1996

Na+-H+ Exchange and Na+-Li+ Exchange in Human Erythrocytes: An Nmr Investigation

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Na^+-H^+ EXCHANGE AND Na^+-Li^+ EXCHANGE IN HUMAN ERYTHROCYTES

AN NMR INVESTIGATION

A DISSERTATION SUBMITTED TO

THE FACULTY OF THE GRADUATE SCHOOL

IN CANDIDACY FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

DEPARTMENT OF CHEMISTRY

BY

YULING CHI

CHICAGO, ILLINOIS

MAY 1996

ACKNOWLEDGEMENTS

I am grateful to my advisor, Dr. Duarte E. Mota de Freitas, for all his brilliant guidance, his encouragement, his financial support and his willingness to help at any time and in many aspects. I would especially like to thank him for teaching me how to think logically, how to identify and solve specific problems, and how to be organized and work efficiently. Under his guidance, the knowledge I have got and the accomplishment I have achieved will be a wonderful starting point in my professional life.

My grateful appreciation goes to my committee members, Dr. David Crumrine, Dr. Patrick M. Henry, and Dr. Vinod K. Bansal, for their excellent suggestions and constructive criticism during the course of this project. I would also like to thank my former committee member, Dr. Charles Thompson for his valuable suggestions on my research projects.

I would like to thank Dr. George Michel in Department of Psychology, DePaul University and Dr. Gerald M. Funk in Department of Mathematical Sciences, Loyola University of Chicago for their help with the statistical analysis.

I am most thankful to Dr. David Crumrine and to the former NMR manager, Christopher Clifford, for helping me solve the NMR problems on Sundays. I would also like to thank the current NMR manager, David French, for his valuable suggestions and willingness to help.

I sincerely thank my labmates, Cherian Zachariah, Wanrong Lin, Chandra Srinivasan, Louis V. Amari, Hanan Hasan, Joyce Nikolakopoulos, Suilan Mo, Dr. Qinfen Rong, Dr.

Ravi Ramasamy, and especially Dr. Elizabeth Dorus for their wonderful suggestions, encouragement, and great help.

I would like to acknowledge the support of all faculty, staff and all the graduate students in the Chemistry Department. I would like to thank the Graduate School for the financial support.

Special thanks go to my family for their special love, their understanding, and their never ending encouragement throughout my life, especially during these four years for me to pursue the Ph.D. degree.

To my dear mother and father

LIST OF PUBLICATIONS

1. Yuling Chi, Duarte Mota de Freitas, Mary Sikora, Vinod K. Bansal: **Correlations of Na^+-Li^+ Exchange Activity with Na^+ and Li^+ Binding and Phospholipid Composition in Erythrocytes of Essential Hypertensive Patients and Normotensive Individuals.** *Hypertension* (In press).

2. Yuling Chi, Suilan Mo, Duarte Mota de Freitas: **Na^+-H^+ and Na^+-Li^+ Exchange Are Mediated By the Same RBC Membrane Transport Protein: An NMR Investigation.** (Manuscript in preparation to be submitted to *Biochemistry*).

3. Duarte Mota de Freitas, Suilan Mo, Yuling Chi: **Na^+-H^+ Exchange Across Human Erythrocyte Membranes as Probed by Multinuclear NMR Spectroscopy.** *Biophysical Journal*, 64: A401, 1993 (Abstract).

4. Duarte Mota de Freitas, Suilan Mo, Yuling Chi: **Na^+-H^+ Exchange Across Human Erythrocyte Membranes as Probed by Multinuclear NMR Spectroscopy.** *Journal of Inorganic Biochemistry*, 51: 570, 1993 (Abstract).

5. Yuling Chi, Duarte Mota de Freitas, Mary Sikora, Vinod K. Bansal: **Correlations of Na^+-Li^+ Exchange Activity with Na^+ and Li^+ Binding and Phospholipid Composition in Erythrocytes of Essential Hypertensive Patients and Normotensive Individuals.** *American Journal of Hypertension*, 8: 122A, 1995 (Abstract).

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HEPES	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
HMA	5-(N,N-hexamethylene)-amiloride
HPLC	high-performance liquid chromatography
Ht	hematocrit
^{7}Li	lithium-7 isotope
MES	2-[N-morpholino]ethanesulfonic acid
MIA	5-(N-methyl-N-isobutyl)-amiloride
MIR	modified inversion recovery
MOPS	3-[N-morpholino]propanesulfonic acid
mOsm	mmol/kg
NHE	Na^{+}-H^{+} exchange
NMR	nuclear magnetic resonance
PC	phosphatidyl choline
PE	phosphatidyl ethanolamine
PEp	PE plasmalogen
PI	phosphatidyl inositol
P_i	inorganic phosphate
PS	phosphatidyl serine
PW	pulse width
RBC	red blood cell
^{23}Na	sodium-23 isotope
SEM	standard error of mean
Sph	sphingomyelin

SR	shift reagent
SW	spectral width
T_1	spin-lattice relaxation time
T_2	spin-spin relaxation time
τ_c	correlation time
TLC	thin-layer chromatography
TRIS	tris-(hydroxymethyl)-aminomethane
TTHA	triethylenetetraminehexaacetate
UV/Vis	ultraviolet-visible spectrophotometry

ABSTRACT

Na^+-Li^+ exchange was proposed as the mode of Na^+-H^+ exchange in human body. ^7Li NMR investigation of Li^+ efflux across H^+- and Li^+-loaded RBC ghosts in the presence and absence of a pH gradient indicated that Na^+-H^+ exchange and Na^+-Li^+ exchange competed against each other for transport by the same membrane protein when they occurred simultaneously.

Amiloride and its analogues are specific Na^+-H^+ exchange inhibitors. Significant inhibitory effects of these inhibitors on the kinetic parameters of Na^+-Li^+ exchange suggested that both Na^+-H^+ and Na^+-Li^+ exchange are mediated by the same transport protein. Phloretin is an effective Na^+-Li^+ exchange inhibitor. Both phloretin and HMA inhibited Li^+ efflux from Li^+-loaded RBC ghosts. These data also proved that the protein mediating Na^+-Li^+ exchange is the same as the protein mediating Na^+-H^+ exchange.

Detailed investigation of the interactions of H^+, Li^+, and Na^+ with RBC membranes showed that these three cations bind to the same binding site of the RBC membrane. The observation of similar amiloride binding constants to the RBC membranes with Na^+ and Li^+ present indicated that amiloride had the similar inhibitory effects on Na^+ and Li^+ binding to RBC membranes. All of these results suggested that Na^+-Li^+ exchange and Na^+-H^+ exchange are mediated by the same RBC membrane protein.

It has been reported that both Na^+-H^+ and Na^+-Li^+ exchange activities were enhanced in essential hypertension. In this study we found that Na^+ binding to the RBC membranes

from essential hypertensives was weaker than from normotensives. Using ^{31}P NMR, we found that the percentage of PS was higher for the hypertensive group than for the normotensive group; the percentage of PE was lower for the hypertensive than for the normotensive individuals. In intact RBCs, increased kinetic parameters were observed in essential hypertension. Therefore we concluded that changes in lipid-protein interactions in the RBC membrane of hypertensive patients appear to be responsible for weaker Na^+ binding to the membrane, and for the faster rates of RBC Na^+-Li^+ exchange.

CHAPTER I

INTRODUCTION

I.1. Na^+-H^+ Exchange in Human Erythrocytes

Na^+-H^+ exchange was first discovered in 1976. Not only because it is an ubiquitous transport system, but also because it is involved in a variety of cellular functions, a lot of attention has been devoted to the investigation of Na^+-H^+ exchange (Nakhoul and Boron, 1988). It is a transport system widely distributed in erythrocytes which plays an important role in regulating intracellular pH, cellular volume, and transmembrane ion transport (Nakhoul and Boron, 1988). Figure 1 shows how Na^+-H^+ exchange coupled with chloride-bicarbonate exchange results in net movement of NaCl into the cell to regulate intracellular pH and cell volume (Nakhoul and Boron, 1988). The role of Na^+-H^+ exchange in stimulus-response coupling and cell proliferation is depicted in Figure 2, in which there is a potential link between Na^+-H^+ exchange, Ca^{2+} mobilization, and protein kinase C (Rosskopf et al., 1993).

The Na^+-H^+ exchanger has been found to be an integral membrane protein in all eukaryotic cells (Rosskopf et al., 1993). The Na^+-H^+ exchanger promotes an electroneutral 1:1 exchange of intracellular H^+ for extracellular Na^+ driven by the inwardly-directed Na^+ gradient established by the Na^+/K^+ ATPase (Rosskopf et al., 1993). Na^+-H^+ exchange is one of the two independent pathways for passive sodium movement across the vesicle (Wigham

Figure 1. Schematic Diagram of Na^+-H^+ Exchange Coupled with Chloride-bicarbonate Exchange to Regulate Intracellular pH and Cell Volume (adapted from reference by Nakhoul and Boron, 1988).

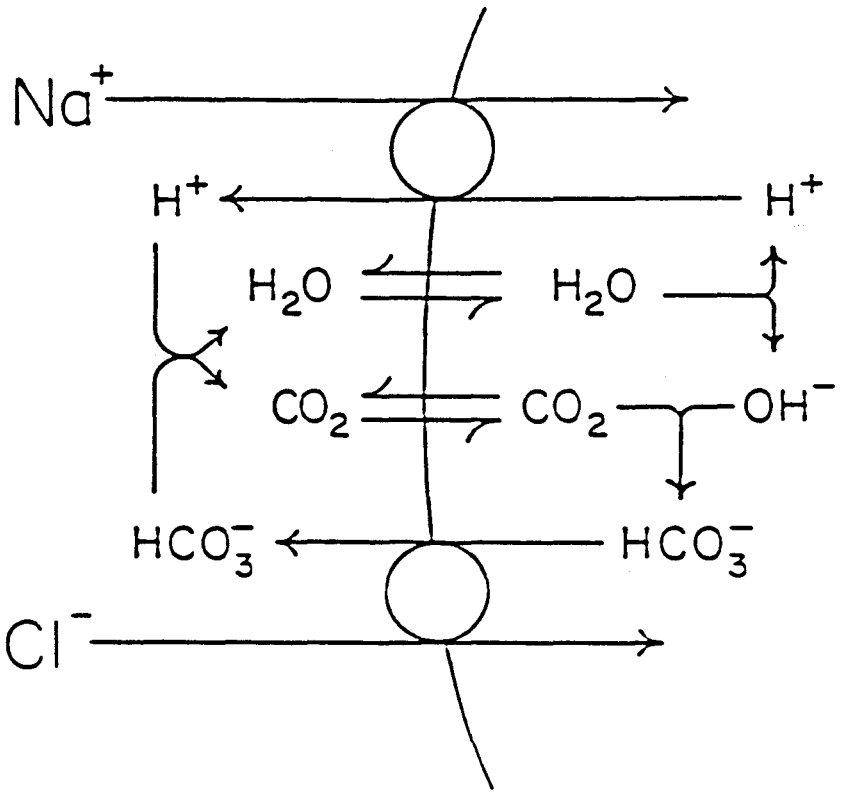
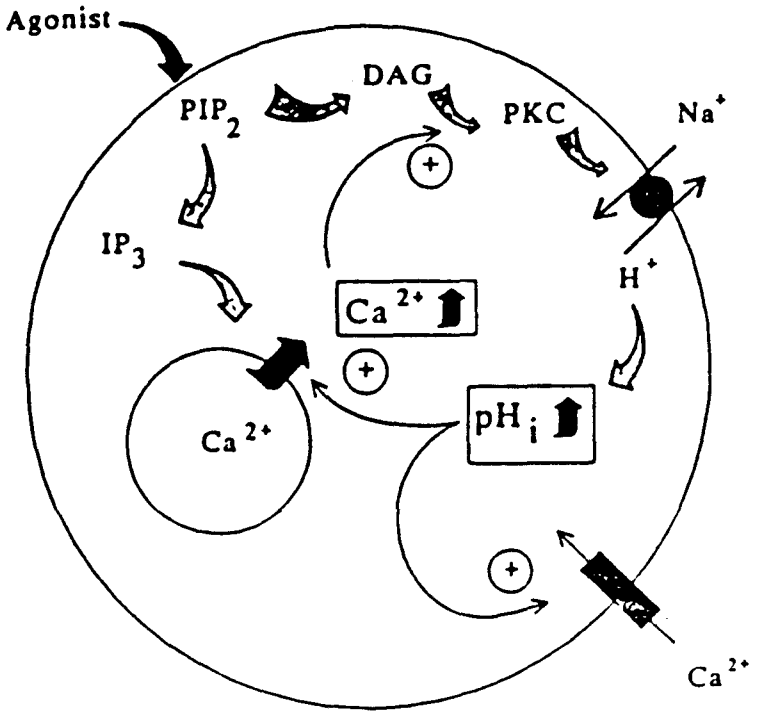

Na+
H+
H+
H2O
H2O
CO2
CO2
OH−
HCO3−
HCO3−
Cl−

Figure 2. Interaction Between Na^+-H^+ Exchange and Ca^{2+} Mobilization (adapted from reference by Rosskopf et al., 1993).


Agonist
PIP2
DAG
PKC
Na+
IP3
H+
Ca2+
Ca2+
pHi
Ca2+

et al., 1994). There are two types of Na^+-H^+ exchanger which have been reported, one is pH-dependent and the other is pH-independent (Kuroda et al., 1994). Na^+-H^+ exchange removes H^+ from the cell in exchange for extracellular Na^+. It is activated by the transmembrane pH gradient. Internal H^+, independent of its role as a substrate for exchange with external Na^+, has an important modifier role as an allosteric activator of the Na^+-H^+ exchanger. Allosteric behavior with respect to internal H^+ is a property that enhances the ability of plasma membrane Na^+-H^+ exchangers to extrude intracellular acid loads and thereby contributes to the regulation of intracellular pH (Aronson et al., 1982). The presence of the proton activation site has been revealed by investigating the dependence of Na^+-H^+ exchange activity on internal H^+ which is very sigmoidal and has a high Hill coefficient (n = 2-3) (Canessa, 1989); Na^+-H^+ exchange activity greatly increased with an increase of pH in the assay medium. pH did not affect the K_m value of the Na^+-H^+ exchange, but did increase the V_{max} value (Kuroda et al., 1994). Sodium has low-affinity single class binding sites on the outside of red blood cell (RBC) membranes. The number of Na^+-H^+ exchanger sites was estimated at 500 per human platelet and 2000 per rat thymocyte (Rosskopf et al., 1993). Kinetic studies indicated that amiloride can block 60 to 80 % of H^+-induced Na^+ influx (Escobales and Canessa, 1986). Further investigations indicated that amiloride strongly inhibits the pH-dependent exchanger (Kuroda et al., 1994). The activity of the exchanger appears to be modulated by many biological agents, including hormones, growth factors, and tumor-promoting agents. Na^+-H^+ exchange modes are also regulated by physiological levels of insulin (Canessa et al., 1993).

Molecular biological studies resulted in the cloning of the Na^+-H^+ exchanger by genetic complementation of mutants lacking Na^+-H^+ exchange activity with human genomic DNA. Several NHE (Na^+-H^+ exchange) isoforms have been found. NHE-1 is a

phosphoglycoprotein of 815 amino acids (Sardet et al., 1989). The gene encoding for NHE-1 is located on the short arm of chromosome 1 (Dudley et al., 1990; Lifton et al., 1990; Mattei et al., 1988), and its structure, including intron-exon organization, has recently been determined (Miller et al., 1991). Besides NHE-1, Collins et al. (Collins et al., 1993) have cloned a second isoform of the Na^+/H^+ exchanger, NHE-2. Recently, the isoform, NHE-3, has been cloned. It encoded a protein of 834 amino acids with a calculated relative molecular weight of 92,906 (Brant et al., 1995). The putative isoform, NHE-4, has been postulated (Rosskopf et al., 1993).

It was first found in 1952 that the Na^+ content in arterial tissue is elevated in essential hypertension (Tobian and Binion, 1952). Na^+ extrusion systems are very important because high intracellular Na^+ is unfavorable for the cells. One of the major Na^+ extrusion systems in Vibrios, the respiratory Na^+ pump, only functions under alkaline pH conditions. Therefore, another Na^+ extrusion system, the Na^+-H^+ exchange, must be crucial for the cells, especially under neutral pH conditions where the respiratory Na^+ pump may not function (Kuroda et al., 1994). Na^+-H^+ exchange may raise blood pressure by increasing intracellular Na^+ concentration or pH in renal proximal tubules and vascular smooth muscle (Nakhoul and Boron, 1988). It has been found that the increased Na^+-H^+ activity in platelets is significantly positively correlated with the occurrence of essential hypertension in humans (Livne et al., 1987). Na^+-H^+ exchange can be up-regulated by salt-sensitive elevation of blood pressure (Pontremoli et al., 1992). Recent research suggested that the Na^+-H^+ exchange pathway provides an important new mechanism involved in ischemic/reperfusion damage. There is evidence supporting the involvement of Na^+-H^+ exchange in reperfusion (Pierce and Meng, 1992). Increased Na^+-H^+ exchange activity can occur by an increase in the number of transport sites or by an increased turnover induced by increased

phosphorylation activated by cytosolic Ca^{2+} and protein kinases A and C (Canessa et al., 1993). The maximal rate of Na^+-H^+ exchange is related to the fluidity of the membrane and serum cholesterol (Carr et al., 1993).

Because the RBC membrane transport protein is asymmetric with higher ionic affinities on the intracellular side of the membrane than on the extracellular side (Hannaert and Garay, 1986; Sarkadi et al., 1978), and because the affinities of H^+ and Na^+ to RBC membranes are highly asymmetric ($H^+ \gg Na^+$), the outside membrane is not saturated by Na^+ during the process of Na^+-H^+ exchange. The rate measured by the standard method (Canessa, 1989) is not the maximal rate. Na^+-H^+ exchange follows first order kinetics (Carr et al., 1993). The rate constant, as a characteristic of an ion transport system, is expressed in the following way:

$$v = k[M^+] \quad (1)$$

where v is the flux rate of the metal ion M^+, $[M^+]$ is the external M^+ concentration in the influx transport process or the internal concentration in the efflux transport experiments. Replaced by the Michaelis-Menten equation, equation (1) can be changed to equation (2):

$$v = V_{max}[M^+]/(K_m + [M^+]) \quad (2)$$

where V_{max} is the real maximal rate, and K_m is dissociation constant of the metal ion M^+ to the RBC membranes. In the case of Na^+-H^+ exchange, the kinetic parameters can also be analyzed by the following equation (Carr et al., 1993):

$$[Na^+]/(\text{rate of pH change}) = (K_m + [Na^+])/V_{max} \quad (3)$$

I.2. Na^+-Li^+ Countertransport in Human Erythrocytes

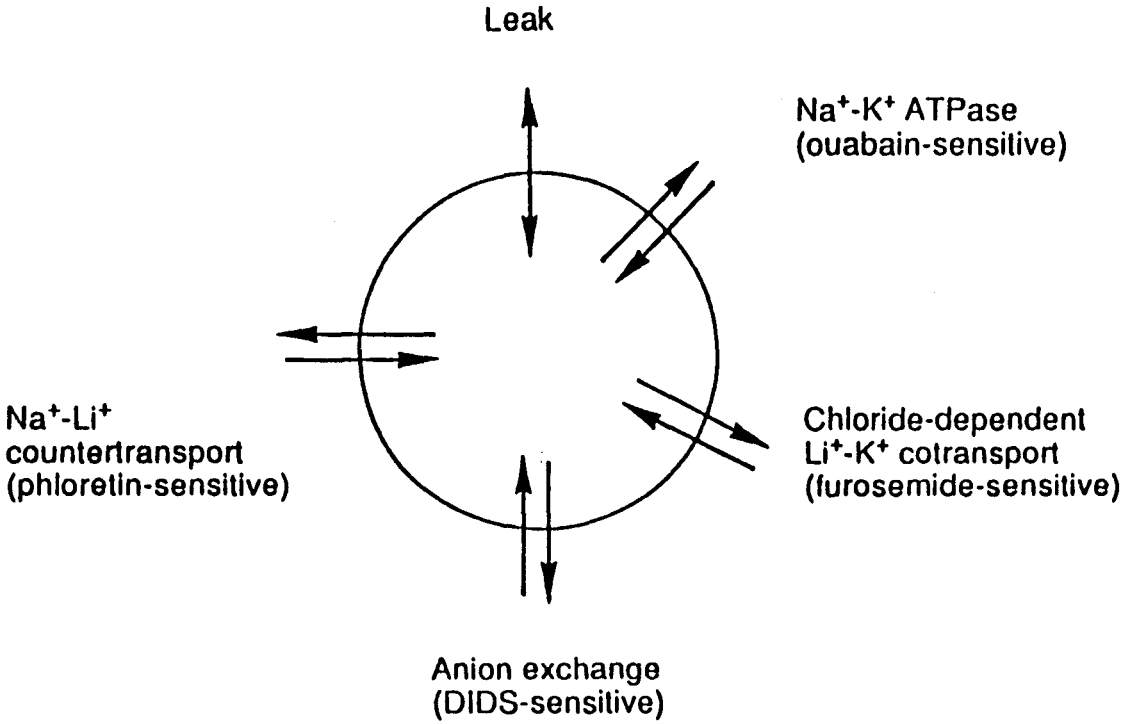
There is no lithium in the human body. After Cade first successfully introduced lithium in the treatment of mania in 1949 (Cade, 1949), many studies have aimed to investigate

lithium transport in the human body. There are five lithium transport pathways in human RBCs (Figure 3): Na^+-Li^+ countertransport, K^+-Li^+-Cl^- cotransport, anion exchange, the Na^+, K^+-ATPase, and a residual Li^+ pathway (Duhm, 1992). Because of its high reproducibility, Na^+-Li^+ countertransport has been studied in the human population in more detail than any other ion transport mechanism. It was found that Na^+-Li^+ countertransport is sensitive to pH and temperature (Rutherford et al, 1992a). It can be inhibited by phloretin (Pandey, et al., 1978). Inhibitin has recently been reported as a specific inhibitor of Na^+-Na^+ and Na^+-Li^+ exchange (Morgan et al., 1989).

Dorus et al. (Dorus et al., 1983) have found evidence for an autosomal major gene locus encoding a polypeptide chain of a RBC membrane protein responsible for Na^+-Li^+ exchange for which a polymorphism occurred. There are strong positive correlations between Na^+-Li^+ countertransport and polygenes or a major gene (Williams et al., 1988); the variation in Na^+-Li^+ countertransport was explained 34.4% by a major locus and 45.9% by polygenic inheritance giving a total of 80.3% due to genetic factors (Hasstedt et al., 1988). Studies on Na^+-Li^+ countertransport and on the family history of hypertension indicated that Na^+-Li^+ countertransport can be a marker of the genetic predisposition to primary hypertension in childhood (Houtman et al., 1993).

In 1980, Canessa et al. (Canessa et al., 1980a) first reported that Na^+-Li^+ countertransport was greater in patients with essential hypertension than in normotensive individuals. High mean levels of Na^+-Li^+ countertransport have been found consistently in essential hypertensives compared with normotensive individuals by many groups all over the world (Motulsky et al., 1987). The abnormal Na^+-Li^+ exchange activity has also been found in other diseases such as hyperlipidemia and diabetes, and it has also been reported for women taking oral contraceptives and in late pregnancy (Rutherford et al., 1992a). Blacks

Figure 3. Li^+ Transport Pathways in Human RBC (adapted from reference by Mota de Freitas et al., 1991).


Leak
Na+-K+ ATPase
(ouabain-sensitive)
Na+-Li+
countertransport
(phloretin-sensitive)
Chloride-dependent
Li+-K+ cotransport
(furosemide-sensitive)
Anion exchange
(DIDS-sensitive)

generally have lower values of Na^+-Li^+ countertransport than whites; elevated countertransport is, however, a characteristic in most black hypertensives (Weder, 1993). A positive association between Na^+-Li^+ countertransport and blood pressure has been found (Turner et al., 1985). It was reported that a person with a high countertransport value was 1.3-1.9 times more likely to develop hypertension than a person with a low value (Laurenzi and Trevisan, 1989). Subjects with an elevated maximal activity for RBC Na^+-Li^+ countertransport are a subset of the population with a genetic lesion that predisposes them to the development of essential hypertension (Weder et al., 1991). Increased RBC Na^+-Li^+ can be considered as a possible intermediate phenotype for hypertension (Weder, 1993). Na^+-Li^+ countertransport has been used to classify essential hypertensives in clinical examination (de al Sierra et al., 1993).

