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NMR OBSERVATION OF ALTERED SODIUM INTERACTION WITH HUMAN ERYTHROCYTE MEMBRANES OF ESSENTIAL HYPERTENSIVES

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SUMMARY:

Sodium-23 nuclear magnetic resonance was utilized to compare sodium binding to erythrocyte ghosts of normotensive and of essential hypertensive individuals. Plots of the reciprocal of the excess longitudinal relaxation rates as a function of total sodium ion concentration indicated tighter and more complex sodium interaction with erythrocyte membrane preparations from normotensives and a weaker, simpler sodium binding with membranes of hypertensives. NMR studies comparing 1) sodium-23 interaction with DIDS inhibition of chloride-35 interaction and 2) competitive effects of cations on the sodium interaction provided evidence for specific sodium binding to the cytoplasmic surface of the erythrocyte ghosts. The results are briefly considered relative to possible mechanisms for essential hypertension.

INTRODUCTION:

A number of reports in the literature indicate altered ion permeabilities (1-5) and Na-K pump activities (6,7) in vascular smooth muscle of hypertensive animal models as well as in essential hypertensive men (8) when compared to appropriate normotensive controls. Ion transport differences between hypertensives and normotensives have also been observed in human erythrocytes (9-11) as well as in erythrocytes of animal models (12-14). Additionally, sodium-lithium countertransport has been found to be increased in the erythrocytes of patients with essential hypertension (15). In the present study on human erythrocyte membranes of males (4 normotensives and 5 hypertensives), sodium binding properties have been examined using sodium-23 nuclear magnetic resonance (NMR). Differences in the sodium binding properties are observed which suggest possible mechanisms for the differences in ion permeabilities and a potential role of the characterization of sodium binding to erythrocyte membranes by NMR as a diagnostic tool.

MATERIALS AND METHODS:

DIDS (4,4' - Diisothiocyano 2,2' - disulfonic acid stilbene) was purchased through Sigma Chemical Company. D_2O (99.7%) was obtained from Merck and Co., Inc., St. Louis, Mo.. All chemicals were reagent grade. Freshly drawn venous blood obtained from human donors was collected in a Fenwal Blood-Pack contain-

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ing 63 ml of anticoagulant citrate phosphate dextrose solution, USP (CPD). Blood that was not immediately prepared was stored at 4°C and used within a 10 day period. Information on the preparations and on the age and blood pressure of the donors is included in Table I.

Membrane Preparation: Unsealed and sealed right-side-out (SRSO) ghosts were prepared by a modified procedure of Steck and Kant (16,17). In order to reduce the initial sodium concentration for the NMR studies, the final wash buffers of the membrane preparations were 40x then 20x the volume of 5 mM KH_2PO_4, pH 7.2 for the unsealed and 100x the volume of 5 mM KH_2PO_4 + 1 mM $MgSO_4$, pH 7.2 for the SRSO ghosts. All buffers in the preparation were at pH 7.2 to maximize the DIDS binding to the anion transport protein (18). The absence of hemoglobin in the unsealed ghosts was checked spectrophotometrically. When necessary, ghost preparations were concentrated by centrifugation at 54,500 x g_{max} for 45 minutes. All operations were performed at 0°-4°C. Protein concentrations were determined by the method of Lowry, et al. (19) immediately following completion of membrane isolation. In addition, the unsealed ghost preparations were 5 μM in $CaCl_2$ for the NaCl titrations.

NMR Studies: ^{23}Na NMR data at 26.3 MHz and ^{35}Cl NMR data at 9.7 MHz were collected on a JEOL FX-100 spectrometer. The longitudinal relaxation times (T_1's) were obtained as previously reported (20). For the titrations, concentrated NaCl, LiCl, $CaCl_2$, or DIDS aliquots were introduced directly into the NMR sample tube using either a Gilson P-20 variable ul or a 5 ul Eppendorf

TABLE I

SAMPLES		BLOOD PRESSURE SYSTOLIC	DIASTOLIC	PREPARATION	CONCENTRATION (MEMBRANE PROTEIN)
A.* Hypertensive (age)					
H-1	29	148	100	Unsealed	5.3
H-2	40	154	108	Unsealed	12.6
H-3	43	170	120	Unsealed	12.7
MEAN		157±9	109±8		
H-4	42	140	90	Sealed right side out (SRSO)	3.6
H-5	58	170	100	Sealed right side out (SRSO)	9.4
B.** Normotensive (age)					
N-1	26	115	70	Unsealed	4.3
N-2	27	110	75	Unsealed	6.3
N-3	49	108	78	Unsealed	6.0
N-4	30	118	70	Unsealed	5.0
MEAN		113±4	73±3		

*Patients had positive family histories for hypertension with no evidence of secondary causes and had never received antihypertensive treatment.

