. $Na^+$-$H^+$ exchange requires both $Na^+$ and $H^+$ gradients to function.** a). $Na^+$-$H^+$ exchange remains inactive when diluted in similar pH. b). NHE activity requires the presence of both $Na^+$ and $H^+$ gradients.

present extracellularly [265]. Clearly, alterations in the ionic environment of the cardiomyocyte will have striking effects on $Na^+$-$H^+$ exchange and pH.

It is thought that the NHE functions in an ATP dependent fashion. The exact mechanism by which the NHE is ATP dependent remains unclear. Phosphorylation by ATP of residues within the cytoplasmic domain has been suggested. Removing segments of the cytoplasmic tail activated by ATP attenuates NHE stimulation. [266]. The ATP dependence of the NHE has been challenged [267-270]. Evidently, further research in this area is necessary.

### *5. Pharmacological properties*

There has been considerable attention surrounding the $Na^+$-$H^+$ exchanger following the discovery of its importance during several pathological situations. For this reason, inhibition of the NHE family has become important clinically. Papillary muscle alkalinization can also be attenuated following NHE inhibition [271]. Several NHE blockers have been used extensively for attenuating the degree of damage following an ischemic event [272-303]. The more potent inhibitors of NHE include amiloride, ethylisopropylamiloride, dimethylamiloride, methylisobutyl amiloride, HOE 642 (cariporide), HOE 694, cimetidine, clonidine and harmaline **(Table 3)**.

#### *a. Amiloride and analogs*

Structurally, the diuretic amiloride consists of a pyrazinoylguanidine moiety. The pyrazine ring contains an amino group on positions 3 and 5 and a chloro group on

position 6. Johnson et al first described NHE inhibition by amiloride (304). NHE inhibition by amiloride has been examined in several cell types (305-308).

The various isoforms of $Na^+$-$H^+$ exchange vary in their degree of inhibition by amiloride. The order of sensitivity to amiloride is as follows: NHE-1 is the most sensitive, followed by NHE-2 and NHE-3 (219,220,309-311). The mechanism of action of amiloride is via competitive inhibition with $Na^+$ for the external surface of the NHE anti porter (259,312,313). Increasing extracellular $Na^+$ decreases the degree of inhibition by amiloride and its analogs. In addition, amiloride analogs also bind to the NHE on an alternate site (314).

The structure of amiloride is important for optimal inhibitory potency. To date, more than one thousand amiloride analogs have been isolated (315). The potency of amiloride for NHE is greatest when position 5 on the pyrazine ring is replaced by a hydrophobic substituent. Kleyman *et al* report a 100nm $IC_{50}$ of amiloride for NHE following this substituent replacement (316). The transport of amiloride and its analogs depends on a number of factors. Inhibition of NHE occurs when amiloride is protonated. (305). The hydrophobicity of amiloride determines the degree to which it will dissipate a $H^+$ gradient across a membrane (225,317). Furthermore, the lipophilic nature of amiloride enables it to accumulate in the cells. This may disrupt the kinases and other modulators causing NHE inhibition (318,319).

Amiloride is a non-specific inhibitor of the NHE. The $IC_{50}$ for inhibition of the NHE is much lower when compared to inhibitory concentrations of amiloride for the epithelial $Na^+$ channel (320), $Na^+$-$Ca^{2+}$ exchange (321), $Na^+$-$K^+$ ATPase (307) and the

**Table 3. Pharmacological properties of mammalian $Na^+$-$H^+$ exchanger isoforms. .** (adapted from Orlowski *et al*; Characteristics of the plasma membrane $Na^+$-$H^+$ exchanger gene family. In: The $Na^+$-$H^+$ exchanger, ed. Larry Fliegel, publisher: R.G. Landes Company, 1996) [221].

| | Inhibition Constants ($K_{0.5}$) | | |
|---|---|---|---|
| **Inhibitor** | **NHE1** | | |
| | **Rat** | **Rabbit** | **Human** |
| *Amilorides family* | | | |
| **Amiloride** | 1.6uM | 1uM | 3uM |
| EIPA | 15nM | 20nM | 22nM |
| DMA | 23nM | | 100nM |
| Benzamil | 120nM | | |
| Others | | | |
| HOE 694 | | | 160nM |
| Cimetidine | 26uM | | 28uM |
| Clonidine | 210uM | | |
| Harmaline | 140uM | | |

$Na^+$-glucose cotransporter (322). Increasing the [amiloride] inhibits voltage gated $Na^+$, $K^+$ and $Ca^{2+}$ channels (323,324), $Na^+$-$Ca^{2+}$ exchange in cardiac cells (324), $Na^+$-glucose, $Na^+$-alanine, $Na^+$-$PO_4^{3-}$ cotransporters (322), $Na^+$-$K^+$ ATPase in vesicles and intact cells (307,325), and the nicotinic receptor (326). Amiloride is capable of inhibiting not only NHE, but also voltage gated $Na^+$ (327) and $Ca^{2+}$ channels (328) and the $Na^+$-$Ca^{2+}$ exchanger, albeit poorly. The $IC_{50}$ of amiloride necessary to inhibit $Na^+$-$Ca^{2+}$ exchange is much higher (303), approximately 1mM (321). Amiloride poorly inhibits the $Na^+$-$K^+$ ATPase at a concentration of 3mM (307). The exact mechanism of how amiloride inhibits the $Na^+$-$K^+$ ATPase is undetermined but it probably interacts with the $Na^+$ binding site. Ultimately, the amiloride family of drugs produces significant changes on myocardial action potential characteristics and contractile function (329). The intensity of these effects is dependent upon the specific amiloride analogue, its concentration, and the duration of exposure to drug (329).

***b. Other $Na^+$-$H^+$ exchange inhibitors***

$Na^+$-$H^+$ exchange is also inhibited by HOE 694, HOE 642 (cariporide), cimetidine (histamine $H_2$-receptor antagonist) (330,331), clonidine ($\alpha$2-agonist) (331,332), harmaline (hallucinogen which inhibits some $Na^+$ transport systems) (331,333,334) and loperamide (opiate antagonist) (335). These agents differ from amiloride and the HOE compounds chemically. However, structurally they bear a slight resemblance to amiloride. Cimetidine and harmaline inhibit NHE with a similar sensitivity as amiloride (219,220).

Cariporide (4-isopropyl-3-methylsulfonylbenzoylguanidine methanesulfonate) (HOE 642) is an important inhibitor of NHE. Cariporide is both structurally and functionally distinct from amiloride. Cariporide is a benzoylguanidine derivative, unlike amiloride which is a pyrazinoylguanidine moiety. It is functionally similar to HOE 694, the NHE inhibitor which was synthesized prior to cariporide. Functionally, it is more specific for the NHE-1 isoform (336,337) than amiloride which is a non-specific inhibitor of NHE and other ion transporters.

Clinically, cariporide is an important NHE inhibitor. It decreases the amount of injury observed following oxidative stress (338), the exposure to lysophosphatidylcholine (an ischemic metabolite) (28) and ischemia-reperfusion challenge (337). Cariporide is currently being examined in the clinical trial GUARDIAN during ischemia-reperfusion injury and has proven to be beneficial (192).

### *6. Other pH regulating mechanisms*

Along with $Na^+$-$H^+$ exchange regulating $pH_i$ within the acidic range, there exist three additional ion exchangers responsible for pH maintenance. The $Na^+$-HC03- co-transporter is known to maintain pH during basal conditions. The $Na^+$-HC03- co-transporter, unlike the $Na^+$-$H^+$ exchanger, transports both ions into the cell. However, it along with the $Na^+$-$H^+$ exchanger, functions exclusively to maintain pH following acidosis (339-341). The activity of the $Na^+$-$HC0_3^-$ co-transporter is optimal at rest (162,329,340,342), as opposed to the $Na^+$-$H^+$ exchanger which is optimal under acidic conditions.

Maintenance of basal pH following alkalosis is also important, and carried out through two other ion-exchangers. The $Cl^-/OH^-$ and $Cl^-/HCO_3^-$ function to decrease intracellular pH following an alkalinic load in the heart [343] **(Figure 6)**. These transporters move $OH^-$ and $HCO_3^-$ out of the cell while $Cl^-$ enters. Conversely, recovery from acidosis in the neonatal myocardium is dependent upon the $Cl^-/HCO3^-$ transporter when the NHE-1 is inhibited [344].

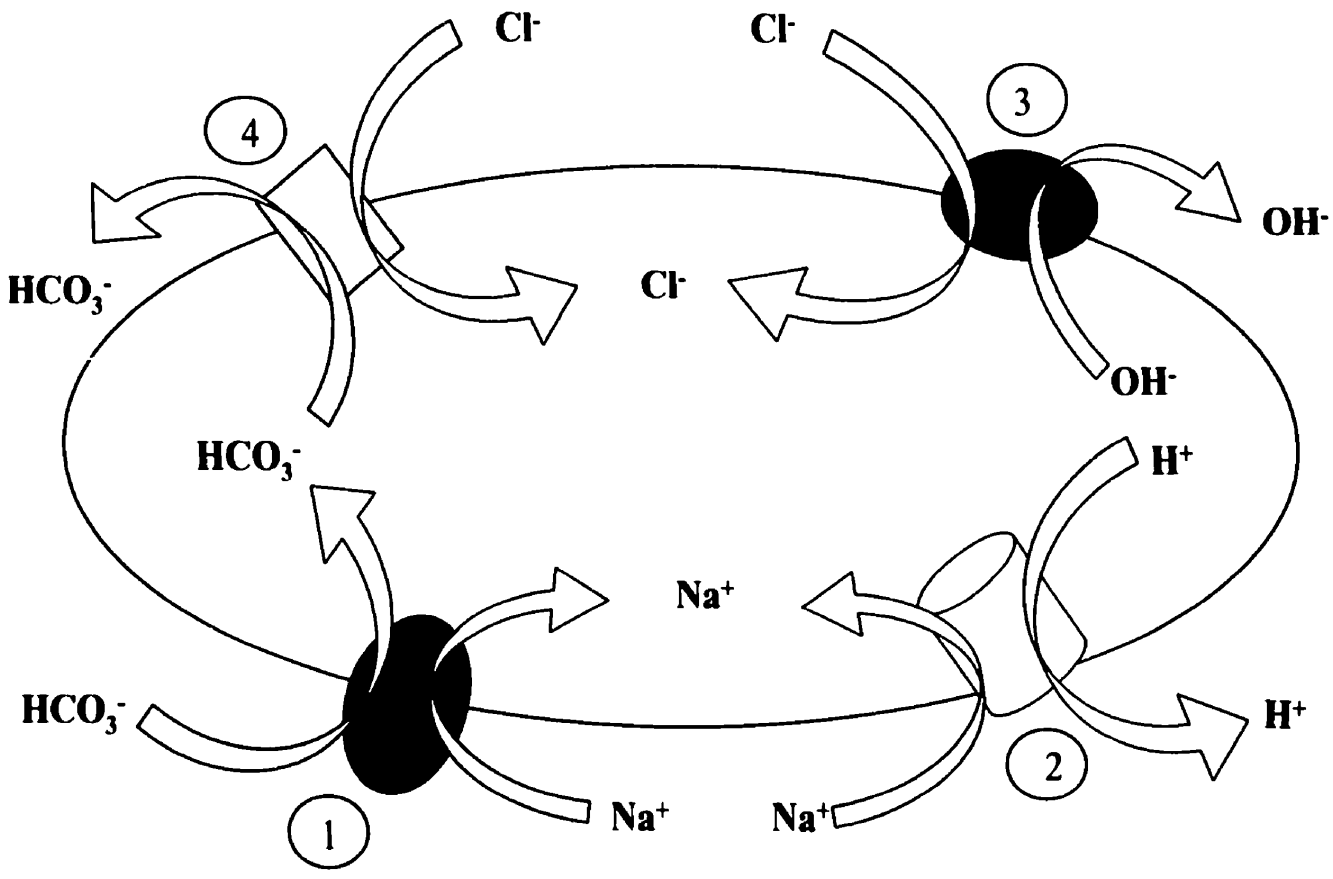


**Figure 6. pH regulators of cardiomyocytes:** 1. $Na^+$-$HCO_3^-$ symport, 2. $Na^+$-$H^+$ exchanger, 3. $Cl^-$-$OH^-$ exchanger and 4. $Cl^-$-$HCO_3^-$ exchanger

## III. Physiological / Pathological Modulation of the $Na^+$-$H^+$ Exchanger

### *1. Humoral factors*

The $Na^+$-$H^+$ exchanger is modulated physiologically by a number of extracellular factors including angiotensin II, endothelin, thrombin, ∝-adrenergic agonists such as phorbol esters (196,271,278,345-353) and enalapril (354). An increase in intracellular $Ca^{2+}$ and subsequent activation of calmodulin stimulate the $Na^+$-$H^+$ exchanger (355,356). Toxic metabolites such as hydrogen peroxide also modulate the activity of the NHE (357). Furthermore, a tyrosine-kinase stimulation of NHE via a receptor coupled mechanism has also been reported (176). Membrane phospholipids have been shown to modulate NHE in whole, intact cardiomyoctes. Karmazyn and colleagues have shown a lysophosphatidylcholine-induced stimulation of $Na^+$-$H^+$ exchange in whole cells. In contrast, Demaureux *et al* report that altering membrane phospholipids has no effect on the NHE (27).

### *2. Cell volume regulation*

Another important function of the NHE is cell volume regulation during osmotic shrinkage (358,359). Under physiological conditions, cells are bathed in an external molarity of 300mOsm/L. This concentration is similar to the intracellular molarity. Ions like $Na^+$ change their intracellular and extracellular concentrations in response to a variety of stimuli. To maintain ionic equilibrium, water will move across the cell membrane. Under normal circumstances, cells will freely accumulate $H_20$ because it will easily pass through the cell membrane. If extracellular [$Na^+$] changes, this can lead to

cell shrinkage or swelling. If severe, cell survival is maintained through ion extrusion. Reduction of cellular volume is accomplished mainly through $K^+$ and $Cl^-$ movement and/or efflux of solutes such as taurine, inositol, betaine or glutamine (360-362). Conversely, cells can increase their intracellular volume. A cell volume increase occurs under two situations. The first occurs during exposure of the cell to a hyperosmotic medium. This exposure leads to cell shrinkage, followed by volume increase. Secondly, cell swelling in a hypoosmolar solution leads to a loss of ions. However, when returned to an anisomolar environment, the cells shrink. To reestablish normal cell size, an increase in cell volume must occur. This type of cell volume increase occurs rapidly (363,364). Parker and co-workers observed an increase in extracellular $H^+$ during cell volume increase (365). Cala *et al* proposed a model for NHE involvement during cell shrinkage (366). Cala *et al's* model suggests NHE activation following cell shrinkage. This causes a subsequent increase in $pH_i$. This alkalinization results in stimulation of the Cl-/HCO3- followed by Cl- and $Na^+$ uptake. This leads to an overall increase in H2O uptake via osmosis (366). This NHE stimulation occurs in a variety of cell types (188,367-371). Prevention of cell volume regulation with NHE inhibition was observed in *Amphiuma* and dog erythrocytes (366).

It is worth noting, however, that not all NHE isoforms respond similarly to hyperosmotic challenge. NHE-2 responds to hyperosmotic challenge in certain cells and not others (268,348). The NHE-3 isoform is inhibited and unaffected by hyper and hypotonicity, respectively (268,348).

### *3. Ischemia-reperfusion injury*

Cardiovascular disease is the leading cause of death worldwide. Ischemic heart disease comprises the largest fraction of this. The prevalence of ischemic heart disease makes it a serious problem facing clinicians today. Approximately 25% of all deaths related to cardiovascular disease are attributable to ischemic lesions. Ischemic heart disease claims the lives of greater than 200,000 Americans below the age of 65 each year with more than 25 times that number who suffer from associated complications (372). Similarly, in Canada, cardiovascular disease accounted for 40% of all deaths in 1988 (373) and 21% (374) of total medical costs in 1986. With approximately $5.2 billion and $11.6 billion spent on direct and indirect medical costs, respectively, cardiovascular disease is the most expensive disease to treat today (374). The mortality rate of individuals suffering from ischemic heart disease is also relatively high with approximately 60% of all cardiovascular deaths attributed to ischemic heart disease (375).

Ischemia is defined as inadequate blood flow to the tissues where oxygen supply does not meet the metabolic demands of the tissue. This causes an accumulation of lactate and [$H^+$] within the heart and a decrease in the ATP energy supply. Under normal conditions, 50%-70% of oxygen consumption in the heart is derived from fatty acids as the primary energy source. In diabetes, 90%-99% of total myocardial oxygen consumption occurs through fatty acid metabolism (376). During ischemia, the level of glycolysis is increased with a concomitant decrease in the metabolism of fatty acids and glucose. Anaerobic glycolysis provides an important source of ATP during ischemia (377). Although ATP is being provided, accumulation of glycolytic products during low-

flow or no-flow ischemia may be detrimental to the heart [378]. This increase in myocardial lactate levels and decrease in intracellular pH leads to myocardial cell injury if uncontrolled [378].

Intracellular pH, therefore, becomes a central issue in ischemia / reperfusion injury. The pH is strictly controlled and maintained despite constant fluctuations in acidic events inside the cell. This control is especially crucial in the myocardium where the internal pH must be regulated at 7.0-7.3 [156,238]. To maintain this [pH]i, the $Na^+$-$H^+$ exchanger regulates the $[H^+]$. The predominant form in the myocardium, NHE-1, extrudes one $H^+$ for the uptake of a single $Na^+$ [255,256]. Events such as ischemia, which are associated with large changes in pH, activate the $Na^+$-$H^+$ exchanger. During ischemia, a large increase in intracellular and extracellular $[H^+]$ occurs. During reperfusion, a $[H^+]$ gradient is established that is thought to stimulate the $Na^+$-$H^+$ exchanger and remove $H^+$ in exchange for extracellular $Na^+$ [379]. This $Na^+$ increase brings $Ca^{2+}$ into the cell through the sarcolemmal $Na^+$-$Ca^{2+}$ exchanger **(Figure 7).** Accumulation of calcium in the myocardium is associated with adverse effects on cardiac structure and performance and is a major cause of cell damage [380-382]. Inhibition of any of the above steps would potentially counteract the deleterious effects of ischemia / reperfusion injury on the heart by ultimately reducing $Ca^{2+}$ accumulation. Many have tested this hypothesis through inhibition of the $Na^+$-$H^+$ exchanger during an ischemia / reperfusion episode with amiloride analogues and other

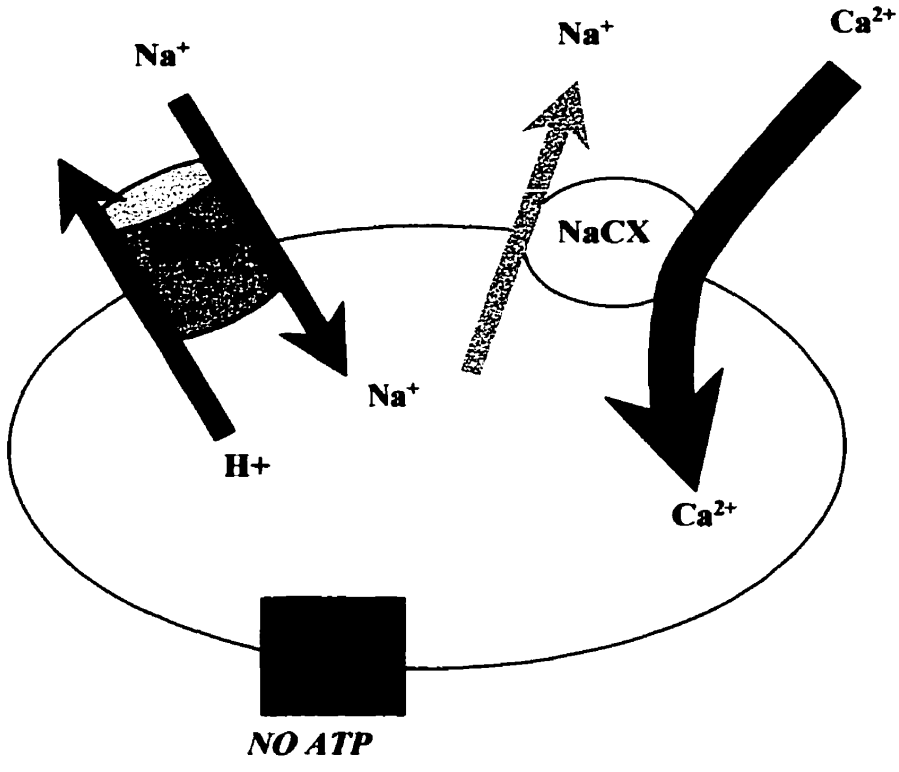


**Figure 7. Ischemia-reperfusion injury and the role of the $Na^+$-$H^+$ exchanger.** The NHE becomes active during reperfusion causing excess $Na^+$ to accumulate intracellularly. This $Na^+$ stimulates the NaCX to function in reverse allowing $[Ca^{2+}]_i$ increase. This causes $Ca^{2+}$ induced cell damage. The NaK pump remains inactive as ATP stores are diminshed.

related inhibitors (272-303). Inhibition of the $Na^+$-$H^+$ exchanger will decrease the amount of $Ca^{2+}$ accumulated and protect the myocardium from an otherwise damaging injury (257,383-385).

Pierce and colleagues have conclusively demonstrated the beneficial effects of NHE inhibition during the onset of reperfusion (284,285,386), (387). Many others have confirmed these findings (273,276,278,290,294,337,345). This follows the idea that NHE activation is optimal during the reperfusion phase and inhibition during this phase is critical to prevent $Ca^{2+}$-induced cell damage. Pharmacological inhibition of the NHE becomes increasingly important when the severity and duration of ischemic heart disease is increased.

Interestingly, the first clinical trial of NHE inhibition during ischemia was presented at the 48th Annual Scientific Session of the American College of Cardiology. The study known as GUARDIAN (Guard During Ischemia Against Necrosis) has generated some interesting results involving patient mortality and infarction following by-pass surgery (192). Patients were selected based on the presence of unstable angina and non-Q wave myocardial infarctions. They required either a percutaneous or by-pass surgical intervention. Each patient was given between 20 and 100 mg of cariporide (HOE 642) or placebo every 8 hours for 2 –7 days prior to treatment. Patients were assessed at day 36 with a follow up scheduled at 6 months. Although the results failed to show a substantial reduction in mortality and infarction, other subgroups within the trial did show favorable results. These data provided encouraging results and an incentive to examine NHE inhibition in more detail. A clinical trial involving HOE 642 (Cariporide) was shown to improve left ventricular function when used prophylactically prior to

angioplasty [388]. ESCAMI (Evaluation of the Safety and the Cardioprotective Effects of Eniporide in Acute Myocardial Infarction ), has been initiated to evaluate the effectiveness of EMD 96785 (Eniporide) prior to balloon angioplasty. The results are yet to be fully announced.

### *4. Cardiac hypertrophy*

Protein synthesis and cell proliferation is affected by $pH_i$. The involvement of NHE as a downstream regulator of many hypertrophic stimuli has been suggested [389]. Inhibition of the NHE is, therefore, a potential target to attenuate these events. This approach has been used to prevent restenosis observed after carotid artery balloon angioplasty [390,391]. Inhibition of the NHE prevents stretch-induced activation of protein synthesis [392]. Peiro *et al* have demonstrated the involvement of NHE in vascular smooth muscle hypertrophy [393]. Cardiac hypertrophy was inhibited by the NHE blocker cariporide [394]. Although NHE stimulation has effects on hypertrophy, hypertrophic conditions also influence the NHE. Recently, Ito *et al* have shown that cardiac hypertrophy diminishes the degree of NHE stimulation caused by angiotensin II and endothelin [350]. This decrease in stimulation is thought to occur via abnormal protein kinase C signaling. Although this was observed in aortic banded rats, similar findings were demonstrated in infarcted rabbit myocardium [395].

### *5. Diabetes*

#### *a. Pathology of ischemia in diabetes*

Cardiovascular dysfunction is a major cause of death in patients with diabetes as well[396]. Diabetics will suffer a myocardial infarction at a rate of two-and-a-half to

five times greater than non-diabetics [397,398]. Along with having a poorer prognosis, [397,398] the long term mortality rate (3-5 years) following an acute myocardial infarction is 2-3 times higher [399-404] with women and young diabetics showing the most pronounced detrimental effects [405,406]. It is interesting to note that women suffering from diabetes, who are normally at a lower risk of developing coronary artery disease, are at a greater disadvantage and more vulnerable to re-infarction and death after myocardial infarction. In 1981 the number of estimated deaths from diabetes in the United States was 34,750, which corresponds to a mortality rate of 15.2/100,000 [407]. Diabetes is also implicated as a contributory cause in a substantial proportion of deaths from other diseases, particularly those of the cardiovascular-renal system. Diabetes is ranked seventh among the leading causes of death in the United States and is particularly associated with deaths in the elderly. As one considers our aging population, a decrease in the prevalence of ischemic heart disease in the diabetic population does not seem likely, thereby placing a larger task on researchers and clinicians in the future.

Paradoxically, despite the clinical data that supports an increased susceptibility to ischemia / reperfusion injury in diabetes, many laboratories have shown an increased resistance of hearts from diabetic animals to ischemia [291,408-416]. If the above schema **(Figure 8)** is correct, then it may be used to detect the mechanisms which may be responsible for the increased resistance of the diabetic heart to ischemia / reperfusion challenge. Several points within the cascade of events shown in **Figure 8** may be altered by the diabetic state and are worthy of discussion.

The $Na^+$-$H^+$ exchanger plays a central role within the schema of events illustrated in **Figure 8**. If pharmacological inhibition of the exchanger protects the heart from

ischemia / reperfusion injury, then the resistance observed in diabetic hearts to ischemia / reperfusion injury may involve an endogenous inhibition of the $Na^+$-$H^+$ exchanger. Interestingly, a reduced [pH]i recovery was observed in diabetic cardiac papillary muscle in response to an acid load (291). This reduced recovery was similar to control hearts perfused with a $Na^+$-$H^+$ exchange blocker (411). This suggests that $Na^+$-$H^+$ exchange was depressed in diabetic hearts. This was directly demonstrated by Pierce and colleagues who found a depressed $Na^+$-$H^+$ exchange in cardiac sarcolemmal vesicles harvested from diabetic rats (430). This, therefore, suggests that a depression in $Na^+$-$H^+$ exchange is present during diabetes and may play an important role in protecting the heart from ischemia / reperfusion injury through the $Ca^{2+}$ overload cascade proposed in **Figure 8**. Interestingly, the membrane phospholipids in diabetic cardiac sarcolemma are altered.

***b. Possible explanations for inhibited NHE-1 during diabetes.***

As shown in **Figure 8**, intracellular [$H^+$] is a central feature that initiates a cascade of ionic changes that ultimately ends in cardiac injury. Anything that alters the generation of [$H^+$] within the cardiomyocytes in response to the ischemic challenge will affect this entire cascade and cardiac viability. For example, the degree to which lactate and $H^+$ will accumulate largely depends on the myocardial glycogen stores present prior to ischemia. Depletion of these glycogen stores, within the myocardium, has been shown to be beneficial to the heart. Less glycogen leads to a decrease in lactate and $H^+$ accumulation that causes the $Na^+$-$H^+$ exchanger to become less activated. In diabetes, attenuation of cardiac glycolytic metabolism is observed under basal conditions (417-420). It has been

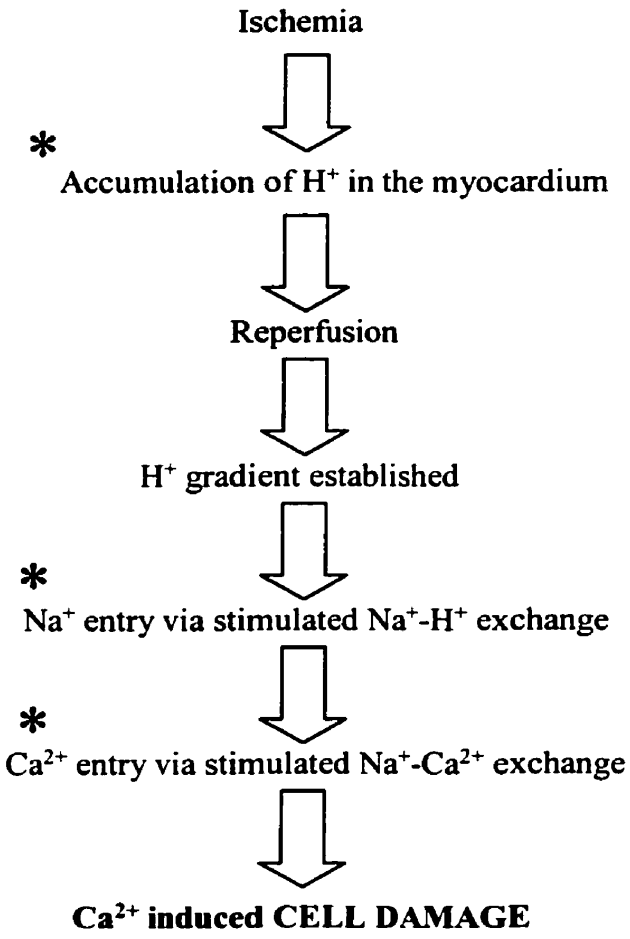


**Figure 8. The central role for $Na^+$-$H^+$ exchange in ischemic – reperfusion injury.** Diabetes (*) alters several steps in this cascade to provide resistance from ischemia – reperfusion injury.

proposed that the decrease of glucose metabolism observed in diabetics may be due to an attenuated insulin dependent glucose uptake or elevated fatty acid levels (408,420,421). This decrease in glucose metabolism leads to less [$H^+$] and lactate production. Less [$H^+$] accumulation does not stimulate $Na^+$-$H^+$ exchange activity and protects the myocardium via an attenuation of $Ca^{2+}$ influx **(see Figure 8).** Glucose metabolism is further dependent upon glycogen stores within the myocardium. Pre-ischemic glycogen stores are reported to be increased in a diabetic myocardium (422). The relationship between degree of damage following ischemia and levels of pre-ischemic glycogen stores within the diabetic myocardium are not clearly established (378,423).