Prior to 1990, the maximal rates of Na^+-Li^+ exchange in human RBCs were generally assayed by measuring the rates of Li^+ efflux from Li^+-loaded RBCs into Na^+-containing and Na^+-free media; the maximal rates of RBC Na^+-Li^+ exchange were then calculated by subtraction of the measured rates of Li^+ transport in the Na^+-free medium from those measured in the Na^+-containing medium (Canessa et al., 1980a). It was recently found that, under the standard transport assay conditions, Li^+ is present at a saturating concentration, but Na^+ may not be (Rutherford et al., 1990; Rutherford et al., 1992a). The dissociation constants (K_m) for extracellular Na^+ are, for at least some individuals, of the same order of magnitude as the extracellular Na^+ concentration (140 mM) used in the standard transport assay, suggesting that the RBC Na^+-Li^+ exchange protein is far from saturated with Na^+ on the extracellular side of the RBC membrane. The rates obtained by using the standard transport assay conditions may not therefore be maximal rates of RBC Na^+-Li^+ exchange; variations in Na^+ affinity (K_m) and maximal velocity (V_{max}) could change the observed rates.

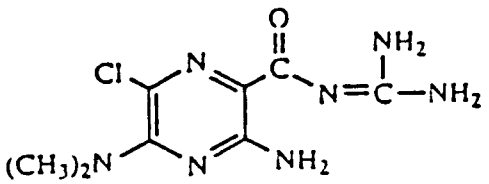
Only by varying the Na^+ concentration in an isotonic suspension medium, can one measure the true kinetic parameters of RBC Na^+-Li^+ exchange with equation (2) (Hannaert and Garay, 1986; Rutherford et al., 1990; Rutherford et al., 1992a).

Both RBC Na^+-H^+ and Na^+-Li^+ exchangers are elevated in essential hypertensive patients relative to normotensive individuals (Semplicini et al., 1989). Na^+-Li^+ countertransport is readily observable in human RBC (Canessa et al., 1987; Semplicini et al., 1989), and it is also considered that in the red cell, an enhanced Na^+-Li^+ countertransport rate is the best reproducible finding among the Na^+ transport systems in primary hypertension (Hilton, 1986). It has been proposed that RBC Na^+/Li^+ exchange may be a mode of operation of Na^+/H^+ exchange (Canessa et al., 1988). Recent genetic studies also proposed hypotheses that Na^+-H^+ is highly regulated by insulin levels therefore potentially connecting the genetics of Na^+-Li^+ countertransport to hyperinsulinemia and "dyslipidemic hypertension" (Williams et al., 1994). But whether or not Na^+-H^+ and Na^+-Li^+ exchange are mediated by the same membrane protein is still unknown.

I.3. Na^+-H^+ and Na^+-Li^+ Exchange Inhibitors

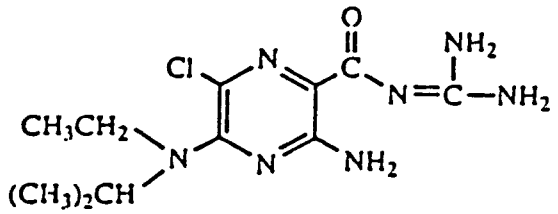
Amiloride is a specific inhibitor of Na^+-H^+ exchange systems. The inhibitory activities of amiloride and its analogues have been used to characterize the Na^+-H^+ exchanger. Amiloride is a weak base (pK_a = 8.7) (Kleyman and Cragoe, 1988). The structure of amiloride is shown in Figure 4. It is a pH-dependent inhibitor because only its protonated form can interact with Na^+ channels (Kleyman and Cragoe, 1988). It can block 60-80% of the H^+-induced Na^+ entry and has small effects in the absence of pH gradients (Dubinsky and Frizzell, 1983; Escobales and Canessa, 1986). The protonated form of amiloride can enter cells and exert additional effects (Benos et al., 1983). A high concentration (0.1-1 mM) of

Figure 4. Structures of Amiloride, 5-(N,N-hexamethylene)-amiloride (HMA), 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), and 5-(N-methyl-N-isobutyl)-amiloride (MIA).



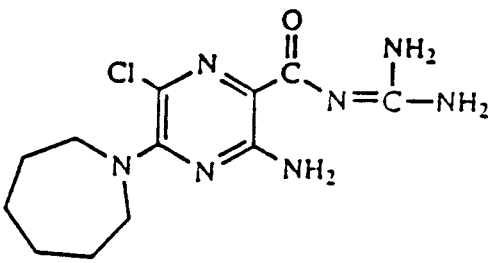
Amiloride

$C_6H_8ClN_7O$; M.W. 229.65
Apparent K_i: 83.8 μM



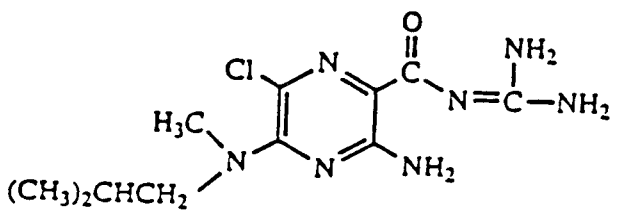
EIPA

$C_{11}H_{18}ClN_7O$; M.W. 299.76
Apparent K_i: 0.38 μM



HMA

$C_{12}H_{18}ClN_7O$; M.W. 311.8
Apparent K_i: 0.16 μM



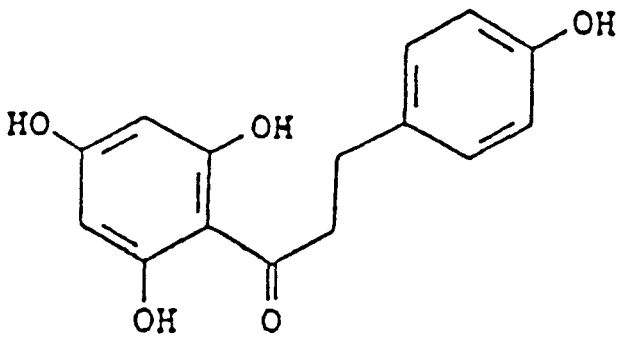
MIA

$C_{11}H_{18}ClN_7O$; M.W. 299.76
Apparent K_i: 0.44 μM

amiloride is required to inhibit Na^+-H^+ exchangers (Dubinsky and Frizzell, 1983). Many amiloride analogues such as 5-(N,N-hexamethylene)-amiloride (HMA), 5-(N-ethyl-N-isopropyl)-amiloride (EIPA) and 5-(N-methyl-N-isobutyl)-amiloride (MIA) (structures are shown in Figure 4) have been found to be 100-1000-fold more potent than amiloride (Kleyman and Cragoe, 1988). Studies on structure-activity relationships of amiloride analogues indicated that the guanidinium moiety of amiloride is the active group that recognizes and binds to the Na^+ transport site of the exchanger (Simchowitz and Cragoe, 1986). Replacements in the 6-Cl position reduced the inhibitory activity (Simchowitz and Cragoe, 1986). Increasing the number of carbon atoms in the alkyl chain increased the activity, and branched alkyl groups were more effective than straight chains (Simchowitz and Cragoe, 1986). The lipid concentration present in the experiments critically determined the inhibitory concentrations, because the inhibition activity increased with a decrease in lipid concentration (Davies and Solioz, 1992). Molecular studies on the topology of the amiloride binding site showed that the amiloride binding site on proteins appear to be a pocket formed by planar aromatic amino residues, including tyrosine, phenylalanine, and histidine into which amiloride, a large planar molecule, inserts (Lin et al., 1994).

Phloretin (shown in Figure 5), a dianionic-type organic compound, strongly inhibits Na^+-dependent Li^+ efflux (Pandey et al., 1978). Due to its dianionic characteristics, phloretin affects the voltage-dependent ion conductance. It blocks the ion channel more strongly at negative membrane potentials than at positive membrane potentials (Klusemann and Meves, 1992). Although it also affects potassium channels, phloretin is still an effective Na^+-Li^+ inhibitor. Other studies have shown that phloretin is a specific glucose transport inhibitor which has been used to inhibit tumor cell growth (Nelson and Falk, 1993 a and b). Phloretin is also considered a competitive inhibitor (Nelson and Falk, 1993 b).

Figure 5. Structure of Phloretin.



$C_{15}H_{14}O_5$; M.W. 274.26

Phloretin

The effects of both amiloride and phloretin on both Na^+-H^+ exchange and Na^+-Li^+ countertransport have been tested by the standard assay. It was found that amiloride and its analogues, HMA and EIPA, decreased RBC Na^+-H^+ exchange (Escobales and Canessa, 1985; Escobales and Canessa, 1986; Kleyman and Cragoe, 1988), but not RBC Na^+-Li^+ exchange (Pandey et al., 1978); phloretin inhibits human RBC Na^+-Li^+ exchange, but not RBC Na^+-H^+ exchange (Pandey et al., 1978). However, amiloride inhibits both Na^+-H^+ and Na^+-Li^+ exchange in bovine vascular smooth muscle (Kahn et al., 1989). It is possible that amiloride and its analogues change the kinetic parameters V_{max} or K_m but do not change V_{std} for RBC Na^+-Li^+ exchange. To test the inhibitory activity of amiloride and its analogues on Na^+-Li^+ exchange across intact RBCs, we propose to address whether or not Na^+-H^+ and Na^+-Li^+ are mediated by the same membrane protein.

I.4. RBCs and RBC Ghosts

Whole human blood contains plasma and erythrocytes. The sodium, potassium, magnesium, and calcium concentrations in the plasma are 138, 4.0, 0.86, and 2.0 mM, whereas the concentrations of those ions in erythrocytes are 10, 88, 2.5, and 4.6 mM (Touyz et al., 1992). Erythrocytes are separated from plasma by red blood cell membranes. The human erythrocyte membrane is composed of 49% protein, 43% lipid and 8% carbohydrate. For lipid, 32% is phospholipid and 11% is cholesterol. The main phospholipid components are phosphatidyl choline (PC), phosphatidyl ethanolamine (PE), phosphatidyl serine (PS), phosphatidyl inositol (PI), and sphingomyelin (Sph). The head groups of the phospholipids PI and PS are negatively charged, and these two anionic phospholipids reside primarily in the inner leaflet of the RBC membrane. PC, PE, and Sph are neutral. PC and Sph are predominantly present in the outer leaflet, whereas PE is predominantly restricted to the inner

leaflet.

Na^+-Li^+ countertransport activity is mediated by an RBC membrane protein (Motulsky et al., 1987), and so is Na^+-H^+ exchange. Measurements of Na^+ or Li^+ movements across resealed ghosts, instead of through intact RBCs, simplify the study of RBC transport proteins, because resealed RBC ghosts are post-hemolytic residues of RBCs which are devoid of intracellular structure and contain primarily cell membranes (Schowch and Passow, 1973). Ghosts are ideal models to study ion transport without the interferences of hemoglobin and other factors existing inside the red blood cells. Ghosts have the advantage over intact RBCs of being amenable to be loaded with the required concentrations of ions for the measurements of ion exchange, and they have been used successfully to study lithium transport (Duhm et al., 1976).

There are two kinds of hypotonic ghosts that have been made: white ghosts, and resealed ghosts (as shown in Figure 6). The value of resealed ghosts to characterize transport properties of the RBC membrane is that resealed erythrocyte ghosts can maintain most of the characteristics of the native erythrocyte membranes (Klonk and Deuticke, 1992), and that they can recover their permeabilities to metal ions such as Li^+, Na^+ and K^+ (Hoffman, 1992). The permeability of resealed ghosts to hemoglobin and small ions during hemolysis and resealing depends on the conditions at and after hemolysis such as temperature, pH, and ionic strength (Hoffman, 1992). The purity of the resealed ghosts is generally assumed to be related to the retention of hemoglobin, which in turn depends on the conditions during hemolysis and resealing. The number of holes through which hemoglobin can escape during hemolysis can range from one to many (Hoffman, 1992). For ghosts in which only one hole was seen, the size of the hole ranged from below 100 Å to 1 μm or more (Hoffman, 1992). The permeability of membrane to hemoglobin is determined by the number of holes, the size

Figure 6. Electron Micrograph of An Erythrocyte Ghost (adapted from reference by Mathews and van Holde, 1990).

of the holes, and the opening time of the holes (Hoffman, 1992). There are many factors controlling the yield of resealed ghosts, such as temperature, pH, ionic strength, and the presence of alkali metal ions (Bodemann and Passow, 1972).

Ionic movements through RBCs are mainly related to the fluidity of the membrane and the interactions between ions and membrane proteins and phospholipids. The interactions between ions and membranes can be addressed by testing the amounts of free and bound ions in RBC membrane suspensions. Exposure of white ghosts (exposed RBC membrane) to ions makes it possible to directly test these interactions. The composition and the function of the RBC membranes are demonstrated by membrane constituency, activity of enzymes, organization of the membrane constituents, permeability, etc. It is generally agreed that there is no measurable loss of cholesterol or phospholipid from the membrane during the preparation of white ghosts (Schwoch and Passow, 1973), but considerable structural changes occur in the lipids. The loss of membrane proteins depends on ionic strength and pH, and the release of all membrane proteins is not equal. White ghosts still contain a large number of enzymes, even though some loosely bound enzymes are lost in the preparation. The loss of enzymes is also affected by osmolarity and pH (Schwoch and Passow, 1973).

I.5. Essential Hypertension

Hypertension is a physiological phenomenon, and high blood pressure is considered to be an important risk factor in stroke, and in heart and renal disease (Motulsky et al, 1987). Essential hypertensive patients have only high blood pressure (diastolic pressure equal or higher than 90 mm Hg) but do not suffer from hypothyroidism, diabetes mellitus, hyperlipidemia, obesity, pregnancy, or other conditions which could affect blood pressure. Essential hypertension is one subgroup of hypertension in which genetic factors are deemed

to play an important role in its development (Saruta, 1994). Studies on familial aggregation of blood pressure, twin studies, population-based family studies, and adoption studies are concordant, and indicated that 20% to 40% of the population variance in blood pressure is generally genetically determined (Lifton, 1993). Two genetic loci promoting human hypertension have been well established. One is the GRA (glucocorticoid-remediable aldosteronism), a rare "determinant" gene which seems to produce severe hypertension and early strokes; the other is AGT (angiotensinogen), a very common "susceptibility" gene which seems to predispose patients to hypertension but may need other genetic and environmental influences to be fully expressed (Williams et al., 1994). The role of AGT as a molecular basis of human hypertension is well described (Jeunemaitre et al., 1992).

As previously mentioned, essential hypertensive patients have elevated Na^+-H^+ and Na^+-Li^+ exchange activities. The Na^+-H^+ as well as the Na^+-Li^+ exchanger genes are used as the best-characterized cause and intermediate phenotypes in the linkage studies for the investigation of genetic factors in essential hypertension (Lifton, 1993). In vitro, the rate of RBC Na^+-Li^+ is one possible marker of essential hypertension which is supported by parent-offspring and sib-sib correlations > 0.3 and by nonsignificant spouse correlations (Hasstedt et al., 1988). Na^+-Li^+ exchange rates have been used to investigate abnormalities in RBCs from essential hypertensive patients (Canessa et al., 1980a). The abnormal Na^+-Li^+ countertransport activity may be caused by an increase in the number of countertransporters, by structural changes in the transport protein, or by changes in the phospholipid environment around the transport protein. Changes in the number of countertransporters and in the transport protein structure are controlled by genetic factors. Changes in the transporter protein structure and the phospholipid environment can also result in the different affinities of Na^+ or Li^+ to the RBC membranes of hypertensive patients. Significantly lower Na^+

binding constants to exposed RBC membranes in essential hypertensive patients have been reported (Ong and Cheung, 1986; Urry et al., 1980). However, no information about Li^+ interactions with RBC membranes was reported prior to our investigation. Phospholipid compositions extracted from RBC membranes of essential hypertensive patients were measured by two-dimensional thin-layer chromatography, 2D-TLC (Preiss et al., 1982). No significant differences were found by the 2D-TLC method between hypertensive patients and normal individuals. The amounts of PS and PI were, however, not separately reported in that study, and the saturated phospholipid compositions could not be detected by the 2D-TLC method. It is therefore possible that abnormal phospholipid compositions exist in essential hypertension that have gone undetected by the analytical methods previously used. Higher PC/Sph ratios and abnormal molecular species in PC and PE that correlated with enhanced Na^+-Li^+ activity were found in hyperlipidemic patients (Engelmann et al., 1992 a; Engelmann et al., 1992 b; Engelmann et al., 1993). Alterations of the fatty acid composition of some phospholipids in essential hypertensive patients have been found to cause abnormal RBC Na^+-Li^+ exchange activity (Engelmann and Duhm, 1991; Engelmann et al., 1993; Ollerenshaw et al., 1987).

Normal erythrocytes contain 2.5 mM Mg^{2+}, which is essential for the use of ATP and other metabolic processes. Intracellular Mg^{2+} ions exist in two forms: as free Mg^{2+}, and as a Mg^{2+}-ATP complex which serves as a donor of high-energy phosphate (Bock et al., 1985). Free Mg^{2+} is known to regulate the activity of various enzymes involved in macromolecular synthesis, glycolysis, respiration, and in RBC membrane transport processes (Ramasamy and Mota de Freitas, 1989). It has been reported that untreated essential hypertensive patients had lower intracellular free magnesium (192 $\pm$ 8 μM) than normal controls (261 $\pm$ 9.8 μM), whereas medication-treated essential hypertensives had increased intracellular magnesium (237

± 7.8 μM) (Resnick et al., 1984). Ca^{2+} is responsible for vascular smooth muscle contractility and peripheral resistance. Lower free intracellular magnesium levels may lead to an increased intracellular Ca^{2+} concentration which induces high blood pressure. In this dissertation, I investigate whether the lower free intracellular magnesium levels in RBCs from essential hypertensive patients were related to other abnormalities associated with essential hypertension such as Na^+-Li^+ countertransport activity, Na^+ and Li^+ interactions with RBC membranes, and phospholipid composition in RBC membranes.

I.6. Techniques

Several methods such as atomic absorption (Canessa, 1989), fluorescence (Carr et al., 1993), and flame photometry (Nissen et al., 1989) have been used to measure Na^+-Li^+ and Na^+-H^+ exchange activities. The atomic absorption method has been used most frequently, because of its high selectivity and sensitivity, and of its simplicity. Each element absorbs radiation at a specific wavelength and it is not prone to interference. Very small amounts of ions in biological systems can be detected. Reproducible results have been obtained for the analysis of Na^+-Li^+ exchange, but it is difficult to obtain reproducible results for Na^+-H^+ exchange by the atomic absorption method, because it is difficult to obtain intracellular Na^+ concentrations completely separated from extracellular Na^+ (Canessa, 1989). The atomic absorption method requires physical separation of intra- and extracellular ions by centrifugation and cell lysing. It is possible to induce errors by the invasive nature of this method because of nonspecific ion binding to membranes and cell metabolites, and additional ion transport during sample processing (Mota de Freitas et al., 1991).

Nuclear magnetic resonance (NMR) spectroscopy provides a non-invasive method to monitor ion transport in biological systems, because it can simultaneously visualize the intra-

and extracellular ion pools during the ion transport process without lysing the RBCs. Shift reagents (SRs) make it possible to clearly separate intra- and extracellular signals. Shift reagents, discovered by Hinckley in 1969 (Hinckley, 1969), give shifts without significant line broadening (Friebolin, 1993). Negatively-charged shift reagents are soluble in aqueous solution but are insoluble in hydrophobic solutions. Due to the hydrophobic nature of cell membranes, SRs are not soluble in RBC membranes; in addition, they are repelled by the negatively charged head groups of phospholipids. SRs remain in the extracellular compartment during NMR experiments and produce a large hyperfine shift for the extracellular Na^+ or Li^+ ions because lanthanide SRs are paramagnetic and the extracellular ions are subject to a pseudocontact shift. Thus intracellular and extracellular signals are effectively separated. The most popular lanthanide shift reagents are $Dy(TTHA)^{3-}$, $Tm(DOTP)^{5-}$ and $Dy(PPP)_2^{7-}$ (Mota de Freitas, 1993). $Dy(TTHA)^{3-}$ and $Tm(DOTP)^{5-}$ induce downfield shifts whereas $Dy(PPP)_2^{7-}$ induces an upfield shift. The opposite direction of the pseudocontact shifts induced by $Dy(TTHA)^{3-}$ or $Tm(DOTP)^{5-}$ and by $Dy(PPP)_2^{7-}$ for Na^+ and Li^+ are due to the different locations of the Na^+ and Li^+ relative to the cones around the effective magnetic axes of these SRs. SRs have been successfully used to discriminate intra- and extracellular Na^+ and Li^+ signals (Gupta and Gupta, 1982; Mota de Freitas et al., 1990).

Besides the SR method, the Modified Inversion Recovery (MIR) method can be used to resolve intra- and extracellular resonances (Mota de Freitas, 1993). Based on the transmembrane difference in the T_1 values the modified inversion recovery pulse sequence, $(D_1\text{-}180°\text{-}D_2\text{-}60°\text{-}AQ)_n$, is applied to eliminate the extracellular alkali NMR resonance and selectively observe the intracellular NMR resonance (Mota de Freitas, 1990; Mota de Freitas, 1993).

Visibility of intracellular alkali metal NMR resonances is a matter of concern, because

it may be less than 100% for some nuclei (Mota de Freitas, 1993). It has been shown that lithium is 100% visible in NMR experiments of RBC suspensions by comparing the measurements of intracellular lithium concentrations by using both atomic absorption and NMR methods (Mota de Freitas et al., 1990). The visibility of intracellular Na^+ by NMR is, however, only 75% (Nissen et al., 1989). The NMR-invisible intracellular Na^+ ions may interact strongly with negatively charged phospholipids on the cytoplasmic side of the erythrocyte membrane. A subpool of intracellular Na^+ experiences large enough quadrupolar broadening to cause the disappearance of the corresponding resonance, whereas intracellular Li^+ does not lead to partial NMR invisibility presumably because of the small quadrupolar broadening experienced by Li^+ (Mota de Freitas et al., 1990). Another possible reason for the different visibility properties of Li^+ and Na^+ resonances is that the binding sites of Li^+ and Na^+ to RBC membranes are different (Nissen et al., 1989).

Measurements of ionic movements across resealed RBC ghosts can also be obtained by the SR method as for intact RBCs.

^{31}P NMR has been used successfully to measure free intracellular Mg^{2+} without destroying RBCs (Bock et al., 1985). Well-defined αP, βP and γP resonances of ATP are present in ^{31}P NMR spectra of RBCs. The relative chemical shifts of the ^{31}P resonances of erythrocyte ATP are predominantly determined by the interactions between magnesium and ATP.

NMR is considered to be a sensitive and powerful technique for the study of molecular dynamics in biological systems. There are two states in which ions can exist in biological systems, in free motion or in slow motion. NMR relaxation measurements, in particular, provide information about molecular motions. In relaxation methods, the ($180° - \tau - 90°$) pulse sequence is the most commonly used sequence to accurately measure T_1 (longitudinal

relaxation time) (Urry et al., 1989). The properties of the nucleus, the surrounding lattice, and the interactions between the two determine the relaxation mechanism. Correlation time, τ_c, is a parameter that is used to describe molecular motion. For the extreme narrowing condition ($\omega^2\tau_c^2 \ll 1$), the spin-lattice ($1/T_1$) and spin-spin ($1/T_2$) relaxation rates are equal and undergo a single exponential decay expressed by equation (4):

$$1/T_1 = 1/T_2 = 3(2I+3)\chi^2\tau_c/40I_2(2I - 1) \quad (4)$$

where χ is the product of the quadrupolar coupling constant, e^2qQ/h, and the asymmetry factor, $1 + \eta/3$ (eq is the electric field gradient, eQ is the electric quadrupole moment, h is the Plank's constant, and η is fractional enhancement in NOE). In a homogeneous magnetic field, the reciprocal of T_2 is proportional to the line width at half-intensity of the signal, $\Delta\nu_{1/2}$, and is written as

$$1/T_2 = \pi\Delta\nu_{1/2} \quad (5)$$

The nuclear spin quantum number for both ^{23}Na and ^{7}Li is 3/2; both nuclei have finite quadrupole moments. The relaxation and exchange times in the bound state are much shorter than those in the free state, and the relaxation decay is biexponential (Mota de Freitas, 1993). For I = 3/2 nuclides and for two-site exchange when one site (the hydrated ion in aqueous solution) is under the extreme narrowing condition and the other site (a membranous peptide binding site) is not, the time dependencies of the longitudinal, $M_Z(t)$, and transverse, $M_T(t)$, magnetizations can be expressed by equations (6) and (7), respectively (Detelier, 1987; Harris, 1983; Urry et al., 1989):

$$M_Z(t) = M_Z(0)[0.2\exp(-t/T_1') + 0.8\exp(-t/T_1'')] \quad (6)$$

$$M_T(t) = M_T(0)[0.6\exp(-t/T_2') + 0.4\exp(-t/T_2'')] \quad (7)$$

where the single and double primed symbols denote the relaxation times for the two components. With p_b, the mole fraction of bound ions being very small compared to p_f, the

mole fraction of free ions in solution, T_1', T_1^*, T_2', and T_2^* can be given by the following equations:

$$1/T_1' = 1/T_{1f} + [p_b\chi^2/10][\tau_c/(1 + \omega^2\tau^2)] \quad (8)$$

$$1/T_1^* = 1/T_{1f} + [p_b\chi^2/10][\tau_c/(1 + 4\omega^2\tau^2)] \quad (9)$$

$$1/T_2' = 1/T_{2f} + [p_b\chi^2\tau_c/20][1 + 1/(1+ \omega^2\tau^2)] \quad (10)$$

$$1/T_2^* = 1/T_{2f} + [p_b\chi^2\tau_c/20][1/(1+ 4\omega^2\tau^2) + 1/(1+ \omega^2\tau^2)] \quad (11)$$

where f and b subscripts refer to free and bound states.