**Normotensive subjects had negative family histories for hypertension.

pipette fitted with 0.022 mm (ID) teflon tubing. Five or 20% D_2O was added to the membrane preparations in order to provide an internal NMR lock. All data was collected at 30 ± 2°C and samples were kept at 4°C when not in use.

The longitudinal relaxation rate, $R_1(=1/T_1)$, is used in plotting the data. Specifically following James and Noggle (21,22), the reciprocal of the excess longitudinal relaxation rate, i.e. the difference between the rates in the presence and absence of binding, $\Delta R^{-1} = (R_1-R_{1f})^{-1}$ where R_{1f} is 17.5 sec^{-1} for sodium-23 in solution, is plotted as a function of total ion concentration. Additionally in order to compare preparations with different concentrations of binding sites, the difference rate is divided by the protein concentration, i.e. $\Delta r = (mg/ml)/(R_1-R_{1f})$. For simple binding processes when the site concentration is much less than the ion concentration, the plot is linear and the intercept on the negative sodium axis of such plots gives the dissociation constant, the reciprocal of which is the binding constant. Furthermore, it has recently been demonstrated that non-linear slopes can be interpreted in terms of multiple binding with the possibilities of negative or positive cooperative binding being apparent in the data (20,23-24).

RESULTS:

The James-Noggle plots for unsealed erythrocyte ghosts from hypertensive individuals are nicely reproducible with an apparent binding constant of approximately 200 M^{-1}. Interestingly, the slopes decrease slightly with increasing sodium concentration. This could be due to the presence of independent classes of sites: One class with the tighter binding constant of 200 M^{-1} and a second class with a weaker binding constant. Alternatively, it could be due to interacting sites showing some negative cooperativity as was found for the two binding sites of the gramicidin channel (20,23-24). Also there is observed a steeper slope for data on hypertensives in their fifth decade of life than for a hypertensive in the third decade of life. Since these difference relaxation rate plots are normalized to total protein concentration, the occurrence of the same x-axis intercept yet different slopes could arise due to a decrease with advancing age in the number of sodium binding sites per milligram membrane protein.

In an effort to begin localization of the observable sodium ion binding site (or sites), a comparison on the same preparation of sodium ion interaction with the effect of DIDS on inhibition of chloride ion interaction (25) was considered. Chloride binding to different preparations of sealed-right-side-out (SRSO) ghosts varied presumably because the extent of resealing varied. With different preparations, however, as the chloride binding was increasingly blockable, it was found that the detectable Na interaction was correspondingly less, that is, the more effective DIDS in blocking chloride ion interaction on a given preparation the less the sodium interaction for that sample. The most dramatic example is given in Table II. With 10 mM NaCl in the absence of membranes, the sodium-23 T_1 is 57 msec and that of chloride-35 is 37 msec. For the SRSO ghost preparation of Table II, which was about 6 μM in anion

TABLE II

HUMAN ERYTHROCYTES (HYPERTENSIVE)

SEALED-RIGHT-SIDE-OUT(SRSO)-GHOSTS

SYSTEM	Sodium-23 T_1 (msec)	Chloride-35 T_1 (msec)
1. 10 mM NaCl	57	37
2. SRSO-ghosts+10mM NaCl	58	28
3. 2 + 5μM DIDS	57	35
4. 2 + 10μM DIDS	57	36

transport protein, the T_1 for ^{35}Cl was 28 msec. On addition of 5 μM DIDS the T_1 became 35 msec and on further addition to 10 μM DIDS the T_1 raised to 36 msec, that is, the chloride interaction could be essentially completely blocked by DIDS in this preparation. On the other hand, there was no detectable sodium ion interaction with this preparation as also shown in Table II. This provides evidence for the observed Na interaction sites with unsealed ghosts being on the cytoplasmic surface of the membrane.