The mechanism responsible for the inhibition of the $Na^+$-$H^+$ exchanger is not completely understood, however, several possibilities exist **(Table 4)**. For example, if the number of $Na^+$-$H^+$ exchanger proteins were decreased, this may result in lower activity. This decreased expression could be detected by a change in the amount of mRNA expressed for the exchanger in the diabetic heart. However, Pierce et al compared mRNA levels from 1 and 8 week diabetic hearts and found no change when compared to controls (424).

This finding was recently supported by others (418) where the mRNA levels remained unchanged even after a short ischemic event. The mRNA levels reported during diabetes, therefore, suggest that the depressed $Na^+$-$H^+$ exchanger may instead involve a defect at the translational level. Such an alternative mechanism may involve hormonal changes that occur during diabetes. Thyroid hormones are reported to activate the $Na^+$-$H^+$ exchanger in renal brush-border membranes (425). Thyroid hormones are known to be depressed in

**Table 4. Possible Mechanisms of $Na^+$-$H^+$ Exchange Depression and Consequent Resistance to Ischemia / Reperfusion Injury during Diabetes**

| **Postulated Mechanisms** |
|---|
| 1. Levels of $Na^+$-$H^+$ exchange mRNA |
| 2. Altered levels of Thyroid Hormones and Insulin |
| 3. Intracellular Calcium |
| 4. Alteration in Membrane Phospholipid Environment |
| 5. Type and Duration of Diabetes |

chemically-induced diabetic hearts [426]. Thus, it is possible that this may contribute to a depressed cardiac $Na^+$-$H^+$ exchange observed in chronic diabetes. Furthermore, stimulation of the $Na^+$-$H^+$ exchanger by insulin has also been reported in frog skeletal muscle fibers [427]. Therefore, a lack of both of these hormones in IDDM may directly or indirectly inhibit this exchanger conferring cardioprotection following ischemia / reperfusion injury.

Calcium has also been shown to modulate the $Na^+$-$H^+$ exchanger by two pathways: 1) a calcium / calmodulin binding site on the $Na^+$-$H^+$ exchanger and 2) a calcium / calmodulin dependent protein kinase II which phosphorylates and activates the exchanger. Increases in $[Ca^{2+}]$ stimulate the $Na^+$-$H^+$ exchanger and a decrease inhibits it through diminished phosphorylation [428]. $Ca^{2+}$ levels were found to be depressed in both acute and chronic diabetes (see [429] for review). If depressed $Ca^{2+}$ levels are present intracellularly prior to ischemia, this may serve to inhibit the $Na^+$-$H^+$ exchanger **(Figure 8)**. If $[Ca^{2+}]i$ is reduced during ischemia because of a depressed $Na^+$-$H^+$ exchange and $Na^+$-$Ca^{2+}$ exchange (see above), then one would expect the $Ca^{2+}$ / calmodulin augmentation of $Na^+$-$H^+$ exchange to be less than optimal as well.

Little is known about factors that regulate the $Na^+$-$H^+$ exchanger at the membrane level. The phospholipid environment may alter the activity of the $Na^+$-$H^+$ exchanger during diabetes. Others have reported striking effects on other ion transporters within the myocardium following the addition of phospholipases, enzymes that hydrolyze specific phospholipids [5]. Phospholipids within the sarcolemmal membrane from the myocardium of diabetic animals are altered when compared to non-diabetic animals

**(Table 5).** Levels of phosphatidic acid, lysophosphatidylcholine and phosphatidylethanolamine are significantly changed in sarcolemmal membranes from diabetic animals (200). It is reasonable to suggest, therefore, that a change in phospholipid content within the diabetic rat sarcolemmal membrane may contribute to the depression in $Na^+$-$H^+$ exchange activity. **Table 5** also indicates elevated levels of lysophosphatidylcholine from 6-8 week old diabetic rats (200). Because this lipid has been shown to enhance the activity of the $Na^+$-$H^+$ exchanger in non-diabetics (28), it is also unlikely that this lipid provides a mechanism for the resistance observed in diabetics. It is apparent that the phospholipid environment may be a modulator of $Na^+$-$H^+$ exchange but, at present, its role in modulating the exchanger during diabetes remains unclear.

As shown in **Figure 8**, the $Na^+$-$Ca^{2+}$ exchanger is another important protein involved in ischemia / reperfusion injury. A decrease in $Na^+$-$Ca^{2+}$ exchange should provide cardioprotection by decreasing $Ca^{2+}$ entry. Less $Ca^{2+}$ entry during ischemia leads to reduced damage in the myocardium. A depressed cardiac sarcolemmal $Na^+$-$Ca^{2+}$ exchange in diabetes has been reported (202,430).

Clinical data supports an increased susceptibility to ischemic injury in diabetics (426,431-433). In contrast, experimental data reports a resistant myocardium. Diabetes mellitus can be subdivided into Type I, insulin-dependent diabetes mellitus (IDDM) and Type II, non-insulin dependent diabetes mellitus (NIDDM). NIDDM is the predominant form of diabetes in the human population (426). Clinically, these types of diabetes differ from one another. NIDDM individuals suffer from elevated insulin and cholesterol levels, hypertension, hypertriglyceridemia, obesity and impaired glucose tolerance. IDDM have depressed insulin levels are usually not obese. The response of the NIDDM

**Table 5. Alterations of Phospholipid Species in the Diabetic Cardiac Sarcolemma**

| Phospholipid Species | Diabetic Membrane |
|---|---|
| Phosphatidylcholine | N/C |
| Lysophosphatidylcholine | INCREASED |
| Phosphatidylethanolamine | DECREASED |
| Sphingomyelin | N/C |
| Phosphatidylserine | N/C |
| Phosphatidylinositol | N/C |
| Diphosphatidylglycerol | N/C |
| Phosphatidic Acid | DECREASED |

N/C = no significant change

Reference: (200-202)

model to ischemia / reperfusion injury is consistent with the clinical findings which support a hypersensitive response. Pierce et al [434] demonstrated a poorer recovery of developed tension in hearts from 6 and 9-month-old NIDDM rats following both severe and moderate ischemic insults. Surprisingly, a mild resistance was observed in these NIDDM rats at 3 months of age [434]. This age dependent sensitivity to ischemia / reperfusion injury is similar in obese, NIDDM diabetic humans which makes the JCR-LA model an ideal model to study diabetes-induced myocardial dysfunction. The status of the cardiac $Na^+$-$H^+$ exchanger or the $Na^+$-$Ca^{2+}$ exchanger in these NIDDM rats is unknown at this time. The type of diabetes and the duration of diabetes, therefore, may also be important factors to consider regarding mechanisms by which the myocardium suffers damage after ischemia. However, this remains controversial as both resistance and hypersensitivity has been observed in acute and chronic IDDM (see [435] for review). Further work in both types of diabetes is clearly necessary.

### *6. Tumor growth*

The microenvironment surrounding tumors is metabolically deficient. This leads to acidosis [436-440]. Maintenance of the pH therefore becomes critical in order to sustain tumor growth. The pH regulating capacity of the NHE has been implicated in maintaining tumorigenesis. Pouyssegur *et al* report the inability of NHE deficient cells to produce tumors [441]. Rotin and colleagues have further implicated the presence of NHE as a necessary component to tumor growth [367]. In a recent study, Reshkin and colleagues illustrate how serum deprived tumor cells upregulate NHE activity to augment

tumor invasion [442]. This is in contrast to the NHE downregulation seen in normal serum deprived cells. This points to a critical role for the NHE during proliferation and maintenance of tumorigenesis. Interestingly, phorbol esters, which also stimulate NHE, act to maintain and promote tumor growth.

*7. Apoptosis*

Programmed cell death occurs in myocardium subjected to an ischemia-reperfusion event [443]. Veinot et al have reported apoptosis to be the initial mechanism of cell death following a myocardial infarction. Furthermore, the degree of apoptosis is increased following reperfusion [444]. The exact mechanism responsible for apoptosis remains unclear. However, it is interesting that inhibition of NHE decreases the degree of apoptosis following ischemia. NHE inhibitors attenuate apoptosis in metabolically inhibited cardiomyocytes [445], ischemic myocardium [446] and during in vivo coronary artery occlusion [447]. Therapeutic interventions aimed at the NHE, therefore, not only prevent immediate damage, but can attenuate the extent of programmed cell death observed in ischemic myocardium.

# B. MATERIALS AND METHODS

## I. Materials

| Product | Source |
|---|---|
| Acetic Acid | Fisher Scientific (Napean, ON) |
| Acetone | Fisher Scientific (Napean, ON) |
| Adenosine Triphosphate | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Adsorbant papers (TLC) | Fisher Scientific (Napean, ON) |
| Alamethecin | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Ammonia ($NH_3$) | Fisher Scientific (Napean, ON) |
| Ammonium Hydroxide ($NH_4OH$ | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Ammonium Molybdate | Malinckrodt Inc. (Parls, Kentucky) |
| ANS | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Bovine serum albumin | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Calcium Chloride ($^{45}Ca$) | Mandel Scientific Co. Ltd (Guelph, ON) |
| CAPS | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| CHES | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Chloroform | Fisher Scientific (Napean, ON) |
| Coomassie Brilliant Blue | Sigma-Aldrich Canada Ltd. (Oakville, ON) |
| Cupric sulfate | Sigma-Aldrich Canada Ltd. (Oakville, ON) |
| Deoxyribonuclease I (DNAse) | Worthington Biochem Corp. (Lakewood, NJ) |
| Dithiotreitol | Sigma-Aldrich Canada Ltd. (Oakville, ON) |
| Docosahexanoic Acid | Doosan Serdary Laboratories (Toronto, ON) |

| | |
|---|---|
| EGTA | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Eicosapentanoic Acid | Doosan Serdary Laboratories (Toronto, ON) |
| Folic Acid and Cioclaten's Phenol Reagent | Sigma-Aldrich Canada Ltd. (Oakville, ON) |
| HEPES | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Hydrochloric Acid(HCl) | Fisher Scientific (Napean, ON) |
| Linoleic Acid | Doosan Serdary Laboratories (Toronto, ON) |
| Linolenic Acid | Doosan Serdary Laboratories (Toronto, ON) |
| Lysophosphatidic Acid | Doosan Serdary Laboratories (Toronto, ON) |
| Lysophosphatidylcholine | Doosan Serdary Laboratories (Toronto, ON) |
| Lysophosphatidylethanolamine | Doosan Serdary Laboratories (Toronto, ON) |
| Lysophosphatidylinositol | Doosan Serdary Laboratories (Toronto, ON) |
| Magnesium Chloride | Fisher Scientific (Napean, ON) |
| Methanol | Fisher Scientific (Napean, ON) |
| MES | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Millipore Filters | Fisher Scientific (Napean, ON) |
| Phospholipase $A_2$ | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Phospholipase C | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Phospholipase D | Calbiochem, (La Jolla, CA) |
| Potassium Chloride | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Potassium Diphosphate | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| *p*-nitrophenol phosphate | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| SDS | Bio Rad Laboratories. (Hercules, CA) |

| | |
|---|---|
| Sodium Azide | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Sodium Carbonate | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Sodium Chloride ($^{22}$Na) | Mandel Scientific Co. Ltd (Guelph, ON) |
| Sodium Chloride | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Sodium Bisufite | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Sodium Hydroxide | Fisher Scientific (Napean, ON) |
| Sodium Sulfite | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Sodium Tartarate | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Sucrose | Fisher Scientific (Napean, ON) |
| Sulfuric Acid | Fisher Scientific (Napean, ON) |
| TAPS | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Tetrasodium Pyrophosphate | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Thin Layer Chromatography Plates (K6F-Silica) | Fisher Scientific (Napean, ON) |
| Trizma Base | Sigma-Aldrich Canada Ltd. (Oakville,ON) |
| Trizma Maleate | Sigma-Aldrich Canada Ltd. (Oakville,ON) |

The Millipore filters, thin layer chromatography plates and organic solvents were supplied by Fisher Scientific. A 0.1mL aliquot of 1000 μCi $^{22}$Na was supplied by NEN Life Sciences. Phospholipase D (*Streptomyces chromofuscus)* was obtained from Calbiochem, La Jolla CA. Phospholipids were purchased from Doosan Serdary, Toronto

ON. Deoxyribonuclease I was supplied by Worthington Biochemical Corporation, Lakewood, NJ. All other materials, including phospholipase C *(Clostridium perfringes)*, were supplied by Sigma, St. Louis, MO. Plasmalogen phosphatidic acid was generously supplied by Dr. David A. Ford of St. Louis University School of Medical Biochemistry, St. Louis, USA.

## II. Methods

### *1. Sarcolemmal membrane preparation*

Pigs (65-85 kg) were anaesthetized with telazol using a dose of 1 mL / 23 kg animal body weight. Hearts were removed and immediately placed in an ice cold water bath. The right and left ventricles were separated and left ventricle used for the sarcolemmal preparation. Between 50-60g of the left ventricle was excised and endocardium and epicardium removed. A 175 mL solution **(solution 1)** consisting of 0.25M sucrose, 1mM Dithiothreitol (DTT) , 20mM Tris [hydroxymethylamino] methane (TRIS), 0.25mM Tetrasodium Pyrophosphate and 1M KCl (pH 7.4) was added. Tissue was scissor minced and homogenized in a cold waring blender, 2X 10 seconds at high speed. The final volume was brought to 320mL with **solution 1** and centrifuged at 48,400 X *g* for 35 minutes. During the spin, 1-2g of the remaining left ventricle was removed and placed into 20mL of 0.25M sucrose, 1mM DTT and 20mM TRIS **(solution 2)**. This was minced, homogenized in a glass teflon Wheaton homogenizer, filtered through several layers of gauze and stored in a test tube in the fridge until day 2. From previous spin, supernatant was discarded and resultant pellets were resuspended in a small volume of **solution 1**. Each pellet was homogenized in a cold waring blender at high speed for 1X

10 seconds and recentrifuged at 48,400 X *g* for 35 minutes. Resultant pellets were resuspended in **solution 1** and glass teflon homogenized in a cold Wheaton Homogenizer. Homogenate was then added to an Erlenmeyer flask containing 135,000 Units Activity of Deoxyribonuclease I (DNAse I) in 10 mL of **solution 2**. This was then incubated for 45 minutes at 30 °C with intermittent shaking. Following incubation, 10 ml aliquots of the homogenate was removed into centrifuge tubes and subject to polytron for 10-15 second bursts at a setting of 20. This was repeated for the total homogenate volume and pooled together. Homogenate was then centrifuged for 15 minutes at 17,400 X *g* and supernatant was recentrifuged for 45 minutes at 175,000 X *g*. During spin, several discontinuous sucrose gradients were layered in the following volumes; 4mL of 11% sucrose (w/v), 6mL of 26% sucrose (w/v), 5mL of 29% sucrose (w/v), 4mL of 32% sucrose (w/v) and 4mL of 34% sucrose (w/v). Following centrifugation, the pellet was carefully homogenized in a cold Wheaton homogenizer to avoid bubble formation. Approximately 4 mL of this homogenate, which was resuspended in 48% sucrose, was carefully added to the bottom of 6 discontinuous sucrose gradients using a glass pasteur pipette and a 30mL syringe. This homogenate was spun overnight in an SW28 swing bucket rotor at 24K. Following overnight centrifugation, membrane fractions were collected. Collection of the top fraction, F1, constitutes an 11% sucrose medium. At the 11%-26% interface, sarcolemma was identified as a concentrated cloudy protein band deemed F2. For our purposes, $Na^+$-$H^+$ exchange was carried out on this F2, sarcolemma fraction. The fractions at the 26%-32% (F3) and 32%-34% (F4) were also collected. The band of interest (F2) was diluted with a suspension medium containing 200mM sucrose, 25 mM Mes, 8 mM KOH and centrifuged for 2 hrs at 175 000 X g. These pellets were

resuspended in the same sucrose, Mes solution in protein concentrations of 1-3 mg / mL. Purity and enzyme activity of all fractions was determined using specific marker assays. $K^+$-stimulated *p*-nitrophenylphosphatase activity of the sarcolemmal vesicles was 11.5 ± 2.0 μmol / mg per h (n = 7). $Na^+$-$K^+$ ATPase activity was 10. 9 ± 3.4 and 35.2 ± 10.2 μmol / mg per h in the absence and presence of 12.5 μg / mL alamethicin respectively **(Table 6)**. The cardiac sarcolemmal vesicles were frozen in liquid $N_2$, and stored at –80 °C for subsequent analysis, while the remaining fractions were discarded.

***2. $Na^+$-$H^+$ exchange measurement***

$H^+$-dependent $Na^+$ uptake was examined in control and phospholipase D treated vesicles as described elsewhere in detail (448). Briefly, 5μL of $^{22}Na$ (0.1 μCi) was added to the bottom of a polystyrene tube containing 25μL uptake medium ( 200mM sucrose, 30 mM Ches, 40 mM KOH, 0.1 mM EGTA and 0.1 mM $Na^+$ (pH 10.61). $Na^+$ was diluted 100 fold in uptake medium to generate a 0.1mM $Na^+$ solution (100μL 1mM $Na^+$ in 900μL uptake medium). A 20μL aliquot of sarcolemmal membrane protein (11 μg) was placed on the side of the tube following a predetermined equilibration time at 25°C. $Na^+$-$H^+$ exchange was initiated by vortexing the mixture generating final assay concentrations of 180 mM sucrose, 10 mM Mes, 17.5 mM Ches, 17 mM KOH, 0.05 mM EGTA 0.05 mM $Na^+$ and a final extravesicular pH of 9.33. Calibration of all assay media was done carefully using an Orion 82-10 pH electrode to ensure accuracy. Following a preset time (2-5 seconds), 3 mL of stop solution (100 mM KCl, 20 mM Hepes, pH 7.5) was added to the polystyrene tube to arrest the reaction. KCl was added to our stop

**Table 6. $Na^+$-$K^+$ ATPase and $K^+$-*p*NPPase activities in cardiac sarcolemmal vesicles isolated from healthy animals.**

| Fraction | $K^+$-*p*NPPase *(µmol/mg per h)* | $Na^+$-$K^+$ ATPase *(µmol/mg per h)* |
|---|---|---|
| H | 0.1 ± .06 | 0.2 ± .05 |
| **F2** | **11.5 ± 2.0** | **†10.9 ± 3.4** |
| | | ‡35.2 ± 10.2 |
| F3 | 2.8 ± .9 | 1.7 ± .9 |
| F4 | 0.6 ± .09 | 0.7 ± .1 |

Values are represented as means ± S.E. of 7 different animals. F2 represents the purified cardiac sarcolemmal vesicles. $Na^+$-$K^+$ ATPase activity in the †absence and ‡presence of 12.5 µg / mL alamethicin. Homogenate (H), fraction 3 (F3) and fraction 4 (F4).

media to prevent non-specific $Na^+$ binding to the Millipore filters and the extravesicular surface of sarcolemmal vesicles. The reaction mixture was filtered rapidly through 0.45 μM Millipore filters, followed by an additional 2 X 3 mL wash with the same stop solution. Total stop solution was therefore 9 mL and volumes greater than 9 mL did not affect $^{22}Na$ uptake, whereas, less than 9 mL resulted in greater background values. Filters were removed, placed in vials with scintillant, dried and radioactivity quantified using scintillation spectroscopy. Blank samples were included to deduct non-specific retention of $Na^+$ to the Millipore filters. Blanks were treated in a similar manner except 3 mL ice-cold stop solution was added immediately prior to the inclusion of 20 μL sarcolemmal protein. Blank counts were approximately 10% of total counts per minute (cpm) and subtracted accordingly.

### *3. Passive efflux of $Na^+$*

$Na^+$-$H^+$ exchange was carried out as mentioned above. However, uptake of $Na^+$ was permitted for a reaction time of 1 minute. Following uptake, 450μL of an efflux medium consisting of 20μM dimethylamiloride (DMA), $H_20$, a sucrose solution (200mM sucrose, 25mM Mes, 8mM KOH pH 5.5) and an uptake solution in a volume ratio of 30,270,1188 and 1485μL respectively was added. This efflux solution contained no $Na^+$, creating an optimum gradient for $Na^+$ to passively exit the vesicles. DMA was added as a precautionary measure to ensure the $Na^+$-$H^+$ exchanger was inoperable. Passive efflux was measured for 2 and 15 seconds following the addition of efflux medium, stopped with 9mL of stop solution (as mentioned above) and subsequently filtered. Controls were

permitted an uptake time of 1 minute, however, no efflux media was added. The effects of phospholipase D on membrane fluidity were quantified through the use of this experiment. Further variations in the $Na^+$-$H^+$ exchange protocol involved increasing the extravesicular pH and $Na^+$ while keeping all other parameters identical.

***4. Treatment with phospholipase D***

Cardiac sarcolemmal vesicles (100μg) were exposed to 2.54 μL of phospholipase D (32.5 U/mL) for 50 min ± 10 min at 25°C. Therefore, unless otherwise specified, the final phospholipase D / mg sarcolemmal protein ratio was 0.825 U / mg protein. Control tubes were treated in a similar manner except the enzyme was denatured in boiling water for 60 minutes prior to its use in the assay. Following treatment, $Na^+$-$H^+$ exchange activity was examined immediately, as previously mentioned.

***5. Treatment with lysophospholipids***

Several lysophospholipids were incubated with sarcolemmal vesicles between 1 and 11 minutes. Lysophospholipids were suspended in 200mM sucrose, 25mM Mes and 8mM KOH (pH 5.5) to yield final concentration of 10mM lysophospholipid. This was diluted 100-1000 fold to yield a final concentrations of 10, 25, 50 and 100μM lysophospholipid. Pre-incubation of lysophospholipids was carried out over 3 minutes with a subsequent $Na^+$ uptake time of 2, 5 and 30 seconds.

***6. Treatment with plasmalogen phosphatidic acid***

Approximately 285μL of a 4.78 mg/mL plasmalogen phosphatidic acid solution was aliquoted and dried with $N_2$ gas. Complete removal of the chloroform from the phosphatidic acid was essential. To ensure dryness, 500μL of methanol was added to the partially dried phospholipid and subsequently evaporated with $N_2$ gas. The phosphatidic acid was then resuspended in 2mL of 200mM sucrose, 25mM Mes, 8mM KOH pH 5.5 to yield a final phospholipid concentration of 1mM. To promote dissolution of the phosphatidic acid into solution, a probe sonicator was used at an amplitude setting of 45-50. It was essential to keep the phosphatidic cold as sonication would result in a excessive heat generation. Following dissolution, pre-determined concentrations of phosphatidic acid were used and pre-incubated with our sarcolemmal vesicles for 1 to 5 minutes. Following pre-incubation, $Na^+$-$H^+$ exchange was carried out as mentioned above.

***7. Treatment with fatty acids***

Several fatty acids were incubated with sarcolemmal vesicles. Fatty acids were initially removed into chloroform yielding a stock concentration of 10mM. An aliquot of this stock was removed and dried with $N_2$ gas. A pre-determined volume of 200mM sucrose, 25mM Mes, 8mM KOH pH 5.5 was added to yield a working stock of 1mM fatty acid. These were subsequently diluted 100-1000 fold to yield final fatty acid concentrations of 10, 25, 50 and 100μM. $H^+$-dependent $Na^+$ uptake was examined for 2, 5 and 30 seconds following a 3 minute pre-incubation time.

***8. Phospholipid separation***

Sarcolemmal vesicles (1 mg) were incubated with boiled and unboiled phospholipase D (0.825 U/mg protein) for 60 minutes at 25°C. The treated sarcolemma was centrifuged

in the tabletop ultracentrifuge for 15 minutes at 80K. Pellets were removed using 300μL $H_20$ and resuspended in a 2:1 chloroform/methanol solution. Pellets were homogenized in a glass-glass Wheaton homogenizer to ensure complete dissolution of the phospholipids into the chloroform/methanol/$H_20$ solution (2:1:5%). This was then placed in glass conical tubes at 4°C for overnight extraction of phospholipids. The following day, 1 mL of 0.01N HCl was added to each sample and centrifuged at 1000 X *g* for 10 minutes. The upper phase was removed from each tube following the spin to remove any contaminating protein. Lipids were centrifuged 3X 10 minutes at 1000 X *g* following the addition of 1-1.5 mL chloroform:methanol:0.01N HCl (3:48:47). The aqueous upper phase was removed following each spin. Following the final spin, 0.5mL of a neutral solution consisting of chloroform:methanol:$H_2O$ (3:48:47) was added and immediately removed and discarded. Approximately 1 drop of $NH_4OH$ was added to the phospholipids to buffer any HCl which may have been present. This was followed by the addition of approximately 15-20 drops of HPLC grade methanol to clear the cloudy suspension. Phospholipids were stored at 4°C overnight to allow further extraction. During this time, K6F silica gel thin layer chromatography (TLC) plates were marked for solvent fronts and activated overnight in the oven at 100°C. To spot the TLC plates, 0.9mL of the clear phospholipid suspension was dried using $N_2$. The phospholipids were never dried to completeness as this accelerated the degradation process. To these partially dried phospholipids, 100μL of a chloroform:methanol:$H_20$ solution (75:25:2) was added. This was then spotted on the origin 2 cm from the edge of the TLC plate (refer to diagram). Spots were dried with $N_2$ gas. This was repeated 2X 50μL with the same chloroform:methanol:$H_20$ solution and spotted on the previous 100μL. The

phospholipids were separated using 2-D thin layer chromatography. The first solvent phase was chloroform:methanol:ammonium hydroxide 65:25:4 and the second dimension was run in 75:15:30:15:7.5 chloroform:methanol:acetone:acetic acid:$H_20$ to ensure complete separation of phospholipids. Phospholipid spots were dried for approximately 1-2 hours following each dimension. Acid hydrolysis using 12N HCl was carried out between dimension 1 and 2 to improve phosphatidic acid separation from phosphatidylinositol, phosphatidylserine and lysophosphatidylcholine.

***9. Phospholipid quantification***

Following separation, the phospholipids were stained with a medium containing 30% methanol, 0.03% Coomassie Blue, 0.6% NaCl and 70% $H_20$. Staining was carried out for 30 minutes and followed by a destaining period of 10 minutes using a 30% methanol, 0.6% NaCl and 70% H2O medium. TLC plates were then subsequently dried and quantified as a percentage of control using the Molecular Analyst program on the Bio Rad Densitometer. Background was subtracted from each phospholipid individually.

***10. Protein assay***

Protein concentration was measured using the method described by previously (121). Briefly, duplicate tubes were prepared for both standards and experimental tubes. Standard tubes consisted of Bovine Serum Albumin (BSA) (0.1 mg/mL) and $H_20$ at concentrations between 0 and 0.1 mg/mL BSA. Sarcolemmal fractions (H, $P_3$, F1, F2, F3 and F4, see above) were diluted 10 fold and sarcolemmal membranes diluted 100 fold. All tubes contained 3 mL of a media consisting of 2% $Na_2CO_3$ and 0.1N NaOH, 10%

SDS, 1% $CuSO_4$ and 2% Na Tartarate. This was followed by the addition of Folic Acid and Cioclaten's Phenol Reagent (1:1 dilution). Absorbance was measured by the spectrophotometer at 750 nm to determine protein concentration.

### *12. $Na^+$-$K^+$ ATPase assay*

$Na^+$-$K^+$ ATPase activity was determined by the method described previously (121). Standard, blanks, controls and experimental tubes were prepared in duplicates. Standard tubes contained $KH_2PO_4$ and $H_2O$ only, whereas blanks, controls and experimental tubes contained a media consisting of 0.25M Tris, 600mM NaCl, 17.5mM $MgCl_2$, 5mM EGTA, 25mM $NaN_3$ and 30mM ATP, 37°C @ pH 7.0. Final reaction volume was 1mL and was carried out in a 37°C water bath. Reaction time was 14 minutes. Inorganic phosphate produced was measured using Fiske and SubbaRow and ANS reagent as described previously (121). Absorbance was measured at 690nm where the values of KCl free tubes were subtracted from KCl containing tubes giving net $Na^+$-$K^+$ ATPase activity in $\mu mol/P_i/mg/hr$.

### *13. $K^+$-nitrophenylphosphatase assay*

P-nitrophenylphosphatase activity was measured as described elsewhere (121). Briefly, duplicate tubes were prepared for standard, control, blank and experimental tubes. Standard tubes contained *p*-nitrophenol substrate and $H_2O$. All other tubes contained a *cocktail* consisting of 0.25M Tris, 25mM $MgCl_2$, 5mM EGTA, pH 7.8 @ 37°C $^+/-$ protein and 200mM KCl. Once prepared, tubes were placed into a 37°C water bath for 5-7 minutes to allow p-nitrophenol liberation. Tubes were subsequently placed

into an ice bath for 30 minutes and 2mL of 1N NaOH added. Absorbance was measured at 410nm where values from KCl-free tubes (controls) were subtracted from KCl containing tubes. Specific activity was calculated as μmol/mg/hr.

### *14. Statistics*

Data are expressed as a mean ± S.E. Statistical determination was done using a Student *t*-test and was considered significant at $p < 0.05$.

# C. RESULTS

The effect on $Na^+$-$H^+$ exchange of phospholipids in the cardiac sarcolemmal membrane was determined using two phosphatidylcholine-specific phospholipases. Phospholipase D, which hydrolyses phosphatidylcholine into phosphatidic acid, and phospholipase C, which hydrolyses phosphatidylcholine into diacylglycerol were used. Thin layer chromatography was employed for both phospholipase C and D treated vesicles in order to separate the phospholipid classes. **Figure 9** illustrates the membrane phospholipid alterations observed following phospholipase D addition. Phospholipase D activity generated a statistically significant decrease in phosphatidylcholine in treated cardiac sarcolemmal vesicles when compared to controls. A significant increase in phosphatidic acid generation following phospholipase D treatment was also observed when compared to controls over a series of samples **(Table 7)**.

$Na^+$-$H^+$ exchange was examined as a function of varying concentrations of phospholipase D. There was a significant depression of $Na^+$-$H^+$ exchange when cardiac sarcolemmal vesicles were treated with 0.4, 0.8 and 1.6 U phospholipase D / mg sarcolemma **(Figure 10)**. A 30-60% decrease in $^{22}Na^+$ uptake was observed when measured as a function of increasing phospholipase D concentrations.