In the extreme narrowing condition, a Lorentzian lineshape which is characterized by a $\Delta\nu_{1/8}/\Delta\nu_{1/2}$ ratio of $7^{1/2}$ is observed. But in most biological samples a non-Lorentzian line shape is observed, which is resolved into a narrow Lorentzian curve, due to the slow relaxation component and accounting for 40% of the total signal intensity, and a broad Lorentzian curve, originating from the fast relaxation component and responsible for the remaining 60% of the total signal intensity (Mota de Freitas, 1993). There are two methods for determining relaxation time constants, one is curve resolution with $1/T_2 = \pi\Delta\nu_{1/2}$, and the other is the null method (Urry et al., 1989).

Phospholipid compositions in RBC membranes have been previously analyzed by two-dimensional thin-layer chromatography (Preiss et al., 1982; Sengupta et al., 1981) or high-performance liquid chromatography (Kurumi et al., 1991). The visualization reagents used in thin-layer chromatography or the ultraviolet absorbance measurements used in high-performance liquid chromatography (HPLC) result in detection of only unsaturated fatty acids by these methods (Pearce et al., 1991; Spillman et al., 1983). High-resolution ^{31}P NMR was introduced by Meneses and Glonek (Meneses and Glonek, 1988) to measure phospholipid compositions of cell membranes. The ^{31}P NMR method has the advantage of being sensitive to total, and not just unsaturated, phospholipid. After improving the composition of the

solvent system used for dissolving the phospholipid extracts (Edzes et al., 1992), very good resolution for phospholipid extract samples has been achieved by ^{31}P NMR.

CHAPTER II

STATEMENT OF THE PROBLEMS

The purpose of this thesis is to investigate whether or not Na^+-H^+ and Na^+-Li^+ exchange are mediated by the same RBC membrane transport protein, and to address the correlations of Na^+-Li^+ exchange activity with Na^+ and Li^+ binding to RBC membranes and their phospholipid compositions, as well as with total phospholipid concentrations, and intracellular free Mg^{2+} concentrations in RBCs from essential hypertensive patients and normotensive individuals.

Na^+-H^+ exchange occurs in the human body. It is very important in regulating intracellular pH, cellular volume, and transmembrane ion transport (Nakhoul and Boron, 1988). Na^+-Li^+ exchange is observable in human RBCs, and has been considered as the most reproducible Na^+ transport pathway in primary hypertension (Hilton, 1986). Na^+-Li^+ exchange has been used as a marker of genetic predisposition to primary hypertension in childhood (Houtman et al., 1993). Both RBC Na^+-H^+ and Na^+-Li^+ exchange rates are higher for hypertensives than for normotensives (Semplcini et al., 1989b), and both Na^+-H^+ and Na^+-Li^+ exchanger genes have been used as the most characterized cause and intermediate phenotypes in the linkage studies for the investigation of genetic factors in essential hypertension (Lifton, 1993). Na^+-H^+ exchange and Na^+-Li^+ countertransport were significantly positively correlated in RBCs from hypertensive patients with insulin-dependent

diabetes mellitus (Semplicini et al., 1989a). It was reported that Na^+-H^+ exchange may potentially connect the genetics of Na^+-Li^+ countertransport to hyperinsulinemia and "dyslipidemic hypertension" (Williams et al., 1994). H, Li, and Na are in the same group in the periodic table, and they are the only substrates for most of the monovalent cation transport system (Jennings et al., 1985). But whether or not these two ionic exchange processes are mediated by the same membrane transport protein is still unknown.

Amiloride and its analogues, HMA, EIPA, and MIA, are specific Na^+-H^+ exchange inhibitors, whereas phloretin is a very effective Na^+-Li^+ exchange inhibitor. Na^+-H^+ and Na^+-Li^+ exchange are mediated by the same transport system in sarcolemmal vesicles from bovine superior mesenteric artery, because both Na^+-H^+ and Na^+-Li^+ exchange in sarcolemmal vesicles were inhibited by EIPA (Kahn et al., 1989). However a difference was found between Na^+-H^+ exchange and Na^+-Li^+ countertransport in human RBCs, because Na^+-Li^+ countertransport was not inhibited by amiloride (Kahn, 1987). Inhibitors were therefore previously used to address whether or not Na^+-H^+ and Na^+-Li^+ exchange were mediated by the same RBC transport system. The inhibitory activities of the inhibitors were, however, tested by measuring the standard rates of RBC Na^+-H^+ or Na^+-Li^+ exchange under the standard assay conditions (Canessa et al., 1980a; Canessa et al., 1992; Kahn, 1987; Kahn, 1989). In the standard assay conditions, the extracellular side of the RBC membranes were, however, far from saturated with Na^+ (Canessa et al., 1992; Rutherford et al., 1990; Rutherford et al., 1992a). It is possible that the transport inhibitors change V_{max} and K_m but do not change V_{std}. In this study the transport inhibitors were used to test their effects on all of the kinetic parameters of RBC Na^+-Li^+ exchange.

Na^+-H^+ and Na^+-Li^+ exchange are operational in membranes present in intact RBCs. Resealed RBC ghosts are devoid of hemoglobin and of other factors, and still maintain most

transport characteristics of the native erythrocyte membranes (Klonk and Deuticke, 1992). They can be loaded with the required concentrations of ions. They are ideal models to investigate ion transport without the interference of hemoglobin and of other factors. To test whether or not there is competition between Na^+-Li^+ and Na^+-H^+ exchange when they occur simultaneously is another way to address this question. Comparison of Li^+ efflux rates across Li^+-loaded resealed RBC ghosts induced by an outside Na^+ gradient in the absence of a pH gradient with the rates in the presence of a pH gradient will tell us whether or not Li^+ and H^+ interact with the same membrane transport protein; furthermore these studies will tell us whether or not Na^+-H^+ and Na^+-Li^+ exchange compete against each other. Both Na^+-H^+ and Na^+-Li^+ exchange inhibitors, HMA and phloretin, will be applied to the Li^+ efflux rate measurements to investigate their inhibitory effects.

The rate of ion transport depends on the interactions between ions and the RBC membranes. To fully address the interactions in detail, the inhibitory activities of amiloride will be investigated by testing the inhibitory binding constants to RBC membranes for both Na^+ and Li^+ binding to RBC membranes. The same inhibitory binding constants of amiloride for both Na^+ and Li^+ at the same pH would indicate that RBC Na^+-H^+ and Na^+-Li^+ exchange are mediated by the same RBC membrane transport protein.

Atomic absorption methods will be used to measure Na^+-Li^+ exchange kinetic parameters. ^7Li NMR will be applied to measure Na^+-induced Li^+ efflux rates across Li^+-loaded resealed RBC ghosts in the absence and in the presence of a pH gradient with and without transport inhibitors. The inhibitory binding constants of amiloride will be generated by James-Noggle plots from ^{23}Na T_1 and ^7Li T_1 relaxation measurements.

As mentioned before, RBC Na^+-Li^+ exchange rates are higher for essential hypertensive patients than for normotensive individuals (Canessa et al., 1980a). There are several reasons

which can cause the abnormal Na^+-Li^+ exchange activity in essential hypertension: changes in the number of countertransporter molecules; changes in the transport protein structure; and the abnormal phospholipid environments around the transporter. Hyperlipidemia generally occurs along with hypertension, and the medications used for lowering lipid are suggested for use in hypertension. Therefore the abnormal phospholipid environments around the transport protein are considered to be the most important reasons for causing the abnormal Na^+-Li^+ exchange activity in essential hypertension. The affinities of Na^+ and Li^+ to RBC membranes may be directly related to the Na^+-Li^+ exchange rates. The probable abnormal phospholipid compositions may affect the affinities of Na^+ and Li^+ to RBC membranes. It was also found that essential hypertensive patients have lower intracellular free Mg^{2+} concentrations than normal individuals (Gupta and Gupta, 1987; Resnick et al., 1984).

The kinetic parameters of RBC Na^+-Li^+ exchange will be measured by atomic absorption methods. Na^+ and Li^+ binding constants to RBC membranes will be obtained by James-Noggle plots from the observed ^{23}Na T_1 and ^7Li T_1 values. ^{31}P NMR will be applied to measure phospholipid composition and intracellular Mg^{2+} concentrations. The correlations of RBC Na^+-Li^+ exchange activity with Na^+ and Li^+ binding to RBC membranes and their phospholipid compositions, as well as with total phospholipid concentrations, and intracellular free Mg^{2+} concentrations in RBCs from essential hypertensive patients and normotensive individuals will be determined from Spearman correlation matrices.

CHAPTER III

EXPERIMENTAL APPROACH

III.1.Materials

III.1A. Reagents

Choline chloride, glucose, sucrose, magnesium chloride ($MgCl_2$) hexahydrate, lithium chloride (LiCl), sodium chloride (NaCl), dimethyl sulfoxide (DMSO), methanol (anhydrous) (MeOH), chloroform ($CHCl_3$), deuterated chloroform ($CDCl_3$), potassium chloride (KCl), deuterium oxide (99.8% D_2O), sodium hydroxide, triethylenetetraamine hexacetic acid (H_6TTHA), and dysprosium chloride ($DyCl_3$) were supplied by Aldrich (Wisconsin). 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), [Ethylenedinitrilo]tetraacetic acid (EDTA, 99%), butylated hydroxytoluene (BHT), tris(hydroxymehtyl)aminomethane (TRIS), 2-[N-morpholino]ethanesulfonic acid (MES), 3-[N-morpholino]propanesulfonic acid (MOPS), albumin bovine serum (ABS), ouabain, phloretin, bumetanide, 4,4'-diisothiocyanatostilbene-2,2'-disulfonic acid (DIDS), methazolamide, the detergent octyl-βD-gluco pyranoside, and the calcium ionophore A23187 were purchased from Sigma Chemical Company (St. Louis, MO). Methylphosphonic acid (MeP) was purchased from Alfa Products (Ward Hill, MA). Amiloride, 5-(N,N-hexamethylene)-amiloride (HMA), 5-(N-ethyl-N-isopropyl)-amiloride (EIPA), and 5-(N-methyl-N-isobutyl)-amiloride (MIA) were supplied from Research Biochemicals International (Natick, MA). The protein assay dye reagent was obtained from Bio-Rad Chem. Co..

III.1B. Blood Samples

Fresh packed red blood cells (RBCs) were supplied from a blood bank (Chicago Chapter of Life Source). Whole blood samples from three male and seven female essential hypertensive patients (not having hypothyroidism, diabetes mellitus, hyperlipidemia, obesity, pregnancy, or other condition which could affect blood pressure) and from three male and seven female normal individuals were obtained through the Hypertension and Renal Section of the Loyola University Medical Center. Normal individuals were matched with the patients according to gender, race and age. Because RBC Na^+-Li^+ exchange activity is elevated in white, but not in black, hypertensive patients (Canessa et al., 1991; Canessa et al., 1993), all subjects used in this study were Caucasians. The protocols for the experiments were approved by the Institutional Review Board Committee. The selection of essential hypertensive patients used in this study was based on medical history. Some of the patients were receiving antihypertensive medication (verapramil, triamterene, atenolol, enalapril, clonidine, and/or hydrochlorothiazide) at the time of blood drawing. All normotensive individuals had a diastolic blood pressure $\leq$ 80 mmHg at the time of blood drawing. All patients and normotensive individuals used in this study had no dietary restriction and ate salt freely; they were instructed to fast for a minimum of 12 hr prior to blood drawing. Triglyceride and cholesterol concentrations were measured for all samples by the clinical laboratory of the Loyola University Medical Center.

III.2.Sample Preparation

III.2A. Preparation of Li^+-loaded RBCs

Washed RBCs were added to a lithium loading solution (150 mM LiCl, 10 mM glucose, and 10 mM HEPES, pH 7.4) at 10% hematocrit and incubated at 37°C for 3 hours. After

incubation, extracellular Li^+ was removed from the Li^+-loaded RBCs by washing four times with CWS (Canessa et al., 1980a).

III.2B. Preparation of H^+- and Li^+-loaded Resealed RBC Ghosts

RBCs were washed three times using CWS. Ice-cold packed RBCs were added to ice-cold hemolyzing medium (5 mM TRIS-MES, pH 6.0, 4 mM $MgCl_2$, 21 mM LiCl, 70 $\pm$ 10 mOsM) at 10% hematocrit. After mixing for 10 minutes at 0°C-2°C, the isotonicity was restored to 300 $\pm$ 10 mOsm by adding choline chloride. Subsequent to stirring for an additional 10 minutes at 0°C, the suspensions were incubated for 1 hour at 37°C to reseal the ghosts. The pH was adjusted to 6.0 during the incubation. After incubation, the ghosts were washed twice with CWS (pH 6.0) at 2 °C (Bodemann and Passow, 1972; Duhm et al., 1976).

III.2C. Preparation of Unsealed RBC Membranes

Unsealed RBC membranes were prepared by lysing the washed packed RBCs with 5 mM HEPES buffer, pH 8.0 (5H8). The membranes were washed three more times with 5H8 and centrifuged at 51948 g at 4°C until the membranes were pale white (Steck and Kant, 1974).

III.2D. Preparation of Shift Reagents

For the preparation of 5 mL of 0.125 M $Na_3DyTTHA$, 0.2356 g $DyCl_3$ and 0.3090 g H_6TTHA were added to 3 mL water and titrated with 30 μL aliquots of 2.5 M NaOH until all of the salts were dissolved while stirring. The pH was maintained lower than 8.0 during the titration. Then the solution was transferred to a 5 mL volumetric flask and made up to 5 mL. Tetramethylammonium hydroxide was used instead of NaOH in case sodium had to

be excluded from the experiments (Pike et al., 1983).

III.2E. Preparation of Mg^{2+}-Saturated RBCs

Washed packed RBCs were added to a Mg^{2+}-loading solution (112.5 mM choline, 75 mM sucrose, 10 mM glucose, 20 mM $MgCl_2$, 4 mg/L A23187, and 10 mM HEPES, pH 7.4) at 15% hematocrit, and were incubated at 37 °C for 45 minutes. After incubation, Mg^{2+}-loaded RBCs were packed by centrifugation at 3928 g and washed four times using CWS at 4 °C (Bock et al., 1985; Flatman, 1988).

III.2F. Preparation of Mg^{2+}-Depleted RBCs

Washed packed RBCs were added to a Mg^{2+}-depleting solution (112.5 mM choline, 75 mM sucrose, 10 mM glucose, 20 mM EDTA, 4 mg/L A23187, and 10 mM HEPES, pH 7.4) at 15% hematocrit, and were incubated at 37 °C for 45 minutes. After incubation, Mg^{2+}-loaded RBCs were packed by centrifugation at 3928 g and washed four times using CWS at 4 °C (Flatman, 1988).

III.2G. Extraction of Phospholipids

The phospholipids in the RBC membrane were extracted as follows: 17 mL methanol was added to 1 mL membrane and mixed for 10 minutes. Then 33 mL chloroform was added, and the sample was mixed for an additional 15 minutes. All extracting solvents contained 50 mg/L of butylated hydroxytoluene (BHT) as an antioxidant. The resulting extract was filtered through a sintered glass funnel, and was washed with 50 mL of a chloroform, methanol mixture in the ratio of 2:1. The filtrate was washed with 0.2 times its total volume with 0.74% KCl to remove all non-lipid impurities. The bottom chloroform layer was collected

and dried in a rotary evaporator at 30°C.

III.3.Instrumentation

III.3A. Nuclear Magnetic Resonance Spectrometer

^{23}Na, ^{7}Li, and ^{31}P measurements were obtained at 79.4, 116.5, and 121.4 MHz, respectively, on a Varian VXR-300 NMR spectrometer. The instrument was equipped with 10 mm multinuclear probes and a variable temperature unit. All NMR experiments were run under identical gain settings.

^{7}Li NMR spectra for Li^{+} efflux across resealed RBC ghosts were obtained using a flip angle of 60° (18 μs), an acquisition time of 0.981 s, a 30 s delay, a spectral width of 4500.5 Hz, and 29 transients, with 10 Hz spinning and at 37 °C.

^{31}P NMR spectra for measuring the chemical shift difference between the αP and the βP resonances of ATP to determine intracellular free Mg^{2+} concentrations were obtained using a flip angle of 60° (7 μs), an acquisition time of 1.37 s, a 0 s delay, a spectral width of 10000.0 Hz, and 1500 transients, without spinning and at 37 °C.

^{31}P NMR spectra for analyzing phospholipid compositions in the extracts of RBC membranes were obtained using a flip angle of 60° (7 μs), an acquisition time of 1.37 s, a 4 s delay, a spectral width of 1051.3 Hz, and 15000 transients, with 16 Hz spinning and at 37 °C.

^{23}Na and ^{7}Li spin-lattice relaxation time (T_1) measurements were conducted by the inversion recovery method (180°-τ-90°) with 16 Hz spinning and at 37 °C. The delay time was at least five times the value of T_1.

III.3B. Atomic Absorption Spectrophotometer

Li^+ and Na^+ absorption were measured at 670.8 and 589.0 nm, respectively, on a Perkin Elmer 5000 spectrophotometer equipped with a flame source and a graphite furnace. The slit width was set at 1.4 high for both Li^+ and Na^+. The fuel and oxidant were acetylene and compressed air. The flow rate for fuel and oxidant were 45 and 50, respectively.

III.3C. UV/Vis Spectrophotometer

The protein and hemoglobin concentrations were measured at 595 nm and 415 nm, respectively on an IBM UV/Vis 9420 or on a JASCO MODEL V-500 UV/VIS spectrophotometer.

III.3D. Centrifuge

A Savant refrigerated centrifuge, model HSC 1000, was used for separating and washing RBCs. Resealed RBC ghosts and unsealed RBC membranes were prepared by using a Beckman J2-21 refrigerated centrifuge equipped with fixed angle rotors, JA-10 and JA-20.

III.3E. Osmometer

The osmolarity of the suspension medium was checked with a Wescor Vapor Pressure Osmometer (Wescor Inc., Logan, UT).

III.3F. Hemofuge

Hematocrits were measured by an IEC model MB IM116 hemofuge.

III.4.Data Analysis

III.4A. Determination of Kinetic Parameters of RBC Na^+-Li^+ Countertransport

The Li^+ loaded RBCs were suspended at 10% hematocrit in six media containing 150, 100, 70, 40, 20 and 0 mM NaCl that were made isotonic with choline chloride and also contained 10 mM glucose, 0.1 mM ouabain, and 10 mM HEPES, pH 7.4. Aliquots were taken every 20 minutes from each of the Li-loaded RBC suspensions and centrifuged at 10519 g for 1.5 minutes at 4°C. The supernatants were collected and analyzed by AA. The kinetic parameters of Na^+-Li^+ exchange V_{std}, V_{max} and K_m were obtained from the following equations (Canessa et al., 1980a; Rutherford et al., 1990):

$$V = Slope_{NaCl} \times (1-Ht_{NaCl})/Ht_{NaCl} - Slope_{Choline} \times (1-Ht_{Choline})/Ht_{Choline} \quad (12)$$

$$V_{std} = V_{([Na+] = 150\ mM)} - V_{([Choline] = 150\ mM)} \quad (13)$$

$$1/V = K_m/(V_{max} \times [Na^+]) + 1/V_{max} \quad (14)$$

III.4B. Determination of Li^+ Efflux Rates Across H^+- and Li^+- loaded Resealed RBC Ghosts by ^7Li NMR

Li^+-loaded resealed BRC ghosts were added to a NaCl medium containing 120 mM NaCl, 10 mM glucose, 0.1 mM ouabain, 5 mM $Na_3DyTTHA$, 10 mM TRIS-MES for pH 6.0, or 10 mM TRIS-MOPS for 8.0, with or without 0.1 mM phloretin or 0.1 mM HMA at 20% hematocrit. ^7Li NMR measurements were taken every 15 minutes. Extracellular Li^+ concentrations were calculated by the following equation:

$$[Li^+]_{out} = A_{out} \times [Li^+]_s / (A_s \times (1 - Ht)) \quad (15)$$

where A_{out} was the peak area under the extracellular Li^+ NMR resonance, $[Li^+]_s$ and A_s were the known concentration and peak area of a standard Li^+ solution which was measured separately, and Ht was the hematocrit. Li^+ efflux rates were obtained by the equation shown below:

$$Rate = Slope \times (1-Ht)/Ht \quad (16)$$

III.4C. Determination of Na^+ Binding Constants to RBC Membranes from ^7Li T_1 Measurements

The observed ^{23}Na or ^7Li NMR relaxation values measured in RBC membrane suspensions represent the weighted average of free and bound Na^+ or Li^+ ions; these values are thus sensitive to ion binding to the RBC membrane and total intracellular ion concentrations. Free and bound Na^+ and Li^+ are undergoing exchange which is represented by a time-averaged expression:

$$R_1 = (1/T_1) = R_{1free}X_{free} + R_{1bound}X_{bound} \quad (17)$$

James and Noggle introduced graphical methods to calculate metal ion binding constants (James and Noggle, 1969) which express the interactions between metal ions and cell membranes and assume one to one stoichiometry for binding at a microscopic level when [B] ◄ $[M^+]$:

$$(\Delta R)^{-1} = (R_{obs}-R_{free})^{-1}$$

$$= K_b^{-1}\{[B](R_{bound}-R_{free})\}^{-1} + [M^+]\{[B](R_{bound}-R_{free})\}^{-1} \quad (18)$$

where R_1, R_{1free}, and R_{1bound} were the observed, free, and bound relaxation rates of M^+ ions in RBC membrane suspension, X_{free} and X_{bound} were the mole fractions of free and bound M^+, $[M^+]$ and [B] were the total concentration of M^+ and membrane binding sites, respectively. A series of ^7Li T_1 values were measured for unsealed RBC membranes with varying concentrations of Na^+ titrated with Li^+ at increasing concentrations. Apparent Li^+ binding constants K_{Li}^{app} to unsealed RBC membranes were obtained from James-Noggle plots (James and Noggle, 1969).

Because Na^+ and Li^+ competitively bind to RBC membranes, the Li^+ binding constant K_{Li} and the actual Na^+ binding constant $K_{Na(a)}$ were determined by the following equation:

$$1/K_{Li}^{app} = (1 + K_{Na(a)}[Na^+])/K_{Li} \quad (19)$$

The observed Na^+ binding constants to the same membrane preparations were calculated from the observed ^{23}Na T_1 values for the membrane suspensions titrated with Na^+ at increasing concentrations by James-Noggle plots (James and Noggle, 1969).

The ratio of the actual Na^+ binding constant to the observed Na^+ binding constant was calculated by equation (20):

$$r = K_{Na(a)} / K_{Na(o)} \qquad (20)$$

III.4D. Determination of Inhibitor Binding Constants to RBC Membranes in the Presence of Na^+ and Li^+

The inhibitory activity of the inhibitors, such as amiloride, HMA, and phloretin, for Na^+ or Li^+ binding to RBC membranes can be addressed by measuring inhibitor binding constants to RBC membranes in suspensions in the presence of Na^+ or Li^+. As a competitive inhibitor (Potts, 1994), the kinetic behavior of amiloride can be expressed by the equation (21):

$$1/K_M^{app} = (1 + K_I[I])/K_M \qquad (21)$$

where K_M^{app} was the apparent binding constant of the metal ion M^+, K_M was the metal ion binding constant, [I] was the inhibitor concentration, and K_I was the inhibitor binding constant. The apparent Na^+ or Li^+ binding constants to unsealed RBC membranes were calculated from James-Noggle plots (James and Noggle, 1969) from the ^{23}Na or ^7Li T_1 values observed in RBC membrane suspension in the presence of varying concentrations of amiloride titrated with Na^+ or Li^+ at increasing concentrations.

Because Li^+ is 100% visible by ^7Li NMR, the apparent Li^+ binding constants were directly used in equation (21) to generate the inhibitor binding constants. Because Na^+ is not 100% visible by ^{23}Na NMR, the apparent binding constants were corrected by multiplying the

corresponding correction factor from the curve in Figure 8. Then the corrected Na^+ binding constants were used to generate inhibitor binding constants to the RBC membranes in the presence of Na^+ using equation 21.