James-Noggle plots for sodium interaction with erythrocyte membranes prepared as unsealed ghosts from normotensive individuals are seen in Figure 1B where a family of curves is obtained. The simplest curve (curve c) exhibits an immediate steep rise and an intercept indicating an apparent binding constant of approximately 1000 M^{-1}. Curves a and b exhibit a lag before a similar steep rise. The lag is what would be expected for positive cooperative binding, i.e., an increase in binding affinity with an increase in total sodium concentration, $[Na]_T$. For curve a the binding constant for sodium appears to increase by more than one order of magnitude on going from 2 mM to 12 mM sodium ion, whereas 5 mM to 13 mM has been reported for intracellular sodium (26).

When comparing the two sets of curves, those of normotensive with those of hypertensive unsealed erythrocyte ghosts, it should be noted that the normotensive membrane preparations tended to retain more hemoglobin which could be the result of a greater tendency, on the part of the normotensive ghosts, to reseal spontaneously. This raises the question as to whether the lag observed before the steep rise might be due to an increasing leakiness of spontaneously resealed ghosts during the ion titration. While this places in question the cooperative nature of the curve, the result of tighter sodium binding with ghosts of normotensives is retained. With respect to the normotensive curves it is also of interest that erythrocytes from a 62 year old man with documented primary aldosteronism, not on treatment, were found to have binding characteristics similar to those of normotensive subject c in Figure 1B, indicating that the abnormality in essential hypertensives may not exist in secondary hypertension.

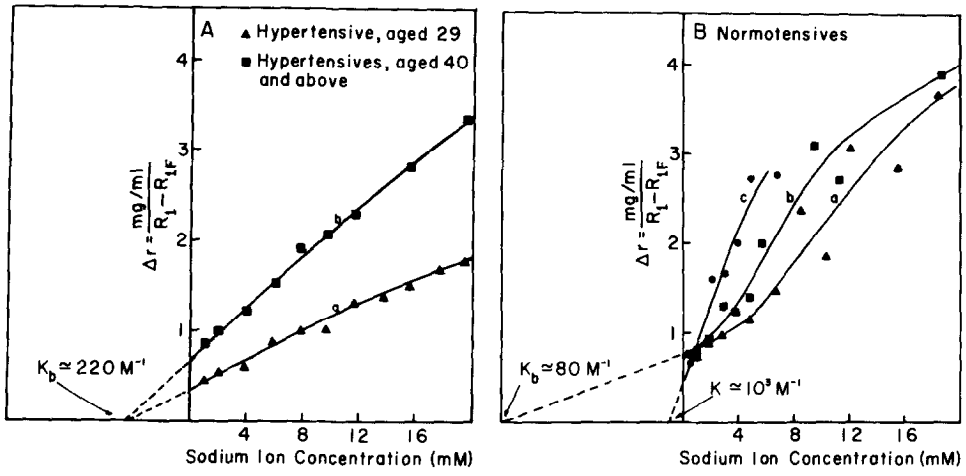

FIGURE 1: Excess Longitudinal Relaxation Rates (R_1-R_{1f}) for sodium-23 normalized to total erythrocyte membrane protein and plotted as the reciprocal, Δr, as a function of total sodium ion concentration for unsealed erythrocyte ghosts of A Essential Hypertensives and B Normotensives. The reciprocal of the negative x-axis intercept gives an apparent binding constant. Relating the plots to the samples of Table I in A curve a is due to sample H-1 and curve b is due to samples H-2 and H-3. In B curve a is due to samples N-1 and N-2, curve b to sample N-3 and curve c to sample N-4. Each sample was run in duplicate. See text for discussion.