$Na^+$-$H^+$ exchange was measured over a number of reaction times as a function of phospholipase D treatment. $Na^+$-$H^+$ exchange was significantly inhibited following phospholipase D treatment at all reaction times except at 1 minute where $Na^+$ uptake saturates **(Figure 11)**.

**Table 7. Membrane phospholipid alterations in phospholipase D treated cardiac sarcolemmal vesicles.**

| Phospholipid classes | % of Control |
|---|---|
| Phosphatidylcholine | 70 ± 5* |
| Phosphatidylethanolamine | 85 ± 6* |
| Sphingomyelin | 92 ± 9 |
| Phosphatidylinositol | 113 ± 7 |
| Phosphatidylserine | 90 ± 13 |
| Phosphatidic Acid | 908 ± 77* |
| Lysophosphatidylcholine | 113 ± 6 |

Sarcolemmal vesicles were incubated with 0.82 U phospholipase D / mg protein for 60 minutes at 25°C (pH 5.5). Phospholipids were extracted and separated on two-dimensional thin layer chromatography as described in Materials and Methods. Data are represented as mean ± S.E. for 3-5 separate experiments. *$P < 0.05$ versus controls.

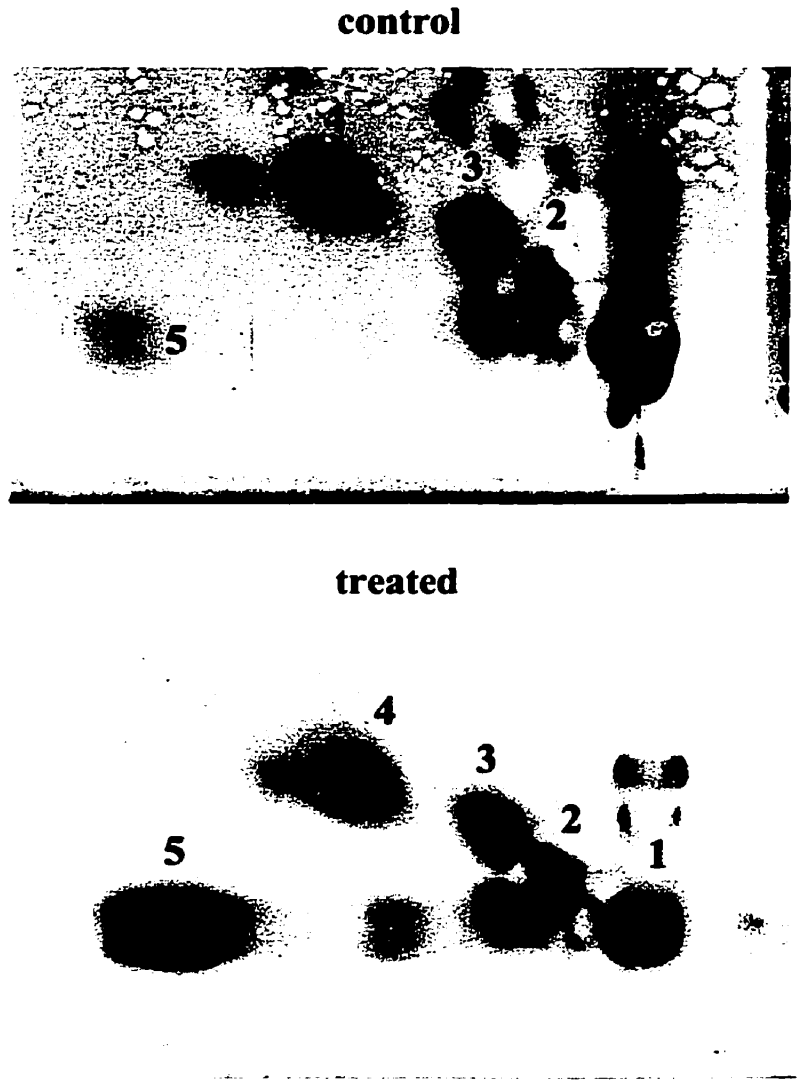


**Figure 9. Thin layer chromatography of control and PLD treated sarcolemmal vesicles.** Sarcolemmal vesicles were incubated with 0.05U/mg PLD for 60 minutes at 25°C. Control contained boiled inactive PLD. 1. Origin, 2. Sphingomyelin, 3. Phosphatidylethanolamine, 4. Phosphatidylcholine, 5. **Phosphatidic acid.**

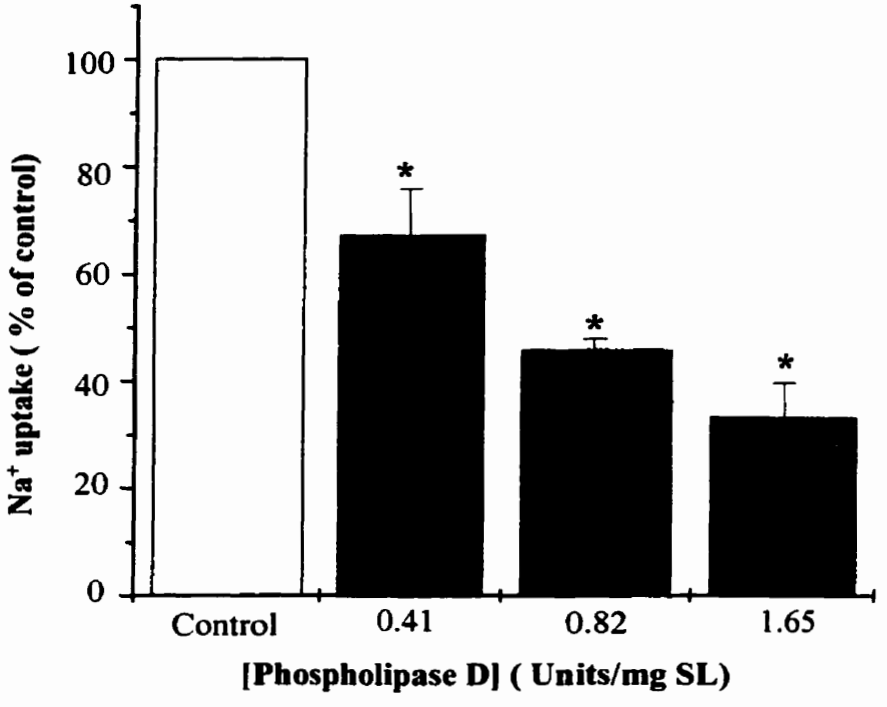


**Figure 10. $H^+$-dependent $Na^+$ uptake as a function of variable concentrations of phospholipase D.** Sarcolemmal vesicles were incubated with phospholipase D for 60 minutes at 25°C (pH 5.5). $Na^+$-$H^+$ exchange was examined for 5 seconds with a final concentration of 0.05 mM $Na^+$, pH 9.33. Data are represented as means ± S.E. of 3 separate experiments. Controls were examined in a similar manner but contained boiled inactive phospholipase D. $*P < 0.05$ versus control.

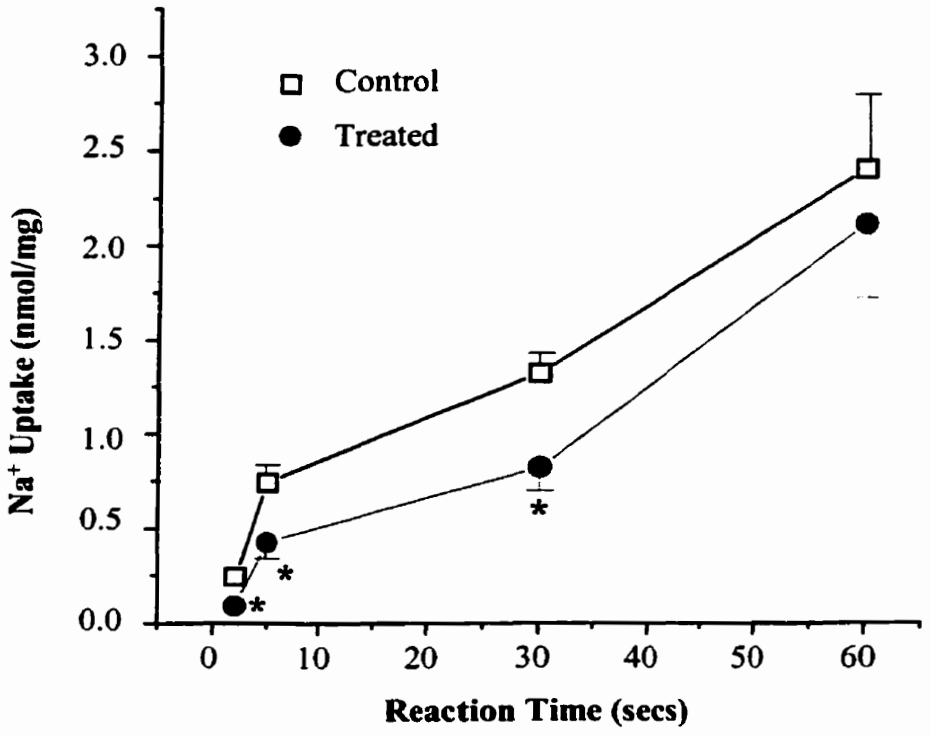


**Figure 11. $H^+$-dependent $Na^+$ uptake in phospholipase D treated sarcolemmal vesicles as a function of reaction time.** Sarcolemmal vesicles were incubated with 0.82 U phospholipase D / mg sarcolemma for 60 minutes at 25°C (pH 5.5). $Na^+$ uptake was examined in a final solution consisting of 0.05 mM $Na^+$, pH 9.33. Data are represented as mean ± S.E. of 4-7 separate experiments. $*P < 0.05$ versus control.

$Na^+$-$H^+$ exchange was also examined at varying extravesicular [$pH_o$]. In control vesicles, $Na^+$ uptake exhibited an appropriate increase as the transsarcolemmal $H^+$ gradient increased, as demonstrated previously (25). Phospholipase D treatment significantly inhibited $Na^+$-$H^+$ exchange at all extravesicular pH values examined (**Figure 12**).

The effect of varying the extracellular [$Na^+$] was studied in control vesicles and vesicles treated with phospholipase D (**Figure 13**). $Na^+$-$H^+$ exchange was inhibited by phospholipase D treatment at all extracellular $Na^+$ concentrations examined (0.05 - 10 mM). These differences were statistically significant when compared to controls that contained the boiled, inactive form of phospholipase D.

Although an inhibition of $Na^+$-$H^+$ exchange was observed, this decrease in activity may be simply due to an increase in the passive ion permeability of the sarcolemmal vesicles. Vesicles were loaded with $^{22}Na$ via $Na^+$-$H^+$ exchange for 1 minute, then rapidly diluted into a medium that was optimal for measuring passive $Na^+$ efflux from the vesicles as the $Na^+$ moved down its transsarcolemmal concentration gradient (25). Consistent with the results from **Figure 11**, the initial load of intravesicular $Na^+$ after 1 minute of $H_i$-dependent uptake was not different between control vesicles and those treated with phospholipase D ($2.40 \pm 0.40$ nmol / mg and $2.11 \pm 0.39$ nmol / mg respectively). Passive $Na^+$ efflux was then initiated and measured after 2 and 15 seconds. This resulted in a 30 and 50% depletion of vesicular $Na^+$ content. Dimethylamiloride (20 μM) was included in the efflux medium to ensure $Na^+$-$H^+$ exchange was not operable.

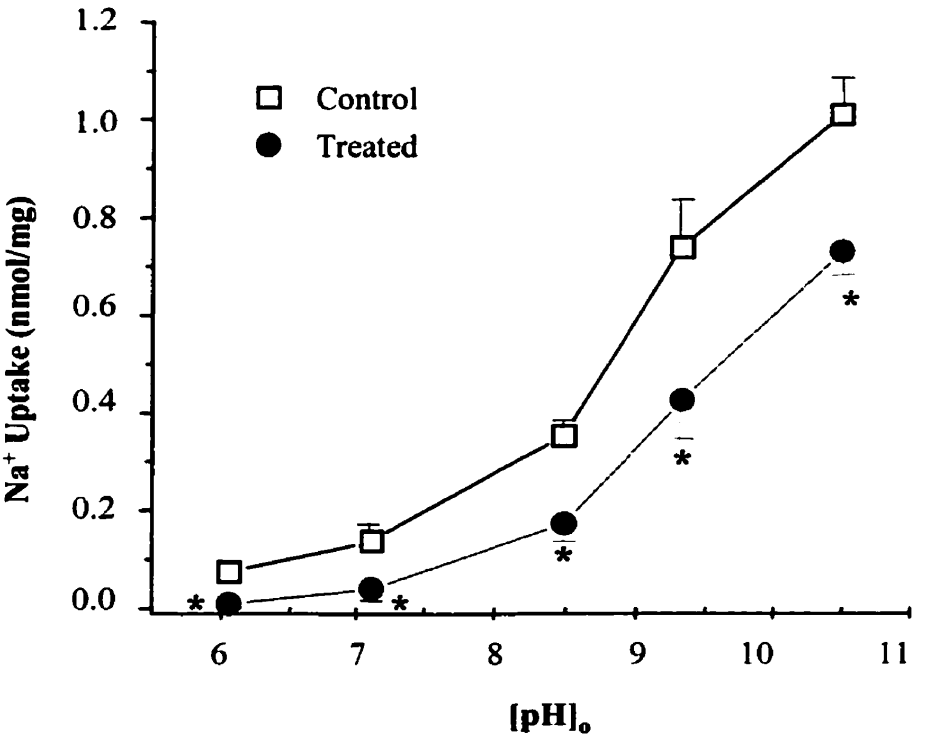


**Figure 12. $H^+$-dependent $Na^+$ uptake in phospholipase D treated sarcolemmal vesicles as a function of $[pH]_o$.** Sarcolemmal vesicles were pre-incubated with 0.82 U phospholipase D / mg protein as described. Final pH of the medium was 9.33, and 0.05 mM Na. $Na^+$ uptake occurred for a period of 5 seconds. Data are from 4-7 separate experiments as means ± S.E. $*P < 0.05$ vs control.

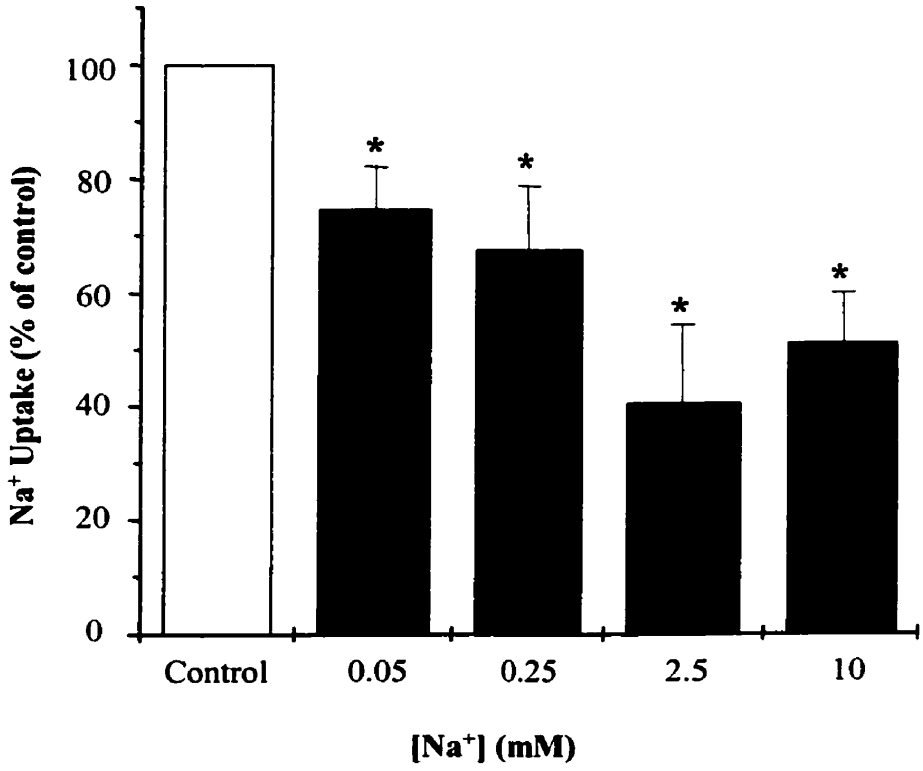


**Figure 13. $H^+$-dependent $Na^+$ uptake in phospholipase D treated sarcolemmal vesicles as a function of extravesicular $Na^+$.** Sarcolemmal vesicles were incubated with 0.82 U phospholipase D / mg protein as described. The reaction time was for 5 seconds in a medium containing a final pH 9.33. Data from 4-7 separate experiments are represented as mean ± S.E. $*P < 0.05$ versus control.

There was no statistically significant difference observed in passive $Na^+$ efflux from phospholipase D treated vesicles and control vesicles (**Figure 14**).

Treatment of cardiac sarcolemmal vesicles with phospholipase D generates phosphatidic acid but also diminishes the total phosphatidylcholine pool (**Table 6**). In order to identify whether a phosphatidic acid increase or phosphatidylcholine decrease may be responsible for the change in $Na^+$-$H^+$, cardiac sarcolemmal vesicles were treated with a phosphatidylcholine-specific phospholipase C. Phospholipase C also depletes the phosphatidylcholine pool but generates diacylglycerol instead of phosphatidic acid. As shown in **Figure 15**, $Na^+$-$H^+$ exchange was unaffected by phospholipase C treatment over a number of reaction times. In addition, passive permeability of $Na^+$ in the cardiac sarcolemmal vesicles was unaffected following phospholipase C treatment **(Figure 16).**

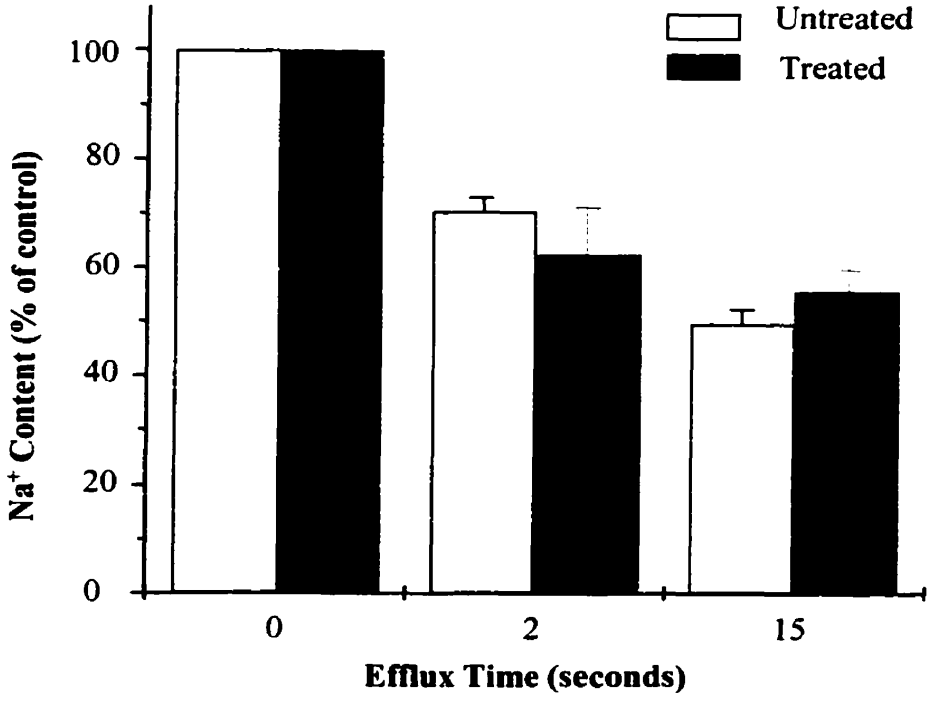


**Figure 14. Passive permeability of $Na^+$ from control and phospholipase D treated sarcolemmal vesicles.** Control and treated sarcolemmal vesicles were incubated with inactive and active phospholipase D, respectively, for 60 minutes at 25°C in pH 5.5. Following incubation, $H^+$-dependent $Na^+$ uptake was initiated for 1 minute in a medium containing 1 mM $Na^+$. Passive efflux was carried out in a $Na^+$ free medium and terminated at 2 and 15 seconds. Data are represented as mean ± S.E. for 3 separate experiments.

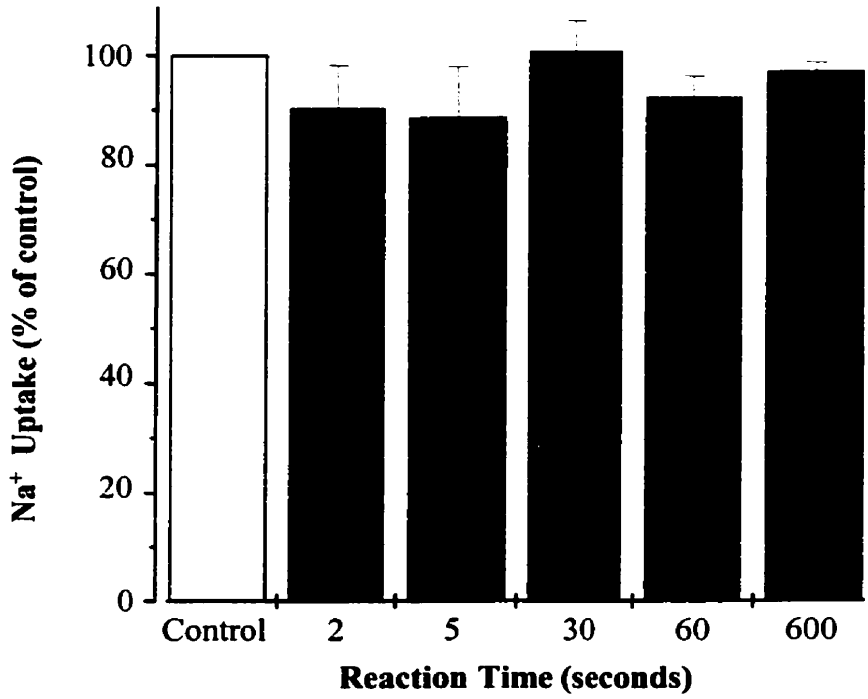


**Figure 15. $H^+$-dependent $Na^+$ uptake in phospholipase C treated sarcolemmal vesicles as a function of reaction time.** Sarcolemmal vesicles were incubated with 0.05 U phospholipase C / mg protein for 60 minutes at 25°C in pH 5.5. Following treatment, $Na^+$ uptake occurred for 2 seconds to 10 minutes in a final medium consisting of 0.05 mM $Na^+$, pH 9.33. Controls were examined in similar manner but contained boiled inactive phospholipase C. Data are represented as mean ± S.E. of 3-4 separate experiments. $*P < 0.05$ versus control.

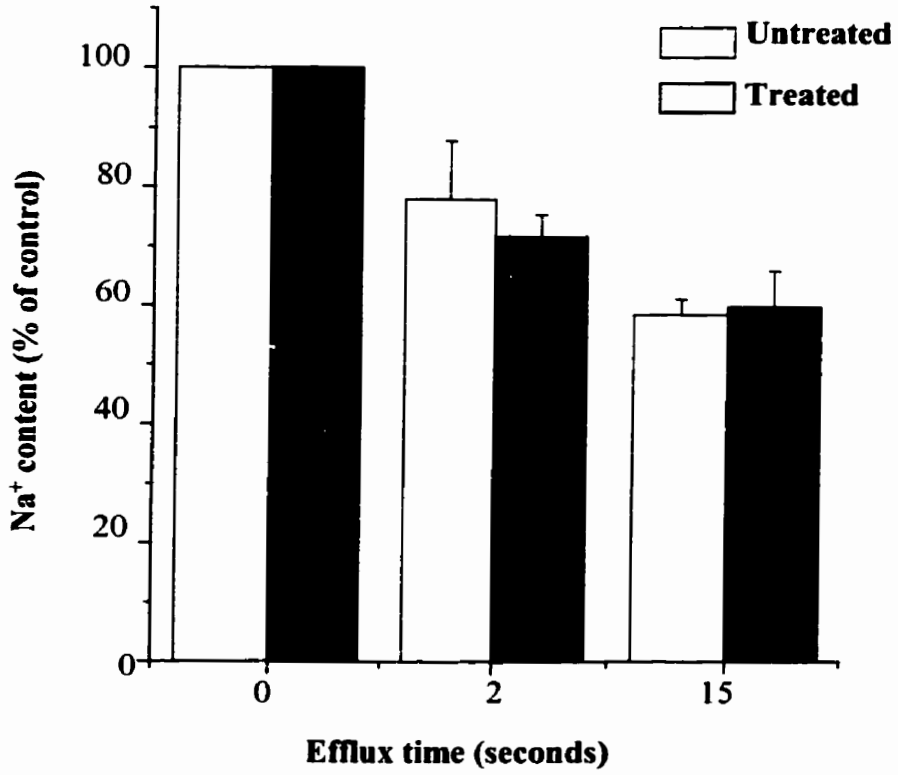


**Figure 16. Passive permeability from control and phospholipase C treated sarcolemmal vesicles.** Control and treated sarcolemmal vesicles were pre-incubated with inactive and active phospholipase C, respectively, for 60 minutes at 25°C in pH 5.5. Following incubation, $H^+$-dependent $Na^+$ uptake was initiated for 1 minute in a medium containing 1mM Na+. Passive efflux was carried out in a $Na^+$ free medium and terminated at 2 and 15 seconds. Data are represented as mean ± S.E. for 3 separate experiments.

The effects of certain fatty acids on $Na^+$-$H^+$ exchange were also investigated. Gamma (γ)- linolenic acid and linoleic acid did not affect $Na^+$-$H^+$ exchange activity when compared to controls. These results were in contrast to those obtained following the addition of eicosapentanoic acid (EPA) and docosahexanoic acid (DHA) **(Table 8)**. The addition of 10-100μM EPA resulted in a dose dependent inhibition of the cardiac $Na^+$-$H^+$ exchanger **(Figure 17)**. A statistically significant inhibition was present at 50 and 100μM EPA. This inhibition was consistent across variations in reaction time **(Figure 18)** and extracellular pH **(Figure 19)**. The addition of 10-100μM DHA also resulted in a dose-dependent inhibition of $Na^+$-$H^+$ exchange activity **(Figure 20)**. Cardiac $Na^+$-$H^+$ exchange was inhibited by 20-50% following DHA treatment. The greatest inhibition was observed at 100μM DHA. Therefore, examination of DHA on $Na^+$-$H^+$ exchange across variable reaction times was carried out at 100μM. The inhibition was statistically significant when examined across 2, 5, 30 and 60 seconds of uptake **(Figure 21)**.

The membrane destabilizing effect of fatty acids has been well documented [6,8]. For this reason the inhibition was thought to result from an increase in ion permeability. To determine the nature of this observed inhibition, passive ion permeability following ion loading of the vesicles was examined. At 100μM DHA and EPA, there was no statistically significant difference between control and PUFA treated vesicles **(Figure 22)**. Therefore, it can be concluded that the PUFA dependent inhibition of cardiac $Na^+$-$H^+$ exchange was not due to an increase in ion permeability.

**Table 8. $H^+$-dependent $Na^+$ uptake in PUFA treated cardiac sarcolemmal vesicles.**

| Linoleic Acid | Linolenic Acid | Eicosapentanoic Acid | Docosahexanoic Acid |
|---|---|---|---|
| 99 ± 5 | 96 ± 1 | 76 ± 3* | 66 ± 8* |

Sarcolemmal vesicles were incubated with 50μM of either linoleic, linolenic acid, eicosapentanoic acid or docosahexanoic acid at 25°C (pH 5.5). $H^+$-dependent $Na^+$ uptake was carried out for 5 seconds in final solution consisting of 0.05mM $Na^+$, pH 9.33. Data are represented as a percentage of control ± S.E.
*$P < 0.05$.

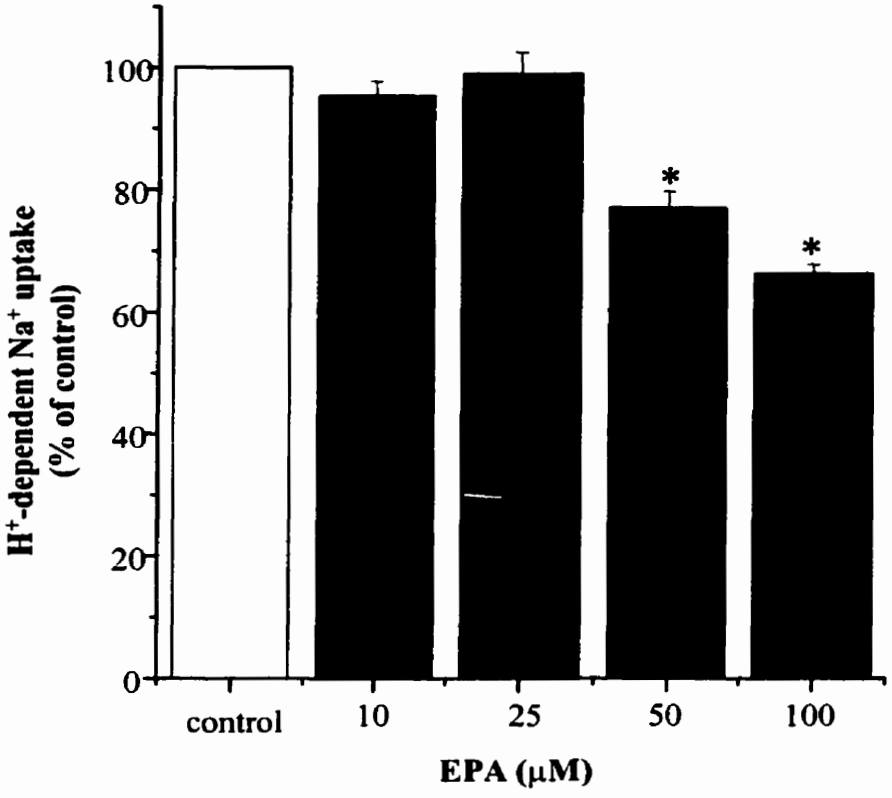


**Figure 17. $H^+$-dependent $Na^+$ uptake in sarcolemmal vesicles as a function of variable concentrations of eicosapentanoic acid.** Sarcolemmal vesicles were incubated with variable concentrations of eicosapentanoic acid at 25°C (pH 5.5). $Na^+$-$H^+$ exchange was examined for 5 seconds with a final concentration of 0.05mM $Na^+$, pH 9.33. Data are represented as means ± S.E. of 3-5 separate experiments. Controls were examined in a similar fashion but contained fatty acid vehicle only. $*P < 0.05$ versus control.