III.4E. Determination of Intracellular Free Mg^{2+} Concentration from ^{31}P NMR Chemical Shifts

Fresh blood was washed three times with CWS. ^{31}P NMR measurements were taken for washed and packed RBCs. The chemical shift difference between the αP and the βP resonances was used to determine the portion of total intracellular ATP that exists as the Mg^{2+} complex; the free Mg^{2+} concentration, which is related to the apparent dissociation constant of MgATP, can be obtained by the following equation (Gupta and Gupta, 1987):

$$\phi = [ATP]_f/[ATP]_t = (\delta_{\alpha\beta}^{cell} - \delta_{\alpha\beta}^{MgATP})/(\delta_{\alpha\beta}^{ATP} - \delta_{\alpha\beta}^{MgATP}) \quad (22)$$

$$[Mg^{2+}]_f = K_D^{MgATP}[(1 - \phi)/\phi] \quad (23)$$

where K_D^{MgATP} was the apparent dissociation constant of MgATP, $\delta_{\alpha\beta}^{cell}$ was the chemical shift difference between the αP and βP resonances of intracellular ATP. $\delta_{\alpha\beta}^{ATP}$ and $\delta_{\alpha\beta}^{MgATP}$ were the values of this difference for magnesium-depleted and magnesium-saturated RBCs, respectively, measured by ^{31}P NMR. $[ATP]_t$ was the total ATP concentration in the cell, and $[ATP]_f$ was the sum of the concentrations of all ATP species not chelated to magnesium. K_D^{MgATP} is temperature dependent. At 37 °C, the value of K_D^{MgATP} was determined to be 38 $\pm$ 4 μM, but at 25 °C, it was found to be 45 $\pm$ 8 μM (Gupta and Gupta, 1987; Resnick et al., 1984).

III.4F. Quantitation of Phospholipid Compositions and Total Phospholipid in Human RBC Membranes by ^{31}P NMR

The purified phospholipids were suspended in 3 mL of the solvent mixture solution ($CDCl_3$: MeOH : 0.2 M EDTA = 125 : 8 : 3). The sample was placed in a 10 mm NMR tube and was measured using ^{31}P NMR. The areas of each peak of the phospholipid extracts were integrated using the integration software of the NMR spectrometer, and the percentage of each phospholipid composition was calculated (Edzes et al., 1992). A known amount of pure PC was added to the same extract and was measured again using ^{31}P NMR. The increased peak area corresponding to the added pure PC was used to calculate the total phospholipid by the following equation:

$$TP = \{(A_{PC} / A_s) \times (W_{PC} / V_s) \times V\} / \{V_m \times [Pro] \times PC\%\} \quad (24)$$

where TP was the total phospholipid, W_{PC} was the known amount of the added PC, A_{PC} and V were the area of PC from the ^{31}P NMR spectrum and the total volume of the solvents used to dissolve the phospholipid extract, respectively, A_s and V_s were the increased area of PC from the measured ^{31}P NMR spectrum and the volume of the phospholipid sample when the known amount of PC was added, respectively, V_m was the volume of membrane used, [Pro] was the protein concentration of the membrane, and PC% was the percentage of PC in the total phospholipid extract.

III.4G. Protein Concentration Determination

The membrane protein concentration was measured by the "Bradford Assay" method (Bollag and Edelstein, 1991) with the detergent hexyl-β-D-glucopyranoside or octyl-β-D-glucopyranoside (Fanger, 1987) at 595 nm by UV/Vis. 5 μL membranes was added to 45 μL of 5 mM HEPES solution, pH 8.0, with 10 μL 50% detergent, and incubated 5 minutes at room temperature. Then 2.5 mL of dye reagent, which was purchased from Bio-Rad Chem. Co., was diluted with 4 fold deionized water was added to the membrane suspension.

The dye reagent was filtered through a Whatman No. 1 paper before it was added to the membrane suspension.

III.4H. Statistical Analysis

Each essential hypertensive patient was matched to a normotensive individual according to age, race and gender. The statistical significance of the differences between hypertensive patients and normal individuals for the kinetic parameters of RBC Na^+-Li^+ exchange, Na^+ and Li^+ binding constants, phospholipid compositions and total phospholipid concentrations of RBC membranes, and intracellular free Mg^{2+} in intact RBCs were analyzed by one-way analysis of variance (ANOVA) with Tukey's conservative correction. $p \leq 0.05$ was considered significantly different. Correlation coefficients among these parameters were obtained by using Spearman correlation; correlation coefficients ≥ 0.40 were considered significant ($p \leq 0.05$).

CHAPTER IV

RESULTS

IV.1. Na^+-Li^+ Exchange Activity in Intact Human RBCs with and without Inhibitors

The Na^+-Li^+ countertransport rates and the Na^+ dissociation constants to intact RBCs in the presence and absence of inhibitors measured by AA spectroscopy along with the statistical analysis are shown in Tables 1 and 2. Amiloride had a significant effect on V_{max} (0.23 ± 0.01 vs. 0.34 ± 0.05 mmol Li^+/L cells · h, $p = 0.04$, $n = 3$), but had no significant effects on V_{std} and K_m. HMA had significant effects on V_{max} and K_m (V_{max} and K_m were 0.20 ± 0.03 mmol Li^+/L cell · h and 48 ± 17 mM with HMA vs. 0.34 ± 0.05 mmol Li^+/L cell · h, and 117 ± 13 mM w/o inhibitors, $p \leq 0.03$, $n = 3$), but had no significant effects on V_{std}. Phloretin had significant effects on V_{std}, V_{max}, and K_m (V_{std}, V_{max}, and K_m were 0.079 ± 0.014, 0.10 ± 0.01 mmol Li^+/L cell · h and 45 ± 13 mM with phloretin vs. 0.19 ± 0.04, 0.34 ± 0.05 mmol Li^+/L cell · h, and 117 ± 13 mM without inhibitors, $p \leq 0.03$, $n = 3$). From Tables 1 and 2 we can see that HMA, an amiloride analogue, is more effective than amiloride. HMA, which is a Na^+-H^+ exchange inhibitor, and phloretin, which is a Na^+-Li^+ exchange inhibitor both inhibited the Na^+-Li^+ exchange activity in intact RBCs.

IV.2. Li^+ Efflux Across H^+- and Li^+-Loaded Resealed RBC Ghosts

The pH value inside the ghosts was 6.0. When H^+- and Li^+-loaded resealed RBC

Table 1. Na^+-Li^+ Countertransport Rates (mmol Li^+/L RBCs · h), and Na^+ Dissociation Constants (mM) in the Presence and Absence of Inhibitors.

	w/o Inhibitor			w/0.1 mM Amiloride			w/0.1 mM HMA			w/0.1 mM Phloretin		
	1	2	3	1	2	3	1	2	3	1	2	3
V_{std}	0.23	0.16	0.19	0.16	0.18	0.20	0.23	0.11	0.22	0.099	0.073	0.065
V_{max}	0.41	0.29	0.32	0.25	0.22	0.23	0.20	0.17	0.24	0.11	0.10	0.089
K_m	115	134	103	113	39.2	54.6	48.9	67.1	26.7	26.5	55.7	52.9
R^2	0.99	0.99	0.97	0.97	0.99	0.97	0.97	0.99	0.96	0.99	0.99	0.99

Table 2. Student's *t*-test *p* Values, and Average Values for Na^+-Li^+ Countertransport Parameters for Intact RBCs in the Presence and Absence of Amiloride, HMA and Phloretin.

	w/o Inhibitor ($n = 3$)	w/0.1 mM Amiloride ($n = 3$)	p^*	w/0.1 mM HMA ($n = 3$)	p^*	w/0.1 mM Phloretin ($n = 3$)	p^*
V_{std}	0.19±0.03	0.18±0.02	0.60	0.19±0.05	0.89	0.079±0.014	0.01
V_{max}	0.34±0.05	0.23±0.01	0.04	0.20±0.03	0.03	0.10±0.01	0.03
K_m	117±13	69±32	0.12	48±17	0.01	45±13	0.01

*$p \leq 0.05$ is considered significantly different.

ghosts were suspended in NaCl medium at pH 8.0, there was a pH gradient. Both Li^+ and H^+ from inside the ghosts exchanged with Na^+ from the outside medium. Both Na^+-Li^+ and Na^+- H^+ exchange occurred simultaneously. As shown in Figure 7, Li^+ came out more slowly when Na^+-H^+ exchange also occurred at the same time, with or without inhibitors. Both HMA and phloretin decreased Li^+ efflux. The Li^+ efflux rates were calculated and are shown in Table 3. Li^+ efflux rates across resealed H^+- and Li^+-loaded RBC ghosts were significantly lower in the presence of a pH gradient than in its absence (0.48 $\pm$ 0.09 vs. 0.81 $\pm$ 0.09 without inhibitors; 0.28 $\pm$ 0.05 vs. 0.49 $\pm$ 0.08 with 0.1 mM HMA; 0.25 $\pm$ 0.04 vs. 0.41 $\pm$ 0.06 mmol Li^+/L cell·h with phloretin) presumably due to the competition between Li^+ and H^+ for transport by the same transport protein. HMA significantly decreased Li^+ efflux rates (0.49 $\pm$ 0.08 vs. 0.81 $\pm$ 0.09 in the absence of a pH gradient; 0.28 $\pm$ 0.05 vs. 0.48 $\pm$ 0.09 mmol Li^+/L cell·h in presence of a pH gradient). Phloretin also significantly decreased Li^+ efflux rates (0.41 $\pm$ 0.06 vs. 0.81 $\pm$ 0.09 in the absence of a pH gradient; 0.25 $\pm$ 0.04 vs. 0.41 $\pm$ 0.06 mmol Li^+/L ghosts·h in the presence of a pH gradient).

IV.3.Na^+ Visibility in RBC Membranes and the Correction Curve

IV.3A. Na^+ Visibility in RBC Membranes

^{23}Na NMR visibility in RBC membrane suspension was measured by comparing the signal intensities of Na^+ in RBC membranes with those in water solutions at the same Na^+ concentrations. Table 4 shows results from two trials for the Na^+ visibility and ^{23}Na T_1 values in RBC membranes by ^{23}Na NMR without and with 150 mM LiCl. When there was no LiCl, sodium is not 100% visible. The lower the Na^+ concentration, the lower the visibility. ^{23}Na T_1 values at lower Na^+ concentrations were lower than ^{23}Na free T_1 when

Figure 7. ^{7}Li NMR Spectra of Li^{+} Efflux Across H^{+}- and Li^{+}-loaded Resealed RBC Ghosts in NaCl Media. Acquisitions were accumulated every 15 min. The pH inside of the resealed ghosts was 6.0 for all of the cases. The pH outside of the resealed ghosts was 6.0 for the sets of spectra a, b, and c. The pH outside of the resealed ghosts was 8.0 for the sets of spectra d, e, and f. There was no inhibitor in NaCl media for the sets a and d. There was 0.1 mM HMA in the NaCl media for the sets b and e. There was 0.1 mM phloretin in the NaCl media for the sets c and f.

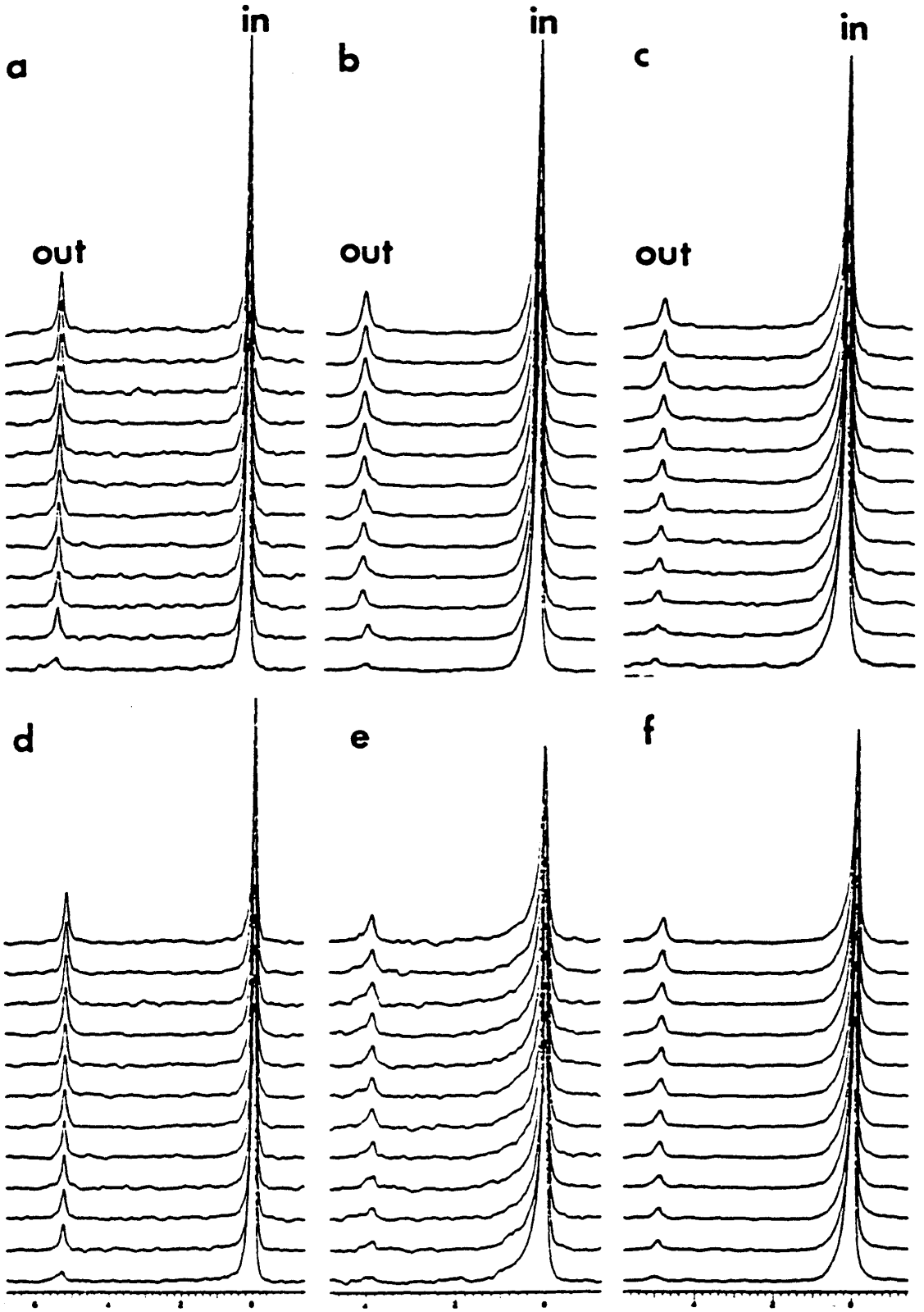

a
in
out
b
in
out
c
in
out
d
e
f

Table 3. Li^+ Efflux Rates (mmol Li^+/L Ghosts · h) Across H^+- and Li^+-loaded Resealed RBC Ghosts in NaCl Medium with and without Inhibitors. (n = 3)

$pH_{(out)}$	w/o Inhibitor	w/0.1 mM HMA	w/0.1 mM Phloretin
6	0.81 ± 0.09	0.49 ± 0.08	0.41 ± 0.06
8	0.48 ± 0.09	0.28 ± 0.05	0.25 ± 0.04

All $pH_{(in)}$: 6.

Table 4. ^{23}Na Visibility in RBC Membrane Suspensions by NMR.

$[Na^+]$/mM	Membranes/w/o LiCl*		Membranes/w/150 mM LiCl*	
	Visibility (%)	T_1/ms	Visibility (%)	T_1/ms
4	52.8 ± 0.4	39.9 ± 3.0	100.2 ± 3.0	61.4 ± 1.0
12	78.6 ± 0.5	46.9 ± 2.0	99.8 ± 5.0	62.5 ± 2.0
20	95.2 ± 3.2	50.5 ± 0.6	96.0 ± 5.6	63.8 ± 2.0
500	91.6 ± 2.8	65.1 ± 0.4	92.4 ± 2.1	65.3 ± 0.2

*Average of the values were obtained from two trials.

there was no LiCl present. After 150 mM LiCl was added to the same membrane suspension at the same Na^+ concentrations, ^{23}Na T_1 values were close to ^{23}Na free T_1, and Na^+ was fully visible at any Na^+ concentrations thereafter.

IV.3B. Actual Na^+ Binding Constants to RBC Membranes

The sodium-23 nucleus is not 100% visible by ^{23}Na NMR. The observed ^{23}Na T_1 values are not the actual T_1 values. Therefore the Na^+ binding constants calculated by James-Noggle plots from the observed T_1 values are not the actual Na^+ binding constants. The actual Na^+ binding constants were obtained by measurements of ^7Li T_1 values in RBC membrane suspensions in the presence of varying concentrations of Na^+ at increasing concentrations of Li^+. Tables 5 through 9 show ^7Li T_1 measurements for RBC membranes in the presence of varying concentrations of NaCl. The observed Na^+ binding constants were obtained from the observed ^{23}Na T_1 values. At a given Na^+ concentration, when the Li^+ concentrations increased, ^7Li T_1 values also increased. When Na^+ concentrations were elevated, ^7Li T_1 values increased for the same Li^+ concentrations, and apparent Li^+ binding constants decreased.

IV.3C. Ratio of the Actual Na^+ Binding Constants to the Observed Na^+ Binding Constants to RBC Membranes and the Correction Curve

Both the observed and the actual Na^+ binding constants differed from batch to batch of human blood obtained from the Life Source. The observed and the actual Na^+ binding constants show, however, the same trends. The blood that gave higher observed Na^+ binding constants also gave higher actual Na^+ binding constants. The ratios of the actual Na^+ binding constant to the observed Na^+ binding constant are listed in Table 10. All of the ratios are

Table 5. ^{7}Li T_1 Measurements (s) for RBC Membranes in the Presence of Varying Concentrations of NaCl. (Trial 1)

			[Na^+]/mM		
[Li^+]/mM	0	2	4	6	10
2	5.0±0.7	5.9±0.7	5.7±0.5	8.4±0.6	9.2±0.6
4	5.9±0.9	8.1±0.6	8.0±0.6	9.0±0.3	9.6±0.6
6	6.4±0.2	8.1±0.3	9.2±0.4	9.0±0.3	10.1±0.3
8	7.5±0.3	8.2±0.2	8.8±0.2	10.9±1.0	10.3±0.3
10	9.1±0.8	9.2±0.2	9.2±0.2	10.1±0.2	10.6±0.3
12	9.2±0.5	10.0±0.4	9.5±0.2	10.5±0.2	12.3±0.6
500	19.5±0.8	23.0±2.4	22.9±3.2	22.2±1.8	21.5±1.9
K_{Li}^{app}/M^{-1}	174	158	107	54	39
r^2	0.98	0.88	0.88	0.98	0.98

$K_{b(Li)} = 283\ M^{-1}$ $K_{Na(i)} = 618\ M^{-1}$ $r^2 = 0.94$

$K_{Na(o)}^{*} = 254\ M^{-1}$

*$K_{Na(o)}$ was obtained by ^{23}Na T_1 measurements for the same membrane preparation.

Protein concentration was 6.11 ± 1.70 mg/mL.

Table 6. ^{7}Li T_1 Measurements (s) for RBC Membranes in the Presence of Varying Concentrations of NaCl. (Trial 2)

			[Na^+]/mM		
[Li^+]/mM	0	2	4	6	10
2	5.1±0.3	6.2±0.4	6.5±0.7	6.6±0.4	7.5±0.5
4	5.6±0.2	6.5±0.2	6.0±0.2	8.1±0.5	7.8±0.2
6	5.7±0.3	6.7±0.4	7.2±0.3	8.9±0.8	8.5±0.2
8	7.0±0.6	7.0±0.2	8.3±0.4	8.6±0.3	8.6±0.2
10	7.7±0.4	8.8±0.7	8.8±0.2	9.6±0.7	9.4±0.5
12	7.6±0.2	8.8±0.6	9.0±0.3	10.3±0.7	11.3±1.2
500	17.3±0.3	18.3±0.8	17.7±0.7	21.1±2.4	20.9±2.0
K_{Li}^{app}/M^{-1}	171	122	109	85	64
r^2	0.90	0.82	0.94	0.74	0.96

$K_{b(Li)} = 175\ M^{-1}$ $K_{Na(i)} = 182\ M^{-1}$ $r^2 = 0.99$

$K_{Na(o)}^{*} = 153\ M^{-1}$

*$K_{Na(o)}$ was obtained from ^{23}Na T_1 measurements for the same membrane preparation.

Protein concentration was 6.11 ± 1.70 mg/mL.

Table 7. ^{7}Li T_1 Measurements (s) for RBC Membranes in the Presence of Varying Concentrations of NaCl. (Trial 3)

	[Na^+]/mM				
[Li^+]/mM	0	2	4	6	10
2	6.3±0.5	6.4±0.4	6.9±0.5	7.8±0.6	7.6±0.1
4	6.8±0.2	7.0±0.2	6.6±0.2	8.8±0.3	9.0±0.7
6	6.9±0.2	7.6±0.6	7.3±0.1	8.7±0.3	9.7±0.6
8	8.3±0.8	7.7±0.2	8.3±0.3	9.4±0.4	8.8±0.3
10	8.9±0.5	8.2±0.5	8.5±0.1	9.5±0.6	9.8±0.3
12	8.5±0.5	8.5±0.2	9.2±0.6	9.3±0.3	9.8±0.4
500	18.8±1.8	18.0±0.6	17.8±0.8	18.1±1.1	17.3±0.4
K_{Li}^{app}/M^{-1}	161	119	101	68	84
r^2	0.96	0.91	0.98	0.91	0.79

$K_{b(Li)} = 174\ M^{-1}$ $K_{Na(i)} = 234\ M^{-1}$ $r^2 = 0.94$

$K_{Na(o)}^{*} = 181\ M^{-1}$

*$K_{Na(o)}$ was obtained from ^{23}Na T_1 measurements for the same membrane preparation.

Protein concentration was 6.11 ± 1.70 mg/mL.

Table 8. ^{7}Li T_1 Measurements (s) for RBC Membranes in the Presence of Varying Concentrations of NaCl. (Trial 4)

	[Na^+]/mM				
[Li^+]/mM	0	2	4	6	10
2	3.2±0.2	4.0±0.2	4.1±0.1	5.4±0.4	6.0±0.3
4	4.3±0.2	4.9±0.2	4.8±0.1	6.4±0.5	7.2±0.3
6	4.7±0.2	5.4±0.1	5.9±0.3	6.6±0.3	8.0±0.4
8	5.2±0.2	6.3±0.4	6.5±0.4	6.9±0.2	7.9±0.3
10	6.2±0.4	6.7±0.4	6.9±0.2	7.4±0.2	8.9±0.7
12	6.4±0.3	6.7±0.1	7.4±0.5	8.0±0.2	8.4±0.4
500	16.2±0.4	17.2±0.9	16.5±0.4	17.8±1.1	16.7±0.6
K_{Li}^{app}/M^{-1}	222	150	193	94	75
r^2	0.98	0.95	0.99	0.97	0.81

$K_{b(Li)} = 208\ M^{-1}$ $K_{Na(i)} = 185\ M^{-1}$ $r^2 = 0.99$

$K_{Na(o)}^{*} = 153\ M^{-1}$

*$K_{Na(o)}$ was obtained from ^{23}Na T_1 measurements for the same membrane preparation.

Protein concentration was 6.11 ± 1.70 mg/mL.

Table 9. ^{7}Li T_1 Measurements (s) for RBC Membranes in the Presence of Varying Concentrations of NaCl. (Trial 5)

			$[Na^+]$/mM		
$[Li^+]$/mM	0	2	4	6	10
2	5.6±0.6	5.7±0.8	7.6±0.5	8.4±1.5	10.9±1.0
4	6.5±0.2	6.9±0.4	7.0±0.3	7.7±0.7	10.0±0.6
6	7.3±0.2	7.0±0.1	7.9±0.2	8.2±0.3	10.8±0.3
8	7.7±0.3	8.5±0.3	9.2±0.3	9.8±0.5	11.6±0.3
10	8.4±0.3	9.2±0.1	10.8±0.4	10.6±0.6	11.5±0.1
12	8.9±0.1	10.8±0.3	10.5±0.2	10.9±0.4	11.7±0.1
500	20.0±0.7	20.4±0.8	20.8±0.6	22.0±1.8	20.6±0.9
K_{Li}^{app}/M^{-1}	191	162	129	99	52
r^2	0.95	0.99	0.88	0.83	0.81

$K_{b(Li)} = 272\ M^{-1}$ $K_{Na(i)} = 372\ M^{-1}$ $r^2 = 0.94$

$K_{Na(o)}^{*} = 215\ M^{-1}$

*$K_{Na(o)}$ was obtained from ^{23}Na T_1 measurements for the same membrane preparation.

Protein concentration was 6.11 ± 1.70 mg/mL.

Table 10. Ratio of Actual Na^+ Binding Constants to Observed Na^+ Binding Constants to RBC Membranes.

	1	2	3	4	5
$K_{Na(a)}/M^{-1}$	618	182	234	185	372
$K_{Na(o)}/M^{-1}$	254	151	181	153	215
Ratio ($K_{Na(a)}/K_{Na(o)}$)	2.43	1.20	1.29	1.21	1.73

higher than 1.00. From Table 10 we generated the correction curve shown in Figure 8. At lower binding constants, the correction factors were close to 1.00, and the actual Na^+ binding constants were very close to the observed Na^+ binding constants.

