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L M Resnick, ..., R Marion, J H Laragh

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Research Article

To study the ionic basis of salt sensitivity in hypertension, 19F-, 13P-, and 23Na-nuclear magnetic resonance techniques were used to measure cytosolic free calcium (Cai), pH (pHi), free magnesium (Mgi), and sodium (Nai) in erythrocytes of essential hypertensive subjects (n = 19). Individuals were studied for 2 mo each on low- (UNaV < 50 meq/d) and high-(UNaV > 200 meq/d) salt diets, with the concomitant administration of nifedipine (10 mg t.i.d.) or placebo tablets for 1 mo of each diet. Salt loading elevated Cai and Nai while suppressing Mgi and pHi; these changes occurred predominantly in salt-sensitive subjects (n = 9). Nifedipine blunted the pressor response to salt loading > 50% (delta diastolic BP [high-low salt vs placebo] = 5 +/- 2 vs 14 +/- 2 mmHg, P < 0.05) and reversed salt-induced ionic changes, lowering Cai and elevating Mgi and pHi. Regardless of the definition of salt sensitivity, continuous relationships were observed between the pressure response to salt loading, the levels of Cai (r = 0.726, P < 0.001), Nai (r = 0.747, P < 0.001), and pHi (r = -0.754, P < 0.001), and the salt-induced change in Mgi (r = -0.757, P < 0.001). Altogether, these results emphasize the reciprocal and coordinate nature of intracellular ionic changes in response to dietary salt loading and calcium channel [...]



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Intracellular Ionic Consequences of Dietary Salt Loading in Essential Hypertension

Relation to Blood Pressure and Effects of Calcium Channel Blockade

Lawrence M. Resnick, Raj K. Gupta, Beatrice DiFabio, Mario Barbagallo, Samuel Mann, Rosemarie Marion, and John H. Laragh

Division of Endocrinology/Hypertension, Wayne State University Medical Center, Detroit, Michigan 48201; Cardiovascular Center, New York Hospital-Cornell Medical Center, New York 10021; and Department of Physiology and Biophysics, Albert Einstein College of Medicine, Bronx, New York 10463

Abstract

To study the ionic basis of salt sensitivity in hypertension, ¹⁹F-, ³¹P-, and ²³Na-nuclear magnetic resonance techniques were used to measure cytosolic free calcium (Ca_i), pH (pH_i), free magnesium (Mg_i), and sodium (Na_i) in erythrocytes of essential hypertensive subjects (n = 19). Individuals were studied for 2 mo each on low- $(U_{Na}V < 50 \text{ meq/d})$ and high- (UNaV > 200 meq/d) salt diets, with the concomitant administration of nifedipine (10 mg t.i.d.) or placebo tablets for 1 mo of each diet. Salt loading elevated Ca_i and Na_i while suppressing Mg_i and pH_i; these changes occurred predominantly in salt-sensitive subjects (n = 9). Nifedipine blunted the pressor response to salt loading > 50% (Δ diastolic BP [high-low salt vs placebo] = 5 ± 2 vs 14 ± 2 mmHg, P < 0.05) and reversed salt-induced ionic changes, lowering Ca_i and elevating Mg_i and pH_i. Regardless of the definition of salt sensitivity, continuous relationships were observed between the pressure response to salt loading, the levels of Ca_i (r = 0.726, P < 0.001), Na_i (r = 0.747, P < 0.001), and pH_i (r = -0.754, P < 0.001), and the salt-induced change in Mg_i (r = -0.757, P < 0.001). Altogether, these results emphasize the reciprocal and coordinate nature of intracellular ionic changes in response to dietary salt loading and calcium channel blockade in essential hypertension. They suggest that salt sensitivity is mediated by cellular calcium accumulation from the extracellular space, in association with magnesium depletion and acidification. Lastly, interpretation of intracellular ion measurements in the future will require concurrent assessment of dietary salt intake. (J. Clin. Invest. 1994. 94:1269-1276.) Key Words: NMR spectroscopy • intracellular ions • pH • calcium • magnesium

Introduction

Epidemiological as well as direct intervention studies repeatedly have demonstrated that altered dietary intake of mineral salts such as sodium chloride can result, in at least some "sensitive" individuals, in clinically significant differences in blood pressure. Current theories differ, however, as to how increased availability of salt to the organism as a whole results in the vasoconstrictor and other organ system responses characteristic of clinical salt-sensitive hypertension. Common to many current formulations is the proposal that an initially sodium chloride–derived signal is somehow translated at the cellular level to a calcium signal, resulting in an increased cytosolic free calcium and, thus, in increased vascular smooth muscle tone and elevated blood pressure (1). However, the relations between pressor responses to salt and the concomitant alterations in the different intracellular ionic species remain unclear.

Our group has used nuclear magnetic resonance $(NMR)^1$ spectroscopic techniques to study intracellular cations in peripheral blood and has demonstrated abnormalities of intracellular free magnesium (2), intracellular pH (3), and cytosolic free calcium levels (4) in hypertension and allied metabolic disorders. These data complement earlier studies in essential hypertensive subjects where we observed salt-induced alterations of extracellular serum ionized calcium and of various calcium regulatory hormones that in turn were reversed and or prevented by calcium channel blockade (5, 6). These studies led us to hypothesize that salt-sensitive hypertensive disease is critically dependent on enhanced cellular calcium accumulation from the extracellular space.