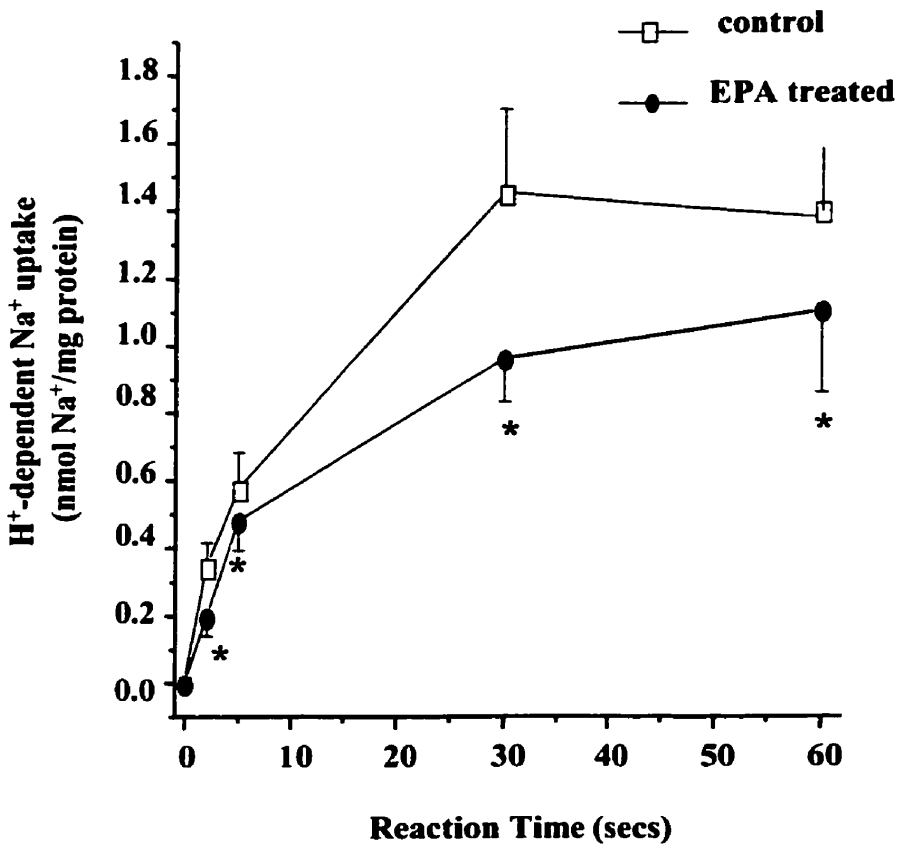


**Figure 18. $H^+$-dependent $Na^+$ uptake as a function of variable reaction time in eicosapentanoic acid treated sarcolemmal vesicles.** Sarcolemmal vesicles were incubated with 100μM of eicosapentanoic acid at 25°C (pH 5.5). $Na^+$ uptake was examined in a final solution consisting of 0.05mM $Na^+$, pH 9.33. Data are represented as mean ± S.E. of 3-5 separate experiments. $*P < 0.05$ versus control.

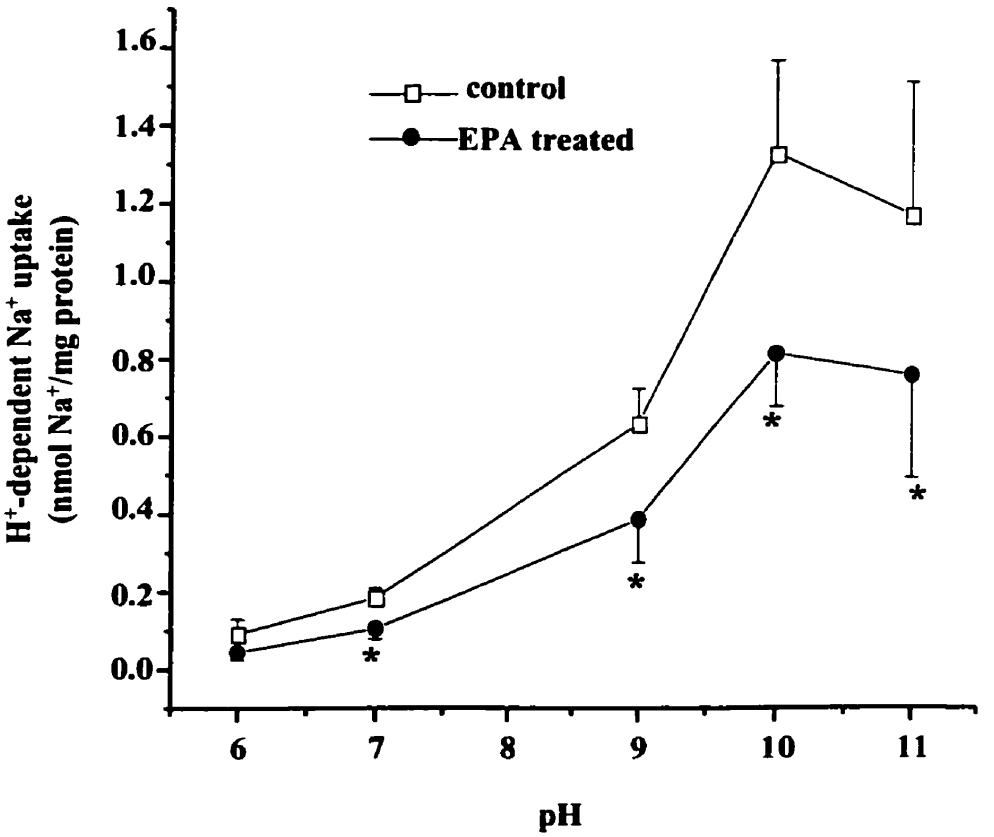


**Figure 19. $H^+$-dependent $Na^+$ uptake in eicosapentanoic acid treated sarcolemmal vesicles as a function of $[pH]_o$.** Sarcolemmal vesicles were incubated with 100μM eicosapentanoic acid as described. $Na^+$ uptake occurred for a period of 5 seconds, while extravesicular pH was varied. Final $Na^+$ concentration for the medium was 0.05mM. Data are from 4-7 separate experiments as means ± S.E. $*P < 0.05$ vs control.

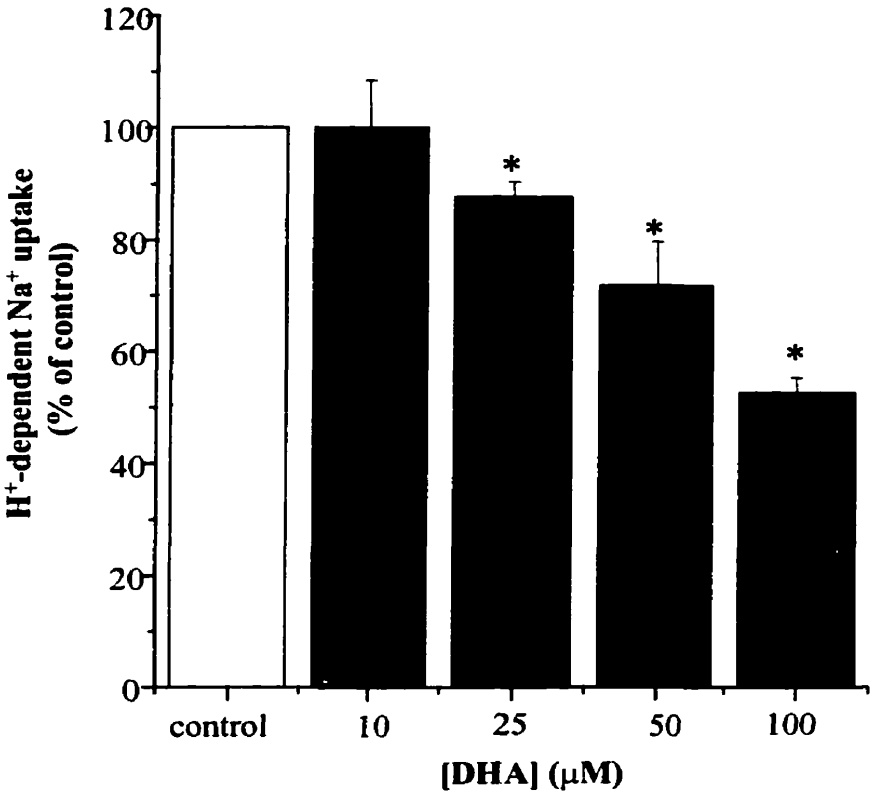


**Figure 20. $H^+$-dependent $Na^+$ uptake in sarcolemmal vesicles as a function of variable concentrations of docosahexanoic acid.** Sarcolemmal vesicles were incubated with variable concentrations of eicosapentanoic acid at 25°C (pH 5.5). $Na^+$-$H^+$ exchange was examined for 5 seconds with a final concentration of 0.05mM $Na^+$, pH 9.33. Data are represented as means ± S.E. of 3-5 separate experiments. Controls were examined in a similar fashion but contained fatty acid vehicle only. $*P < 0.05$ versus control.

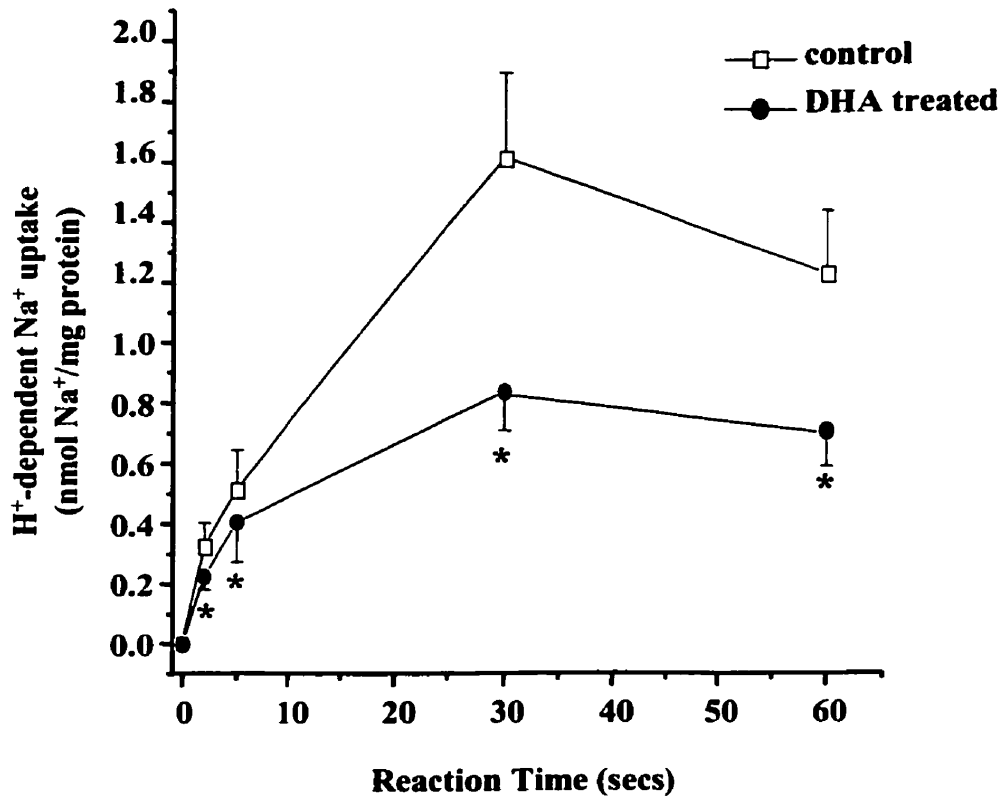


**Figure 21. $H^+$-dependent $Na^+$ uptake as a function of variable reaction time in docosahexanoic acid treated sarcolemmal vesicles.** Sarcolemmal vesicles were incubated with 100μM of docosahexanoic acid at 25°C (pH 5.5). $Na^+$ uptake was examined in a final solution consisting of 0.05mM $Na^+$, pH 9.33. Data are represented as mean ± S.E. of 3-5 separate experiments. $*P < 0.05$ versus control.

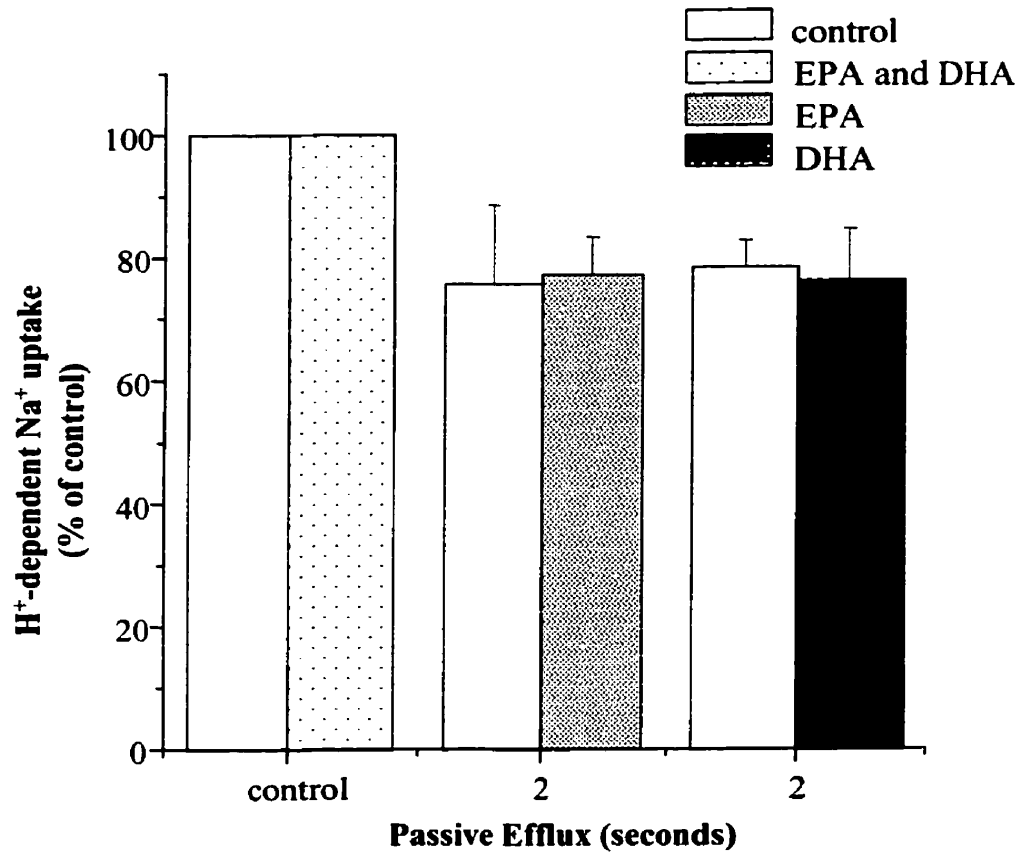


**Figure 22. Passive efflux of $Na^+$ from sarcolemmal vesicles treated with eicosapentanoic acid and docosahexanoic acid.** Control and treated sarcolemmal vesicles were incubated with PUFAs (EPA and DHA) and PUFA vehicle, respectively, at 25°C in pH 5.5. Following incubation, $H^+$-dependent $Na^+$ uptake was initiated for 1 minute in a medium containing 1mM $Na^+$. Passive efflux was carried out in a $Na^+$ free medium and terminated at 2 seconds. Data are represented as mean ± S.E. for 3-4 separate experiments.

The effect of lyosphosphatidylcholine (LPC) on the activity of the $Na^+$-$H^+$ exchanger was examined. Different concentrations of LPC (10, 25 50 and 100μM LPC) were incubated with sarcolemmal vesicles. **Figure 23** shows no statistically significant difference at any concentration of LPC. When $Na^+$-$H^+$ exchange was examined at various reaction times (2, 5 and 30 seconds), no concentration of LPC produced a statistically significant change in $Na^+$-$H^+$ exchange activity **(Figure 24).**

The effects of plasmalogen LPC, lysoplasmenylcholine ($LP_EC$), on $Na^+$-$H^+$ exchange activity were also examined. At concentrations of 10, 25, 50 and 100μM $LP_EC$, there was no change in $Na^+$-$H^+$ exchange activity **(Figure 25).** This finding was consistent across variable reaction times **(Figure 26).**

To further analyze the dependence of the $Na^+$-$H^+$ exchanger on membrane phospholipids, a number of lysophospholipids were incubated with sarcolemmal vesicles. Lysophosphatidylserine (LPS), lysophosphatidylinositol (LPI) and lysophosphatidylethanolamine (LPE) were pre-incubated with cardiac sarcolemmal vesicles for 2 minutes. Incubation with LPS, LPI and LPE produced no statistically significant changes in $Na^+$-$H^+$ exchange activity **(Figure 27).** When examined at variable reaction times, the results were similar **(Figure 28).**

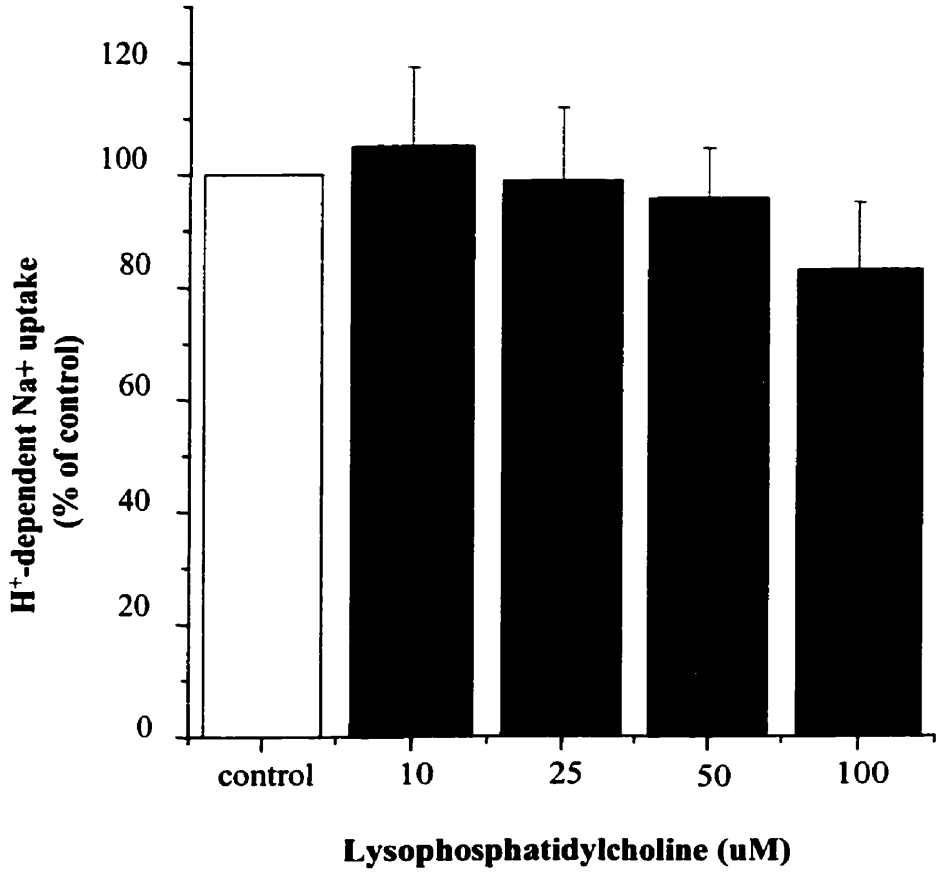


**Figure 23. H+-dependent $Na^+$ uptake in LPC treated sarcolemmal vesicles.** Sarcolemmal vesicles were pre-incubated with 10, 25, 50 and 100uM LPC for 3 minutes. H+-dependent Na+ uptake was examined for 5 seconds in pH 10.61 at T=25°C.

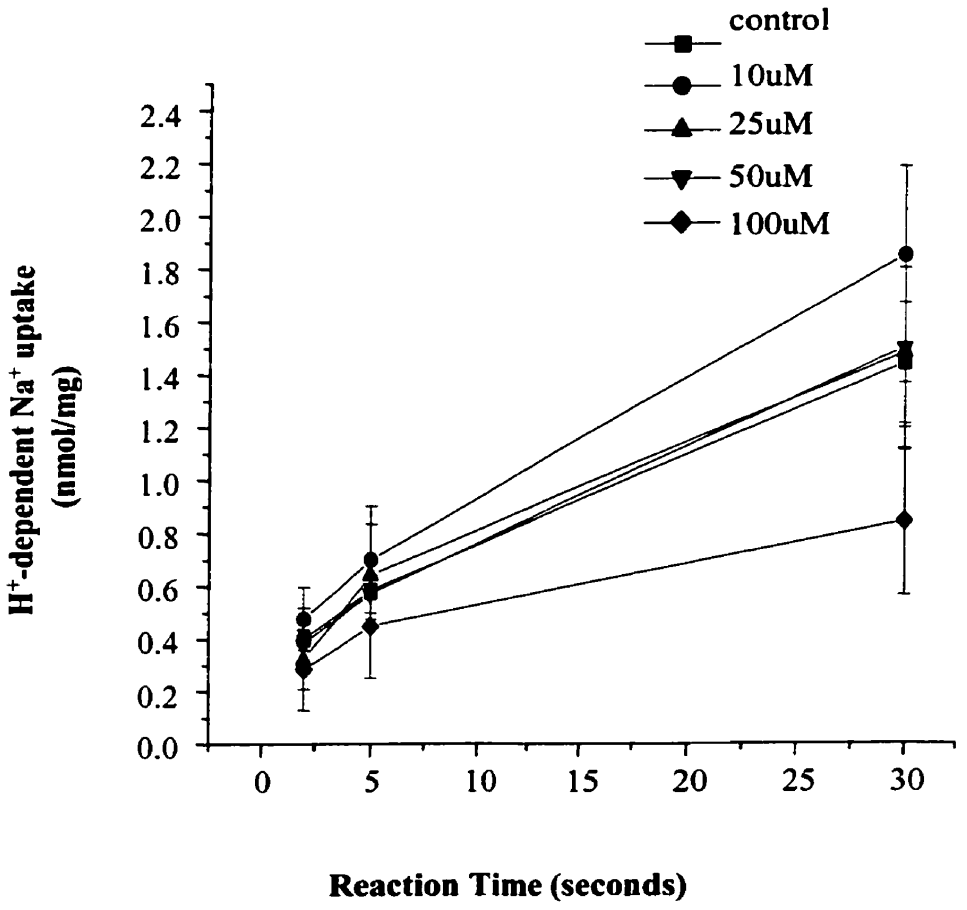


**Figure 24. $H^+$-dependent $Na^+$ uptake in LPC treated vesicles as a function of reaction time.** Sarcolemmal vesicles were pre-incubated with 50uM LPC for 3 minutes at pH 5.5, T = 25°C. Reaction was carried out in pH 10.61 media for 2, 5 and 30 seconds, $[Na^+]_f = 0.05mM$

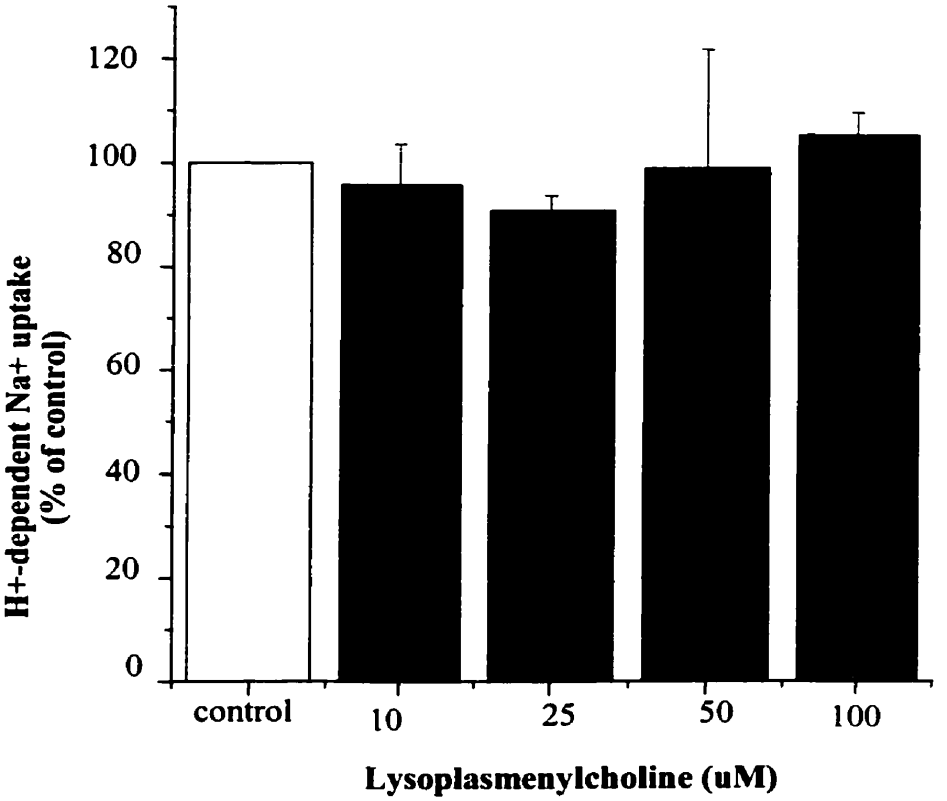


**Figure 25. $H^+$-dependent $Na^+$ uptake in LPeC treated sarcolemmal vesicles.** Sarcolemmal vesicles were pre-incubated with 10, 25, 50 and 100uM of LPeC for 3 minutes in pH 5.5, T=25°C. $H^+$-dependent $Na^+$ uptake was examined for 5 seconds in pH 10.61, [$Na^+$] 0.05mM.

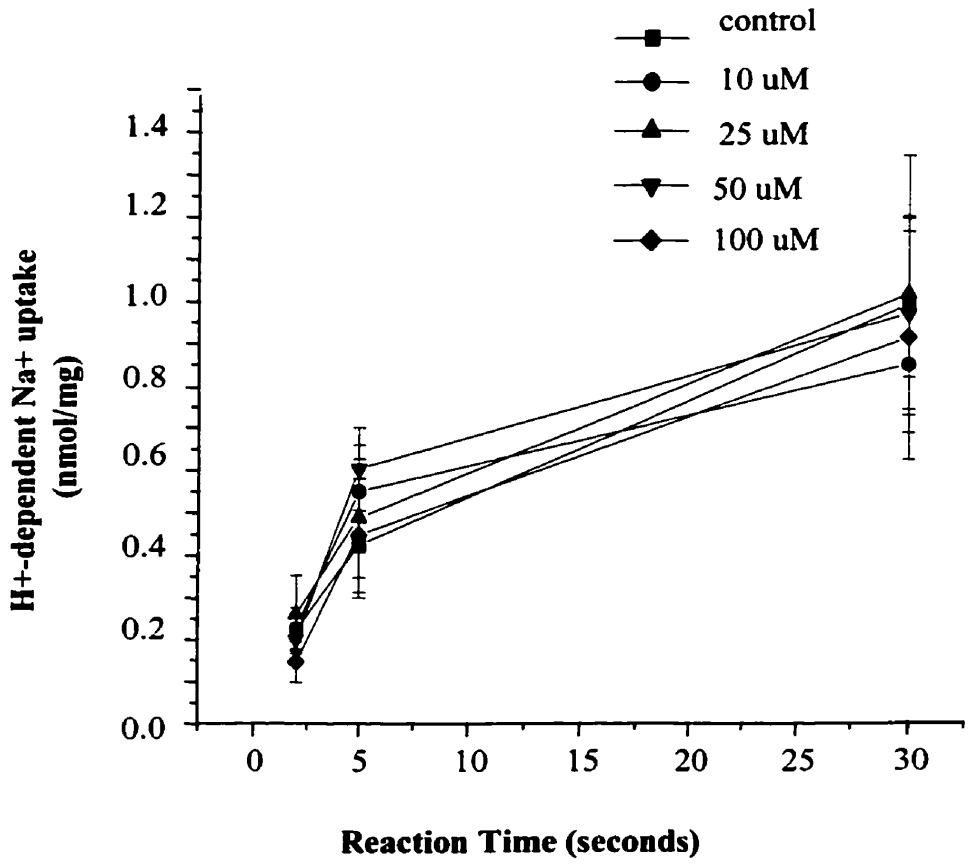


**Figure 26. $H^+$-dependent $Na^+$ uptake in lysoplasmenylcholine (LPeC) treated sarcolemmal vesicles as a function of reaction time and [LPeC].** Sarcolemmal vesicles were pre-incubated with10, 25, 50 and 100uM LPeC for 3 minutes in pH 5.5, T=25°C. $H^+$-dependent $Na^+$ uptake was examined at 2, 5 and 30 seconds in pH 10.61, [$Na^+$] 0.05mM.

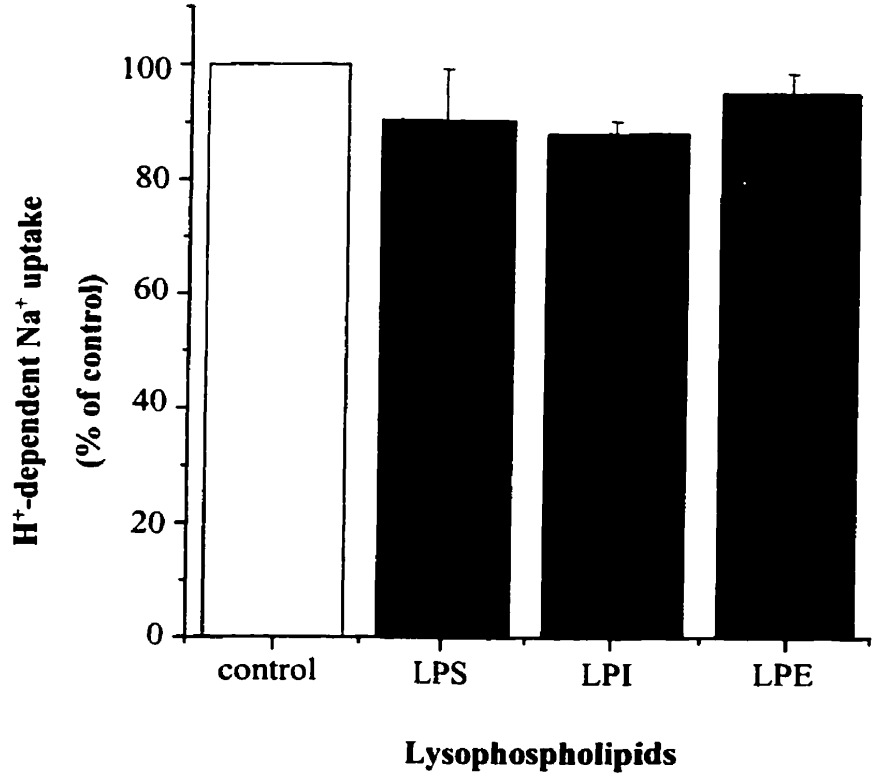


**Figure 27. $H^+$-dependent $Na^+$ uptake as a function of variable lysophospholipids.** Sarcolemmal vesicles were pre-incubated with 50uM lysophosphatidylserine (LPS), lysophosphatidylinositol (LPI) and lysophosphatidylethanolamine (LPE) for 2 minutes. $Na^+$ uptake was examined in a final solution consisting of 0.05mM [$Na^+$], pH 9.33 for 5 seconds. Data are represented as mean ± S.E. of 3 separate experiments.