IV.4. Inhibitory Activities of Amiloride and Its Analogues on Na^+ and Li^+ Interactions with RBC Membranes

IV.4A. Inhibitory Effects of Amiloride and Its Analogues on ^{23}Na and ^{7}Li Relaxation Times

The inhibitory effects of amiloride and its analogues, EIPA, HMA and MIA on both ^{23}Na and ^{7}Li spin lattice relaxation times were tested by measuring ^{23}Na and ^{7}Li T_1 with increasing concentrations of these inhibitors. As shown in Tables 11 and 12, and Figure 9, at the same Na^+ and Li^+ concentration ^{23}Na and ^{7}Li T_1 values increased as the concentration of all these inhibitors increased. All of these inhibitors bound to the protein in the RBC membranes, which also contain the Na^+ and Li^+ binding sites, and resulted in more free Na^+ and Li^+. HMA had the highest inhibitory effects though the effects of all of these inhibitors were of the same order of magnitude.

IV.4B. Amiloride Inhibitory Activities on Na^+ and Li^+ Binding to RBC Membranes

Tables 13 through 20 show ^{23}Na and ^{7}Li T_1 measurements for RBC membrane suspensions with increasing concentrations of Na^+ or Li^+ in the presence of varying concentrations of amiloride (0, 0.5, 1.0, 1.5, 2.0, 2.5 mM) at pH 7.0 and 8.0. One of the two trials of both ^{23}Na and ^{7}Li T_1 measurements at pH 8.0 is ploted in Figure 10. At the same amiloride concentrations, ^{23}Na or ^{7}Li T_1 values increased as Na^+ or Li^+ concentrations increased. At the same Na^+ or Li^+ concentrations, as amiloride concentration elevated, both

Figure 8. Correction Factor for Na^+ Binding Constants to RBC Membranes. pH value was 7.6 $\pm$ 0.2. Protein concentration of the membrane samples was 6.11 $\pm$ 1.70 mg/mL.

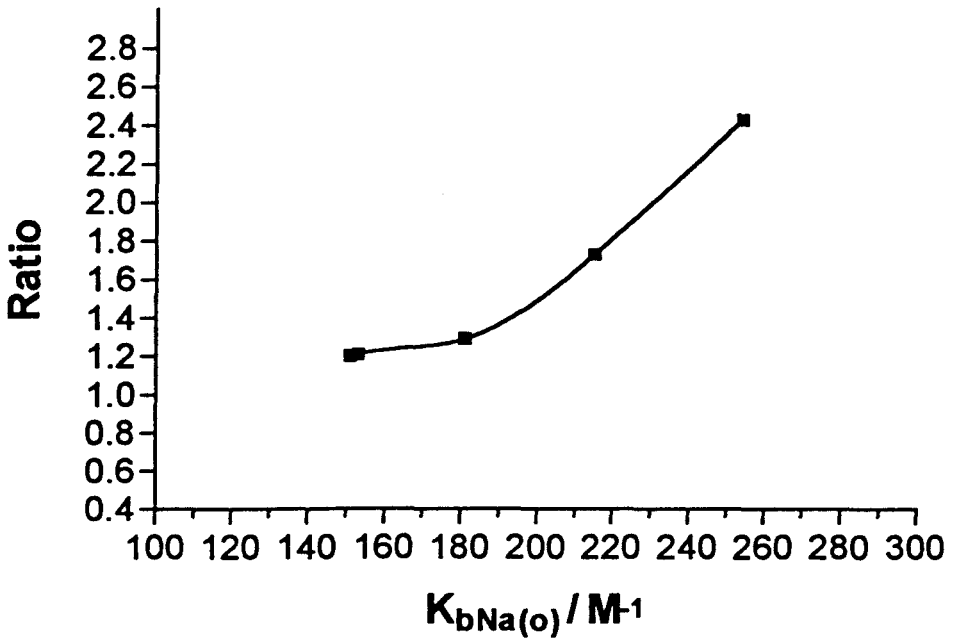

Ratio
2.8
2.6
2.4
2.2
2.0
1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
100 120 140 160 180 200 220 240 260 280 300
$K_{bNa(o)}$ / M^{-1}

Table 11. ^{23}Na T_1 Measurements (ms) for Membrane Samples in the Presence of Amiloride, EIPA, HMA, and MIA at pH 8.0.

Inhibitors	Concentration/mM						
	0	1.0	2.0	3.0	4.0	5.0	6.0
Amiloride	42.8±1.0	44.7±0.9	48.2±1.0	50.3±2.0	55.0±1.0	55.1±3.0	56.1±1.0
EIPA	44.3±3.0	45.7±0.9	48.3±2.0	49.2±2.0	52.3±2.0	53.1±2.0	56.1±2.0
HMA	45.9±2.0	50.6±2.0	49.3±1.0	51.5±2.0	53.9±2.0	55.3±2.0	57.6±2.0
MIA	44.2±1.0	45.2±1.0	49.5±3.0	47.9±2.0	53.4±2.0	52.6±2.0	52.5±2.0

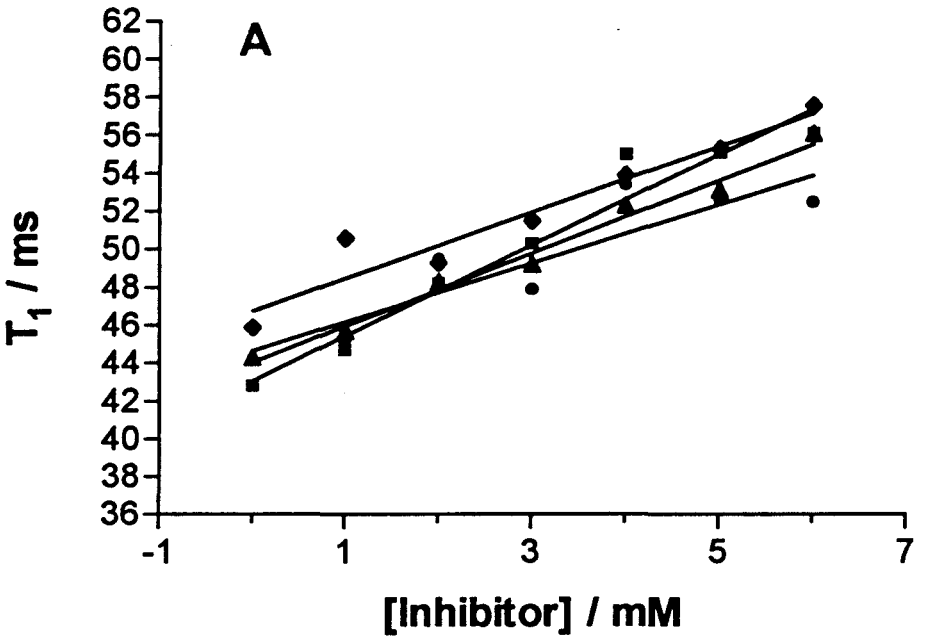
The Na^+ and protein concentrations in all membrane samples were 4 mM and 5.83 ± 0.02 mg/mL, respectively.

Table 12. ^{7}Li T_1 Measurements (s) for Membrane Samples in the Presence of Amiloride, EIPA, HMA, and MIA at pH 8.0.

Inhibitors	Concentration/mM						
	0	1.0	2.0	3.0	4.0	5.0	6.0
Amiloride	7.5±0.2	8.2±0.1	9.4±0.2	10.2±0.1	10.9±0.1	11.9±0.4	12.7±0.3
EIPA	7.1±0.3	9.5±0.2	11.5±0.3	11.9±0.2	12.7±0.2	14.4±0.1	15.9±0.9
HMA	7.9±0.1	10.4±0.3	11.6±0.2	13.5±0.3	16.7±1.0	17.3±0.3	17.2±0.6
MIA	7.7±0.1	9.9±0.3	11.9±0.2	12.3±0.4	14.2±0.2	15.9±0.4	15.7±0.8

The Li^+ and protein concentrations in all membrane samples were 4 mM and 5.01 ± 0.02 mg/mL, respectively.

Figure 9. ^{23}Na (panel A) and ^{7}Li (panel B) T_1 Relaxation Times with Amiloride (squares), EIPA (triangles), HMA (diamonds), and MIA (dots). Na^+ and Li^+ concentrations were 4 mM. Protein concentration in the membrane suspensions was 5.83 $\pm$ 0.02 mg/mL. pH was 8.0.


A
T_1 / ms
62
60
58
56
54
52
50
48
46
44
42
40
38
36
-1
1
3
5
7
[Inhibitor] / mM

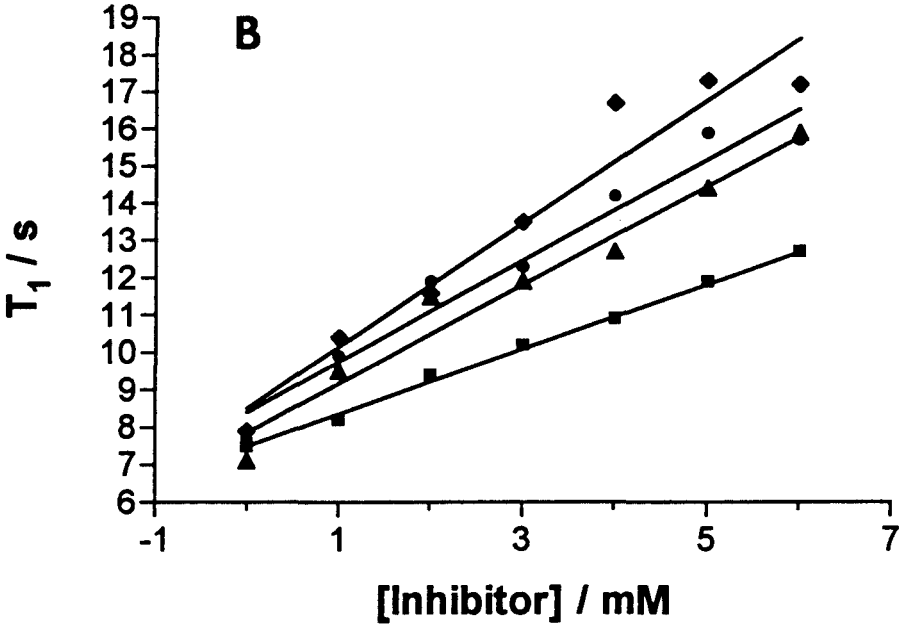

B
T_1 / s
19
18
17
16
15
14
13
12
11
10
9
8
7
6
-1
1
3
5
7
[Inhibitor] / mM

Table 13. ^{23}Na T_1 Measurements (ms) for RBC Membranes in the Presence of Varying Concentrations of Amiloride at pH 7.0. (Trial 1)

	[Amiloride]/mM					
$[Na^+]$/mM	0	0.5	1.0	1.5	2.0	2.5
2	33.8±0.6	35.6±5.0	40.6±2.0	41.3±8.0	47.1±7.0	46.6±7.0
4	39.0±0.6	40.0±4.0	42.9±1.0	44.1±5.0	50.2±2.0	47.4±4.0
6	43.3±0.7	45.0±4.0	47.9±2.0	46.0±2.0	51.9±2.0	48.3±3.0
8	46.0±1.0	45.0±2.0	47.3±1.0	47.8±0.6	55.4±2.0	49.9±2.0
10	46.1±1.0	46.6±1.0	49.7±1.0	49.7±0.3	53.9±1.0	52.3±2.0
12	49.1±0.8	47.8±0.7	51.7±1.0	51.4±1.0	54.8±0.8	51.6±1.0
$K_{Na(a)}^{app}/M^{-1}$	338	206	234	207	152	107
r^2	0.97	0.93	0.94	0.99	0.98	0.89

$K_{b(Na)} = 284\ M^{-1}$ $K_{Ami(Na)} = 1068\ M^{-1}$ $r^2 = 0.87$

Protein concentration was 7.03 ± 1.44 mg/mL.

Table 14. ^{23}Na T_1 Measurements (ms) for RBC Membranes in the Presence of Varying Concentrations of Amiloride at pH 7.0. (Trial 2)

	[Amiloride]/mM					
$[Na^+]$/mM	0	0.5	1.0	1.5	2.0	2.5
2	40.1±1.0	45.9±3.0	40.9±2.0	50.7±2.0	47.4±2.0	51.1±0.6
4	45.7±2.0	45.4±1.0	47.4±1.0	50.4±1.0	48.3±2.0	53.5±2.0
6	47.2±0.8	49.0±0.9	51.0±0.6	52.3±0.8	55.3±0.9	52.8±0.9
8	48.2±0.5	50.8±1.0	50.3±1.0	53.5±0.5	54.8±0.8	56.3±1.0
10	49.6±0.7	58.2±0.7	52.5±0.9	53.7±0.8	58.0±0.8	56.1±0.5
12	54.2±1.0	54.0±0.4	55.7±0.3	56.6±1.0	55.9±1.0	59.3±0.6
$K_{Na(a)}^{app}/M^{-1}$	352	220	272	143	124	108
r^2	0.87	0.95	0.81	0.89	0.88	0.82

$K_{b(Na)} = 240\ M^{-1}$ $K_{Ami(Na)} = 973\ M^{-1}$ $r^2 = 0.84$

Protein concentration was 7.03 ± 1.44 mg/mL.

Table 15. ^{7}Li T_1 Measurements (s) for RBC Membranes in the Presence of Varying Concentrations of Amiloride at pH 7.0. (Trial 1)

	[Amiloride]/mM					
$[Li^+]$/mM	0	0.5	1.0	1.5	2.0	2.5
2	7.2±0.7	9.3±0.4	9.2±0.8	8.8±1.0	12.1±0.7	14.1±1.4
4	8.8±0.2	8.6±0.2	12.4±0.7	10.5±0.4	11.8±.07	11.7±0.8
6	9.9±0.3	10.9±0.4	10.1±0.5	11.2±0.4	13.1±0.7	11.8±0.6
8	10.5±0.6	10.0±.02	12.3±0.8	12.7±0.3	12.5±0.3	12.1±0.4
10	10.4±0.2	11.7±0.6	11.1±0.4	12.2±0.2	12.5±0.3	13.0±0.6
12	11.7±0.6	11.8±0.6	12.7±0.2	13.2±0.5	13.7±0.1	14.4±0.4
K_{Li}^{app}/M^{-1}	150	97	84	80	50	83
r^2	0.93	0.84	0.81	0.83	0.82	0.92

$K_{b(Li)} = 154\ M^{-1}$ $K_{Ami(Li)} = 1004\ M^{-1}$ $r^2 = 0.98$

Protein concentration was 7.03 ± 1.44 mg/mL.

Table 16. ^{7}Li T_1 Measurements (s) for RBC Membranes in the Presence of Varying Concentrations of Amiloride at pH 7.0. (Trial 2)

	[Amiloride]/mM					
[Li]/mM	0	0.5	1.0	1.5	2.0	2.5
2	5.5±0.1	5.9±0.3	5.9±0.3	7.2±0.5	7.7±0.7	8.1±0.7
4	6.3±0.3	6.7±0.4	6.1±0.3	7.2±0.2	7.5±0.3	8.6±0.3
6	6.6±0.3	8.0±0.4	6.5±0.1	7.5±0.3	8.2±0.4	9.1±0.3
8	7.5±0.2	8.0±0.3	7.3±0.2	8.1±0.4	9.0±0.7	9.2±0.4
10	8.1±0.4	8.1±0.2	7.9±0.1	8.7±0.4	8.4±0.2	9.2±0.9
12	9.1±0.4	9.5±0.3	7.8±0.3	8.9±0.4	9.8±0.7	10.4±0.9
K_{Li}^{app}/M^{-1}	152	116	77	57	58	51
r^2	0.96	0.89	0.94	0.95	0.81	0.84

$K_{b(Li)} = 153\ M^{-1}$ $K_{Ami(Li)} = 924\ M^{-1}$ $r^2 = 0.93$

Protein concentration was 7.03 ± 1.44 mg/mL.

Table 17. ^{23}Na T_1 Measurements (ms) for RBC Membranes in the Presence of Varying Concentrations of Amiloride at pH 8.0. (Trial 1)

	[Amiloride]/mM					
$[Na^+]$/mM	0	0.5	1.0	1.5	2.0	2.5
2	32.3±4.0	34.5±3.0	39.1±2.0	40.6±3.0	43.8±2.0	49.0±4.0
4	41.8±1.0	42.7±3.0	45.3±2.0	48.9±1.0	46.5±1.0	50.0±1.0
6	44.6±1.0	39.3±2.0	46.1±1.0	51.4±1.0	48.9±0.6	53.7±2.0
8	47.1±1.0	43.3±0.7	49.0±1.0	52.3±0.3	49.4±7.0	53.8±1.0
10	49.3±0.5	46.0±0.4	48.2±0.4	54.3±0.6	50.8±0.6	53.9±1.0
12	50.1±0.7	47.4±0.4	50.0±0.7	54.9±0.9	53.0±0.7	55.1±1.0
$K_{Na(a)}^{app}/M^{-1}$	474	341	188	232	155	140
r^2	0.97	0.98	0.90	0.98	0.97	0.82

$K_{b(Na)}$ =273 M^{-1} $K_{Ami(Na)}$ = 878 M^{-1} r^2 = 0.94

Protein concentration was 7.03 ± 1.44 mg/mL.

Table 18. ^{23}Na T_1 Measurements (ms) for RBC Membranes in the Presence of Varying Concentrations of Amiloride at pH 8.0. (Trial 2)

	[Amiloride]/mM					
$[Na^+]$/mM	0	0.5	1.0	1.5	2.0	2.5
2	36.8±2.0	40.4±2.0	43.1±2.0	44.4±1.0	46.0±0.9	48.3±2.0
4	40.5±0.4	43.4±1.0	44.8±1.0	47.0±1.0	47.7±0.4	50.2±2.0
6	43.7±0.7	44.2±0.6	49.0±0.8	49.2±0.6	52.1±0.4	52.9±1.0
8	45.9±0.1	47.5±0.5	49.1±0.4	49.7±0.6	54.2±0.7	53.3±1.0
10	47.9±0.3	48.3±0.8	49.6±0.7	51.0±0.6	53.7±0.5	55.1±0.5
12	49.2±0.5	50.6±0.7	51.1±1.0	52.8±0.8	56.6±0.8	55.7±0.5
$K_{Na(a)}^{app}/M^{-1}$	290	218	144	166	185	110
r^2	0.99	0.97	0.91	0.97	0.94	0.98

$K_{b(Na)} = 174\ M^{-1}$ $K_{Ami(Na)} = 584\ M^{-1}$ $r^2 = 0.89$

Protein concentration was 7.03 ± 1.44 mg/mL.

Table 19. ^{7}Li T_1 Measurements (s) for RBC Membranes in the Presence of Varying Concentrations of Amiloride at pH 8.0. (Trial 1)

	[Amiloride]/mM					
[Li]/mM	0	0.5	1.0	1.5	2.0	2.5
2	4.8±0.5	5.7±0.2	5.7±0.4	6.3±0.5	7.1±0.6	7.8±0.5
4	6.4±0.5	6.0±0.1	6.7±0.5	7.2±0.2	8.2±0.3	8.3±0.3
6	6.7±0.1	7.1±0.1	7.9±0.4	7.8±0.1	8.9±0.1	8.9±0.2
8	7.3±0.2	7.5±0.1	7.5±0.3	8.0±0.1	9.0±0.1	8.8±0.1
10	7.9±0.1	7.8±0.1	8.1±0.1	8.8±0.1	9.4±0.1	9.3±0.1
12	8.4±0.1	8.2±0.1	8.8±0.2	8.8±0.1	10.1±0.1	9.9±0.1
K_{Li}^{app}/M^{-1}	163	92	90	72	80	51
r^2	0.99	0.95	0.89	0.97	0.95	0.94

$K_{b(Li)} = 147\ M^{-1}$ $K_{Ami(Li)} = 742\ M^{-1}$ $r^2 = 0.96$

Protein concentration was 7.03 ± 1.44 mg/mL.

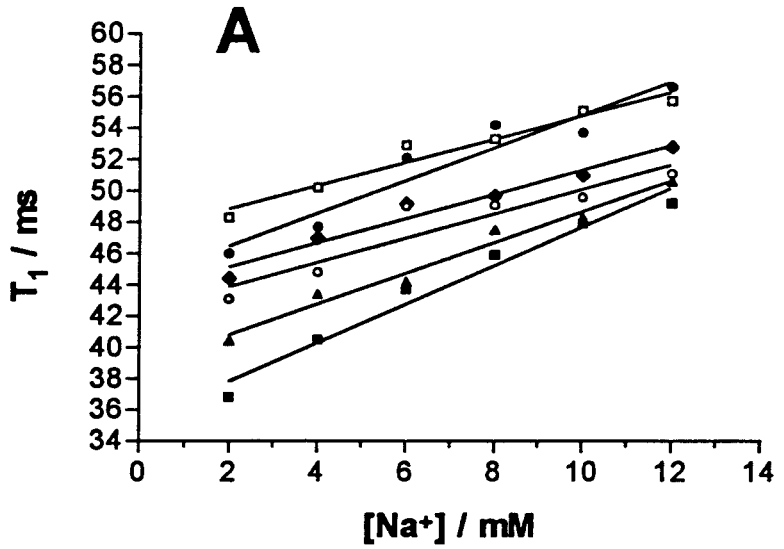
Table 20. ^{7}Li T_1 Measurements (s) for RBC Membranes in the Presence of Varying Concentrations of Amiloride at pH 8.0. (Trial 2)

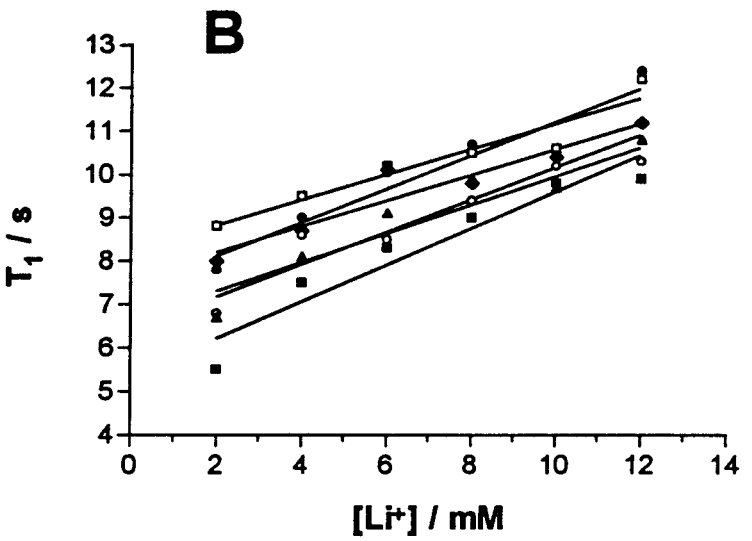
	[Amiloride]/mM					
[Li]/mM	0	0.5	1.0	1.5	2.0	2.5
2	5.5±0.5	6.7±0.5	6.8±0.5	8.0±0.8	7.8±0.3	8.8±0.6
4	7.5±0.4	8.1±0.4	8.6±0.4	8.7±0.3	9.0±0.4	9.5±0.2
6	8.3±0.3	9.1±0.5	8.5±0.3	10.1±0.6	10.2±0.4	10.2±0.6
8	9.0±0.2	9.9±0.3	9.4±0.4	9.8±0.2	10.7±0.5	10.5±0.2
10	9.8±0.2	9.7±0.3	10.2±0.4	10.4±0.4	10.2±0.3	10.6±0.2
12	9.9±0.3	10.8±0.2	10.3±0.5	11.2±0.4	12.4±0.4	12.2±0.2
K_{Li}^{app}/M^{-1}	172	131	104	99	91	59
r^2	0.95	0.93	0.95	0.94	0.97	0.92

$K_{b(Li)}$ = 183 M^{-1} $K_{Ami(Li)}$ = 689 M^{-1} r^2 = 0.87

Protein concentration was 7.03 ± 1.44 mg/mL.

Figure 10. ^{23}Na (panel A) and ^{7}Li (panel B) T_1 Measurements with 0 mM (solid squares), 0.5 mM (triangles), 1.0 mM (circles), 1.5 mM (diamonds), 2.0 mM (dots), 2.5 mM (open squares) Amiloride. Protein concentration in RBC membrane suspensions was 7.03 $\pm$ 1.44 mg/mL. pH was 8.0.


A
T_1 / ms
[Na^+] / mM


B
T_1 / s
[Li^+] / mM

^{23}Na and ^{7}Li T_1 values increased. The ^{23}Na $T_{1(free)}$ values for calculating the Na^+ apparent binding constants at pH 7.0 and pH 8.0 were 67.1 ± 0.8 ms and 65.8 ± 0.4 ms, respectively. The ^{7}Li $T_{1(free)}$ values for calculating the Li^+ apparent binding constants at pH 7.0 and pH 8.0 were 20.6 ± 0.9 s and 21.0 ± 0.9 s, respectively. The protein concentration in the RBC membrane suspensions was 7.03 ± 1.44 mg/mL.