Addition of vanadate (2 mM) had no effect on the sodium T_1 values whereas addition of LiCl, KH_2PO_4, $MgSO_4$ and $CaCl_2$ all resulted in longer relaxation times with the order of effectiveness being $Ca^{+2} >> Mg^{+2} > Li^{+} > K^{+}$. The observation of sodium binding in the presence of a 200 times greater concentration of K^{+} and a 200 fold difference in the competitive effect of Ca^{+2} over that of Mg^{+2} and the magnitude of the apparent binding constants argue for specific sodium binding. Also, using the same sample as for curve a of Figure 1A, at 5 mM NaCl, addition of 20 mM LiCl only raises the Δr to 0.9 indicating that the affinity for Na^{+} is greater than for Li^{+}. This is because an equivalent amount of a competitive ion with the same binding constant would be expected to double the slope whereas a four-fold greater concentration of Li^{+} does not double the slope.

DISCUSSION:

Comparison of Na interaction with Cl interaction using sealed and partially sealed ghosts provides evidence that the observed Na binding site is accessible only from the cytoplasmic side of the membrane; comparison of the competitive effects of other cations on the observed sodium interaction provides evidence that the sodium binding is specific; and comparison of sodium interaction with erythrocyte membranes from hypertensive individuals with those from normotensive individuals indicates a decreased affinity and/or loss of positive cooperativity for the hypertensives. It is now appropriate to consider possible binding sites. In doing so it is useful to note that the sensitivity for a given sodium binding

depends on the electric field gradient at the binding site. The electric field gradient at the site is responsible for the enhanced relaxation rate. The experimental quantity ΔR_1 is $\sum_i P_i(R_{1i}-R_{1f})$ where P_i and R_{1i} are the mole fraction of ions and the relaxation rates, respectively, at site i. Since each term in the summation is the product of two quantities, when the difference quantity is large for a given site the sensitivity can be great even when there are other binding sites of similar affinity but with small electric field gradients. For example, sodium binding to gramicidin channels in phospholipid micelles shows two binding constants (~100 M^{-1} and 1 M^{-1}). This is uncomplicated by an apparent binding constant to the polar groups of the micelle of the order of 1 M^{-1} because $R_{1m}-R_{1f}$ for this interaction is negligible. On the other hand the difference is so large for binding in the channel that the presence of channels can be detected in a suspension several hundred nanomolar in channels. For favorable binding sites with large electric field gradients resulting in a large R_{1i} ($\sim 10^4$) the possibility exists that binding with site concentrations in the tenths of μM range can be detected. Four possible sodium interaction sites that have been implicated in hypertension are the sodium-potassium adenosine-triphosphatase also called the sodium pump (7), the sodium-lithium countertransport system (15), the sodium-calcium exchange system (27) and the sodium-potassium cotransport system (28). The sodium-lithium countertransport system does not seem to be a good candidate as its affinity for Li is 20 times greater than for Na and it does not "accept any monovalent or divalent cations" (15) whereas Li appears to bind less tightly to the observed site and Ca, Mg and K also bind at the site. With respect to the sodium pump, a sodium-23 James-Noggle plot of partially purified Na-K ATPase from brain has been reported with an upward curvature (22) similar to that of curves a and b of Figure 1B. This requires consideration that the data on normotensive membranes could reflect a positive cooperativity which would be an effective way to achieve the rate-limiting loading of three sodium ions on the cytoplasmic surface before an ATP driven exchange for two potassium ions could occur.

A decrease in intracellular sodium binding, particularly if the binding is required for a mechanism of sodium extrusion from the cell, can result in an increase in sodium activity within the cell. Assuming that the effects observed in erythrocytes are reflected in smooth muscle cells, the increased intracellular concentration of sodium can, directly by the Na-Ca exchange system, lead to an increase in intracellular Ca which would in turn increase the state of contraction of smooth muscle. Alternatively an increase in intracellular Na and altered permeability could result in a decrease in the transmembrane potential in smooth muscle which would in turn increase the probability of ion leaks through voltage dependent channels. Again a resulting increase in intracellular Ca would increase

vasoconstriction. It reamins to be determined, of course, whether the observations on erythrocytes can be mechanistically related to smooth muscle cells. From the results presented here, it is apparent that the potential of sodium-23 NMR as a diagnostic tool requires a population survey to classify adequately the differences in sodium interaction and to determine, for example, if indeed there is a reduction in binding sites with age and whether this might relate to the common middle age onset of hypertension.

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