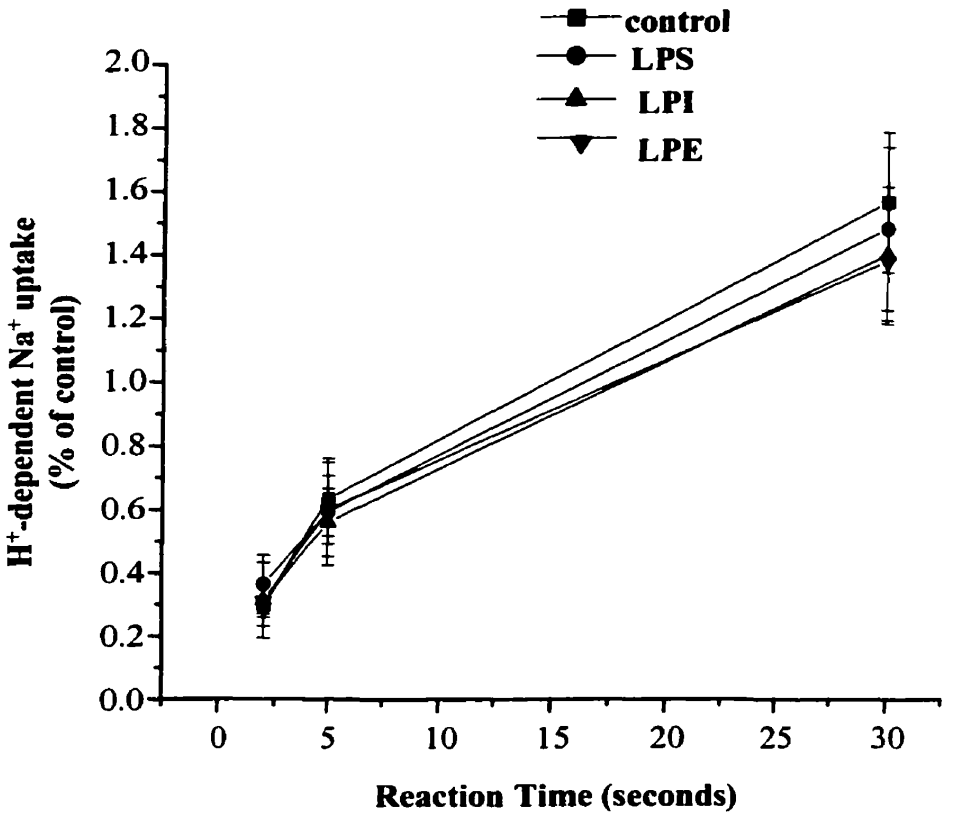


**Figure 28. Time course in $H^+$-dependent $Na^+$ uptake in lysophosphatidylserine (LPS), lysophosphatidylinositol (LPI) and lysophosphatidylethanolamine (LPE) treated sarcolemmal vesicles.** Sarcolemmal vesicles were pre-incubated with 50uM LPS, LPI and LPE for 2 minutes at 25°C (pH 5.5). $Na^+$ uptake was examined in a final solution consisting of 0.05mM $Na^+$, pH 9.33. Data are represented as mean ● S.E. of 3 separate experiments.

# D. DISCUSSION

This study demonstrates that the cardiac sarcolemmal $Na^+$-$H^+$ exchanger requires a suitable phospholipid environment for optimal activity and that phospholipase D-induced changes in the exchanger-associated phospholipids inhibit the exchanger activity. Following phospholipase D treatment, all parameters of $Na^+$-$H^+$ exchange ($Na^+$, $pH_o$ and reaction time dependency) were inhibited. The striking difference between the effects of phospholipase C and D on $Na^+$-$H^+$ exchange strongly implicates phosphatidic acid as the lipid moiety responsible for the effects on $Na^+$-$H^+$ exchange. Changes in the membrane phosphatidylcholine content by up to 20 % with phospholipase C treatment did not alter $Na^+$-$H^+$ exchange. However, phospholipase D inhibited $Na^+$-$H^+$ exchange by 30 - 60% when compared to controls. Phospholipase D hydrolyses phosphatidylcholine as well but instead generates phosphatidic acid. However, it is also possible that phospholipase D action may inhibit $Na^+$-$H^+$ exchange through an alteration of the phosphatidylcholine / phosphatidic acid ratio [449]. Another potential explanation for the observed inhibition of the $Na^+$-$H^+$ exchanger could be as a result of an increase in passive $Na^+$ permeability of the vesicles. However, when passive efflux was examined, no difference between control and phospholipase D treated cardiac sarcolemmal vesicles was observed. Alternatively, a decrease in the total volume of the vesicles may also account for the observed inhibition. However, when $H^+$-dependent $Na^+$ uptake was examined as a function of time, there was no difference between control and phospholipase D treated vesicles following 1 minute of

$Na^+$ uptake. Since total intravesicular $Na^+$ loading capacity was unaltered, this suggests vesicular size and volume were not changed in a meaningful manner.

In contrast to the conclusions of others (27), our data argue persuasively that membrane phospholipids can modulate $Na^+$-$H^+$ exchange activity. The previous work did not directly examine the effects of endogenous phospholipids on $Na^+$-$H^+$ exchange (27). Instead, their conclusions were based on effects observed where phospholipid flippase activity was stimulated. The flippase allows for preferential distribution of negatively charged phospholipids to the inner leaflet of the phospholipid bilayer (450). Our results demonstrating a lack of effect of changes in membrane phosphatidylcholine content on $Na^+$-$H^+$ exchange are, therefore, consistent with the conclusions of Demareux *et al* (27). However, the present results extend these observations to identify clear, class dependent effects of specific phospholipids on the exchanger. The degree of inhibition generated by phospholipase D treatment in the present study is also similar to that observed previously by the $Na^+$-$K^+$ ATPase and $Ca^{2+}$ ATPase (5). The $Na^+$-$Ca^{2+}$ exchanger also exhibits a phospholipid-specific modulatory response (121).

The interaction of phospholipids with the $Na^+$-$H^+$ exchanger has relevance to ischemia-reperfusion injury where exchange activity is altered. $Na^+$-$H^+$ exchange is stimulated during the acidic load which occurs in ischemia-reperfusion injury (379,451). Interestingly, others have shown that sarcolemmal phospholipase D is depressed following 30 minutes of post-ischemic reperfusion (449). Our results may partly explain why prior exposure of the myocardium to a brief ischemic event offers a cardioprotective effect to future episodes of ischemia. Preconditioning has been reported to increase the amount of phospholipase D activity within the myocardium (452). Our data would

suggest that this phospholipase D action would inhibit $Na^+$-$H^+$ exchange and may account for the cardioprotection observed following preconditioning [453-455]. Inhibition of the $Na^+$-$H^+$ exchanger during ischemia is beneficial to the myocardium [284]. In summary, in contrast to previous suggestions, the cardiac $Na^+$-$H^+$ exchanger is sensitive to the membrane phospholipid environment. The exchanger appears to be affected by specific phospholipid classes, namely phosphatidic acid. This effect may play an important role in the cardioprotective effects observed in ischemic preconditioning.

The PUFA study demonstrates that specific fatty acids can significantly inhibit the activity of the cardiac sarcolemmal $Na^+$-$H^+$ exchanger. Following DHA and EPA treatment, all of the parameters of $Na^+$-$H^+$ exchange that were examined were altered (fatty acid concentration, reaction time and extravesicular pH). Two observations support the contention that the inhibition of the exchanger was not artifactual but reflects a real, direct interaction of the lipids with the exchanger. First, the inhibition of exchange by DHA and EPA was not due to an increase in the passive permeability characteristics of the membrane. Fatty acids are known to compromise the passive permeability of membranes [73]. An increase in sarcolemmal leakiness would inhibit the capacity of the vesicles to maintain an ionic load and give the appearance of a direct inhibitory effect on $Na^+$-$H^+$ exchange. However, DHA and EPA did not affect passive ion permeability under the assay conditions employed in the present study. Second, the effects of the PUFA's on $Na^+$-$H^+$ exchange were specific to the PUFA tested. Following exposure to 50μM LA and LNA, the activity of the $Na^+$-$H^+$ exchanger remained unaltered. This lack of effect of LA and LNA on cardiac $Na^+$-$H^+$ exchange agrees with the findings of Gore et al [81] that enrichment of cells with LA or LNA did not alter $Na^+$-$H^+$ exchange. In contrast, the

addition of DHA and EPA significantly inhibited $H^+$-dependent $Na^+$ uptake. This difference in the effects of specific fatty acids on $Na^+$-$H^+$ exchange implies a structural specificity of the effect. DHA and EPA are longer chain fatty acids than LA and LNA. The capacity of the cis double bonds found in PUFA's together with the longer chain length may induce a sufficient disturbance in membrane order near the exchanger to alter its activity. This is also consistent with previous studies of the effects of phospholipases on cardiac sarcolemmal $Na^+$-$H^+$ exchange. Although treatment of sarcolemmal vesicles with phospholipase D resulted in a significant change in $Na^+$-$H^+$ exchange, there was no effect after phospholipase C treatment (see results). Again, this implies the $Na^+$-$H^+$ exchanger is sensitive to specific alterations in its lipid environment.

Our current results may have important clinical significance from two perspectives. First, the concentrations of PUFA's that induced an effect on the exchanger in the present study are within the range expected to be found in plasma from individuals consuming a diet enriched in PUFA [458] and in pathological conditions like ischemia [78]. Secondly, our results have mechanistic importance in pathological conditions like ischemia/reperfusion. The $Na^+$-$H^+$ exchanger is known to play a critical role in the initiation of arrhythmias and damage during ischemia-reperfusion challenge [257,260,384,385]. Pharmacological inhibition of $Na^+$-$H^+$ exchange significantly reduces ischemia-induced arrhythmias, contractile dysfunction, damage and necrosis [284,285,302,303,329,379,459]. Billman has reported the anti-arrhythmic effects of DHA and EPA following ischemia [65,78]. Although inhibition of $Ca^{2+}$ or $K^+$ channels has been suggested as mechanisms by which PUFA's exert their anti-arrhythmic effect [79,80] our results provide a clear and important alternative mechanistic explanation. By

inhibiting the $Na^+$-$H^+$ exchanger, $Ca^{2+}$ overload will be reduced or prevented (386), and the arrhythmias, contractile dysfunction, damage and necrosis associated with the ischemic/reperfusion insult will be avoided. This conclusion, however, is limited to DHA and EPA and cannot explain the cardioprotective actions of other PUFA's (62,63).

In summary, our study is the first to demonstrate a potent effect of DHA and EPA on $Na^+$-$H^+$ exchange. Our results provide insight into the cardioprotective action of PUFAs like DHA and EPA during ischemia/reperfusion and further insight into the interactions of lipids with the exchanger. Given their anti-hyperlipidemic and anti-arrhythmic actions, PUFA's may become an essential recommendation as a nutritional supplement with beneficial cardiovascular effects.

Lysolipids are another species of lipid that may alter $Na^+$-$H^+$ exchange. Karmazyn et al (456) have shown that LPC stimulated NHE-1 in cardiomyocytes. Others have reported inhibitory affects of LPC on other ion transporters as well. The $Na^+$-$Ca^{2+}$ exchange in sarcolemmal vesicles (457), $Na^+$-$K^+$ ATPase (25) and the $Na^+$-$HCO_3^-$ in ventricular myocytes (21) are all inhibited by LPC. However, Yamaguchi et al reported no effect of LPC on the cardiac $Na^+$-$H^+$ exchanger (21). Therefore it is unclear if LPC does alter $Na^+$-$H^+$ exchange. If it does stimulate $Na^+$- $H^+$ exchange, three potential mechanisms may account for this action. LPC may alter $Na^+$-$H^+$ exchange via a direct effect on the exchanger or, through an indirect signaling mechanism, or via some combianation of both of these effects. Karmazyn and colleagues (456) hypothesized that LPC stimulation of $Na^+$-$H^+$ exchange occurred via a protein kinase pathway. To address the direct effect of lysolipids on $Na^+$-$H^+$ exchanger, we have examined the effects of LPC in isolated sarcolemmal membranes instead of cardiomyocytes. Using a sarcolemmal

membrane preparation removes the influence of kinases or other second messengers. In our study, there was no effect of lysophospholipids on the cardiac sarcolemmal $Na^+$-$H^+$ exchanger. Furthermore, lysophosphatidylycholine, lysophosphatidylserine, lysophosphatidylinositol and lysophosphatidylethanolamine had no effect.

The length of fatty acid carbon chain could contribute to the inability of these lysolipids to alter $Na^+$-$H^+$ exchange function. LPC, LPE, LPS and LPI all contain short carbon chains. The length of the fatty acid chain appears to play a significant role as long chain fatty acids significantly affect NHE activity when compared to shorter chain fatty acids

Interestingly, the result of studies evaluating the effects of phospholipase $A_2$ (generating LPC) are in agreement with the results observed here (a lack of effect on $Na^+$-$H^+$ exchange). Our study, therefore, is the first of its kind to evaluate the direct effects of individual pure lysolipids on cardiac $Na^+$-$H^+$ exchange and clearly rules out a direct effect of these lipids on $Na^+$-$H^+$ exchange.

# E. REFERENCES


1. **Lehninger AL, Cox MM**. *Principles of Biochemistry*. New York: Worth Publishers; 1993.

2. **Gorter E, Grendel F**. On bimolecular layers of lipids on the chromocytes of the blood. *J. Exp. Med*. 1925;41:439-43.

3. **White DA**. *in Form and Function of Phospholipids*. Amsterdam: Elsevier; 1973.

4. **Op den Kamp JAF**. Lipid asymmetry in membranes. *Annu. Rev. Biochem*. 1979;48:47-71.

5. **Philipson KD, Nishimoto AY**. Stimulation of $Na^+$-$Ca^{2+}$ Exchange in Cardiac Sarcolemmal Vesicles by Phospholipase D.*J Biol Chem* . 1983;1:16-19.

6. **Philipson KD, Frank JS, Nishimoto AY**. Effects of phospholipase C on the $Na^+$-$Ca^{2+}$ exchange and $Ca^{2+}$ permeability of cardiac sarcolemmal vesicles. *J Biol Chem* . 1983;258:5905-5910.

7. **Luciani S**. Reconstitution of the sodium-calcium exchanger from cardiac sarcolemmal vesicles. *Biochim Biophys Acta*. 1984;772:127-34.

8. **Vemuri R, Philipson KD**. Phospholipid composition modulates the $Na^+$-$Ca^{2+}$ exchange activity of cardiac sarcolemma in reconstituted vesicles. *Biochim Biophys Acta*. 1988;937:258-68.

9. **Soldati L, Longoni S, Carafoli E**. Solubilization and reconstitution of the $Na^+/Ca^{2+}$ exchanger of cardiac sarcolemma. Properties of the reconstituted system and tentative identification of the protein(s) responsible for the exchange activity. *J Biol Chem*. 1985;260:13321-7.

10. **Barzilai A, Spanier R, Rahamimoff H**. Isolation, purification, and reconstitution of the $Na^+$ gradient- dependent $Ca^{2+}$ transporter ($Na^+$-$Ca^{2+}$ exchanger) from brain synaptic plasma membranes. *Proc Natl Acad Sci U S A*. 1984;81:6521-5.

11. **Vemuri R, Longoni S, Philipson KD**. Ouabain treatment of cardiac cells induces enhanced $Na^+$-$Ca^{2+}$ exchange activity. *Am J Physiol*. 1989;256:C1273-6.

12. **Niggli V, Adunyah ES, Carafoli E**. Acidic phospholipids, unsaturated fatty acids, and limited proteolysis mimic the effect of calmodulin on the purified erythrocyte $Ca^{2+}$ - ATPase. *J Biol Chem*. 1981;256:8588-92.

13. **Niggli V, Zurini M, Carafoli E**. Purification, reconstitution, and molecular characterization of the $Ca^{2+}$ pump of plasma membranes. *Methods Enzymol*. 1987;139:791-808.

14. **Roelofsen B, van Deenen LL.** Lipid requirement of membrane-bound ATPase. Studies on human erythrocyte ghosts. *Eur J Biochem.* 1973;40:245-57.

15. **Cullis PR, Hope MJ.** Physical properties and functional roles of lipids in membranes. In: Vance DE, Vance J, ed. *Biochemistry of Lipids, Lipoproteins and Membranes*: Elsevier Science Publishers; 1991:1-41.

16. **Burnashev NA, Undrovinas AI, Fleidervish IA, Rosenshtraukh LV.** Ischemic poison lysophosphatidylcholine modifies heart sodium channels gating inducing long-lasting bursts of openings. *Pflugers Arch.* 1989;415:124-6.

17. **Oishi K, Zheng B, Kuo JF.** Inhibition of Na,K-ATPase and sodium pump by protein kinase C regulators sphingosine, lysophosphatidylcholine, and oleic acid. *J Biol Chem.* 1990;265:70-5.

18. **Kiyosue T, Arita M.** Effects of lysophosphatidylcholine on resting potassium conductance of isolated guinea pig ventricular cells. *Pflugers Arch.* 1986;406:296-302.

19. **Eddlestone GT.** ATP-sensitive K channel modulation by products of PLA2 action in the insulin-secreting HIT cell line. *Am J Physiol.* 1995;268:C181-90.

20. **Lundbaek JA, Andersen OS**. Lysophospholipids modulate channel function by altering the mechanical properties of lipid bilayers. *J Gen Physiol*. 1994;104:645-73.

21. **Yamaguchi S, Tamagawa M, Nakajima N, Nakaya H**. Selective impairment of HCO3(-)-dependent pHi regulation by lysophosphatidylcholine in guinea pig ventricular myocardium. *Cardiovasc Res*. 1998;37:179-86.

22. **Undrovinas AI, Fleidervish IA, Makielski JC**. Inward sodium current at resting potentials in single cardiac myocytes induced by the ischemic metabolite lysophosphatidylcholine. *Circ Res*. 1992;71:1231-41.

23. **Sato T, Kiyosue T, Arita M**. Inhibitory effects of palmitoylcarnitine and lysophosphatidylcholine on the sodium current of cardiac ventricular cells. *Pflugers Arch*. 1992;420:94-100.

24. **Clarkson CW, Ten Eick RE**. On the mechanism of lysophosphatidylcholine-induced depolarization of cat ventricular myocardium. *Circ Res*. 1983;52:543-56.

25. **Karli JN, Karikas GA, Hatzipavlou PK, Levis GM, Moulopoulos SN**. The inhibition of $Na^+$ and $K^+$ stimulated ATPase activity of rabbit and dog heart sarcolemma by lysophosphatidyl choline. *Life Sci*. 1979;24:1869-75.

26. **Pitts BJ, Okhuysen CH**. Effects of palmitoyl carnitine and LPC on cardiac sarcolemmal $Na^+$-$K^+$- ATPase. *Am J Physiol*. 1984;247:H840-6.

27. **Demareux N, Romanek R, Orlowski J, Grinstein S**. ATP dependence of $Na^+$-$H^+$ exchange: Nucleotide specificity and assessment of the role of phospholipids. *J Gen Physiol* 1997;109:117-128.

28. **Hoque AN, Haist JV, Karmazyn M**. $Na^+$-$H^+$ exchange inhibition protects against mechanical, ultrastructural, and biochemical impairment induced by low concentrations of lysophosphatidylcholine in isolated rat hearts. *Circ Res*. 1997;80:95-102.

29. **Kume N, Gimbrone MA, Jr.** Lysophosphatidylcholine transcriptionally induces growth factor gene expression in cultured human endothelial cells. *J Clin Invest*. 1994;93:907-11.

30. **Tokumura A, Iimori M, Nishioka Y, Kitahara M, Sakashita M, Tanaka S**. Lysophosphatidic acids induce proliferation of cultured vascular smooth muscle cells from rat aorta. *Am J Physiol*. 1994;267:C204-10.

31. **Williams SD, Ford DA**. Activation of myocardial cAMP-dependent protein kinase by lysoplasmenylcholine. *FEBS Lett*. 1997;420:33-8.

32. **Subbaiah PV, Chen CH, Bagdade JD, Albers JJ**. Substrate specificity of plasma lysolecithin acyltransferase and the molecular species of lecithin formed by the reaction. *J Biol Chem*. 1985;260:5308-14.

33. **Rabini RA, Galassi R, Fumelli P, Dousset N, Solera ML, Valdiguie P, Curatola G, Ferretti G, Taus M, Mazzanti L**. Reduced $Na^{(+)}$-$K^{(+)}$-ATPase activity and plasma lysophosphatidylcholine concentrations in diabetic patients. *Diabetes*. 1994;43:915-9.

34. **van Bilsen M, van der Vusse GJ, Willemsen PH, Coumans WA, Roemen TH, Reneman RS**. Lipid alterations in isolated, working rat hearts during ischemia and reperfusion: its relation to myocardial damage. *Circ Res*. 1989;64:304-14.

35. **Otani H, Prasad MR, Engelman RM, Cordis GA, Das DK**. Enhanced phosphodiesteratic breakdown and turnover of phosphoinositides during reperfusion of ischemic rat heart.*Circ Res* . 1988;63:930-936.

36. **Chien KR, Han A, Sen A, Buja LM, Willerson JT**. Accumulation of unesterified arachidonic acid in ischemic canine myocardium. Relationship to a phosphatidylcholine deacylation- reacylation cycle and the depletion of membrane phospholipids. *Circ Res*. 1984;54:313-22.

37. **Saffitz JE, Corr PB, Lee BI, Gross RW, Williamson EK, Sobel BE.** Pathophysiologic concentrations of lysophosphoglycerides quantified by electron microscopic autoradiography. *Lab Invest.* 1984;50:278-86.

38. **Katz AM, Messineo FC.** Lipid-membrane interactions and the pathogenesis of ischemic damage in the myocardium. *Circ Res.* 1981;48:1-16.

39. **Prinzen FW, Van der Vusse GJ, Arts T, Roemen TH, Coumans WA, Reneman RS.** Accumulation of nonesterified fatty acids in ischemic canine myocardium. *Am J Physiol.* 1984;247:H264-72.

40. **Steenbergen C, Jennings RB.** Relationship between lysophospholipid accumulation and plasma membrane injury during total in vitro ischemia in dog heart. *J Mol Cell Cardiol.* 1984;16:605-21.

41. **Weglicki WB, Low MG.** Phospholipases of the myocardium. *Basic Res Cardiol.* 1987;82:107-12.

42. **Corr PB, Cain ME, Witkowski FX, Price DA, Sobel BE.** Potential arrhythmogenic electrophysiological derangements in canine Purkinje fibers induced by lysophosphoglycerides. *Circ Res.* 1979;44:822-32.

43. **Arnsdorf MF, Sawicki GJ**. The effects of lysophosphatidylcholine, a toxic metabolite of ischemia, on the components of cardiac excitability in sheep Purkinje fibers. *Circ Res*. 1981;49:16-30.

44. **Pogwizd SM, Onufer JR, Kramer JB, Sobel BE, Corr PB**. Induction of delayed afterdepolarizations and triggered activity in canine Purkinje fibers by lysophosphoglycerides. *Circ Res*. 1986;59:416-26.

45. **Nakaya H, Tohse N**. Electrophysiological effects of acetyl glyceryl ether phosphorylcholine on cardiac tissues: comparison with lysophosphatidylcholine and long chain acyl carnitine. *Br J Pharmacol*. 1986;89:749-57.

46. **Panagia V, Singh JN, Anand-Srivastava MB, Pierce GN, Jasmin G, Dhalla NS**. Sarcolemmal alterations during the development of genetically determined cardiomyopathy. *Cardiovasc Res*. 1984;18:567-72.

47. **Rabini RA, Galassi R, Fumelli P, Dousset N, Solera ML, Valdiguie P, Curatola G, Ferretti G, Taus M, Mazzanti L**. Reduced $Na(^{+})$-$K(^{+})$-ATPase activity and plasma lysophosphatidylcholine concentrations in diabetic patients. *Diabetes*. 1994;43:915-919.

48. **Shinitzky M, Inbar M**. Microviscosity parameters and protein mobility in biological membranes. *Biochim Biophys Acta*. 1976;433:133-49.

49. **Chapman D**. Recent studies of lipids, lipid cholesterol and membrane system. In: Chapman DaW, D.F., ed. *Biological Membranes*: Academic Press, New York, 1973; 1973.

50. **Huang C**. Configurations of fatty acyl chains in egg phosphatidylcholine-cholesterol mixed bilayers. *Chem Phys Lipids*. 1977;19:150-8.

51. **Huang CH**. A structural model for the cholesterol-phosphatidylcholine complexes in bilayer membranes. *Lipids*. 1977;12:348-56.

52. **Suckling KE, Blair HA, Boyd GS, Craig IF, Malcolm BR**. The importance of the phospholipid bilayer and the length of the cholesterol molecule in membrane structure. *Biochim Biophys Acta*. 1979;551:10-21.

53. **Colbeau A, Nachbaur J, Vignais PM**. Enzymic characterization and lipid composition of rat liver subcellular membranes. *Biochim Biophys Acta*. 1971;249:462-92.

54. **Yeagle PL**. Cholesterol and the cell membrane. *Biochim Biophys Acta*. 1985;822:267-87.

55. **Yeagle PL**. Lanosterol and cholesterol have different effects on phospholipid acyl chain ordering. *Biochim Biophys Acta*. 1985;815:33-6.

56. **Klappauf E, Schubert D**. Band 3-protein from human erythrocyte membranes strongly interacts with cholesterol. *FEBS Lett.* 1977;80:423-5.

57. **Kutryk MJ, Pierce GN**. Stimulation of sodium-calcium exchange by cholesterol incorporation into isolated cardiac sarcolemmal vesicles. *J Biol Chem.* 1988;263:13167-13172.

58. **Albert CM, Hennekens CH, O'Donnell CJ, Ajani UA, Carey VJ, Willett WC, Ruskin JN, Manson JE**. Fish consumption and risk of sudden cardiac death . *Jama.* 1998;279:23-8.

59. **de Lorgeril M, Renaud S, Mamelle N, Salen P, Martin JL, Monjaud I, Guidollet J, Touboul P, Delaye J**. Mediterranean alpha-linolenic acid-rich diet in secondary prevention of coronary heart disease. *Lancet.* 1994;343:1454-9.

60. **Singh RB, Niaz MA, Sharma JP, Kumar R, Rastogi V, Moshiri M**. Randomized, double-blind, placebo-controlled trial of fish oil and mustard oil in patients with suspected acute myocardial infarction: the Indian experiment of infarct survival--4. *Cardiovasc Drugs Ther.* 1997;11:485-91.

61. **Siscovick DS, Raghunathan TE, King I, Weinmann S, Wicklund KG, Albright J, Bovbjerg V, Arbogast P, Smith H, Kushi LH, et al.** Dietary intake

and cell membrane levels of long-chain n-3 polyunsaturated fatty acids and the risk of primary cardiac arrest. *Jama*. 1995;274:1363-7.

62. **McLennan PL, Abeywardena MY, Charnock JS**. Influence of dietary lipids on arrhythmias and infarction after coronary artery ligation in rats. *Can J Physiol Pharmacol*. 1985;63:1411-7.

63. **McLennan PL, Bridle TM, Abeywardena MY, Charnock JS**. Dietary lipid modulation of ventricular fibrillation threshold in the marmoset monkey. *Am Heart J*. 1992;123:1555-61.

64. **Hock CE, Beck LD, Bodine RC, Reibel DK**. Influence of dietary n-3 fatty acids on myocardial ischemia and reperfusion. *Am J Physiol*. 1990;259:H1518-26.

65. **Billman GE, Hallaq H, Leaf A**. Prevention of ischemia-induced ventricular fibrillation by omega 3 fatty acids. *Proc Natl Acad Sci U S A*. 1994;91:4427-30.

66. **Burr ML**. Fish and the cardiovascular system. *Prog Food Nutr Sci*. 1989;13:291-316.

67. **Burr ML, Fehily AM, Gilbert JF, Rogers S, Holliday RM, Sweetnam PM, Elwood PC, Deadman NM**. Effects of changes in fat, fish, and fibre intakes on

death and myocardial reinfarction: diet and reinfarction trial (DART). *Lancet.* 1989;2:757-61.

68. **Siscovick DS, Raghunathan TE, King I, Weinmann S, Wicklund KG, Albright J, Bovbjerg V, Arbogast P, Smith H, Kushi LH, et al.** Dietary intake and cell membrane levels of long-chain n-3 polyunsaturated fatty acids and the risk of primary cardiac arrest. *Jama.* 1995;274:1363-7.

69. **Stiefel P, Ruiz-Gutierrez V, Gajon E, Acosta D, Garcia-Donas MA, Madrazo J, Villar J, Carneado J.** Sodium transport kinetics, cell membrane lipid composition, neural conduction and metabolic control in type 1 diabetic patients. Changes after a low-dose n-3 fatty acid dietary intervention. *Ann Nutr Metab.* 1999;43:113-20.