The apparent Na^+ and Li^+ binding constants at pH 7.0 and 8.0 were listed in Tables 13 through 20. The actual Na^+ binding constants were obtained by multiplying the apparent Na^+ binding constants with the corresponding correction factor from the correction curve shown in Figure 8. Figure 11 shows the apparent Li^+ binding constants and the actual Na^+ binding constants at pH 7.0 and 8.0. At pH values of 7.0 and 8.0, both Na^+ and Li^+ binding constants decreased when amiloride concentration was increased. Both Na^+ and Li^+ binding constants were higher at pH 8.0 than at pH 7.0.

Table 21 shows the amiloride binding constants to RBC membranes with Na^+ or Li^+ present at pH 7.0 and 8.0. The amiloride binding constants in the presence of Na^+ and Li^+ are higher at pH 7.0 than at pH 8.0. Statistical analysis shows that there is no significant difference between amiloride inhibitory binding constant to RBC membranes with Na^+ and Li^+ (1021 ± 48 vs. 964 ± 40 at pH 8.0; 731 ± 147 vs. 716 ± 27 M^{-1} at pH 7.0, $p \geq 0.05$). Amiloride had the same inhibitory effects on both Na^+ and Li^+ binding to RBC membranes.

IV.5. Correlation of Na^+-Li^+ Exchange Activity with Na^+ and Li^+ Binding, Phospholipid Composition and Total Phospholipid, and Free Mg^{2+} Concentration in RBCs from Essential Hypertensive and Normotensive Individuals

IV.5A. Kinetic Parameters of Na^+-Li^+ Exchange in Intact RBCs

Figure 11. Amiloride Inhibitory Activities on Na^+ and Li^+ Binding to RBC Membranes. Squares, $K_{bNa(a)}$, pH 7.0; triangles, $K_{bNa(a)}$, pH 8.0; dots, K_{bLi}, pH 7.0; diamonds, K_{bLi}, pH 8.0.

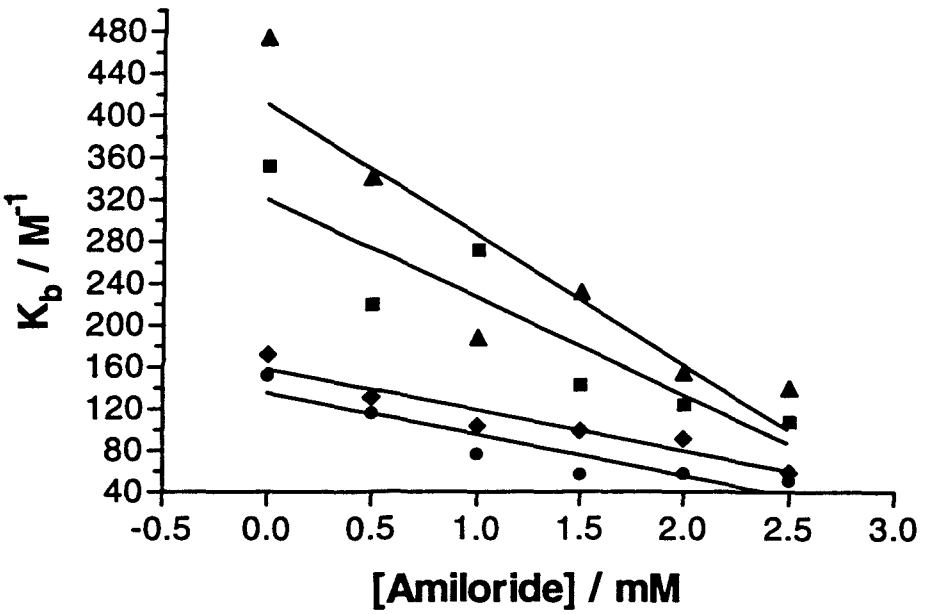

480
440
400
360
320
280
240
200
160
120
80
40
K_b / M^{-1}
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
[Amiloride] / mM

Table 21. Amiloride Inhibitory Activity on Na^+ and Li^+ Binding to RBC Membranes.

pH	$K_{Ami(Na)}/M^{-1}$			$K_{Ami(Li)}/M^{-1}$		
	1	2	mean ± SEM	1	2	mean ± SEM
7.0	1068	973	1021 ± 48	1004	924	964 ± 40
8.0	878	584	731 ± 147	742	689	716 ± 27

The V_{std}, V_{max} and K_m values of Na^+-Li^+ exchange in intact RBCs from essential hypertensive and normotensive individuals are listed in Tables 22 and 23, respectively. As shown in Table 24 and in Figure 12, the rates of RBC Na^+-Li^+ exchange measured by the standard method (V_{std}), and the kinetic parameters V_{max} and K_m which we measured by varying the extracellular Na^+ concentration, were significantly higher for essential hypertensive patients than for normotensive individuals (V_{std}, V_{max}, and K_m were 0.32 ± 0.09, 0.66 ± 0.17 mmol Li^+/L cell·h, and 160 ± 62 mmol/L for hypertensives vs. 0.21 ± 0.06, 0.32 ± 0.14 mmol Li^+/L cell·h, and 86 ± 69 mmol/L for normotensives, $n = 10$, $p < 0.05$).

IV.5B. Na^+ and Li^+ Interactions with RBC Membranes

Figure 13 shows the ^{23}Na and ^7Li T_1 values observed upon titration with Na^+ (panel A) or Li^+ (panel B) of unsealed membranes prepared from the RBCs of a hypertensive patient and those from the RBCs of a matched normotensive individual at the same membrane protein concentration. For the same Na^+ concentrations, the ^{23}Na T_1 values were significantly higher ($p < 0.05$) for the unsealed membrane suspension from RBCs of a hypertensive patient than from those of the matched normotensive individual. For a given Li^+ concentration, however, the ^7Li T_1 values were not significantly different ($p > 0.05$) for the patient and the matched normotensive individual. Similar results were obtained for the rest of the matched pairs.

Na^+ and Li^+ binding constants to the RBC membranes from ten essential hypertensive and ten normotensive individuals are listed in Tables 22 and 23. $K_{b(Na)}$ values were observed $K_{b(Na)}$ values corrected by multiplying the corresponding correction factors from the curve in Figure 8. One-way ANOVA with Tukey's conservative correction showed that Na^+ binding constants to RBC membranes from hypertensives were significantly lower than those from normotensives (317 ± 85 M^{-1} vs. 465 ± 112 M^{-1}, $n = 10$, $p < 0.005$) (Table 24 and Figure

Table 22. Na^+-Li^+ Exchange Rates (in mmol Li^+/L RBCs×h), Na^+ Dissociation Constants (mM) to Outside Sites of RBCs and Na^+, Li^+ Binding Constants (M^{-1}) to RBC membranes, Phospholipid Compositions (%) and Total Phospholipid (mg/100 mg tp[a]), Free Mg^{2+} Concentrations (μM) in RBC of Essential Hypertensive Patients.

Patients	V_{std}	V_{max}	K_m	$K_{b(Na)}$	$K_{b(Li)}$	PC	PI	PS	Sph	PE	TP[b]	$[Mg^{2+}]_f$
42/W/F	0.29	0.54	241	193	264	31.8	1.3	15.5	21.5	29.9	49	237
63/W/F	0.41	0.88	174	436	248	29.5	2.3	15.4	22.6	30.3	44	194
56/W/F	0.21	0.64	258	231	250	28.2	2.3	16.7	24.6	28.2	71	210
76/W/F	0.33	0.80	176	262	248	31.1	2.3	15.2	22.7	28.6	84	264
72/W/M	0.46	0.63	67	267	144	30.1	3.0	16.0	25.1	25.8	84	167
69/W/F	0.26	0.66	204	246	186	32.2	2.7	14.6	22.1	28.3	93	232
49/W/M	0.29	0.48	81	405	196	30.0	2.0	16.2	21.6	30.2	69	194
57/W/M	0.29	0.84	172	411	225	28.8	2.5	15.6	23.6	29.5	59	198
81/W/F	0.46	0.81	127	310	324	30.3	2.1	14.0	22.7	31.0	63	192
56/W/F	0.19	0.30	100	402	196	29.4	2.1	13.9	22.9	31.7	61	200

[a]tp refers to total protein. [b]TP refers to total phospholipid.

Table 23. Na^+-Li^+ Exchange Rates (in mmol Li^+/L RBCs×h), Na^+ Dissociation Constants (mM) to Outside Sites of RBCs and Na^+, Li^+ Binding Constants (M^{-1}) to RBC membranes, Phospholipid Composition (%) and Total Phospholipid (mg/100 mg tp[a]), Free Mg^{2+} Concentrations (μM) in RBC of Matched Normotensive Individuals.

Controls	V_{std}	V_{max}	K_m	$K_{b(Na)}$	$K_{b(Li)}$	PC	PI	PS	Sph	PE	TP[b]	$[Mg^{2+}]_f$
31/W/F	0.28	0.50	94	703	232	29.1	2.5	14.2	21.2	33.0	68	254
47/W/F	0.10	0.14	119	473	228	24.8	1.9	15.4	24.9	33.0	45	161
41/W/F	0.14	0.16	31	490	299	28.1	1.3	14.5	21.5	34.7	68	233
53/W/F	0.22	0.36	39	498	308	31.3	1.2	12.1	21.7	33.7	67	298
50/W/M	0.18	0.25	99	400	165	26.2	1.6	14.7	24.4	33.1	26	237
52/W/F	0.17	0.28	81	358	242	28.2	2.3	12.9	25.9	30.8	23	215
30/W/M	0.31	0.43	54	256	189	28.7	2.0	13.0	21.8	34.5	80	254
32/W/M	0.25	0.28	45	465	230	32.8	1.8	11.7	25.3	28.4	67	276
65/W/F	0.24	0.60	272	452	214	28.1	1.5	14.0	23.9	32.5	52	222
46/W/F	0.18	0.21	22	553	219	29.5	1.9	15.9	21.9	30.8	65	259

[a]tp refers to total protein. [b]TP refers to total phospholipid.

Table 24. Na^+-Li^+ Exchange Parameters, Na^+ and Li^+ Binding Constants, Phospholipid Composition and Total Phospholipid, and Free Mg^{2+} Concentration in RBCs and Clinical Characteristics of Essential Hypertensive and Normotensive Individuals.

	Normotensives (n = 10)	Hypertensives (n = 10)	p
V_{std}, mmol/L cell·h	0.21±0.06	0.32±0.09	< 0.01
V_{max}, mmol/L cell·h	0.32±0.14	0.66±0.17	< 0.001
K_m, mmol/L	86±69	160±62	< 0.05
$K_{b(Na)}$, M^{-1}	465±112	317±85	< 0.005
$K_{b(Li)}$, M^{-1}	233±41	228±48	> 0.05
PC[a]	28.7±2.2	30.1±1.2	> 0.05
PI[a]	1.8±0.40	2.3±0.40	> 0.05
PS[a]	13.8±1.3	15.3±0.9	< 0.05
Sph[a]	23.3±1.7	22.9±1.1	> 0.05
PE[a]	32.5±1.8	29.4±1.6	< 0.001
Total phospholipid, mg/100 mg tp[b]	56±18	68±15	> 0.05
$[Mg^{2+}]_f$, μM	241±36	209±27	< 0.05
Triglycerides, mg/dL	158±70	174±71	> 0.05
Cholesterol, mg/dL	203±28	225±43	> 0.05
Diastolic pressure, mm Hg	75±4	83±10	< 0.05
Systolic pressure, mm Hg	121±9	139±16	< 0.01
Age, y	45±10	62±12	< 0.05

Values are mean±SEM. Statistical analysis performed with one-way ANOVA with Tukey's conservative correction. $p \leq .05$ was considered significantly different.
[a]Percentage of total phospholipid. [b]total protein.

Figure 12. Kinetic Parameters of Na^+-Li^+ Exchange in Intact RBCs from Ten Essential Hypertensive (circles) and Ten Normotensive (squares) Individuals. (A) and (B), Na^+-Li^+ exchange rates, V_{std} and V_{max}, respectively; (C), affinity constants of Na^+-Li^+ exchange in RBCs, K_m.

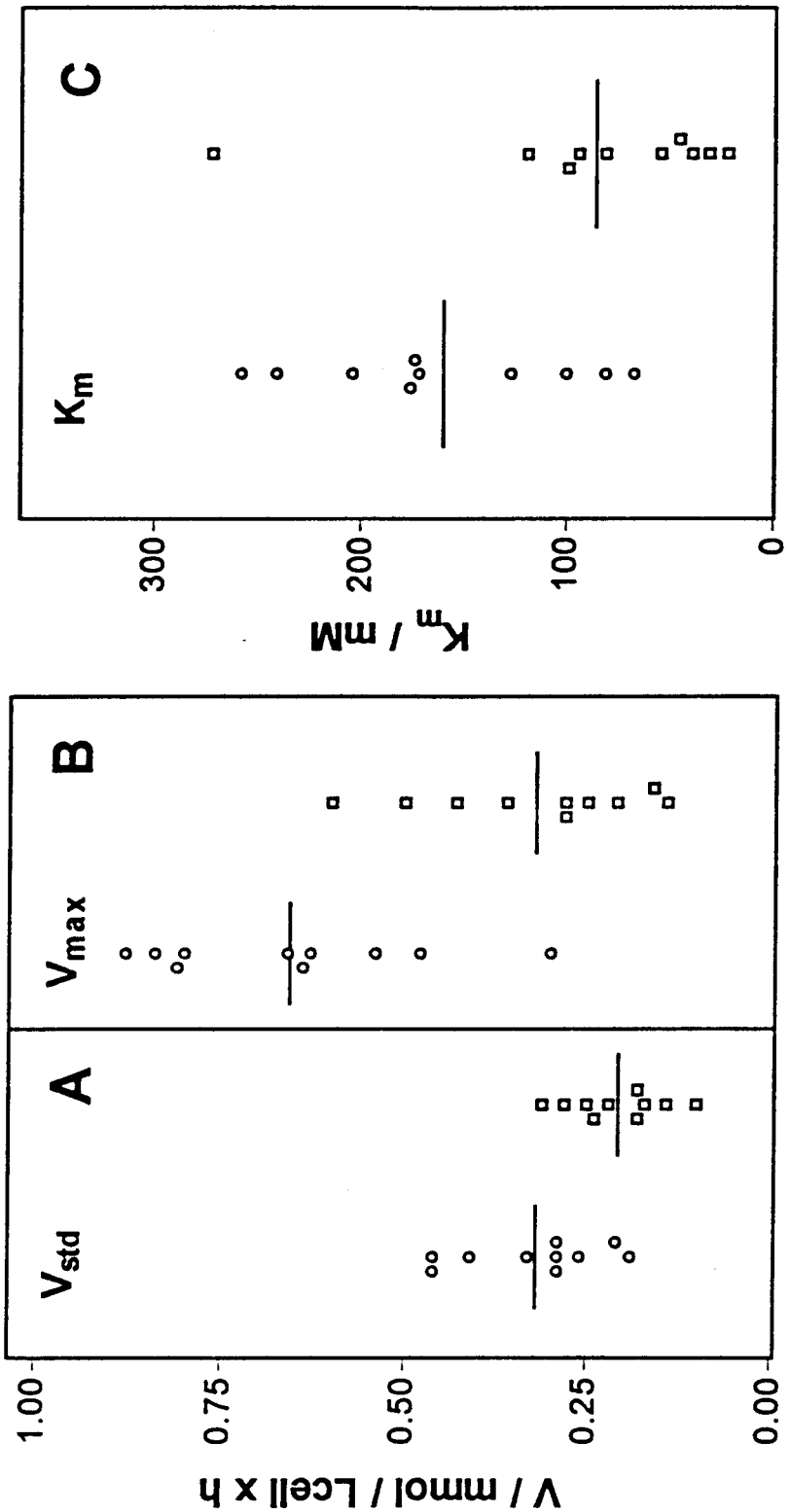
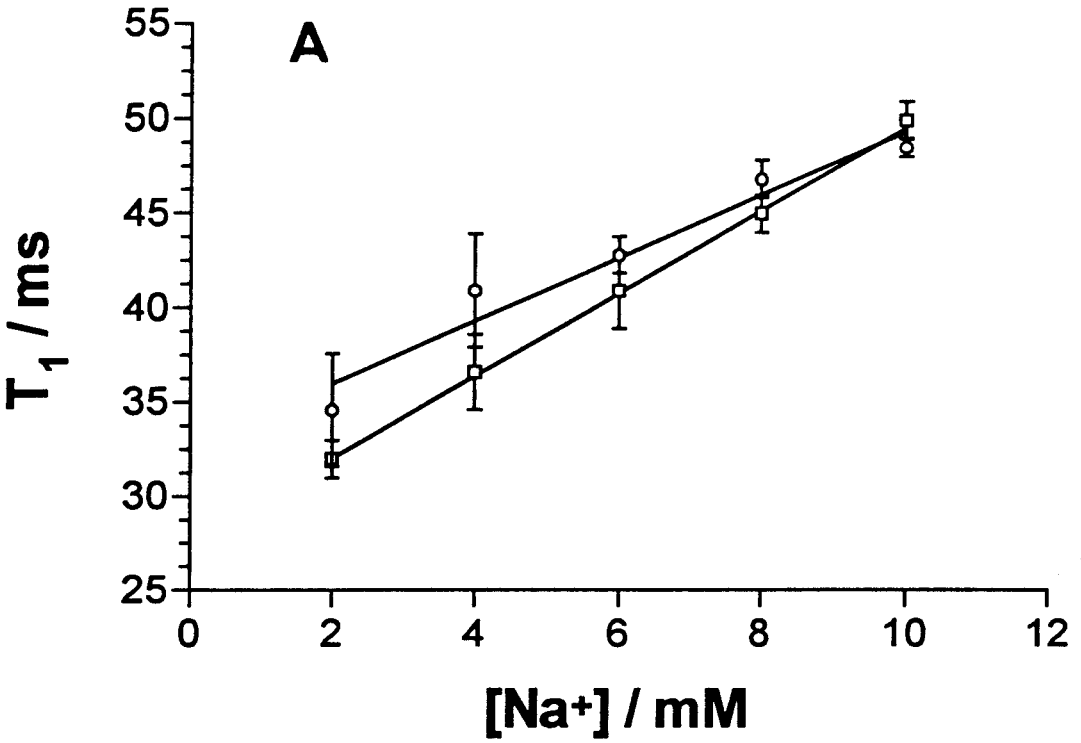
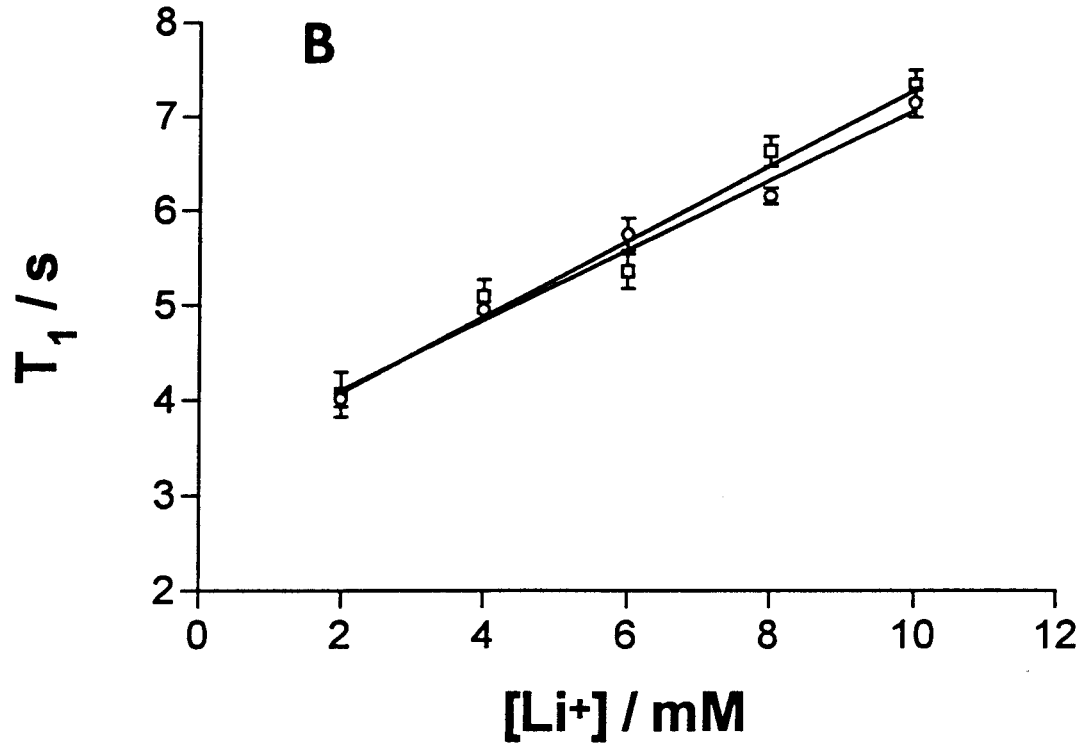

A
B
C
V_{std}
V_{max}
K_m
V / mmol / Lcell x h
1.00
0.75
0.50
0.25
0.00
K_m / mM
300
200
100
0

Figure 13. ^{23}Na (A) and ^{7}Li (B) T_1 Measurements in the RBC Membrane Suspensions from An Essential Hypertensive (circles) and the Matched Normotensive (squares) Individual.


A
55
50
45
40
35
30
25
T_1 / ms
0
2
4
6
8
10
12
[Na+] / mM


B
8
7
6
5
4
3
2
T_1 / s
0
2
4
6
8
10
12
[Li+] / mM

14), indicating that Na^+ was bound to the RBC membranes from hypertensives more weakly than from normotensives. No significant difference was found between the two groups for the Li^+ binding constants to RBC membranes (Table 24 and Figure 14).

IV.5C. Phospholipid Composition and Total Phospholipid

Figure 15 shows a typical ^{31}P NMR spectrum of a phospholipid extract from the RBC membranes from an essential hypertensive patient. The assignments of the phospholipid resonances are indicated in the spectrum and were obtained as described in Methods. Two types of PE are present in human RBC membranes, and they can be discriminated by ^{31}P NMR spectroscopy: regular PE has an alkyl ether on the glyceride backbone, whereas PE plasmalogen has an alkenyl ether. The areas under the two PE resonances were added to yield the PE composition.

The values of phospholipid compositions and total phospholipid in the RBC membranes from ten essential hypertensive and normotensive individuals are listed in Tables 22 and 23. Table 24 and Figure 16 show that the percentage of PS in RBC membranes was significantly higher for essential hypertensive patients than for normotensive individuals ($15.3 \pm 0.9\%$ vs. $13.8 \pm 1.3\%$, $n = 10$, $p < 0.05$), whereas the percentage of PE was significantly lower ($29.4 \pm 1.6\%$ vs. $32.5 \pm 1.8\%$, $n = 10$, $p < 0.001$). No statistically significant differences ($p \leq 0.05$) were found between the patient and control groups for the percentages of the other phospholipids.

The total phospholipid concentrations in the RBC membranes from hypertensive patients and normotensive individuals were also measured by the ^{31}P NMR method; there was no statistically significant difference between the RBC membranes of the two groups in their total phospholipid concentrations (Table 24).

Figure 14. Na^+ (A) and Li^+ (B) Binding Constants to the RBC Membranes from Ten Essential Hypertensive (circles) and Ten Normotensive (squares) Individuals.

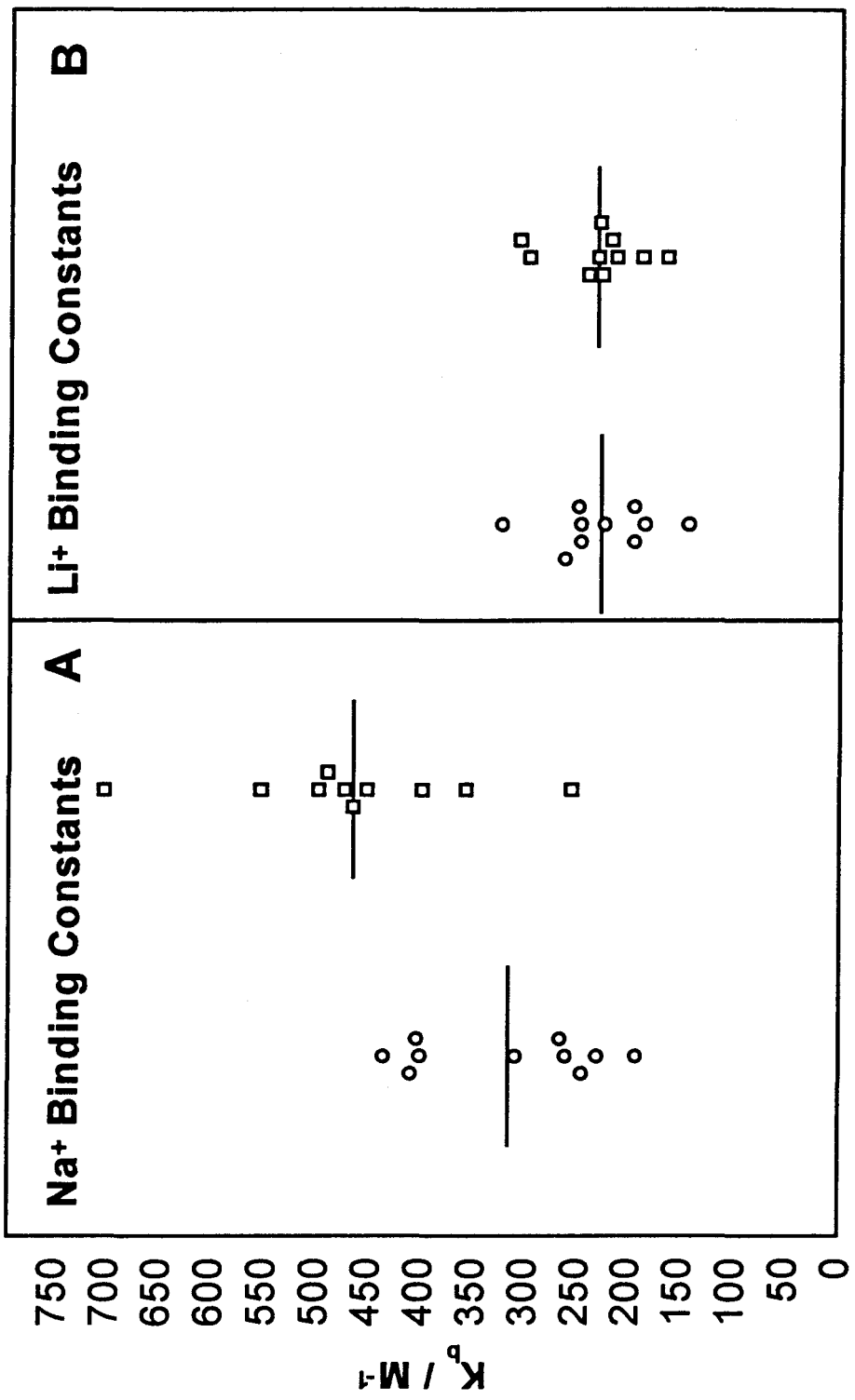

A
Na+ Binding Constants
B
Li+ Binding Constants
750
700
650
600
550
500
450
400
350
300
250
200
150
100
50
0
Kb / M-1

Figure 15. ^{31}P NMR Spectrum of the Phospholipid Extracts from the RBC Membranes from An Essential Hypertensive Individual. The solvent mixture ratio of chloroform/methonal/0.2 mM EDTA was 125:8:3. The assignments of each resonance are: PEp, PE plasmalogen; PE, phosphatidyl ethanolamine; Sph, sphingomyelin; PS, phosphatidyl serine; PI, phosphatidyl inositol; AAPC, alkylacyl PC; PC, phosphatidyl choline. The positions of the resonances (chemical shifts) are reported relative to that of PC set at -0.84 ppm (parts per million).

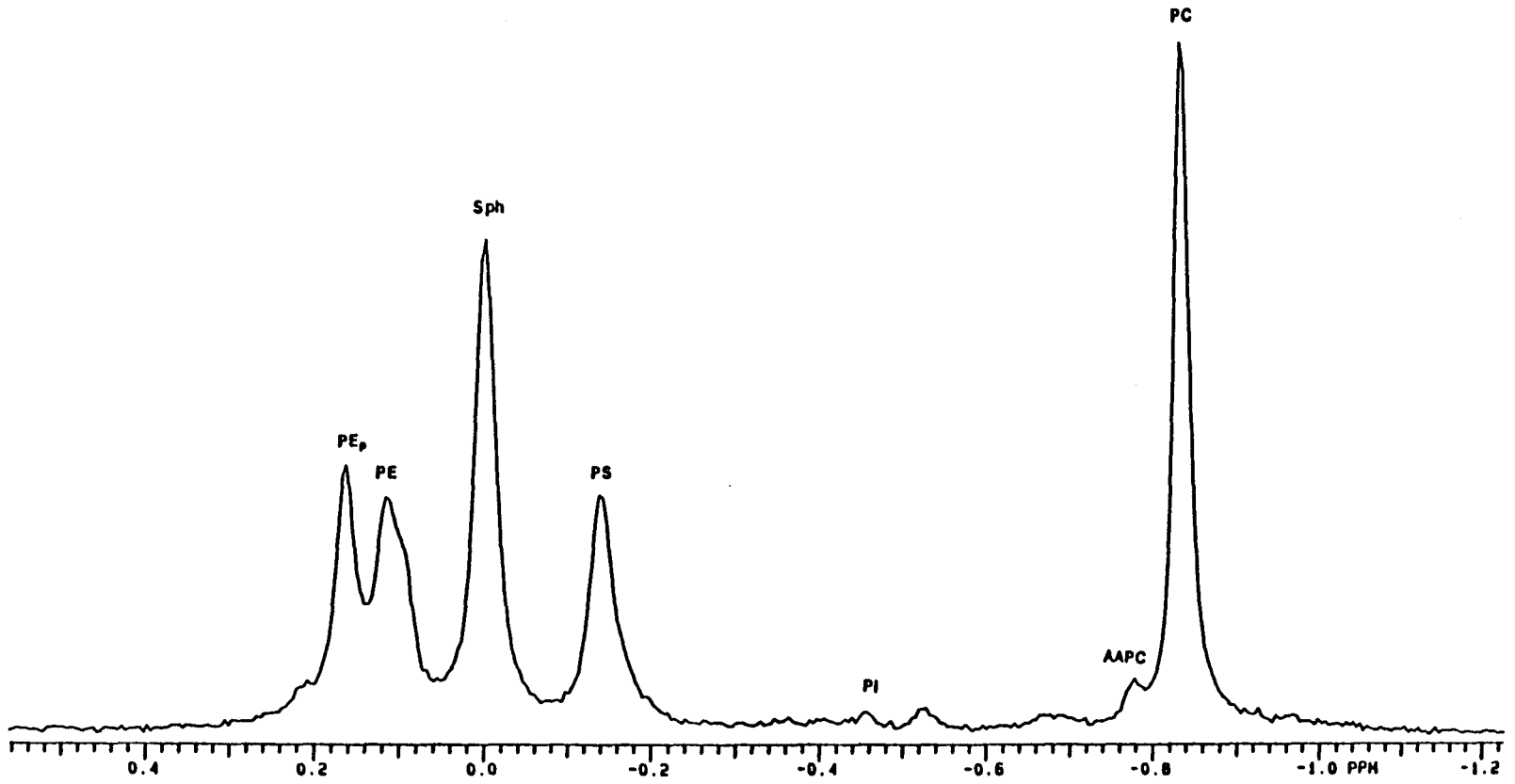
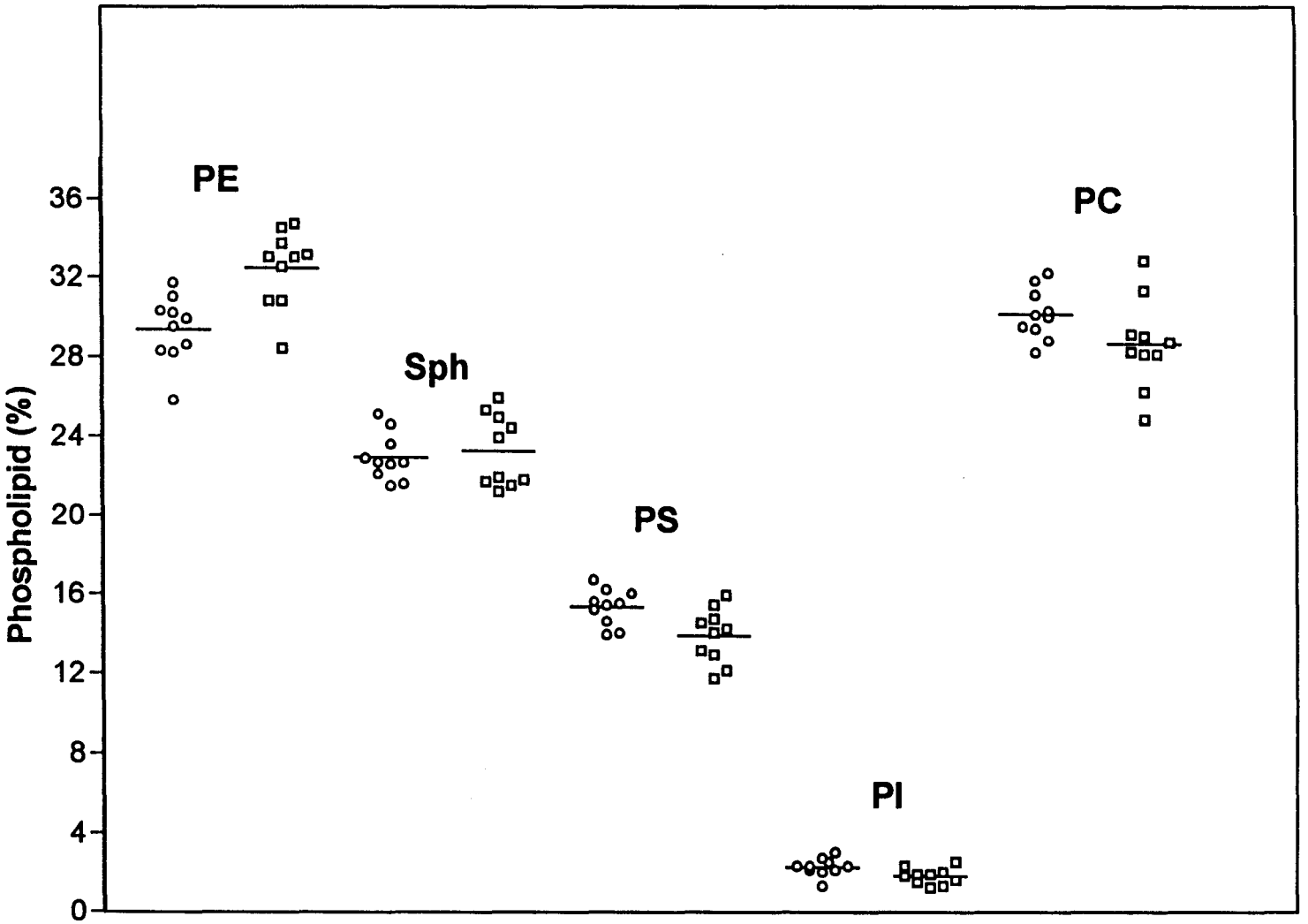

PC
Sph
PE$_p$
PE
PS
AAPC
PI
0.4
0.2
0.0
-0.2
-0.4
-0.6
-0.8
-1.0 PPM
-1.2

Figure 16. Phospholipid Composition of the RBC Membranes from Ten Essential Hypertensive (circles) and Ten Normotensive (squares) Individuals.


PE
Sph
PS
PI
PC
Phospholipid (%)
0
4
8
12
16
20
24
28
32
36

IV.5D. Free Mg^{2+} Concentrations in RBCs

Free Mg^{2+} concentrations in the RBCs from ten essential hypertensive and ten normotensive individuals were measured by ^{31}P NMR and are listed in Tables 22 and 23. One-way ANOVA with Tukey's conservative correction showed that free Mg^{2+} concentration was significantly lower for the hypertensive group than for the normotensive group (Table 24).

IV.5E. Demography and Clinical Characteristics

Demography of ten essential hypertensive and ten normotensive individuals are listed in Tables 25 and 26, respectively. Clinical characteristics, triglyceride and cholesterol levels, blood pressure values for these hypertensive and normotensive individuals were obtained from the Hypertension Section, Loyola University Medical Center and are listed in Tables 25 and 26, respectively. One-way ANOVA did not show significant differences between hypertensive and normotensive groups for triglycerides and cholesterol levels (Table 24). Both diastolic and systolic blood pressures are significantly higher for the hypertensive group than for the normotensive group (Table 24). The average value of age for the hypertensive group was significantly higher than for the normotensive group (Table 24).

IV.5F. Power Analysis of Sample Size

Power analysis of the sample size which needed in this clinical study is listed in Table 27. A sample size of ten for each group was sufficient to yield statistically significant differences with a power level of 0.90, and an α value of 0.05, for all of the RBC parameters (V_{std}, V_{max}, K_m, $K_{b(Na)}$, PS, and PE) which were also statistically significantly different by one-way ANOVA with Tukey's conservative correction. For a power level of 0.95, however,

Table 25. Demography of Essential Hypertensive Patients.

Patients	Age (y)	Race	Sex	Weight (LBs)	BP(S/D) (mm Hg)	Smoker	Triglycerides (mg/dL)	Cholesterol (mg/dL)
1	42	W	F	157	110/70	N	68	250
2	63	W	F	154	144/94	N	136	333
3	56	W	F	218	130/92	N	188	212
4	76	W	F	162	150/90	N	154	254
5	72	W	M	234	160/90	N	238	178
6	69	W	F	165	130/62	N	153	205
7	49	W	M	175	120/80	N	119	179
8	57	W	M	164	138/80	N	153	205
9	81	W	F	171	160/80	N	343	218
10	56	W	F	223	150/90	N	188	212

BP(S/D), blood pressure (systolic/diastolic); W, white; M, male; F, female; N, no

Table 26. Demography of Normotensive Controls.

Controls	Age (y)	Race	Sex	Weight (LBs)	BP(S/D) (mm Hg)	Smoker	Triglycerides (mg/dL)	Cholesterol (mg/dL)
1	31	W	F	165	120/80	N	104	196
2	47	W	F	140	138/80	N	96	203
3	41	W	F	160	120/80	N	112	158
4	53	W	F	195	132/76	N	349	250
5	50	W	M	170	112/70	N	130	210
6	52	W	F	133	110/70	N	133	202
7	30	W	M	225	122/78	N	165	242
8	32	W	M	195	124/72	N	191	164
9	65	W	F	143	120/78	N	130	215
10	46	W	F	129	110/70	N	174	188

BP(S/D), blood pressure (systolic/diastolic); W, white; M, male; F, female; N, no

Table 27. Sample Size for Analysis of the Kinetic Parameters of Na^+-Li^+ Exchange in Intact RBCs, Na^+ and Li^+ Binding Constants to RBC Membranes, Phospholipid Compositions and Total Phospholipid in RBC Membranes, and Free Mg^{2+} Concentration in the RBCs Between Essential Hypertensive and Normotensive Groups.

Parameters	n[a] (0.90)[b]	n[a] (0.95)[b]
V_{std}	10	15
V_{max}	5	6
K_m	6	8
$K_{b(Na)}$	10	14
$K_{b(Li)}$	1675	2452
PC	33	48
PI	14	21
PS	10	17
Sph	275	403
PE	7	10
Total Phospholipid	41	59
$[Mg^{2+}]_f$	20	30
Triglycerides	406	594
Cholesterol	58	84
Diastolic blood pressure	22	32
Systolic blood pressure	10	15

All samples were analyzed with an α value of 0.05. [a]Sample size; [b]power level

only V_{max}, K_m, and PE values were sufficient to yield statistically significant differences between the two groups with a value of 0.05 for α.

IV.5G. Correlation Analysis

For the patient group, Spearman correlation showed that the V_{max} values were positively correlated with the V_{std} values (r = 0.49, p = 0.03), that $K_{b(Na)}$ values were negatively correlated with the K_m values (r = -0.61, p = 0.01), that the $K_{b(Na)}$ values were positively correlated with the PE values (r = 0.52, p = 0.02), and that free Mg^{2+} concentrations were negatively correlated with the V_{std} (r = -0.55, p = 0.01), negatively correlated with K_m values (r = -0.76, p = 0.01) and positively correlated with the $K_{b(Na)}$ values (r = 0.59, p = 0.01). For the control group, Spearman correlation showed that the V_{max} values were positively correlated with the V_{std} values (r = 0.82, p = 0.01), that the $K_{b(Na)}$ values were negatively correlated with the K_m values (r = -0.43, p = 0.04), that the $K_{b(Na)}$ values were positively correlated with the PE values (r = 0.42, p = 0.04) and that free Mg^{2+} concentrations were negatively correlated with V_{std} values (r = -0.58, p = 0.01), negatively correlated with K_m values (r = -0.63, p = 0.01) and positively correlated with $K_{b(Na)}$ (r = 0.40, p = 0.05) (Tables 28 and 29).

Table 28. Spearman Correlation of Na^+-Li^+ Exchange Parameters with Na^+ and Li^+ Binding, Phospholipid Composition and Total Phospholipid, and Free Mg^{2+} concentration in RBCs from Essential Hypertensive Patients.*

	V_{std}	V_{max}	K_m	$K_{b(Na)}$	$K_{b(Li)}$	PC	PI	PS	Sph	PE	TP	$[Mg^{2+}]_f$
V_{std}	1.00											
V_{max}	0.49 (0.03)	1.00										
K_m	0.38	0.24	1.00									
$K_{b(Na)}$ 0.24	0.35	-0.61	1.00 (0.01)									
$K_{b(Li)}$	0.16	0.32	0.05	-0.22	1.00							
PC	0.30	-0.04	0.14	-0.47 (0.03)	-0.02	1.00						
PI	0.17	0.42 (0.05)	-0.06	0.06	-0.56 (0.01)	-0.05	1.00					
PS	0.01	-0.10	0.07	-0.09	-0.07	-0.39	0.11	1.00				
Sph	0.05	0.11	-0.24	0.09	-0.21	-0.60 (0.01)	0.60 (0.01)	0.20	1.00			
PE	-0.03	-0.04	-0.27	0.52 (0.02)	0.33	-0.10	-0.68 (0.01)	-0.58 (0.02)	-0.39	1.00		
TP	-0.05	-0.18	-0.02	-0.45 (0.04)	-0.47 (0.03)	0.35	0.51 (0.03)	0.07	0.24	-0.66 (0.01)	1.00	
$[Mg^{2+}]_f$	-0.55 (0.01)	-0.12	-0.76 (0.01)	0.59 (0.01)	0.21	0.30	-0.17	-0.15	-0.34	-0.17	0.14	1.00

*Correlation coefficients ≥ 0.40 are considered significant ($p \leq 0.05$). Values in parentheses are p values for significance.

Table 29. Spearman Correlation of Na^+-Li^+ Exchange Parameters with Na^+ and Li^+ Binding, Phospholipid Composition and Total Phospholipid, and Free Mg^{2+} concentration in RBCs from Normotensive Individuals.*

	V_{std}	V_{max}	K_m	$K_{b(Na)}$	$K_{b(Li)}$	PC	PI	PS	Sph	PE	TP	$[Mg^{2+}]_f$
V_{std}	1.00											
V_{max}	0.82 (0.01)	1.00										
K_m	0.02	0.30	1.00									
$K_{b(Na)}$	-0.08	-0.13	-0.43	1.00 (0.04)								
$K_{b(Li)}$		-0.26	-0.09	-0.34	0.34	1.00						
PC		0.37	0.35	0.37	-0.48 (0.03)	0.37	1.00					
PI		0.24	0.14	0.19	-0.10	-0.18	0.04	1.00				
PS		0.03	0.05	0.08	0.31	-0.38	-0.57 (0.02)	0.10	1.00			
Sph		-0.31	-0.26	0.32	-0.58 (0.02)	-0.25	-0.18	0.15	-0.16	1.00		
PE		-0.04	-0.06	-0.10	0.42 (0.04)	0.08	-0.33	-0.34	0.14	-0.65 (0.01)	1.00	
TP		0.58 (0.02)	0.29	-0.45 (0.04)	0.29	0.18	0.44 (0.04)	-0.01	-0.20	-0.76 (0.01)	0.48	1.00
$[Mg^{2+}]_f$		-0.58 (0.02)	0.26	-0.63 (0.01)	0.40 (0.05)	0.16	0.87 (0.01)	-0.21	-0.37	-0.37	-0.02	0.52 (0.01)	1.00

*Correlation coefficients ≥ 0.40 are considered significant ($p \leq 0.05$). Values in parentheses are p values for significance.

CHAPTER V

DISCUSSION

V.1. Na^+-Li^+ Exchange Activity in Intact Human RBCs with and without Inhibitors

The osmolarity inside normal red blood cells is 290 miliosmoles/L. To maintain the cells intact in the process of the experiments, the osmolarities in the media have to be maintained at 300 $\pm$ 10 miliosmoles/L. Therefore the highest Na^+ concentration in the media that can be reached is 150 mM. Before 1990, the maximal rates of Na^+-Li^+ exchange in human RBCs were generally assayed by subtraction of the Li^+ efflux rates of Li^+-loaded RBCs in Na^+-free medium from the rates in 150 mM NaCl medium (Canessa et al., 1980a). Mechanistic studies of RBC Na^+-Li^+ exchange have indicated that the RBC membrane transport protein is asymmetric, with higher Na^+ and Li^+ affinities on the intracellular than on the extracellular side of the membrane (Hannert et al., 1986; Sarkadi et al., 1978). It was recently found that, under standard transport assay conditions, Li^+ is present at a saturating concentration at the internal binding site of the RBC membrane transport protein, but Na^+ may not be saturating at the external site (Rutherford et al., 1990; Rutherford et al., 1992a). The dissociation constants (K_m) for extracellular Na^+ are, at least for some individuals, of the same order of magnitude as the extracellular Na^+ concentration (140 mmol/L) used in the standard transport assay, suggesting that the RBC Na^+-Li^+ exchange protein is far from saturated with Na^+ on the extracellular side of the RBC membrane. The rates under standard

transport assay conditions may not, therefore, be maximal rates of RBC Na^+-Li^+ exchange; variations in Na^+ affinity (K_m) and maximal velocity (V_{max}) could change the observed rates. Only by varying the Na^+ concentration in an isotonic suspension medium can one measure the true kinetic parameters of RBC Na^+-Li^+ exchange (Aronson, 1990; Rutherford et al., 1990; Rutherford et al., 1992a).

It was reported that amiloride had no effect on Na^+-Li^+ exchange in human intact RBCs (Carr et al., 1988; Kahn, 1987). However, the inhibitory effects of amiloride and its analogues were reported only on the standard rates of Na^+-Li^+ exchange. To our knowledge, there have been no reports about the effects on V_{max} and K_m. In this study we found that amiloride significantly decreased V_{max} and HMA had significant effects on both V_{max} and K_m (Tables 1 and 2). HMA had higher inhibitory effects than did amiloride. This is in agreement with the previous reports (Kleyman & Cragoe, 1988; Simchowitz & Cragoe, 1986). Amiloride and its analogues are specific Na^+-H^+ exchange inhibitors. They specifically bind to the Na^+-H^+ transport protein and decrease the exchange rates. Table 1 shows that they have significant effects on Na^+-Li^+ exchange. Gende et al found that EIPA blocked the exchanges of Li^+ or Na^+ with H^+ (Gende & Cingolani, 1993). They also found that thrombin, an activator of Na^+-H^+, could stimulate Li^+ as well as Na^+ exchange (Gende & Cingolani, 1993). All of these results suggest that the Na^+-Li^+ exchange protein is highly likely the same as the Na^+-H^+ exchange protein.

V.2. Li^+ Efflux Across H^+- and Li^+-loaded Resealed RBC Ghosts

Resealed RBC ghosts are devoid of hemoglobin, which is the major nonmembrane protein in the cells, and other factors. Measurements of Li^+ movements across resealed RBC ghosts, instead of through intact RBCs, simplify the study of RBC transport proteins. If Na^+-

H^+ and Na^+-Li^+ exchange are mediated by the same transport protein, they will compete with each other for interaction with the same membrane transport protein. The pH value inside the resealed RBC ghosts was 6.0. When H^+- and Li^+-loaded resealed RBC ghosts were suspended in the NaCl media with a higher pH value (8.0), H^+ came out of the ghosts. Li^+ efflux rates were significantly lower when H^+ efflux occurred simultaneously with and without the inhibitors (Figure 7 and Table 3). This means that H^+ and Li^+ competed to bind to the same membrane protein to be transported out of the ghosts. Previous studies on interactions of lithium and protons with the sodium-proton exchanger in intact RBCs indicated that lithium behaved same as protons (Parker, 1986). Both of them can stimulate Na^+-H^+ exchange when they are enriched inside of the cells and can inhibit Na^+-H^+ when they are enriched outside of the cells (Parker, 1986).

Phloretin significantly decreased the Li^+ efflux rates no matter if there was a pH gradient or not (Figure 7 and Table 3), which is predictable according to the previous work (Pandey et al., 1978). HMA, which is a specific Na^+-H^+ exchange inhibitor, also significantly inhibited the Li^+ efflux across the resealed RBC ghosts. This result is in agreement with the inhibitory effect of HMA on Na^+-Li^+ exchange in the intact RBCs, and furthermore proves that Na^+-Li^+ exchange and Na^+-H^+ exchange are mediated by the same membrane protein.