70. **Rao S, Erasmus RT.** Pilot study on plasma fatty acids in poorly controlled non insulin dependent diabetic Melanesians. *East Afr Med J.* 1996;73:816-8.

71. **Philipson KD, Ward R.** Modulation of $Na^{+}$-$Ca^{2+}$ exchange and $Ca^{2+}$ permeability in cardiac sarcolemmal vesicles by doxylstearic acids. *Biochim Biophys Acta.* 1987;897:152-8.

72. **Xiao YF, Wright SN, Wang GK, Morgan JP, Leaf A.** Coexpression with beta(1)-subunit modifies the kinetics and fatty acid block of hH1(alpha) $Na(^{+})$ channels. *Am J Physiol Heart Circ Physiol.* 2000;279:H35-46.

73. **Philipson KDaW, R.** Effects of Fatty Acids on $Na^{+}$-$Ca^{2+}$ Exchange and $Ca^{2+}$ Permeability of Cardiac Sarcolemmal Vesicles. *J Biol Chem.* 1985;260:9666-9671.

74. **Niggli V, Adunyah ES, Carafoli E.** Acidic phospholipids, unsaturated fatty acids, and limited proteolysis mimic the effect of calmodulin on the purified erythrocyte $Ca^{2+}$ - ATPase. *J Biol Chem.* 1981;256:8588-92.

75. **Taffet GE, Pham TT, Bick DL, Entman ML, Pownall HJ, Bick RJ.** The calcium uptake of the rat heart sarcoplasmic reticulum is altered by dietary lipid. *J Membr Biol.* 1993;131:35-42.

76. **Hallaq H, Sellmayer A, Smith TW, Leaf A.** Protective effect of eicosapentaenoic acid on ouabain toxicity in neonatal rat cardiac myocytes. *Proc Natl Acad Sci U S A.* 1990;87:7834-8.

77. **Hallaq H, Smith TW, Leaf A.** Modulation of dihydropyridine-sensitive calcium channels in heart cells by fish oil fatty acids. *Proc Natl Acad Sci U S A.* 1992;89:1760-4.

78. **Billman GE, Kang JX, Leaf A**. Prevention of ischemia-induced cardiac sudden death by n-3 polyunsaturated fatty acids in dogs. *Lipids*. 1997;32:1161-8.

79. **Xiao YF, Gomez AM, Morgan JP, Lederer WJ, Leaf A**. Suppression of voltage-gated L-type $Ca^{2+}$ currents by polyunsaturated fatty acids in adult and neonatal rat ventricular myocytes. *Proc Natl Acad Sci U S A*. 1997;94:4182-7.

80. **Bogdanov KY, Spurgeon HA, Vinogradova TM, Lakatta EG**. Modulation of the transient outward current in adult rat ventricular myocytes by polyunsaturated fatty acids. *Am J Physiol*. 1998;274:H571-9.

81. **Gore J, Besson P, Hoinard C, Bougnoux P**. $Na(^{+})$-$H^{+}$ antiporter activity in relation to membrane fatty acid composition and cell proliferation. *Am J Physiol*. 1994;266:C110-20.

82. **Kriegsmann J, Muller WD, Richter W, Wunderlich J, Wallukat G**. Demonstration of membrane-associated phospholipase A2 in cultivated heart muscle cells by immunogold-technique in surface replicas. *Acta Histochem*. 1993;95:61-6.

83. **Ross MI, Deems RA, Jesaitis AJ, Dennis EA, Ulevitch RJ**. Phospholipase activities of the P388D1 macrophage-like cell line. *Arch Biochem Biophys*. 1985;238:247-58.

84. **Clark JD, Lin LL, Kriz RW, Ramesha CS, Sultzman LA, Lin AY, Milona N, Knopf JL**. A novel arachidonic acid-selective cytosolic PLA2 contains a $Ca(^{2+})$-dependent translocation domain with homology to PKC and GAP. *Cell*. 1991;65:1043-51.

85. **Hazen SL, Stuppy RJ, Gross RW**. Purification and characterization of canine myocardial cytosolic phospholipase A2. A calcium-independent phospholipase with absolute fl- 2 regiospecificity for diradyl glycerophospholipids. *J Biol Chem*. 1990;265:10622-30.

86. **Hazen SL, Hall CR, Ford DA, Gross RW**. Isolation of a human myocardial cytosolic phospholipase A2 isoform. Fast atom bombardment mass spectroscopic and reverse-phase high pressure liquid chromatography identification of choline and ethanolamine glycerophospholipid substrates. *J Clin Invest*. 1993;91:2513-22.

87. **Hazen SL, Gross RW**. Identification and characterization of human myocardial phospholipase A2 from transplant recipients suffering from end-stage ischemic heart disease. *Circ Res*. 1992;70:486-95.

88. **Ford DA, Hazen SL, Saffitz JE, Gross RW**. The rapid and reversible activation of a calcium-independent plasmalogen-selective phospholipase A2 during myocardial ischemia. *J Clin Invest*. 1991;88:331-5.

89. **Hazen SL, Ford DA, Gross RW**. Activation of a membrane-associated phospholipase A2 during rabbit myocardial ischemia which is highly selective for plasmalogen substrate. *J Biol Chem*. 1991;266:5629-33.

90. **McHowat J, Liu S**. Interleukin-1beta stimulates phospholipase A2 activity in adult rat ventricular myocytes. *Am J Physiol*. 1997;272:C450-6.

91. **McHowat J, Creer MH**. Lysophosphatidylcholine accumulation in cardiomyocytes requires thrombin activation of $Ca^{2+}$-independent PLA2. *Am J Physiol*. 1997;272:H1972-80.

92. **Fiehn W, Hasselbach W**. The effect of phospholipase A on the calcium transport and the role of unsaturated fatty acids in ATPase activity of sarcoplasmic vesicles. *Eur J Biochem*. 1970;13:510-8.

93. **Kim D, Duff RA**. Regulation of $K^+$ channels in cardiac myocytes by free fatty acids. *Circ Res*. 1990;67:1040-6.

94. **Bharatula M, Hussain T, Lokhandwala MF**. Angiotensin II AT1 receptor/signaling mechanisms in the biphasic effect of the peptide on proximal tubular $Na^+$,$K^+$-ATPase. *Clin Exp Hypertens*. 1998;20:465-80.

95. **Williams RL, Katan M**. Structural views of phosphoinositide-specific phospholipase C: signalling the way ahead. *Structure*. 1996;4:1387-94.

96. **Heinz DW, Essen LO, Williams RL**. Structural and mechanistic comparison of prokaryotic and eukaryotic phosphoinositide-specific phospholipases C. *J Mol Biol*. 1998;275:635-50.

97. **Munnik T, Irvine RF, Musgrave A**. Phospholipid signalling in plants. *Biochim Biophys Acta*. 1998;1389:222-72.

98. **Shortridge RD, McKay RR**. Invertebrate phosphatidylinositol-specific phospholipases C and their role in cell signaling. *Invert Neurosci*. 1995;1:199-206.

99. **van Haastert PJ, van Dijken P**. Biochemistry and genetics of inositol phosphate metabolism in Dictyostelium. *FEBS Lett*. 1997;410:39-43.

100. **Rhee SG, Bae YS**. Regulation of phosphoinositide-specific phospholipase C isozymes. *J Biol Chem*. 1997;272:15045-8.

101. **Morris AJ, Scarlata S.** Regulation of effectors by G-protein alpha- and beta gamma-subunits. Recent insights from studies of the phospholipase c-beta isoenzymes. *Biochem Pharmacol.* 1997;54:429-35.

102. **Noh DY, Shin SH, Rhee SG.** Phosphoinositide-specific phospholipase C and mitogenic signaling. *Biochim Biophys Acta.* 1995;1242:99-113.

103. **Singer WD, Brown HA, Sternweis PC.** Regulation of eukaryotic phosphatidylinositol-specific phospholipase C and phospholipase D. *Annu Rev Biochem.* 1997;66:475-509.

104. **Ji QS, Winnier GE, Niswender KD, Horstman D, Wisdom R, Magnuson MA, Carpenter G.** Essential role of the tyrosine kinase substrate phospholipase C-gamma1 in mammalian growth and development. *Proc Natl Acad Sci U S A.* 1997;94:2999-3003.

105. **Wahl MI, Jones GA, Nishibe S, Rhee SG, Carpenter G.** Growth factor stimulation of phospholipase C-gamma 1 activity. Comparative properties of control and activated enzymes. *J Biol Chem.* 1992;267:10447-56.

106. **Yagisawa H, Sakuma K, Paterson HF, Cheung R, Allen V, Hirata H, Watanabe Y, Hirata M, Williams RL, Katan M.** Replacements of single basic

amino acids in the pleckstrin homology domain of phospholipase C-delta1 alter the ligand binding, phospholipase activity, and interaction with the plasma membrane. *J Biol Chem.* 1998;273:417-24.

107. **Rhee SG, Choi KD**. Regulation of inositol phospholipid-specific phospholipase C isozymes. *J Biol Chem.* 1992;267:12393-6.

108. **Heinz DW, Ryan M, Bullock TL, Griffith OH**. Crystal structure of the phosphatidylinositol-specific phospholipase C from Bacillus cereus in complex with myo-inositol. *Embo J.* 1995;14:3855-63.

109. **Moser J, Gerstel B, Meyer JE, Chakraborty T, Wehland J, Heinz DW**. Crystal structure of the phosphatidylinositol-specific phospholipase C from the human pathogen Listeria monocytogenes. *J Mol Biol.* 1997;273:269-82.

110. **Hansen CA, Schroering AG, Robishaw JD**. Subunit expression of signal transducing G proteins in cardiac tissue: implications for phospholipase C-beta regulation. *J Mol Cell Cardiol.* 1995;27:471-84.

111. **Schnabel P, Gas H, Nohr T, Camps M, Bohm M**. Identification and characterization of G protein-regulated phospholipase C in human myocardium. *J Mol Cell Cardiol.* 1996;28:2419-27.

112. **Nosek TM, Williams MF, Zeigler ST, Godt RE.** Inositol trisphosphate enhances calcium release in skinned cardiac and skeletal muscle. *Am J Physiol.* 1986;250:C807-11.

113. **Gilbert JC, Shirayama T, Pappano AJ.** Inositol trisphosphate promotes Na-Ca exchange current by releasing calcium from sarcoplasmic reticulum in cardiac myocytes. *Circ Res.* 1991;69:1632-9.

114. **Quist EE, Foresman BH, Vasan R, Quist CW.** Inositol tetrakisphosphate stimulates a novel ATP-independent $Ca^{2+}$ uptake mechanism in cardiac junctional sarcoplasmic reticulum. *Biochem Biophys Res Commun.* 1994;204:69-75.

115. **Puceat M, Vassort G.** Signalling by protein kinase C isoforms in the heart. *Mol Cell Biochem.* 1996;157:65-72.

116. **De Jonge HW, Van Heugten HA, Lamers JM.** Signal transduction by the phosphatidylinositol cycle in myocardium. *J Mol Cell Cardiol.* 1995;27:93-106.

117. **Chyn TL, Martonosi AN, Morimoto T, Sabatini DD.** In vitro synthesis of the $Ca^{2+}$ transport ATPase by ribosomes bound to sarcoplasmic reticulum membranes. *Proc Natl Acad Sci U S A.* 1979;76:1241-5.

118. **Hilgemann DW, Ball R**. Regulation of cardiac $Na^+$,$Ca^{2+}$ exchange and KATP potassium channels by PIP2. *Science*. 1996;273:956-9.

119. **Goldman SS, Albers RW**. Sodium-potassium-activated adenosine triphosphatase. IX. The role of phospholipids. *J Biol Chem*. 1973;248:867-74.

120. **Schrijen JJ, Omachi A, Van Groningen-Luyben WA, De Pont JJ, Bonting SL**. Studies on ($K^+$ $^+$ $H^+$)-ATPase. IV. Effects of phospholipase C treatment. *Biochim Biophys Acta*. 1981;649:1-12.

121. **Pierce GN, Panagia V**. Role of phosphatidylinositol in cardiac sarcolemmal membrane sodium-calcium exchange. *J Biol Chem*. 1989;264:15344-15350.

122. **Billah MM, Anthes JC**. The regulation and cellular functions of phosphatidylcholine hydrolysis. *Biochem J*. 1990;269:281-91.

123. **Kanfer JN**. The base exchange enzymes and phospholipase D of mammalian tissue. *Can J Biochem*. 1980;58:1370-80.

124. **Chalifour RJ, Kanfer JN**. Microsomal phospholipase D of rat brain and lung tissues. *Biochem Biophys Res Commun*. 1980;96:742-7.

125. **Exton JH**. Regulation of phospholipase D. *Biochim Biophys Acta*. 1999;1439:121-33.

126. **Panagia V, Ou C, Taira Y, Dai J, Dhalla NS**. Phospholipase D activity in subcellular membranes of rat ventricular myocardium. *Biochim Biophys Acta*. 1991;1064:242-50.

127. **Moraru, II, Popescu LM, Maulik N, Liu X, Das DK**. Phospholipase D signaling in ischemic heart. *Biochim Biophys Acta*. 1992;1139:148-54.

128. **Baldini PM, Incerpi S, Zannetti A, De Vito P, Luly P**. Selective activation by atrial natriuretic factor of phosphatidylcholine-specific phospholipase activities in purified heart muscle plasma membranes. *J Mol Cell Cardiol*. 1994;26:1691-700.

129. **Brown HA, Gutowski S, Kahn RA, Sternweis PC**. Partial purification and characterization of Arf-sensitive phospholipase D from porcine brain. *J Biol Chem*. 1995;270:14935-43.

130. **Hammond SM, Altshuller YM, Sung TC, Rudge SA, Rose K, Engebrecht J, Morris AJ, Frohman MA**. Human ADP-ribosylation factor-activated phosphatidylcholine-specific phospholipase D defines a new and highly conserved gene family. *J Biol Chem*. 1995;270:29640-3.

131. **Colley WC, Sung TC, Roll R, Jenco J, Hammond SM, Altshuller Y, Bar-Sagi D, Morris AJ, Frohman MA**. Phospholipase D2, a distinct phospholipase D isoform with novel regulatory properties that provokes cytoskeletal reorganization. *Curr Biol.* 1997;7:191-201.

132. **Okamura S, Yamashita S**. Purification and characterization of phosphatidylcholine phospholipase D from pig lung. *J Biol Chem.* 1994;269:31207-13.

133. **Kobayashi M, Kanfer JN**. Phosphatidylethanol formation via transphosphatidylation by rat brain synaptosomal phospholipase D. *J Neurochem.* 1987;48:1597-603.

134. **Siddiqui RA, Exton JH**. Oleate stimulation of diacylglycerol formation from phosphatidylcholine through effects on phospholipase D and phosphatidate phosphohydrolase. *Eur J Biochem.* 1992;210:601-7.

135. **Hammond SM, Jenco JM, Nakashima S, Cadwallader K, Gu Q, Cook S, Nozawa Y, Prestwich GD, Frohman MA, Morris AJ**. Characterization of two alternately spliced forms of phospholipase D1. Activation of the purified enzymes by phosphatidylinositol 4,5- bisphosphate, ADP-ribosylation factor, and Rho family monomeric GTP- binding proteins and protein kinase C-alpha. *J Biol Chem.* 1997;272:3860-8.

136. **Katayama K, Kodaki T, Nagamachi Y, Yamashita S.** Cloning, differential regulation and tissue distribution of alternatively spliced isoforms of ADP-ribosylation-factor-dependent phospholipase D from rat liver. *Biochem J.* 1998;329:647-52.

137. **Massenburg D, Han JS, Liyanage M, Patton WA, Rhee SG, Moss J, Vaughan M.** Activation of rat brain phospholipase D by ADP-ribosylation factors 1,5, and 6: separation of ADP-ribosylation factor-dependent and oleate- dependent enzymes. *Proc Natl Acad Sci U S A.* 1994;91:11718-22.

138. **Anthes JC, Wang P, Siegel MI, Egan RW, Billah MM.** Granulocyte phospholipase D is activated by a guanine nucleotide dependent protein factor. *Biochem Biophys Res Commun.* 1991;175:236-43.

139. **Olson SC, Bowman EP, Lambeth JD.** Phospholipase D activation in a cell-free system from human neutrophils by phorbol 12-myristate 13-acetate and guanosine 5'-O-(3- thiotriphosphate). Activation is calcium dependent and requires protein factors in both the plasma membrane and cytosol. *J Biol Chem.* 1991;266:17236-42.

140. **Geny B, Fensome A, Cockcroft S.** Rat brain cytosol contains a factor which reconstitutes guanine- nucleotide-binding-protein-regulated phospholipase-D

activation in HL60 cells previously permeabilized with streptolysin O. *Eur J Biochem.* 1993;215:389-96.

141. **Balsinde J, Diez E, Fernandez B, Mollinedo F.** Biochemical characterization of phospholipase D activity from human neutrophils. *Eur J Biochem.* 1989;186:717-24.

142. **Wang P, Anthes JC, Siegel MI, Egan RW, Billah MM.** Existence of cytosolic phospholipase D. Identification and comparison with membrane-bound enzyme. *J Biol Chem.* 1991;266:14877-80.

143. **Huang C, Wykle RL, Daniel LW, Cabot MC.** Identification of phosphatidylcholine-selective and phosphatidylinositol-selective phospholipases D in Madin-Darby canine kidney cells. *J Biol Chem.* 1992;267:16859-65.

144. **Philipson KD.** Interaction of charged amphiphiles with $Na^+$-$Ca^{2+}$ exchange in cardiac sarcolemmal vesicles. *J Biol Chem.* 1984;259:13999-4002.

145. **Xu YJ, Panagia V, Shao Q, Wang X, Dhalla NS.** Phosphatidic acid increases intracellular free $Ca^{2+}$ and cardiac contractile force. *Am J Physiol.* 1996;271:H651-9.

146. **Limas CJ**. Phosphatidate releases calcium from cardiac sarcoplasmic reticulum. *Biochem Biophys Res Commun*. 1980;95:541-6.

147. **Hale CC, Ebeling EG, Hsu FF, Ford DA**. The selective acitivation of the cardiac sarcolemmal sodium-calcium exchanger by plasmalogenic phosphatidic acid produced by phospholipase D. *FEBS Lett*. 1998;422:247-251.

148. **Reich R, Blumenthal M, Liscovitch M**. Role of phospholipase D in laminin-induced production of gelatinase A (MMP-2) in metastatic cells. *Clin Exp Metastasis*. 1995;13:134-40.

149. **Williger BT, Ho WT, Exton JH**. Phospholipase D mediates matrix metalloproteinase-9 secretion in phorbol ester-stimulated human fibrosarcoma cells. *J Biol Chem*. 1999;274:735-8.

150. **Rose K, Rudge SA, Frohman MA, Morris AJ, Engebrecht J**. Phospholipase D signaling is essential for meiosis. *Proc Natl Acad Sci U S A*. 1995;92:12151-5.

151. **Williams SA, Tappia PS, Yu CH, Binaglia L, Panagia V, Dhalla NS**. Subcellular alterations in cardiac phospholipase D activity in chronic diabetes. *Prostaglandins Leukot Essent Fatty Acids*. 1997;57:95-9.

152. **Moraru, II, Jones RM, Popescu LM, Engelman RM, Das DK**. Prazosin reduces myocardial ischemia/reperfusion-induced $Ca^{2+}$ overloading in rat heart by inhibiting phosphoinositide signaling. *Biochim Biophys Acta*. 1995;1268:1-8.

153. **Cohen MV, Liu Y, Liu GS, Wang P, Weinbrenner C, Cordis GA, Das DK, Downey JM**. Phospholipase D plays a role in ischemic preconditioning in rabbit heart. *Circulation*. 1996;94:1713-8.

154. **Trifan OC, Popescu LM, Tosaki A, Cordis G, Das DK**. Ischemic preconditioning involves phospholipase D. *Ann N Y Acad Sci*. 1996;793:485-8.

155. **Gevers W**. Generation of protons by metabolic processes in heart cells. *J Mol Cell Cardiol*. 1977;9:867-74.

156. **Ellis D, Thomas RC**. Direct measurement of the intracellular pH of mammalian cardiac muscle. *J Physiol (Lond)*. 1976;262:755-71.

157. **Harold FM, Papineau D**. Cation transport and electrogenesis by Streptococcus faecalis. II. Proton and sodium extrusion. *J Membr Biol*. 1972;8:45-62.

158. **Harold FM, Papineau D**. Cation transport and electrogenesis by Streptococcus faecalis. I. The membrane potential. *J Membr Biol*. 1972;8:27-44.

159. **Marra MA, Prasad SS, Baillie DL**. Molecular analysis of two genes between let-653 and let-56 in the unc- 22(IV) region of Caenorhabditis elegans. *Mol Gen Genet*. 1993;236:289-98.

160. **Mitchel P**. Proton current flow in mitochondrial systems. *Nature*. 1967;214:1327-8.

161. **Murer H, Hopfer U, Kinne R**. Sodium/proton antiport in brush-border-membrane vesicles isolated from rat small intestine and kidney. *Biochem J*. 1976;154:597-604.

162. **Wallert MA, Frohlich O**. $Na^+$-$H^+$ exchange in isolated myocytes from adult rat heart. *Am J Physiol*. 1989;257:C207-C213.

163. **Karmazyn M, Moffat MP**. Role of $Na^+/H^+$ exchange in cardiac physiology and pathophysiology: mediation of myocardial reperfusion injury by the pH paradox. *Cardiovasc Res*. 1993;27:915-24.

164. **Sardet C, Franchi A, Pouyssegur J**. Molecular cloning, primary structure, and expression of the human growth factor-activatable $Na^+$-$H^+$ antiporter. *Cell*. 1989;56:271-280.

165. **Fliegel L**. *The $Na^+$-$H^+$ Exchanger*. Austin, Texas: R.G. Landes Company; 1996.

166. **Mitchel P**. Coupling of phosphorylation to electron and hydrogen transfer by a chemiosmotic type of mechanism. *Nature*. 1961:144-146.

167. **Mitchel PaM, J.** Respiration-driven proton translocation in rat liver mitochondria. *Biochem J*. 1967:1147-1162.

168. **Kinsella JL, Aronson PS**. Amiloride inhibition of the $Na^+$-$H^+$ exchanger in renal microvillus membrane vesicles. *Am J Physiol*. 1981;241:F374-9.

169. **Aronson PS, Nee J, Suhm MA**. Modifier role of internal $H^+$ in activating the $Na^+$-$H^+$ exchanger in renal microvillus membrane vesicles. *Nature*. 1982;299:161-3.

170. **Schuldiner S, Rozengurt E**. $Na^+/H^+$ antiport in Swiss 3T3 cells: mitogenic stimulation leads to cytoplasmic alkalinization. *Proc Natl Acad Sci U S A*. 1982;79:7778-82.

171. **Frelin C, Vigne P, Lazdunski M**. The amiloride-sensitive $Na^+/H^+$ antiport in 3T3 fibroblasts. *J Biol Chem*. 1983;258:6272-6.

172. **Moolenaar WH, Mummery CL, van der Saag PT, de Laat SW**. Rapid ionic events and the initiation of growth in serum-stimulated neuroblastoma cells. *Cell*. 1981;23:789-98.

173. **Pouyssegur J, Chambard JC, Franchi A, Paris S, Van Obberghen-Schilling.** Growth factor activation of an amiloride-sensitive $Na^+/H^+$ exchange system in quiescent fibroblasts: coupling to ribosomal protein S6 phosphorylation. *Proc Natl Acad Sci U S A.* 1982;79:3935-9.

174. **Pierce GN, Philipson KD.** $Na^+$-$H^+$ exchange in cardiac sarcolemmal vesicles. . 1985;818:109-116.

175. **Goldberg B, Arbel T, Chen J, et a.** Characterization of $Na^+$-$H^+$ antiporter gene of E.coli.*Proc.Natl.Acad.Sci.* 1987;84:2615-2619.

176. **Sardet C, Counillon L, Franchi A, Pouyssegur J.** Growth factors induce phosphorylation of the $Na^+/H^+$ antiporter, glycoprotein of 110 kD. *Science.* 1990;247:723-726.

177. **Taglicht D, Padan E, Schuldiner S.** Overproduction and purification of a functional $Na^+/H^+$ antiporter coded by nhaA (ant) from Escherichia coli. *J Biol Chem.* 1991;266:11289-94.

178. **Jia ZP, McCullough N, Martel R, Hemmingsen S, Young PG.** Gene amplification at a locus encoding a putative $Na^+/H^+$ antiporter confers sodium and lithium tolerance in fission yeast. *Embo J.* 1992;11:1631-40.

179. **Orlowski J, Kandasamy RA, Shull GE**. Molecular cloning of putative members of the Na/H exchanger gene family. cDNA cloning, deduced amino acid sequence, and mRNA tissue expression of the rat Na/H exchanger NHE-1 and two structurally related proteins. *J Biol Chem*. 1992;267:9331-9339.

180. **Klanke CA, Su YR, Callen DF, Wang Z, Meneton P, Baird N, Kandasamy RA, Orlowski J, Otterud BE, Leppert M, et a**. Molecular cloning and physical and genetic mapping of a novel human $Na^+/H^+$ exchanger (NHE5/SLC9A5) to chromosome 16q22.1. *Genomics*. 1995;25:615-622.

181. **Wang Z, Orlowski J, Shull GE**. Primary structure and functional expression of a novel gastrointestinal isoform of the rat Na/H exchanger. *J Biol Chem*. 1993;268:11925-8.

182. **Takaichi K, Wang D, Balkovetz DF, Warnock DG**. Cloning, sequencing, and expression of $Na(^+)$-$H^+$ antiporter cDNAs from human tissues. *Am J Physiol*. 1992;262:C1069-76.

183. **Tse CM, Brant SR, Walker MS, Pouyssegur J, Donowitz M**. Cloning and sequencing of a rabbit cDNA encoding an intestinal and kidney-specific $Na^+/H^+$ exchanger isoform (NHE-3). *J Biol Chem*. 1992;267:9340-6.

184. **Tse CM, Levine SA, Yun CH, Montrose MH, Little PJ, Pouyssegur J, Donowitz M.** Cloning and expression of a rabbit cDNA encoding a serum-activated ethylisopropylamiloride-resistant epithelial $Na^+/H^+$ exchanger isoform (NHE-2). *J Biol Chem.* 1993;268:11917-24.

185. **Tse CM, Ma AI, Yang VW, Watson AJ, Levine S, Montrose MH, Potter J, Sardet C, Pouyssegur J, Donowitz M.** Molecular cloning and expression of a cDNA encoding the rabbit ileal villus cell basolateral membrane $Na^+/H^+$ exchanger. *EMBO J.* 1991;10:1957-1967.

186. **Fliegel L, Haworth RS, Dyck JR.** Characterization of the placental brush border membrane $Na^+/H^+$ exchanger: identification of thiol-dependent transitions in apparent molecular size. *Biochem J.* 1993;289:101-7.

187. **Fafournoux P, Noel J, Pouyssegur J.** Evidence that $Na^+/H^+$ exchanger isoforms NHE1 and NHE3 exist as stable dimers in membranes with a high degree of specificity for homodimers. *J Biol Chem.* 1994;269:2589-96.

188. **Bertrand B, Wakabayashi S, Ikeda T, Pouyssegur J, Shigekawa M.** The $Na^+/H^+$ exchanger isoform 1 (NHE1) is a novel member of the calmodulin-binding proteins. Identification and characterization of calmodulin-binding sites. *J Biol Chem.* 1994;269:13703-9.

189. **Wakabayashi S, Bertrand B, Ikeda T, Pouyssegur J, Shigekawa M.** Mutation of calmodulin-binding site renders the $Na^+/H^+$ exchanger (NHE1) highly $H(^+)$-sensitive and $Ca^{2+}$ regulation-defective. *J Biol Chem.* 1994;269:13710-13715.

190. **Silva NL, Haworth RS, Singh D, Fliegel L.** The carboxyl-terminal region of the $Na^+/H^+$ exchanger interacts with mammalian heat shock protein. *Biochemistry.* 1995;34:10412-20.

191. **Numata M, Petrecca K, Lake N, Orlowski J.** Identification of a mitochondrial $Na^+/H^+$ exchanger. *J Biol Chem.* 1998;273:6951-9.

192. **Erhardt LR.** GUARD During Ischemia Against Necrosis (GUARDIAN) trial in acute coronary syndromes. *Am J Cardiol.* 1999;83:23G-25G.

193. **Padan E, Schuldiner S.** Molecular physiology of the $Na^+/H^+$ antiporter in Escherichia coli. *J Exp Biol.* 1994;196:443-56.

194. **Sardet C, Fafournoux P, Pouyssegur J.** Alpha-thrombin, epidermal growth factor, and okadaic acid activate the $Na^+/H^+$ exchanger, NHE-1, by phosphorylating a set of common sites. *J Biol Chem.* 1991;266:19166-71.

195. **Wakabayashi S, Fafournoux P, Sardet C, Pouyssegur J**. The $Na^+/H^+$ antiporter cytoplasmic domain mediates growth factor signals and controls "$H(^+)$-sensing". *Proc Natl Acad Sci U S A*. 1992;89:2424-8.

196. **Noel J, Pouyssegur J**. Hormonal regulation, pharmacology, and membrane sorting of vertebrate $Na^+$-$H^+$ exchanger isoforms. *Am J Physiol* . 1995;268:C283-C296.

197. **Hendey B, Mamrack MD, Putnam RW**. Thrombin induces a calcium transient that mediates an activation of the $Na^+/H^+$ exchanger in human fibroblasts. *J Biol Chem* . 1989;264:19540-19547.

198. **Hesketh TR, Moore JP, Morris JD, Taylor MV, Rogers J, Smith GA, Metcalfe JC**. A common sequence of calcium and pH signals in the mitogenic stimulation of eukaryotic cells. *Nature*. 1985;313:481-4.

199. **Karmazyn M, Gan XT, Humphreys RA, Yoshida H, Kusumoto K**. The myocardial $Na(^+)$-$H(^+)$ exchange: structure, regulation, and its role in heart disease. *Circ Res*. 1999;85:777-86.

200. **Pierce GN, Kutryk MJ, Dhalla NS**. Alterations in $Ca^{2+}$ binding by and composition of the cardiac sarcolemmal membrane in chronic diabetes. *Proc Natl Acad Sci*. 1983;80:5412-5416.

201. **Williams SA, Tappia PS, Yu CH, Bibeau M, Panagia V**. Impairment of the Sarcolemmal Phospholipase D-Phosphatidate Phosphohydrolase Pathway in Diabetic Cardiomyopathy. *J Mol Cell Card*. 1998:109-118.

202. **Makino N, Dhalla KS, Elimban V, Dhalla NS**. Sarcolemmal $Ca^{2+}$ transport in streptozotocin-induced diabetic cardiomyopathy in rats. *Am J Physiol*. 1987;253:E202-E207.

203. **Villereal ML**. Sodium fluxes in human fibroblasts: kinetics of serum-dependent and serum-independent pathways. *J Cell Physiol*. 1981;108:251-9.

204. **Villereal ML**. Sodium fluxes in human fibroblasts: effect of serum, $Ca^{+}2$, and amiloride. *J Cell Physiol*. 1981;107:359-69.

205. **Muldoon LL, Dinerstein RJ, Villereal ML**. Intracellular pH in human fibroblasts: effect of mitogens, A23187, and phospholipase activation. *Am J Physiol*. 1985;249:C140-8.

206. **Wakabayashi S, Bertrand B, Ikeda T, Pouyssegur J, Shigekawa M**. Mutation of calmodulin-binding site renders the $Na^{+}/H^{+}$ exchanger (NHE1) highly $H(^{+})$-sensitive and $Ca^{2+}$ regulation-defective. *J Biol Chem*. 1994;269:13710-5.

207. **Goss G, Orlowski J, Grinstein S.** Coimmunoprecipitation of a 24-kDa protein with NHE1, the ubiquitous isoform of the $Na^+/H^+$ exchanger. *Am J Physiol.* 1996;270:C1493-502.

208. **Wakabayashi S, Bertrand B, Shigekawa M, Fafournoux P, Pouyssegur J.** Growth factor activation and "$H(^+)$-sensing" of the $Na^+/H^+$ exchanger isoform 1 (NHE1). Evidence for an additional mechanism not requiring direct phosphorylation. *J Biol Chem.* 1994;269:5583-5588.

209. **Levine SA, Montrose MH, Tse CM, Donowitz M.** Kinetics and regulation of three cloned mammalian $Na^+/H^+$ exchangers stably expressed in a fibroblast cell line. *J Biol Chem.* 1993;268:25527-35.

210. **Frohlich O.** The NHE family of $Na^+$-$H^+$ exchangers; its known and putative members, and what can be learned by comparing them with each other. In: *The $Na^+$-$H^+$ Exchanger.* Austin, Texas: R.G Landes; 1996:295-307.

211. **Counillon L, Pouyssegur J, Reithmeier RA.** The $Na^+/H^+$ exchanger NHE-1 possesses N- and O-linked glycosylation restricted to the first N-terminal extracellular domain. *Biochemistry.* 1994;33:10463-9.

212. **Haworth RS, Frohlich O, Fliegel L.** Multiple carbohydrate moieties on the $Na^+/H^+$ exchanger. *Biochem J.* 1993;289:637-40.

213. **Geering K.** Subunit assembly and functional maturation of Na,K-ATPase. *J Membr Biol.* 1990;115:109-21.

214. **Reithmeier RA.** Mammalian exchangers and co-transporters. *Curr Opin Cell Biol.* 1994;6:583-94.

215. **Tse CM, Levine SA, Yun CH, Khurana S, Donowitz M.** $Na^+/H^+$ exchanger-2 is an O-linked but not an N-linked sialoglycoprotein. *Biochemistry.* 1994;33:12954-61.

216. **Yusufi AN, Szczepanska-Konkel M, Dousa TP.** Role of N-linked oligosaccharides in the transport activity of the $Na^+/H^+$ antiporter in rat renal brush-border membrane. *J Biol Chem.* 1988;263:13683-91.

217. **Biemesderfer D, Pizzonia J, Abu-Alfa A, Exner M, Reilly R, Igarashi P, Aronson PS.** NHE3: a $Na^+/H^+$ exchanger isoform of renal brush border. *Am J Physiol.* 1993;265:F736-42.

218. **Tse CM, Levine SA, Yun CH, Brant SR, Pouyssegur J, Montrose MH, Donowitz M.** Functional characteristics of a cloned epithelial $Na^+/H^+$ exchanger (NHE3): resistance to amiloride and inhibition by protein kinase C. *Proc Natl Acad Sci U S A.* 1993;90:9110-4.

219. **Orlowski J**. Heterologous expression and functional properties of amiloride high affinity (NHE-1) and low affinity (NHE-3) isoforms of the rat Na/H exchanger. *J Biol Chem*. 1993;268:16369-77.

220. **Yu FH, Shull GE, Orlowski J**. Functional properties of the rat Na/H exchanger NHE-2 isoform expressed in Na/H exchanger-deficient Chinese hamster ovary cells. *J Biol Chem*. 1993;268:25536-41.

221. **Orlowski JaS, G.** Characteristics of the plasma membrane $Na^+$-$H^+$ exchager gene family. In: *The $Na^+$-$H^+$ Exchanger*. Austin, Texas: R.G. Landes; 1996:123-148.

222. **Schuldiner SaP, E**. Molecular dissectioin of bacterial $Na^+$-$H^+$ antiporters. In: *The $Na^+$-$H^+$ Exchanger*. Austin, Texas: R.G. Landes; 1996:231-254.

223. **Yun CH, Little PJ, Nath SK, Levine SA, Pouyssegur J, Tse CM, Donowitz M**. Leu143 in the putative fourth membrane spanning domain is critical for amiloride inhibition of an epithelial $Na^+/H^+$ exchanger isoform (NHE-2). *Biochem Biophys Res Commun*. 1993;193:532-9.

224. **Wang D, Balkovetz DF, Warnock DG**. Mutational analysis of transmembrane histidines in the amiloride- sensitive $Na^+/H^+$ exchanger. *Am J Physiol*. 1995;269:C392-402.

225. **Aronson PS, Nee J, Suhm MA**. Modifier role of internal $H^+$ in activating the $Na^+$-$H^+$ exchanger in renal microvillus membrane vesicles. *Nature*. 1982;299:161-3.

226. **Gerchman Y, Olami Y, Rimon A, Taglicht D, Schuldiner S, Padan E**. Histidine-226 is part of the pH sensor of NhaA, a $Na^+/H^+$ antiporter in Escherichia coli. *Proc Natl Acad Sci U S A*. 1993;90:1212-6.

227. **Fliegel L, Dyck JR, Wang H, Fong C, Haworth RS**. Cloning and analysis of the human myocardial $Na^+/H^+$ exchanger. *Mol Cell Biochem*. 1993;125:137-143.

228. **Petrecca K, Atanasiu R, Grinstein S, Orlowski J, Shrier A**. Subcellular localization of the $Na^+/H^+$ exchanger NHE1 in rat myocardium. *Am J Physiol*. 1999;276:H709-17.

229. **White RL, Doeller JE, Verselis VK, Wittenberg BA**. Gap junctional conductance between pairs of ventricular myocytes is modulated synergistically by $H^+$ and $Ca^{++}$. *J Gen Physiol*. 1990;95:1061-75.

230. **Spray DC, White RL, Mazet F, Bennett MV**. Regulation of gap junctional conductance. *Am J Physiol*. 1985;248:H753-64.

231. **Brant SR, Yun CH, Donowitz M, Tse CM.** Cloning, tissue distribution, and functional analysis of the human $Na^+/N^+$ exchanger isoform, NHE3. *Am J Physiol.* 1995;269:C198-206.

232. **Mattei MG, Sardet C, Franchi A, Pouyssegur J.** The human amiloride-sensitive $Na^+/H^+$ antiporter: localization to chromosome 1 by in situ hybridization. *Cytogenet Cell Genet.* 1988;48:6-8.

233. **Lifton RP, Sardet C, Pouyssegur J, Lalouel JM.** Cloning of the human genomic amiloride-sensitive $Na^+/H^+$ antiporter gene, identification of genetic polymorphisms, and localization on the genetic map of chromosome 1p. *Genomics.* 1990;7:131-5.

234. **Szpirer C, Szpirer J, Riviere M, Levan G, Orlowski J.** Chromosomal assignment of four genes encoding Na/H exchanger isoforms in human and rat. *Mamm Genome.* 1994;5:153-9.

235. **Firek L, Weingart R.** Modification of gap junction conductance by divalent cations and protons in neonatal rat heart cells. *J Mol Cell Cardiol.* 1995;27:1633-43.

236. **Kieval RS, Bloch RJ, Lindenmayer GE, Ambesi A, Lederer WJ.** Immunofluorescence localization of the Na-Ca exchanger in heart cells. *Am J Physiol.* 1992;263:C545-50.

237. **Frank JS, Mottino G, Reid D, Molday RS, Philipson KD.** Distribution of the $Na(^{+})$-$Ca^{2+}$ exchange protein in mammalian cardiac myocytes: an immunofluorescence and immunocolloidal gold-labeling study. *J Cell Biol.* 1992;117:337-45.

238. **Poole WP.** Measurement of myocardial intracellular pH in pathological states. *J Mol Cell Cardiol.* 1978;10:511-526.

239. **Spitzer KW, Ershler PR, Skolnick RL, Vaughan-Jones RD.** Generation of intracellular pH gradients in single cardiac myocytes with a microperfusion system. *Am J Physiol Heart Circ Physiol.* 2000;278:H1371-82.

240. **Tavi P, Laine M, Voutilainen S, Lehenkari P, Vuolteenaho O, Ruskoaho H, Weckstrom M.** Potentiation of stretch-induced atrial natriuretic peptide secretion by intracellular acidosis. *Am J Physiol.* 1999;277:H405-12.

241. **Winkler MM.** Regulation of protein synthesis in sea urchin egges by intracellular pH. In: Nuccitelli R DO, ed. *Intracellular pH: It's measurement, regulation and utilization in cellular functions.* New York: Alan R. Liss; 1982:325-340.

242. **Fidelman ML, Seeholzer SH, Walsh KB, Moore RD**. Intracellular pH mediates action of insulin on glycolysis in frog skeletal muscle. *Am J Physiol.* 1982;242:C87-93.

243. **Condeelis JS, Taylor DL**. The contractile basis of amoeboid movement. V. The control of gelation, solation, and contraction in extracts from Dictyostelium discoideum. *J Cell Biol.* 1977;74:901-27.

244. **Moody WJ**. The effect of decreased intracellular pH on the electrical properties of invertebrate muscle fibers and oocytes. In: Nuccitelli R DO, ed. *Intracellular pH: its measurement, regulation and utilization in cellular functions.* New York: Alan R. Liss; 1982:427-33.

245. **Pouyssegur J, Franchi A, L'Allemain G, Paris S**. Cytoplasmic pH, a key determinant of growth factor-induced DNA synthesis in quiescent fibroblasts. *FEBS Lett.* 1985;190:115-9.

246. **Madshus IH**. Regulation of intracellular pH in eukaryotic cells. *Biochem J.* 1988;250:1-8.

247. **Trivedi B, Danforth WH**. Effect of pH on the kinetics of frog muscle phosphofructokinase. *J Biol Chem.* 1966;241:4110-2.

248. **Kaila K, Vaughan JR, Bountra C**. Regulation of intracellular pH in sheep cardiac Purkinje fibre: interactions among $Na^+$, $H^+$, and $Ca^{2+}$1. *Can J Physiol Pharmacol*. 1987;65:963-969.

249. **Kaila K, Vaughan-Jones RD**. Influence of sodium-hydrogen exchange on intracellular pH, sodium and tension in sheep cardiac Purkinje fibres. *J Physiol (Lond)*. 1987;390:93-118.

250. **Jacobus WE PI, Lucas SK, Kallman CH, Weisfeldt ML, Flaherty JT.** The role of intracellular pH in the control of normal and ischemic myocardial contractility. In: Nuccitelli R DO, ed. *Intracellular pH: its measurement, regulation, and utilization in cellular functions*. New York: Alan R. Liss; 1982:537-565.

251. **Fabiato A, Fabiato F**. Effects of pH on the myofilaments and the sarcoplasmic reticulum of skinned cells from cardiace and skeletal muscles. *J Physiol Lond*. 1978;276:233-255.

252. **Kohlhardt M, Haap K, Figulla HR**. Influence of low extracellular pH upon the Ca inward current and isometric contractile force in mammalian ventricular myocardium. *Pflugers Arch*. 1976;366:31-8.

253. **Vogel S, Sperelakis N**. Blockade of myocardial slow inward current at low pH. *Am J Physiol*. 1977;233:C99-103.

254. **Kurachi Y**. The effects of intracellular protons on the electrical activity of single ventricular cells. *Pflugers Arch*. 1982;394:264-70.

255. **Grinstein S, Cohen S**. Cytoplasmic [$Ca^{2+}$] and intracellular pH in lymphocytes. Role of membrane potential and volume-activated $Na^+/H^+$ exchange. *J Gen Physiol*. 1987;89:185-213.

256. **Krulwich TA**. $Na^+/H^+$ antiporters. *Biochim Biophys Acta*. 1983;726:245-264.

257. **Lazdunski M, Frelin C, Vigne P**. The sodium/hydrogen exchange system in cardiac cells: its biochemical and pharmacological properties and its role in regulating internal concentrations of sodium and internal pH. *J Mol Cell Cardiol*. 1985;17:1029-1042.

258. **Moolenaar WH, Boonstra J, van der Saag PT, de Laat SW**. Sodium/proton exchange in mouse neuroblastoma cells. *J Biol Chem*. 1981;256:12883-7.

259. **Paris S, Pouyssegur J**. Biochemical characterization of the amiloride-sensitive $Na^+/H^+$ antiport in Chinese hamster lung fibroblasts. *J Biol Chem*. 1983;258:3503-8.

260. **Piwnica WD, Lieberman M.** Microfluorometric monitoring of pHi in cultured heart cells: $Na^+$-$H^+$ exchange. *Am J Physiol.* 1983;244:C422-C428.

261. **Lagadic-Gossmann D, Buckler KJ, Vaughan-Jones RD.** Role of bicarbonate in pH recovery from intracellular acidosis in the guinea-pig ventricular myocyte. *J Physiol.* 1992;458:361-84.

262. **Roos A, Boron WF.** Intracellular pH. *Physiol Rev.* 1981;61:296-434.

263. **Vaughan-Jones RD.** Regulation of chloride in quiescent sheep-heart Purkinje fibres studied using intracellular chloride and pH-sensitive micro-electrodes. *J Physiol.* 1979;295:111-37.

264. **Periyasamy SM, Kakar SS, Garlid KD, Askari A.** Ion specificity of cardiac sarcolemmal $Na^+/H^+$ antiporter. *J Biol Chem.* 1990;265:6035-41.

265. **Pierce GN.** Cationic interactions with $Na^+$-$H^+$ exchange and passive $Na^+$ flux in cardiac sarcolemmal vesicles. *Mol Cell Biochem.* 1987;78:89-94.

266. **Cabado AG, Yu FH, Kapus A, Lukacs G, Grinstein S, Orlowski J.** Distinct structural domains confer cAMP sensitivity and ATP dependence to the $Na^+/H^+$ exchanger NHE3 isoform. *J Biol Chem.* 1996;271:3590-9.

267. **Wakabayashi S, Shigekawa M, Pouyssegur J.** Molecular physiology of vertebrate $Na^+/H^+$ exchangers. *Physiol Rev.* 1997;77:51-74.

268. **Kapus A, Grinstein S, Wasan S, Kandasamy R, Orlowski J.** Functional characterization of three isoforms of the $Na^+/H^+$ exchanger stably expressed in Chinese hamster ovary cells. ATP dependence, osmotic sensitivity, and role in cell proliferation. *J Biol Chem.* 1994;269:23544-52.

269. **Cassel D, Katz M, Rotman M.** Depletion of cellular ATP inhibits $Na^+/H^+$ antiport in cultured human cells. Modulation of the regulatory effect of intracellular protons on the antiporter activity. *J Biol Chem.* 1986;261:5460-6.

270. **Weissberg PL, Little PJ, Cragoe-EJ J, Bobik A.** The pH of spontaneously beating cultured rat heart cells is regulated by an ATP-calmodulin-dependent $Na^+/H^+$ antiport. *Circ Res.* 1989;64:676-685.

271. **Cingolani HE, Alvarez BV, Ennis IL, Camilion de Hurtado MC.** Stretch-induced alkalinization of feline papillary muscle: an autocrine- paracrine system. *Circ Res.* 1998;83:775-80.

272. **Duan J, Moffat MP.** Contractile and electrophysiologic effects of realkalization in cardiac tissues: role of Na/H exchange and increased [Ca]i. *Adv Exp Med Biol.* 1992;311:435-436.

273. **Duan J, Karmazyn M.** Protective effects of amiloride on the ischemic reperfused rat heart. Relation to mitochondrial function. *Eur J Pharmacol.* 1992;210:149-157.

274. **Scholz W, Albus U.** Potential of selective sodium-hydrogen exchange inhibitors in cardiovascular therapy. *Cardiovasc Res.* 1995;29:184-8.

275. **Scholz W, Albus U, Counillon L, Gogelein H, Lang HJ, Linz W, Weichert A, Scholkens BA.** Protective effects of HOE642, a selective sodium-hydrogen exchange subtype 1 inhibitor, on cardiac ischaemia and reperfusion. *Cardiovasc Res.* 1995;29:260-8.

276. **Bond JM, Chacon E, Herman B, Lemasters JJ.** Intracellular pH and $Ca^{2+}$ homeostasis in the pH paradox of reperfusion injury to neonatal rat cardiac myocytes. *Am J Physiol.* 1993;265:C129-C137.

277. **Khandoudi N, Moffat MP, Karmazyn M.** Adenosine-sensitive alpha 1-adrenoceptor effects on reperfused ischaemic hearts: comparison with phorbol ester. *Br J Pharmacol.* 1994;112:1007-16.

278. **Yasutake M, Avkiran M.** Exacerbation of reperfusion arrhythmias by alpha 1 adrenergic stimulation: a potential role for receptor mediated activation of sarcolemmal sodium-hydrogen exchange. *Cardiovasc Res.* 1995;29:222-230.

279. **Tani M, Neely JR.** Role of intracellular $Na^+$ in $Ca^{2+}$ overload and depressed recovery of ventricular function of reperfused ischemic rat hearts. Possible involvement of $H^+$-$Na^+$ and $Na^+$-$Ca^{2+}$ exchange. *Circ Res.* 1989;65:1045-1056.

280. **Karmazyn M, Ray M, Haist JV.** Comparative effects of $Na^+/H^+$ exchange inhibitors against cardiac injury produced by ischemia/reperfusion, hypoxia/reoxygenation, and the calcium paradox. *J Cardiovasc Pharmacol.* 1993;21:172-8.

281. **Hendrikx M, Mubagwa K, Verdonck F, Overloop K, Van HP, Vanstapel F, Van LA, Verbeken E, Lauweryns J, Flameng W.** New $Na(^+)$-$H^+$ exchange inhibitor HOE 694 improves postischemic function and high-energy phosphate resynthesis and reduces $Ca^{2+}$ overload in isolated perfused rabbit heart. *Circulation.* 1994;89:2787-2798.

282. **Klein HH, Pich S, Bohle RM, Wollenweber J, Nebendahl K.** Myocardial protection by $Na(^+)$-$H^+$ exchange inhibition in ischemic, reperfused porcine hearts. *Circulation.* 1995;92:912-917.

283. **Moffat MP, Karmazyn M.** Protective effects of the potent Na/H exchange inhibitor methylisobutyl amiloride against post-ischemic contractile dysfunction in rat and guinea-pig hearts. *J Mol Cell Cardiol.* 1993;25:959-71.

284. **Meng HP, Pierce GN**. Protective effects of 5-(N,N-dimethyl)amiloride on ischemia-reperfusion injury in hearts. *Am J Physiol*. 1990;258:H1615-H1619.

285. **Meng HP, Maddaford TG, Pierce GN**. Effect of amiloride and selected analogues on postischemic recovery of cardiac contractile function. *Am J Physiol*. 1993;264:H1831-H1835.

286. **Murphy E, Perlman M, London RE, Steenbergen C**. Amiloride delays the ischemia-induced rise in cytosolic free calcium. *CircRes*. 1991;68:1250-1258.

287. **Bugge E, Ytrehus K**. Inhibition of sodium-hydrogen exchange reduces infarct size in the isolated rat heart--a protective additive to ischaemic preconditioning. *Cardiovasc Res*. 1995;29:269-274.

288. **Faes FC, Sawa Y, Ichikawa H, Shimazaki Y, Ohashi T, Fukuda H, Shirakura R, Matsuda H**. Inhibition of $Na^+/H^+$ exchanger attenuates neutrophil-mediated reperfusion injury. *Ann Thorac Surg*. 1995;60:377-381.

289. **Myers ML, Karmazyn M**. Improved cardiac function after prolonged hypothermic ischemia with the $Na^+/H^+$ exchange inhibitor HOE 694. *Ann Thorac Surg*. 1996;61:1400-6.

290. **Duff HJ, Lester WM, Rahmberg M.** Amiloride. Antiarrhythmic and electrophysiological activity in the dog. *Circulation.* 1988;78:1469-1477.

291. **Khandoudi N, Bernard M, Cozzone P, Feuvray D.** Intracellular pH and role of $Na^+/H^+$ exchange during ischaemia and reperfusion of normal and diabetic rat hearts. *Cardiovasc Res.* 1990;24:873-878.

292. **Weiss RG, Lakatta EG, Gerstenblith G.** Effects of amiloride on metabolism and contractility during reoxygenation in perfused rat hearts. *Circ Res.* 1990;66:1012-1022.

293. **Dennis SC, Coetzee WA, Cragoe-EJ J, Opie LH.** Effects of proton buffering and of amiloride derivatives on reperfusion arrhythmias in isolated rat hearts. Possible evidence for an arrhythmogenic role of $Na(^+)$-$H^+$ exchange. *Circ Res.* 1990;66:1156-1159.

294. **Sack S, Mohri M, Schwarz ER, Arras M, Schaper J, Ballagi PG, Scholz W, Lang HJ, Scholkens BA, Schaper W.** Effects of a new $Na^+/H^+$ antiporter inhibitor on postischemic reperfusion in pig heart. *J Cardiovasc Pharmacol.* 1994;23:72-78.

295. **Mochizuki S, Ischikawa S, Abe M.** Insulin treatment and myocardial function in isolated perfused hearts from diabetic rat. *Jpn Circ J.* 1984:255-265.

296. **Karmazyn M.** $Na^+/H^+$ exchange inhibitors reverse lactate-induced depression in postischaemic ventricular recovery. *Br J Pharmacol.* 1993;108:50-6.

297. **Hata K, Takasago T, Saeki A, Nishioka T, Goto Y.** Stunned myocardium after rapid correction of acidosis. Increased oxygen cost of contractility and the role of the $Na(^+)$-$H^+$ exchange system. *Circ Res.* 1994;74:794-805.

298. **Myers ML, Mathur S, Li GH, Karmazyn M.** Sodium-hydrogen exchange inhibitors improve postischemic recovery of function in the perfused rabbit heart. *Cardiovasc Res.* 1995;29:209-214.

299. **Ward CA, Moffat MP.** Modulation of sodium-hydrogen exchange activity in cardiac myocytes during acidosis and realkalinisation: effects on calcium, pHi, and cell shortening. *Cardiovasc Res.* 1995;29:247-253.

300. **Ladilov YV, Siegmund B, Piper HM.** Protection of reoxygenated cardiomyocytes against hypercontracture by inhibition of $Na^+/H^+$ exchange. *Am J Physiol.* 1995;268:H1531-H1539.

301. **Kaplan SH, Yang H, Gilliam DE, Shen J, Lemasters JJ, Cascio WE.** Hypercapnic acidosis and dimethyl amiloride reduce reperfusion induced cell death in ischaemic ventricular myocardium. *Cardiovasc Res.* 1995;29:231-238.

302. **Pierce GN, Meng H**. The role of sodium-proton exchange in ischemic/reperfusion injury in the heart. $Na(^{+})$-$H^{+}$ exchange and ischemic heart disease. *Am J Cardiovasc Pathol.* 1992;4:91-102.

303. **Meng H, Pierce GN**. Involvement of sodium in the protective effect of 5-(N,N-dimethyl)-amiloride on ischemia-reperfusion injury in isolated rat ventricular wall. *J Pharmacol Exp Ther.* 1991;256:1094-1100.

304. **Johnson JD, Epel D**. Intracellular pH and activation of sea urchin eggs after fertilisation. *Nature.* 1976;262:661-4.

305. **L'Allemain G, Franchi A, Cragoe E, Jr., Pouyssegur J**. Blockade of the $Na^{+}/H^{+}$ antiport abolishes growth factor-induced DNA synthesis in fibroblasts. Structure-activity relationships in the amiloride series. *J Biol Chem.* 1984;259:4313-9.

306. **O'Donnell ME, Cragoe E, Jr., Villereal ML**. Inhibition of $Na^{+}$ influx and DNA synthesis in human fibroblasts and neuroblastoma-glioma hybrid cells by amiloride analogs. *J Pharmacol Exp Ther.* 1983;226:368-72.

307. **Zhuang Y, Cragoe EJ, Jr., Shaikewitz T, Glaser L, Cassel D**. Characterization of potent $Na^{+}/H^{+}$ exchange inhibitors from the amiloride series in A431 cells. *Biochemistry.* 1984;23:4481-8.

308. **Vigne P, Frelin C, Cragoe EJ, Jr., Lazdunski M.** Structure-activity relationships of amiloride and certain of its analogues in relation to the blockade of the $Na^+/H^+$ exchange system. *Mol Pharmacol.* 1984;25:131-6.

309. **Yun CH, Gurubhagavatula S, Levine SA, Montgomery JL, Brant SR, Cohen ME, Cragoe EJ, Jr., Pouyssegur J, Tse CM, Donowitz M.** Glucocorticoid stimulation of ileal $Na^+$ absorptive cell brush border $Na^+/H^+$ exchange and association with an increase in message for NHE-3, an epithelial $Na^+/H^+$ exchanger isoform. *J Biol Chem.* 1993;268:206-11.

310. **Counillon L, Franchi A, Pouyssegur J.** A point mutation of the $Na^+/H^+$ exchanger gene (NHE1) and amplification of the mutated allele confer amiloride resistance upon chronic acidosis. *Proc Natl Acad Sci U S A.* 1993;90:4508-12.

311. **Counillon L, Scholz W, Lang HJ, Pouyssegur J.** Pharmacological characterization of stably transfected $Na^+/H^+$ antiporter isoforms using amiloride analogs and a new inhibitor exhibiting anti-ischemic properties. *Mol Pharmacol.* 1993;44:1041-5.

312. **Kinsella JL, Aronson PS.** Interaction of $NH4^+$ and $Li^+$ with the renal microvillus membrane $Na^+$-$H^+$ exchanger. *Am J Physiol.* 1981;241:C220-6.

313. **Vigne P, Frelin C, Lazdunski M.** The amiloride-sensitive $Na^+/H^+$ exchange system in skeletal muscle cells in culture. *J Biol Chem.* 1982;257:9394-400.

314. **Ives HE, Yee VJ, Warnock DG.** Mixed type inhibition of the renal $Na^+/H^+$ antiporter by $Li^+$ and amiloride. Evidence for a modifier site. *J Biol Chem.* 1983;258:9710-6.

315. **Cragoe E.** *Amiloride and Epithelial Sodium Transport.* Baltimore-Munich: Urban and Schwarzenberg; 1979.

316. **Kleyman TR, Cragoe-EJ J.** Amiloride and its analogs as tools in the study of ion transport. *J Membr Bio.* 1988;105:1-21.

317. **Dubinsky WP, Jr., Frizzell RA.** A novel effect of amiloride on $H^+$-dependent $Na^+$ transport. *Am J Physiol.* 1983;245:C157-9.

318. **Moolenaar WH, Tertoolen LG, de Laat SW.** Phorbol ester and diacylglycerol mimic growth factors in raising cytoplasmic pH. *Nature.* 1984;312:371-4.

319. **Reuss L, Petersen KU.** Cyclic AMP inhibits $Na^+/H^+$ exchange at the apical membrane of Necturus gallbladder epithelium. *J Gen Physiol.* 1985;85:409-29.

320. **Cuthbert AW, Fanelli GM**. Effects of some pyrazinecarboxamides on sodium transport in frog skin. *Br J Pharmacol.* 1978;63:139-49.

321. **Kaczorowski GJ, Barros F, Dethmers JK, Trumble MJ, Cragoe EJ, Jr.** Inhibition of $Na^+/Ca^{2+}$ exchange in pituitary plasma membrane vesicles by analogues of amiloride. *Biochemistry.* 1985;24:1394-403.

322. **Cook JS, Shaffer C, Cragoe EJ, Jr.** Inhibition by amiloride analogues of $Na^+$-dependent hexose uptake in LLC- PK1/Cl4 cells. *Am J Physiol.* 1987;253:C199-204.

323. **Bhalla RC, Sharma RV**. Competitive interaction of amiloride and verapamil with alpha 1- adrenoceptors in vascular smooth muscle. *J Cardiovasc Pharmacol.* 1986;8:927-32.

324. **Bielefeld DR, Hadley RW, Vassilev PM, Hume JR**. Membrane electrical properties of vesicular Na-Ca exchange inhibitors in single atrial myocytes. *Circ Res.* 1986;59:381-9.

325. **Soltoff SP, Mandel LJ**. Amiloride directly inhibits the Na,K-ATPase activity of rabbit kidney proximal tubules. *Science.* 1983;220:957-8.

326. **Benos DJ, Reyes J, Shoemaker DG**. Amiloride fluxes across erythrocyte membranes. *Biochim Biophys Acta*. 1983;734:99-104.

327. **Velly J, Grima M, Decker N, Cragoe EJ, Jr., Schwartz J**. Effects of amiloride and its analogues on [3H]batrachotoxinin-A 20- alpha benzoate binding, [3H]tetracaine binding and 22Na influx. *Eur J Pharmacol*. 1988;149:97-105.

328. **Benos DJ**. Amiloride: a molecular probe of sodium transport in tissues and cells. *Am J Physiol*. 1982;242:C131-45.