Using resealed RBC ghosts, one can avoid the interferences of hemoglobin and load the required concentrations of ions. But the membrane constituents might change during the preparation, especially in the course of hemolysis. Although no lipids are lost during the preparation of the resealed ghosts, a considerable structural change can occur (Schwoch & Passow, 1973). The fluidity of the phospholipid bilayer also changed. Some enzymes and enzyme activities might be lost during the preparation. Keeping the osmolarity in the range

of 60 -80 miliosmoles/L can retain most of the enzymes and the enzyme activities (Schwoch & Passow, 1973). Ionic strength and pH affect the loss of membrane proteins and their structures (Bodemann & Passow, 1972; Schwoch & Passow, 1973). In order to remove most of the hemoglobin and keep the membranes resealable, the temperature must be maintained at 0 - 2 °C during hemolysis. However, resealed RBC ghosts still contain primary cell membranes and maintain most of the characteristics of the native erythrocyte membranes (Klonk & Deuticke, 1992). The data above on Li^+ efflux across the resealed RBC ghosts still provide strong evidence that Na^+-H^+ exchange and Na^+-Li^+ exchange are mediated by the same membrane protein.

V.3. Na^+ Visibility in RBC Membranes and the Correction Curve

In the intact RBCs the Na^+ visibility is only 75% (Nissen et al., 1989). In the RBC membrane suspensions the visibility varied as Na^+ concentration changed (Table 4). The lower the Na^+ concentration, the lower the visibility. Sodium is a quadrupolar nucleus with a spin of 3/2, which is not under conditions of extreme narrowing (Bull, 1972). When sodium ions were exposed to the RBC membrane suspensions, the ions experienced two states of motion. In other words, they experienced a two-site exchange. One site is the hydrated ion in aqueous solution which is under the extreme narrowing condition and is called the narrow component, and the other site is the membranous peptide binding site which is not under the extreme narrowing condition and is called the broad component. The spin-lattice relaxation time (T_1) is the sum of the relaxation time of these two components (Bull, 1972; Urry e tal., 1989). When the broad components are too broad, they are hardly visible by NMR (Urry e tal., 1989). The NMR-invisible Na^+ is due to the broad components, i.e., the bound Na^+. Increased T_1 values following the additions of 150 mM LiCl confirmed the

release of the bound sodium (Table 4). And after the bound sodium was released by lithium, sodium was about 100% visible.

Actual Na^+ binding constants were addressed by the experiments shown in Tables 5 to 9. We generated the correction curve in the $K_{b(Na)}$ range of 150 - 260 M^{-1}. At lower binding constants, the correction factors were close to 1.00, and the actual Na^+ binding constants were close to the observed Na^+ binding constants; the interactions between Na^+ and the membranes were weak at the lower binding constants and there were more free sodium. For larger binding constants, however, the correction factors were significantly higher than 1.0 because of the larger fraction of invisible bound Na^+. The time-consuming processing of the experiments prevented us to get a correction curve in a wider range. In order to get a more accurate curve, more points will be needed.

V.4. Inhibitory Activities of Amiloride and Its Analogues on Na^+ and Li^+ Interactions with RBC Membranes

As mentioned before, amiloride is a specific Na^+-H^+ exchange inhibitor. It is a weak base (pK_a = 8.7) (Kleyman & Cragoe, 1988). In the 1960's, many amiloride analogues were synthesized. And in the 1980's, a lot of work on the inhibitory effects of amiloride analogues on Na^+-H^+ exchange in the intact RBCs has been done (Kleyman & Cragoe, 1988; Simchowitz & Cragoe, 1986; Vigne et al., 1983). Studies on structure-activity relationships of amiloride analogues indicated that the guanidinium moiety of amiloride is the active group that recognizes and binds to the Na^+ transport site of the exchanger (Simchowitz & Cragoe, 1986). Increasing the number of carbon atoms in the alkyl chain at the 5-N position increased the activity (Simchowitz & Cragoe, 1986). The inhibitory activity can be demonstrated by the apparent K_i value which is the dissociation constant. The lower the

apparent K_I, the higher the activity. For Na^+-H^+ exchange in the intact RBCs, the apparent K_I values of amiloride, HMA, EIPA and MIA are 83.8 μM, 0.16 μM, 0.38 μM and 0.44 μM, respectively (Kleyman & Cragoe, 1988; Simchowitz & Cragoe, 1986). In this study we measured both ^{23}Na and ^{7}Li T_1 values with varying concentrations of these inhibitors (Tables 11 and 12, Figure 9). All of these inhibitors inhibited the interaction of Na^+ with the membranes, which is in agreement with a recent report (Spruth et al., 1995). Spruth et al. found that the amiloride derivatives and Na^+ compete for a common binding site (Spruth et al., 1995). Meanwhile, we also found that all of these inhibitors inhibited the interaction of Li^+ with the membranes. The inhibitory effects of these inhibitors were of the same order of magnitude, though HMA has the highest effects. Therefore we chose amiloride to address its inhibitory constants to RBC membranes for Na^+ and Li^+ binding to RBC membranes. The inhibitory activities of amiloride and its analogues relate to the biological system. Senyk et al. reported that the inhibitory constants for amiloride and EIPA are 8 μM and 1 μM, respectively, in rabbit alveolar type-II Na^+ channels reconstituted in planar lipid bilayers (Senyk et al., 1995). The inhibitory effects of amiloride and EIPA are of the same order of magnitude in these reconstituted Na^+ channels.

The ^{23}Na or ^{7}Li T_1 values increased as Na^+ or Li^+ concentration increased in RBC membrane suspensions at specific amiloride concentrations (Tables 13 to 20 and Figure 10) because there were more free Na^+ or Li^+ as the Na^+ or Li^+ concentration increased. At the same Na^+ or Li^+ concentrations, the ^{23}Na or ^{7}Li T_1 values increased as the amiloride concentration increased. This indicated that amiloride inhibited both Na^+ and Li^+ binding to the RBC membranes and left more free Na^+ or Li^+, resulting in weaker Na^+ and Li^+ binding to the membranes. Therefore both Na^+ and Li^+ binding constants decreased as the amiloride concentration increased at pH values of 7.0 and 8.0 (Tables 13 to 20 and Figure 11).

Both Na^+ and Li^+ binding constants were higher at pH 8.0 than at pH 7.0 (Tables 13 to 20 and Figure 11), because H^+ competed with both Na^+ and Li^+ for binding to the RBC membranes. Another reason is that the inhibitory effect of amiloride is higher at pH 7.0 than at pH 8.0. Amiloride is a weak base and only the protonated form of amiloride can interact with the membrane protein (Kleyman & Cragoe, 1988). Therefore, the lower the pH, the higher the inhibitory activity. For the same reason, amiloride binding constants to RBC membranes were higher at pH 7.0 than at pH 8.0. The most important thing is that there is no significant difference between amiloride binding constants to RBC membranes with Na^+ and Li^+ present. Amiloride had the similar inhibitory effects on both Na^+ and Li^+ binding to RBC membranes. The inhibitor studies proved that Na^+-Li^+ and Na^+-H^+ exchange are mediated by the same RBC membrane protein.

The amiloride binding constants to the exposed RBC membranes at pH 7.0 and 8.0 are much lower than the reciprocal of the amiloride apparent K_i for Na^+-H^+ exchange in intact RBCs. There are several reasons which can explain this difference. Amiloride had high blockage effect when it was applied to the outside face of the membranes, but had no effect when it was applied to the inside face (Small & Morris, 1995). When amiloride was applied to both sides of the membranes, the inhibitory effect must be lower than the effect when it was applied to only the outside of the membranes. Although RBC membranes still maintain the characteristics of the native erythrocyte membranes, Na^+-H^+ and Na^+-Li^+ exchange proteins might change in the amount and in the structure after lysed from intact RBCs (Bodemann & Passow, 1972; Schwoch & Passow, 1973). The distribution, the amount, and the structure of the protein in the exposed membranes might be different from that in intact RBCs. Amiloride inhibitory activity also varies from one isoform to another isoform of the Na^+-H^+ exchanger. For instance, the 50% inhibition concentration values for amiloride and

EIPA of the NHE mutant are significantly higher than that of the wild type NHE1 (Wang et al., 1995).

V.5. Correlation of Na^+-Li^+ Exchange Parameters with Na^+ and Li^+ Binding, Phospholipid Composition and Total Phospholipid, and Free Mg^{2+} Concentration in RBCs from Essential Hypertensive and Normotensive Individuals

The AA-determined rates of RBC Na^+-Li^+ exchange measured under standard assay conditions (V_{std}) and in isotonic media containing varying Na^+ concentrations (V_{max}) were found to be significantly higher for the hypertensive patients than for the normotensive individuals (Tables 22 to 24 and Figure 12); the exchange rates for female were, however, not significantly lower ($p > 0.09$) than for male individuals in both the patient and control groups. The gender variations and the enhanced rates that we observed for the hypertensive patients are not in agreement with those previously reported, presumably because of the small sample size used in our study (Canessa et al., 1980a; Laurenzi and Trevisan , 1989; Ramasamy et al., 1990; Rutherford et al., 1990). Within the patient group, one-way ANOVA analysis did not show significant difference for the AA-determined V_{std} and V_{max} values of RBC Na^+-Li^+ exchange between the subgroup that was taking antihypertensive medication and the subgroup that was not taking any medication; these results are in agreement with those previously reported (Canessa, 1980b; Ramasamy et al., 1990), which show that medications for the regulation of blood pressure do not affect the enhanced rates of RBC Na^+-Li^+ exchange in hypertensive patients. A recent study (De la Sierra et al., 1995) that appeared in print after we collected the blood samples for this study, however, showed that long-term antihypertensive therapy with angiotensin-converting enzyme inhibitors (ACEI) caused a significant decrease in the rates of RBC Na^+-Li^+ exchange. Three of the

ten patients used in our study were receiving ACEI therapy. However, we found no significant differences ($p > 0.23$) in the V_{std} and V_{max} values between these three and the remaining seven patients; this difference between our study and that on the effect of ACEI therapy on RBC Na^+ transport (De la Sierra et al., 1995) might be related to the smaller sample size used in our study. The AA-determined dissociation constants of Na^+ to the extracellular side of RBC membranes (K_m) were also significantly higher for hypertensive patients than for normotensive individuals (Tables 22 to 24 and Figure 12); the K_m values that we obtained for RBCs from both hypertensive patients and normotensive individuals are in general agreement with those previously measured in some other investigations (Canessa et al., 1992; Rutherford et al., 1992b) but not in all previous reports (Rutherford et al., 1990).

The authors of some recent studies (Canessa et al., 1992; Zerbini et al., 1995) have claimed that K_m values in the range of 160 mmol/L for RBC samples from hypertensive patients cannot be obtained accurately from measurements at an extracellular Na^+ concentration of 150 mmol/L. This statement is true only provided that the estimates of K_m are derived from hyperbolic Michaelis-Menten plots (as opposed to K_m estimates originating from linear, double-reciprocal plots of 1/v vs. $1/[Na^+]$). In our study, however, we did not simply conduct single measurements in suspensions containing 150 mmol/L Na^+; we varied the extracellular Na^+ concentration from 0 to 150 mmol/L, and we maintained the isotonicity of all suspension media at 0.15 mol/L with choline chloride (see Methods). The method recently described by Canessa and co-workers (Canessa et al., 1992; Zerbini et al., 1995) involves the use of hypertonic Na^+ media (150-250 mmol/L). We do not favor that method because there were no data in these two studies to support the contention that the ionic permeability of RBC membranes (including that mediated by Na^+-Li^+ exchange) and the viability of the cells were not affected by the harsh, hypertonic conditions.

Na^+ and Li^+ ions are in the fast exchange domain on the ^{23}Na and ^7Li NMR time scales (Mota de Freitas, 1993; Mota de Freitas et al., 1994a; Ong and Cheung, 1986; Urry et al., 1980); the ^{23}Na or ^7Li T_1 values (Figure 13) measured with RBC membrane samples of patients and normal individuals therefore depict the weighted averages of free Na^+ or Li^+ in the suspension medium and of Na^+ or Li^+ bound to the RBC membranes. Free Na^+ or Li^+ exhibits relatively larger T_1 values than does bound Na^+ or Li^+. The ^{23}Na or ^7Li T_1 values increased in the presence of increasing concentrations (0 to 10 mmol/L) of Na^+ or Li^+ in membrane samples of both hypertensive patients and normotensive individuals because of increasing concentrations of free Na^+ or Li^+. These observations confirm that both ^{23}Na and ^7Li T_1 values are sensitive to Na^+ and Li^+ binding to RBC membranes, and that they can be used for the calculation of the Na^+ and Li^+ binding constants to RBC membranes from James-Noggle plots (see Methods). For Na^+ concentrations lower than 10 mmol/L, the amount of Na^+ bound to the same amount of RBC membrane was smaller for hypertensive patients than for normotensive individuals (Figure 13, panel A); for Na^+ concentrations $\geq$ 10 mmol/L, the amount of Na^+ bound to RBC membranes from both hypertensive patients and normotensive individuals appears to be the same, however, because the observed ^{23}Na T_1 values are mostly determined by the fractions of free Na^+. In contrast, for a given Li^+ concentration, the amount of Li^+ bound to the RBC membranes of hypertensive patients was not significantly different from that of normotensive individuals (Figure 13, panel B). Our observed ^{23}Na T_1 data (Figure 13, panel A) and the calculated and corrected $K_{b(Na)}$ values (Figure 14 and Tables 22 to 24) indicate that Na^+ has a weaker affinity for RBC membranes of hypertensive patients than for those of normotensive individuals. However, our observed ^7Li T_1 results (Figure 13, panel B) and the calculated $K_{b(Li)}$ values (Figure 14 and Tables 22 to 24) indicate that Li^+ has a similar affinity for RBC membranes of both hypertensive patients and normotensive

individuals. Our calculated and corrected $K_{b(Na)}$ values are in agreement with those previously calculated from ^{23}Na T_1 values measured in suspensions of RBC membranes from hypertensive patients and normotensive individuals (Ong and Cheung, 1986; Urry et al., 1980). Similarly, our calculated $K_{b(Li)}$ values for RBC membranes from normotensive individuals are in agreement with those found in our previous investigation of lithium-treated bipolar patients and normal individuals (Mota de Freitas et al., 1994a).

The Na^+ binding constants, $K_{b(Na)}$, that we calculated from the ^{23}Na T_1 values measured in Na^+-containing suspensions of unsealed RBC membranes are measures of Na^+ binding to the phosphate head groups of phospholipids and protein binding sites present in the internal and external leaflets of the RBC membrane. In contrast, the AA-determined K_m values which we obtained by varying the extracellular Na^+ concentration in the transport assay are Na^+ dissociation constants to the extracellular side of the membrane Na^+-Li^+ exchange protein. It is therefore not surprising that significant negative correlations existed between the $K_{b(Na)}$ values and the K_m values within the patient and control groups. A comparison of $K_{b(Na)}$ and K_m requires taking the reciprocals of one of these sets of values; the values of $K_{b(Na)}$ are larger than those of $1/K_m$, presumably because of the additional number of membrane binding sites for Na^+ that are probed in the ^{23}Na NMR relaxation measurements relative to the AA-determined transport measurements.

The head groups of the phospholipids PI and PS are negatively charged; these two anionic phospholipids reside primarily in the inner leaflet of the RBC membrane. Our previous ^7Li NMR relaxation studies of Li^+-loaded RBCs and of RBC components in the absence and presence of varying Mg^{2+} concentrations indicate that the primary binding sites for alkali metal ions in intact RBCs are present in the cytoplasmic side of the RBC membrane (Mota de Freitas et al., 1994b; Rong et al., 1993), and that variations in Li^+ or Na^+ binding

constants to RBC membranes may be associated with abnormal phospholipid composition (Mota de Freitas et al., 1994a). Our ^{31}P NMR data (Tables 22 to 24 and Figure 16) indicated that there were significant differences between the hypertensive and normotensive groups in the percentages of PS and PE, but not in those of PC, Sph, and PI. The phospholipid compositions of the human RBC membrane that we measured by ^{31}P NMR spectroscopy are in general agreement with those measured by others by two-dimensional thin-layer chromatography (Preiss et al., 1982; Sengupta et al., 1981) or high-performance liquid chromatography (Kurumi et al., 1991). The slightly different percentage compositions measured for human RBC membranes by the different methods are attributed to the fact that ^{31}P NMR spectroscopy measures the total phosphate directly in each phospholipid headgroup; the visualization reagents used in thin-layer chromatography or the ultraviolet absorbance measurements in high-performance liquid chromatography result in detection of only unsaturated fatty acids by these methods (Pearce et al., 1991; Spillman et al., 1983). These methodologic differences presumably also account for the fact that we found, in this study, significant differences between the RBC membranes from hypertensive and normotensive groups in the percentage compositions of PS and PE, but no significant differences were found previously with thin-layer chromatographic measurements (Preisset et al., 1982).

We also used ^{31}P NMR spectroscopy to measure the total phospholipid concentrations in the RBC membranes from hypertensive patients and normotensive individuals and found that they were not significantly different (Table 24). The total phospholipid concentrations in the RBC membranes that we measured by ^{31}P NMR spectroscopy were, however, in good agreement with those previously reported (Kurumi et al., 1991; Sengupta et al., 1981). The statistically significant differences that we found in the percentages of PS and PE are therefore not associated with variations in the total phospholipid concentrations of the RBC

membranes from the two groups.

Previous ^{31}P and ^{2}H NMR studies of phospholipid suspensions indicated that PS and PI interacted with Li^+ and Na^+ ions (Merchant and Glonek, 1992; Roux. and Bloom, 1990). We found significant differences between the two groups for the PS values by one-way ANOVA with Tukey's conservative correction (Table 24). Because of baseline noise and the small amounts of PI present in RBC membranes (Edzes et al., 1992; Mota de Freitas, 1994a), it is possible only to determine the PI content with an accuracy of $\pm 20\%$; the low accuracy of the PI content measured by the ^{31}P NMR method indicates that differences between the two groups in PI levels and any correlations between the PI content and the kinetic transport parameters should be interpreted with caution. The PS content in RBC membranes is, however, sufficiently high to be determined accurately by ^{31}P NMR spectroscopy. We speculate that the higher PS percentage composition of RBC membranes from hypertensive patients relative to that from normotensive individuals might drive the positively charged extracellular Na^+ ions to traverse the RBC membrane more quickly for essential hypertensives than for normotensives. We previously reported that Li^+-treated bipolar patients had lower Na^+-Li^+ exchange rates, which were negatively correlated with Li^+ binding to RBC membranes, and that they had higher PS contents than did normal individuals (Mota de Freitas et al., 1994a). The higher PS content in the inner leaflet of RBC membranes of lithium-treated bipolar patients is presumably responsible for a stronger interaction with intracellular Li^+ and makes it more difficult for Li^+ to be transported, resulting in lower rates of RBC Na^+-Li^+ exchange (Mota de Freitas et al., 1994a).

Free intracellular magnesium concentrations were significantly lower for essential hypertensive patients than for normotensive individuals (Tables 22 to 24), which is in agreement with previous reports (Gupta and Gupta, 1987; Resnick et al., 1984). Touyz et

al. reported that total intracellular magnesium levels were significantly lower for hypertensives than for normotensives (Touyz et al., 1992). Significantly increased intracellular sodium was observed in the essential hypertensive group (Touyz and Milne 1995). This might be due to the fast transport of Na^+ in essential hypertension. Magnesium appears closely linked to calcium, potassium and sodium balances. Gros et al recently reported that the increases in Na^+ ingest brought a significant drop in Mg^{2+} content in gastrocnemius (Gros et al., 1995). In this study we found negative correlations of free magnesium levels with Na^+-Li^+ exchange activities in essential hypertension. In both the essential hypertensive and the normotensive groups, free magnesium concentrations were negatively correlated with V_{std} and K_m, which follows from the above reports concerning lower free and total intracellular Mg^{2+}, and higher intracellular sodium in hypertension. But to understand whether lower free intracellular magnesium is one of the reasons causing higher Na^+-Li^+ exchange activity, more detailed work needs to be involved; in particular, the effect of antihypertensive medication (Gupta and Gupta, 1987; Resnick et al., 1984) needs to be evaluated.

In this initial, exploratory study, we did not recruit hypertensive patients or normotensive individuals who were on a specific diet. It is therefore possible that the percentages of phospholipids as well as the levels of triglycerides and of cholesterol that we report for RBC membranes were influenced by the type of diet that the subjects were on at the time of blood drawing. It is important to note, however, that all of our subjects were instructed to have fasted for a minimum of 12 hr at the time of blood drawing; we estimate that only approximately 70% of the subjects complied with these instructions. We believe that this precaution which we took in our experimental design should have minimized the contribution of individual diets on the lipid compositions of the RBC membranes that we measured.

In our study, there was a perfect match between the patient and control groups in terms of race and gender. Although we attempted to match each essential hypertensive patient as closely as possible with a normotensive patient of similar age, this was not always achieved, as is apparent from the significantly higher average age of the hypertensive patient group relative to the average age of the normotensive patients (Tables 22 to 24). It is always difficult to obtain a perfectly age-matched sample of hypertensive patients and normotensive individuals because essential hypertension is known to be far more common in the elderly population (Laurenzi and Trevisan, 1989).

We found significant differences between the patient and control groups in both the systolic and the diastolic blood pressure values (Tables 22 to 24). None of our patients had isolated late-onset systolic hypertension. Every patient had diastolic hypertension, and each had been diagnosed to have essential hypertension for a minimum of ten years. The statistically significant differences between the RBC parameters of the samples from hypertensive patients relative to those from normotensive individuals that we found in this study are therefore not related to late-onset systolic hypertension, but rather to long-term diastolic hypertension.

Increased sodium-lithium countertransport activity was previously reported not only in hypertensive patients (Canessa et al., 1980a), but also in hyperlipidemic patients (Carr et al., 1990; Corrocher et al., 1985; Hunt et al., 1986; Rutherford et al., 1992b). Abnormal membrane phospholipid composition in erythrocytes from hyperlipidemic patients has been investigated (Engelmann et al., 1992a; Engelmann et al., 1992b; Engelmann et al.,1993). To avoid the possible interference of hyperlipidemia in our investigation of essential hypertension, we selected patients and normal individuals with normal triglyceride and cholesterol concentrations. The normal average values for the cholesterol and triglyceride

levels at the clinical laboratory of the Loyola University Medical Center were 210 mg/dL and less than 250 mg/dL, respectively. In the sample that we used in our study, there were three essential hypertensive patients and one normotensive individual who had slightly elevated cholesterol levels; elevated triglyceride levels were, however, found for only one normotensive individual. We attribute the slightly elevated cholesterol and triglyceride levels for some of our subjects to non-compliance with the instructions that we gave for fasting at the time of blood drawing (see Methods). The triglyceride and cholesterol data listed in Table 24 indicate, however, that there was no significant difference between the two groups in triglyceride and cholesterol concentrations.

We report here the results of our statistical analysis with one-way ANOVA method with Tukey's conservative correction as well as the Spearman correlation coefficients. The ANOVA and Spearman statistical tests are appropriate for the small sample size used in our study (Siegel, 1956). Despite the small sample size in this study, we were able to obtain statistically significant differences for, and significant correlations within, several RBC parameters (V_{max}, K_m, and PE) by using one-way ANOVA with Tukey's conservative correction and Spearman correlation coefficients; the power analysis that we conducted (Table 27) confirmed that the sample size which we used was sufficient to yield significant differences at the 95% confidence level. Future epidemiologic studies with the methods described in this study should, however, include a minimum sample size of 15 to 20 to confirm whether the significant differences in RBC parameters (V_{std}, $K_{b(Na)}$, and PS) that we observed at a confidence level of 90% also hold for a larger sample size. For free intracellular magnesium concentrations, a larger sample size of 20 (Table 27) is needed to see if the difference between these two groups at the power level of 0.90 is significant. We did not attempt to use a larger sample size because our goal was to search for the RBC

parameters that provide the most information on the origins of elevated rates of Na^+-Li^+ exchange in RBCs from Caucasian, essential hypertensive patients. The time-consuming processing of each sample kept us from investigating a much larger sample size in this initial study. Our focus was not on investigating the mortality, epidemiology, or treatment efficacy for essential hypertensive patients; the data that we accumulated are therefore not sufficient to allow us to draw conclusions about these aspects of essential hypertension in Caucasian patients. Nonetheless, the information that we report here is important and significant because it identified more clearly than in previous studies the molecular abnormalities at the level of RBC membranes for Caucasian, essential hypertensive patients who had elevated rates of RBC Na^+-Li^+ exchange.

Alterations of the fatty acid composition of some phospholipids in essential hypertensive patients have been found to cause abnormal RBC Na^+-Li^+ exchange activity (Duhm, 1992; Engelmann et al., 1991; Engelmann et al., 1992a; Engelmann et al., 1992b; Ollerenshaw et al., 1987). More detailed investigations of the molecular species composition of phospholipids may therefore help us to understand the increased Na^+-Li^+ exchange activity in RBCs from essential hypertensive patients.

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The dissertation is, therefore, accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

2/23/96
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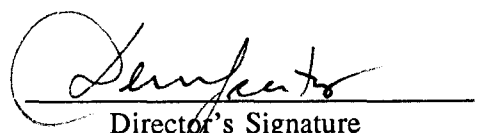
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