329. **Pierce GN, Cole WC, Liu K, Massaeli H, Maddaford TG, Chen YJ, McPherson CD, Jain S, Sontag D**. Modulation of cardiac performance by amiloride and several selected derivatives of amiloride. *J Pharmacol Exp Ther*. 1993;265:1280-91.

330. **Ganapathy V, Balkovetz DF, Miyamoto Y, Ganapathy ME, Mahesh VB, Devoe LD, Leibach FH**. Inhibition of human placental $Na^+$-$H^+$ exchanger by cimetidine. *J Pharmacol Exp Ther*. 1986;239:192-7.

331. **Kulanthaivel P, Leibach FH, Mahesh VB, Cragoe EJ, Jr., Ganapathy V**. The $Na(^+)$-$H^+$ exchanger of the placental brush-border membrane is pharmacologically distinct from that of the renal brush-border membrane. *J Biol Chem*. 1990;265:1249-52.

332. **Ganapathy ME, Leibach FH, Mahesh VB, Devoe LD, Ganapathy V.** Interaction of clonidine with human placental $Na^+$ -$H^+$ exchanger. *Biochem Pharmacol.* 1986;35:3989-94.

333. **Aronson PS, Bounds SE.** Harmaline inhibition of Na-dependent transport in renal microvillus membrane vesicles. *Am J Physiol.* 1980;238:F210-7.

334. **Knickelbein R, Aronson PS, Atherton W, Dobbins JW.** Sodium and chloride transport across rabbit ileal brush border. I. Evidence for Na-H exchange. *Am J Physiol.* 1983;245:G504-10.

335. **Balkovetz DF, Miyamoto Y, Tiruppathi C, Mahesh VB, Leibach FH, Ganapathy V.** Inhibition of brush-border membrane $Na^+$-$H^+$ exchanger by loperamide. *J Pharmacol Exp Ther.* 1987;243:150-4.

336. **Scholz W, Albus U, Lang HJ, Linz W, Martorana PA, Englert HC, Scholkens BA.** Hoe 694, a new $Na^+/H^+$ exchange inhibitor and its effects in cardiac ischaemia. *Br J Pharmacol.* 1993;109:562-568.

337. **Scholz W, Albus U, Counillon L, Gogelein H, Lang HJ, Linz W, Weichert A, Scholkens BA.** Protective effects of HOE642, a selective sodium-hydrogen exchange subtype 1 inhibitor, on cardiac ischaemia and reperfusion. *Cardiovasc Res.* 1995;29:260-268.

338. **Hoque AN, Karmazyn M**. Effect of sodium-hydrogen exchange inhibition on functional and metabolic impairment produced by oxidative stress in the isolated rat heart. *Can J Physiol Pharmacol.* 1997;75:326-34.

339. **Grace AA, Kirschenlohr HL, Metcalfe JC, Smith GA, Weissberg PL, Cragoe EJ, Jr., Vandenberg JI**. Regulation of intracellular pH in the perfused heart by external HCO3- and $Na^+$-$H^+$ exchange. *Am J Physiol.* 1993;265:H289-98.

340. **Kusuoka H, Marban E, Cingolani HE**. Control of steady-state intracellular pH in intact perfused ferret hearts. *J Mol Cell Cardiol.* 1994;26:821-9.

341. **Camilion de Hurtado MC, Perez NG, Cingolani HE**. An electrogenic sodium-bicarbonate cotransport in the regulation of myocardial intracellular pH. *J Mol Cell Cardiol.* 1995;27:231-42.

342. **Fliegel L, Frohlich O**. The $Na^+/H^+$ exchanger: an update on structure, regulation and cardiac physiology. *Biochem J.* 1993;296:273-285.

343. **Xu P, Spitzer KW**. Na-independent Cl(-)-HCO3- exchange mediates recovery of pHi from alkalosis in guinea pig ventricular myocytes. *Am J Physiol.* 1994;267:H85-91.

344. **Nakanishi T, Gu H, Seguchi M, Cragoe EJ, Jr., Momma K.** HCO3(-)-dependent intracellular pH regulation in the premature myocardium. *Circ Res.* 1992;71:1314-23.

345. **Khandoudi N, Ho J, Karmazyn M.** Role of $Na(^{+})$-$H^{+}$ exchange in mediating effects of endothelin-1 on normal and ischemic/reperfused hearts. *Circ Res.* 1994;75:369-378.

346. **Lucchesi P, Berk B.** Regulation of sodium-hydrogen exchange in vascular smooth muscle. *Cardiovasc Res.* 1995;29:172-177.

347. **Yasutake M, Haworth RS, King A, Avkiran M.** Thrombin activates the sarcolemmal $Na(^{+})$-$H^{+}$ exchanger. Evidence for a receptor-mediated mechanism involving protein kinase C. *Circ Res.* 1996;79:705-15.

348. **Yun C, Tse C-M, Nath S, et a.** Structure/function studies of mammalian $Na^{+}$-$H^{+}$ exchanger isoforms-an update. *J Physiol Lond.* 1995;482:1S-6S.

349. **Avkiran M, Snabaitis AK.** Regulation of cardiac sarcolemmal $Na^{+}/H^{+}$ exchanger activity: potential pathophysiological significance of endogenous mediators and oxidant stress. *J Thromb Thrombolysis.* 1999;8:25-32.

350. **Ito N, Kagaya Y, Weinberg EO, Barry WH, Lorell BH**. Endothelin and angiotensin II stimulation of $Na^+$-$H^+$ exchange is impaired in cardiac hypertrophy. *J Clin Invest*. 1997;99:125-35.

351. **Wallert MA, Frohlich O**. Alpha 1-adrenergic stimulation of Na-H exchange in cardiac myocytes. *Am J Physiol*. 1992;263:C1096-102.

352. **Puceat M**. Stimulation of sarcolemmal sodium-hydrogen exchange in cardiac myocytes is not responsible for the positive inotropic action of alpha 1 adrenergic agonists. *Cardiovasc Res*. 1995;29:275-6.

353. **Yokoyama H, Yasutake M, Avkiran M**. Alpha1-adrenergic stimulation of sarcolemmal $Na^+$-$H^+$ exchanger activity in rat ventricular myocytes: evidence for selective mediation by the alpha1A-adrenoceptor subtype. *Circ Res*. 1998;82:1078-85.

354. **Ennis IL, Alvarez BV, Camilion de Hurtado MC, Cingolani HE**. Enalapril induces regression of cardiac hypertrophy and normalization of pHi regulatory mechanisms. *Hypertension*. 1998;31:961-7.

355. **Little PJ, Weissberg PL, Cragoe EJ, Jr., Bobik A**. Dependence of $Na^+/H^+$ antiport activation in cultured rat aortic smooth muscle on calmodulin, calcium,

and ATP. Evidence for the involvement of calmodulin-dependent kinases. *J Biol Chem.* 1988;263:16780-6.

356. **Rood RP, Emmer E, Wesolek J, McCullen J, Husain Z, Cohen ME, Braithwaite RS, Murer H, Sharp GW, Donowitz M.** Regulation of the rabbit ileal brush-border $Na^+/H^+$ exchanger by an ATP- requiring $Ca^{++}$/calmodulin-mediated process. *J Clin Invest.* 1988;82:1091-7.

357. **Sabri A, Byron KL, Samarel AM, Bell J, Lucchesi PA.** Hydrogen peroxide activates mitogen-activated protein kinases and $Na^+$- $H^+$ exchange in neonatal rat cardiac myocytes. *Circ Res.* 1998;82:1053-62.

358. **Grinstein S, Cohen S, Goetz JD, Rothstein A.** Osmotic and phorbol ester-induced activation of $Na^+/H^+$ exchange: possible role of protein phosphorylation in lymphocyte volume regulation. *J Cell Biol.* 1985;101:269-76.

359. **Grinstein S, Rothstein A, Cohen S.** Mechanism of osmotic activation of $Na^+/H^+$ exchange in rat thymic lymphocytes. *J Gen Physiol.* 1985;85:765-87.

360. **Hoffmann EK, Simonsen LO.** Membrane mechanisms in volume and pH regulation in vertebrate cells. *Physiol Rev.* 1989;69:315-82.

361. **Strange K.** Maintenance of cell volume in the central nervous system. *Pediatr Nephrol.* 1993;7:689-97.

362. **Grinstein S, Foskett JK.** Ionic mechanisms of cell volume regulation in leukocytes. *Annu Rev Physiol.* 1990;52:399-414.

363. **Eveloff JL, Warnock DG.** Activation of ion transport systems during cell volume regulation. *Am J Physiol.* 1987;252:F1-10.

364. **Rotin D, Grinstein S.** Impaired cell volume regulation in $Na(^{+})$-$H^{+}$ exchange-deficient mutants. *Am J Physiol.* 1989;257:C1158-65.

365. **Parker JC.** Volume-responsive sodium movements in dog red blood cells. *Am J Physiol.* 1983;244:C324-30.

366. **Cala PM.** Cell volume regulation by Amphiuma red blood cells. The role of $Ca^{+}2$ as a modulator of alkali metal/$H^{+}$ exchange. *J Gen Physiol.* 1983;82:761-84.

367. **Rotin D, Steele-Norwood D, Grinstein S, Tannock I.** Requirement of the $Na^{+}/H^{+}$ exchanger for tumor growth. *Cancer Res.* 1989;49:205-11.

368. **Grinstein S, Woodside M, Sardet C, Pouyssegur J, Rotin D**. Activation of the $Na^+/H^+$ antiporter during cell volume regulation. Evidence for a phosphorylation-independent mechanism. *J Biol Chem*. 1992;267:23823-8.

369. **Grinstein S, Woodside M, Goss GG, Kapus A**. Osmotic activation of the $Na^+/H^+$ antiporter during volume regulation. *Biochem Soc Trans*. 1994;22:512-6.

370. **Winkel GK, Sardet C, Pouyssegur J, Ives HE**. Role of cytoplasmic domain of the $Na^+/H^+$ exchanger in hormonal activation. *J Biol Chem*. 1993;268:3396-400.

371. **Bianchini L, Kapus A, Lukacs G, Wasan S, Wakabayashi S, Pouyssegur J, Yu FH, Orlowski J, Grinstein S**. Responsiveness of mutants of NHE1 isoform of $Na^+/H^+$ antiport to osmotic stress. *Am J Physiol*. 1995;269:C998-1007.

372. **Rutherford JD, Braunwald E, Cohn PF**. Chronic Ischemic Heart Disease. In: Braunwald, E, Ed,. *Heart Disease.A Textbook of Cardiovascular Medicine*. 1988;2:1314-1378.

373. **Foundation HaS**. Deaths from cardiovascular disease. In: *Cardiovascular disease in Canada*; 1991:3-15.

374. **Wigle D, Mao Y, Wong T, Lane R.** Economic burden of illness in Canada. *Chron Dis Can*. 1991;12:1-37.

375. Canadian Task force for Cardiovascular Science. *Canadian Journal of Cardiology*. 1993;9:699-709.

376. **Stanley WC, Lopaschuk GD, McCormack JG**. Regulation of energy substrate metabolism in the diabetic heart. *Cardiovasc Res*. 1997;34:25-33.

377. **Opie LH**. Metabolism of free fatty acids, glucose and catecholamines in acute myocardial infarction. Relation to myocardial ischemia and infarct size. *Am J Cardiol*. 1975;36:938-953.

378. **Neely JR, Grotyohann LW**. Role of glycolytic products in damage to ischemic myocardium. Dissociation of adenosine triphosphate levels and recovery of function of reperfused ischemic hearts. *Circ Res*. 1984;55:816-824.

379. **Pierce GN, Czubryt MP**. The contribution of ionic imbalance to ischemia/reperfusion-induced injury. *J Mol Cell Cardiol*. 1995;27:53-63.

380. **Hearse DJ, Humphrey SM, Bullock GR**. The oxygen paradox and the calcium paradox: two facets of the same problem? *J Mol Cell Cardiol*. 1978;10:641-668.

381. **Nayler WG**. Calcium antagonists and the ischemic myocardium. *Int J Cardiol*. 1987;15:267-285.

382. **Shen AC, Jennings RB**. Kinetics of calcium accumulation in acute myocardial ischemic injury. *Am J Pathol*. 1972;67:441-452.

383. **Piwnica WD, Jacob R, Shigeto N, Horres CR, Lieberman M**. Na/H exchange in cultured chick heart cells: secondary stimulation of electrogenic transport during recovery from intracellular acidosis. *J Mol Cell Cardiol*. 1986;18:1109-1116.

384. **Kim D, Cragoe-EJ, Smith TW**. Relations among sodium pump inhibition, Na-Ca and Na-H exchange activities and Ca-H interactions in cultured chick heart cells. *Circ Res*. 1987:185-193.

385. **Siffert W, Akkerman JWN**. $Na^+/H^+$ exchange and $Ca^{2+}$ influx. *FEBS Lett*. 1989:1-4.

386. **Maddaford TG, Pierce GN**. Myocardial dysfunction is associated with activation of $Na^+/H^+$ exchange immediately during reperfusion. *Am J Physiol*. 1997;273:H2232-H2239.

387. **Hurtado C, Pierce GN**. Inhibition of $Na(^+)/H(^+)$ exchange at the beginning of reperfusion is cardioprotective in isolated, beating adult cardiomyocytes. *J Mol Cell Cardiol*. 2000;32:1897-907.

388. **Rupprecht HJ, Dahl J, Terres W, Seyfarth KM, Richardt G, Schultheibeta HP, Buerke M, Sheehan FH, Drexler H.** Cardioprotective effects of the $Na(^+)/H(^+)$ exchange inhibitor cariporide in patients with acute anterior myocardial infarction undergoing direct PTCA [In Process Citation]. *Circulation.* 2000;101:2902-8.

389. **Dostal DE, Baker KM.** Angiotensin and endothelin: messengers that couple ventricular stretch to the $Na^+/H^+$ exchanger and cardiac hypertrophy [editorial]. *Circ Res.* 1998;83:870-3.

390. **Kranzhofer R, Schirmer J, Schomig A, von Hodenberg E, Pestel E, Metz J, Lang HJ, Kubler W.** Suppression of neointimal thickening and smooth muscle cell proliferation after arterial injury in the rat by inhibitors of $Na(^+)$- $H^+$ exchange. *Circ Res.* 1993;73:264-8.

391. **Mitsuka M, Nagae M, Berk BC.** $Na(^+)$-$H^+$ exchange inhibitors decrease neointimal formation after rat carotid injury. Effects on smooth muscle cell migration and proliferation. *Circ Res.* 1993;73:269-75.

392. **Yamazaki T, Komuro I, Kudoh S, Zou Y, Nagai R, Aikawa R, Uozumi H, Yazaki Y.** Role of ion channels and exchangers in mechanical stretch-induced cardiomyocyte hypertrophy. *Circ Res.* 1998;82:430-7.

393. **Peiro C, Angulo J, Llergo JL, Rodriguez-Manas L, Marin J, Sanchez-Ferrer CF.** Angiotensin II mediates cell hypertrophy in vascular smooth muscle cultures from hypertensive Ren-2 transgenic rats by an amiloride- and furosemide-sensitive mechanism. *Biochem Biophys Res Commun.* 1997;240:367-71.

394. **Yoshida H, Karmazyn M.** $Na(^{+})/H(^{+})$ exchange inhibition attenuates hypertrophy and heart failure in 1-wk postinfarction rat myocardium. *Am J Physiol Heart Circ Physiol.* 2000;278:H300-4.

395. **Skolnick RL, Litwin SE, Barry WH, Spitzer KW.** Effect of ANG II on pHi, $[Ca^{2+}]i$, and contraction in rabbit ventricular myocytes from infarcted hearts. *Am J Physiol.* 1998;275:H1788-97.

396. **Entmacher PS RH, Arks HH.** Longevity of diabetic patients in recent years. *Diabetes.* 1964;13:373-377.

397. **Sievers J, Bloomquist, Biork.** Studies on myocardial infarction in Malmo 1935-1954. *Acta Med Scand.* 1961;169:95-103.

398. **Goldenberg S AM, Blumenthal HT.** Sequelae of arteriosclerosis of the aorta and coronary arteries. *Diabetes.* 1958;7:107.

399. **Clauson BJ BE**. Incidence of fatal coronary disease in non-diabetic and in diabetic persons. *Arch Pathol.* 1949;48:105-109.

400. **Stone PH, Muller JE, Hartwell T, York BJ, Rutherford JD, Parker CB, Turi ZG, Strauss HW, Willerson JT, Robertson T, et al**. The effect of diabetes mellitus on prognosis and serial left ventricular function after acute myocardial infarction: contribution of both coronary disease and diastolic left ventricular dysfunction to the adverse prognosis. The MILIS Study Group. *J Am Coll Cardiol.* 1989;14:49-57.

401. **Lauria MH KJ, Wachs JS, Luria MA.** Survival after recovery from acute myocardial infarction: two and five year prognostic indices. *J Am Med Assoc.* 1979;260:3456-3460.

402. **Abbot RD DR, Kannel WB, Wilson PW.** The impact of diabetes on survival following myocardial infarction, the Framingham study. *J Am Med Assoc.* 1988;260:3456-3460.

403. **Beard CM, Kottke TE, Annegers JF, Ballard DJ**. The Rochester Coronary Heart Disease Project: effect of cigarette smoking, hypertension, diabetes, and steroidal estrogen use on coronary heart disease among 40- to 59-year-old women, 1960 through 1982. *Mayo Clin Proc.* 1989;64:1471-1480.

404. **Howard BV, Lee ET, Fabsitz RR, Robbins DC, Yeh JL, Cowan LD, Welty TK.** Diabetes and coronary heart disease in American Indians: The Strong Heart Study. *Diabetes.* 1996;45 Suppl 3:S6-13.

405. Effect of diabetes mellitus on short and long-term mortality rates of patients with acute myocardial infarction: A statewide study. 1995;130:51-58.

406. **Will JC, Casper M.** The contribution of diabetes to early deaths from ischemic heart disease: US gender and racial comparisons. *Am J Public Health.* 1996;86:576-579.

407. **Krolewski AS, Warram JH.** Epidemiology of Diabetes Mellitus. *Joslin's Diabetes Mellitus.* 1985:12-43.

408. **Gamble J, Lopaschuk GD.** Glycolysis and glucose oxidation during reperfusion of ischemic hearts from diabetic rats. *Biochim Biophys Acta.* 1994;1225:191-199.

409. **Lopaschuk GD, Saddik M, Barr R, Huang L, Barker CC, Muzyka RA.** Effects of high levels of fatty acids on functional recovery of ischemic hearts from diabetic rats. *Can J Physiol Pharmacol.* 1992;263:E1046-E1053.

410. **Tani M, Neely JR.** Hearts from diabetic rats are more resistant to in vitro ischemia: possible role of altered $Ca^{2+}$ metabolism. *Circ Res.* 1988;62:931-940.

411. **Khandoudi N, Bernard M, Cozzone P, Feuvray D.** Mechanisms of intracellular pH regulation during postischemic reperfusion of diabetic rat hearts. *Diabetes.* 1995;44:196-202.

412. **Tilton RG, Daugherty A, Sutera SP, Larson KB, Land MP, Rateri DL, Kilo C, Williamson JR.** Myocyte contracture, vascular resistance, and vascular permeability after global ischemia in isolated hearts from alloxan-induced diabetic rabbits. *Diabetes.* 1989;38:1484-1491.

413. **Liu Y, Thornton JD, Cohen MV, Downey JM, Schaffer SW.** Streptozotocin-induced non-insulin-dependent diabetes protects the heart from infarction. *Circulation.* 1993;88:1273-1278.

414. **Tosaki A, Engelman DT, Engelman RM, Das DK.** Diabetes and ATP-sensitive potassium channel openers and blockers in isolated ischemic/reperfused hearts. *J Pharmacol Exp Ther.* 1995;275:1115-1123.

415. **Tosaki A, Engelman DT, Engelman RM, Das DK.** The evolution of diabetic response to ischemia/reperfusion and preconditioning in isolated working rat hearts. *Cardiovasc Res.* 1996;31:526-536.

416. **Kusama Y, Hearse DJ, Avkiran M.** Diabetes and susceptibility to reperfusion-induced ventricular arrhythmias. *J Mol Cell Cardiol.* 1992;24:411-421.

417. **Garland PB, Randle PJ**. Regulation of glucose uptake by muscles. 10. Effects of alloxan-diabetes, starvation, hypophysectomy and adrenalectomy, and of fatty acids, ketone bodies and pyruvate, on the glycerol output and concentrations of free fatty acids, long-chain fatty acyl-coenzyme A, glycerol phosphate and citrate-cycle intermediates in rat heart and diaphragm muscles. *Biochem J.* 1964;93:678-687.

418. **Dyck JR, Lopaschuk GD**. Glucose metabolism, $H^+$ production and $Na^+/H^+$-exchanger mRNA levels in ischemic hearts from diabetic rats. *Mol Cell Biochem.* 1998;180:85-93.

419. **Goodale WT, Olson RE, Hackel DB**. The effects of fasting and diabetes mellitus on myocardial metabolism in man. *Am J Medicine.* 1959:212-220.

420. **Randle PJ, Hales CN, Garland PB, Newsholme EA**. The glucose fatty acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. *Lancet.* 1963;1:785-789.

421. **Randle PJ**. Alpha-ketoglutarate dehydrogenase complexes and respiratory fuel utilization in diabetes. *Diabetologia.* 1985:479-484.

422. **Higuchi M, Miyagi K, Nakasone J, Sakanashi M.** Role of high glycogen in underperfused diabetic rat hearts with added norepinephrine. *J Cardiovasc Pharmacol.* 1995;26:899-907.

423. **Cross HR, Opie LH, Radda GK, Clarke K.** Is a high glycogen content beneficial or detrimental to the ischemic rat heart? *Circ Res.* 1996:482-491.

424. **Pierce GN, Slotin T, Fliegel L, Gilchrist JSC, Maddaford TG.** Expression of Activity of the Sodium-Hydrogen exchanger in Cardiac Sarcolemma in Health and Disease. *Molecular Biology Intelligence Unit: The $Na^+$-$H^+$ Exchanger.* 1996:217-228.

425. **Kinsella J, Sacktor B.** Thyroid hormones increase $Na^+$-$H^+$ exchange activity in renal brush border membranes. *Proc Natl Acad Sci.* 1985;82:3606-3610.

426. **Pierce GN, Beamish RE, Dhalla NS.** Heart dysfunction in diabetes. *CRC Press, Boca Raton, Fla.* 1988:1-245.

427. **Putnam RW.** Effect of insulin on intracellular pH in frog skeletal muscle fibers. *Am J Physiol.* 1985:C330-C336.

428. **Le PK, Lagadic GD, Feuvray D.** Modulation by pH0 and intracellular $Ca^{2+}$ of $Na(^{+})$-$H^{+}$ exchange in diabetic rat isolated ventricular myocytes. *Circ Res.* 1997;80:253-260.

429. **Hayashi H, Noda N.** Cytosolic $Ca^{2+}$ concentration decreases in diabetic rat myocytes. *Cardiovasc Res.* 1997;34:99-103.

430. **Pierce GN, Ramjiawan B, Dhalla NS, Ferrari R.** $Na(^{+})$-$H^{+}$ exchange in cardiac sarcolemmal vesicles isolated from diabetic rats. *Am J Physiol.* 1990;258:H255-H261.

431. **Hollenberg M, Mangano DT.** Therapeutic approaches to postoperative ischemia. The study of preoperative Ischemia Research Group. *Am J Cardiol.* 1994:30B-33B.

432. **Goldschmid MG, Barrett Connor E, Edelstein SL, et a.** Dyslipidemia and ischemic heart disease mortality among men and women with diabetes. *Circulation.* 1994:991-997.

433. **Takeda R, Shimizu M.** Ischemic heart disease in patients with diabetes mellitus in Japan. *Diabetes Res.Clin.Pract.* 1994:S199-S203.

434. **Maddaford TG, Russell JC, Pierce GN.** Postischemic cardiac performance in the insulin-resistant JCR:LA-cp rat. *Am J Physiol.* 1997:1187-1192.

435. **Paulson DJ.** The diabetic heart is more sensitive to ischemic injury. *Cardiovasc Res.* 1997;34:104-112.

436. **Tannock IF.** The relation between cell proliferation and the vascular system in a transplanted mouse mammary tumour. *Br J Cancer.* 1968;22:258-73.

437. **Tannock IF, Rotin D.** Acid pH in tumors and its potential for therapeutic exploitation. *Cancer Res.* 1989;49:4373-84.

438. **Wike-Hooley JL, Haveman J, Reinhold HS.** The relevance of tumour pH to the treatment of malignant disease. *Radiother Oncol.* 1984;2:343-66.

439. **Vaupel P, Kallinowski F, Okunieff P.** Blood flow, oxygen and nutrient supply, and metabolic microenvironment of human tumors: a review. *Cancer Res.* 1989;49:6449-65.

440. **Newell K, Franchi A, Pouyssegur J, Tannock I.** Studies with glycolysis-deficient cells suggest that production of lactic acid is not the only cause of tumor acidity. *Proc Natl Acad Sci U S A.* 1993;90:1127-31.

441. **Pouyssegur J, Sardet C, Franchi A, L'Allemain G, Paris S**. A specific mutation abolishing $Na^+/H^+$ antiport activity in hamster fibroblasts precludes growth at neutral and acidic pH. *Proc Natl Acad Sci U S A*. 1984;81:4833-7.

442. **Reshkin SJ, Bellizzi A, Albarani V, Guerra L, Tommasino M, Paradiso A, Casavola V**. Phosphoinositide 3-kinase is involved in the tumor-specific activation of human breast cancer cell $Na(^+)/H(^+)$ exchange, motility, and invasion induced by serum deprivation. *J Biol Chem*. 2000;275:5361-9.

443. **Fliss H, Gattinger D**. Apoptosis in ischemic and reperfused rat myocardium. *Circ Res*. 1996;79:949-56.

444. **Veinot JP, Gattinger DA, Fliss H**. Early apoptosis in human myocardial infarcts. *Hum Pathol*. 1997;28:485-92.

445. **Gottlieb RA, Gruol DL, Zhu JY, Engler RL**. Preconditioning rabbit cardiomyocytes: role of pH, vacuolar proton ATPase, and apoptosis. *J Clin Invest*. 1996;97:2391-8.

446. **Chakrabarti S, Hoque AN, Karmazyn M**. A rapid ischemia-induced apoptosis in isolated rat hearts and its attenuation by the sodium-hydrogen exchange inhibitor HOE 642 (cariporide). *J Mol Cell Cardiol*. 1997;29:3169-74.

447. **Humphreys RA, Haist JV, Chakrabarti S, Feng Q, Arnold JM, Karmazyn M.** Orally administered NHE1 inhibitor cariporide reduces acute responses to coronary occlusion and reperfusion. *Am J Physiol.* 1999;276:H749-57.

448. **Massaeli H, Pierce GN.** Methods for measuring sodium-hydrogen exchange in the heart. In: McNeil J, ed. *Biochemical Techniques in the Heart: Methods In Pharmacology.* Boca Raton: CRC Press; 1997:83-100.

449. **Dai J, Liu S, Panagia V.** Alterations of cis unsaturated fatty acid-activated phospholipase D in ischemic-reperfused hearts. *J Mol Cell Cardiol.* 1999;31:A29.

450. **Zachowski A.** Phospholipids in animal eukaryotic membranes: transverse asymmetry and movement. *Biochem Journal.* 1993;294:1-14.

451. **Karmazyn M.** Mechanisms of protection of the ischemic and reperfused myocardium by sodium-hydrogen exchange inhibition. *J Thromb Thrombolysis.* 1999;8:33-8.

452. **Tosaki A, Maulik N, Cordis G, Trifan OC, et a.** Ischemic preconditioning triggers phospholipase D signaling in rat heart. *Am J Physiol.* 1997;273:H1860-H1866.

453. **Kimura Y, Iyengar R, Subramanian R, et a**. Preconditioning of the heart by repeated stunning attenuation of post-ischemic dysfunction. *Basic Res Cardiol.* 1992;87:128-138.

454. **Lawson CS, Coltart DJ, Hearse DJ**. The antiarrhymic action of ischemic preconditioning in rat hearts does not involve functional Gi proteins. *Cardiovasc Res.* 1993;27:681-687.

455. **Li GC, Vasquez BS, Gallagher KP, Lucchesi BR**. Myocardial protection with preconditioning. *Circulation.* 1990;82:609-619.

456. **Hoque AN, Haist JV, Karmazyn M**. $Na(^{+})$-$H^{+}$ exchange inhibition protects against mechanical, ultrastructural, and biochemical impairment induced by low concentrations of lysophosphatidylcholine in isolated rat hearts. *Circ Res.* 1997;80:95-102.

457. **Golfman L HT, Netticadan T, Panagia V, Dhalla N.S.** Modification of cardiac sarcolemmal $Na^{+}$-$Ca^{2+}$ exchange by lysophosphatidylcholine and palmitoylcarnitine. *Cardiovascular Pathobiology.* 1998;2:181-185.

458. **Carluccio MA, Massaro M, Bonfrate C, Siculella L, Maffia M, Nicolardi G, Distante A, Storelli C, De Caterina R**. Oleic acid inhibits endothelial activation

: A direct vascular antiatherogenic mechanism of a nutritional component in the mediterranean diet. *Arterioscler Thromb Vasc Biol.* 1999;19:220-8.

459. **Goel DP, Pierce GN.** Role of the sodium-hydrogen exchanger in ischemia-reperfusion injury in diabetes. *J Thromb Thrombolysis.* 1999;8:45-52.

460. **Singer SJ, Nicolson GL.** The fluid mosaic model of the structure of cell membranes. *Science.* 1972 Feb 18:175(23) 720-731.

461. **Kutryk MJ, Maddaford TG, Ramjiawan B, Pierce GN.** Oxidation of membrane cholesterol alters active and passive transsarcolemmal $Ca^{2+}$ movement. *Circ Res.* 1991 Jan: 68 (1): 18-26.

462. **Piwnica-Worms D, Lieberman M.** Microfluorometric monitoring of $pH_i$ in cultured heart cells: $Na^{+}$-$H^{+}$ exchange. *Am J Physiol.* 1983; 244: C422-C428