To further test this hypothesis and to directly assess the blood pressure and intracellular ionic consequences of dietary salt loading in man, we studied essential hypertensive subjects at extremes of salt intake both in the presence and absence of concurrent therapy with the calcium channel blocking agent, nifedipine. Our results, reported previously in abstract form (7, 8), demonstrate (a) that coordinated, linked changes in intracellular sodium, free calcium, free magnesium, and intracellular pH occur in response to salt loading, (b) that these changes occur predominantly among salt-sensitive individuals, and (c) that they are closely linked to both the pressor effects of salt and the hypotensive effects of calcium channel blockade. Our data also suggest that the previously reported enhanced efficacy of calcium channel blockade in low renin and sodiumvolume forms of hypertension may be explained by their ability to reverse specifically dietary salt-induced changes in cytosolic free calcium and intracellular pH.

This study was presented in part at the American Federation of Clinical Research in Washington D.C. on 7 May 1990 and at the International Society of Hypertension in Montreal, Canada on 22 June 1990.

Address correspondence to Lawrence M. Resnick, M.D., Division of Endocrinology/Hypertension, Wayne State University Medical School, University Health Center-4H, 4201 St. Antoine, Detroit, MI 48201.

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^{1.} Abbreviations used in this paper: DBP, diastolic BP; NMR, nuclear magnetic resonance; PRA, plasma renin activity; RBC, red blood cell; SS and SI, salt-sensitive and -insensitive, respectively.

Methods

Essential hypertensive subjects were recruited and followed at the Hypertension Center of the New York Hospital. The protocol followed was approved by the Committee on Human Rights in Research, and informed consent was obtained from the subjects after the nature of the procedures had been explained. Essential hypertension was diagnosed in untreated subjects on the basis of outpatient blood pressures > 150/ 90 mmHg on at least three occasions and the absence of any history, physical examination, or laboratory evidence of secondary forms of hypertension. Subjects discontinued medications \geq 7 wk before beginning any dietary maneuvers and had been off diuretics for \geq 6 mo.

Dietary methods

During the baseline evaluation period, participants discontinued all foods with potential pharmacological effects and were instructed in keeping food diaries, accurately weighing foods, measuring salt added to food, and estimating food portions of food eaten away from home. Two 24-h food diaries submitted at the end of the baseline period and a diet history questionnaire were used to individualize food intake plans while the low- and high-salt diets were administered.

The low salt diet was achieved by eliminating from the diet all saltcontaining compounds of significance, often substituting foods of similar composition from the same food group. The high salt intake was achieved by adding measured amounts of high-salt bouillon and/or table salt and soy sauce to the baseline daily intake. Subjects were instructed to take salt-containing condiments throughout the day with meals.

Study methods

Drug intervention with nifedipine used a randomized, placebo-controlled, single-blind crossover design in which 2-month periods of low (UNaV < 50 meq/d) and high (UNaV > 200 meq/d) dietary sodium chloride intake were ingested according to the methods described above. On each level of salt intake, subjects were assigned to 1 mo of nifedipine, 10 mg t.i.d., as well as 1 mo of treatment with similarly appearing placebo tablets, also taken t.i.d.

Patients were evaluated at monthly intervals after an overnight fast, 2-4 h after taking their morning pill (nifedipine or placebo) with water. The patient arrived on each occasion with a 24-h urine specimen collected the day before being seen and with two 24-h food diaries, one of which coincided with the day of the 24-h urine collection. An aliquot of the urine specimen was checked for urinary sodium content and, if the total sodium excretion was within the accepted limits for that assigned diet, blood tests were obtained for analysis of intracellular ion content. Urine was analyzed for total sodium, potassium, calcium, magnesium, and creatinine excretion. Blood pressure was obtained manually in the supine, seated, and standing positions by the research nursing staff. Patients in the seated position also had their blood pressure evaluated with an automatic device (Arteriosonde; Roche Diagnostics, Nutley, NJ) set to measure and record pressure every 2 min for 30 min. If urinary sodium excretion was not within the range specified for that diet period, additional time was spent with the dietitian to achieve the goal salt intake, and the patient was given another 7-10 d to comply. Persistent failure to achieve goal sodium chloride excretion levels resulted in the patient being dropped from the study.

At the time of each monthly visit, nifedipine or placebo pills were returned to the research nursing staff who estimated compliance on the basis of pill counts. A new consignment of pills, numerically coded, was allocated to the patient at the beginning of each monthly period.

Serum electrolytes, blood urea nitrogen, creatinine, and urinary electrolytes, calcium, magnesium, and creatinine excretion, were measured by automated techniques. Plasma renin activity and urinary aldosterone excretion were measured by standard radioimmunoassay techniques.

NMR intracellular ion analyses

³¹ P-NMR analysis of Mg_i and pH_i . 10 ml of heparinized blood was spun at 2,000 rpm for 10 min, and the plasma was saved to be used in the analysis of Ca_i (see below). The remaining erythrocyte fraction was

decanted into a 10-mm NMR tube, and ³¹P-NMR spectra were recorded at 81 MHz and 37°C for 30 min on a spectrometer (XL200; Varian Analytical Instruments, Sunnyvale, CA) in the Fourier transform mode and with wide-band proton noise decoupling.

The intracellular free magnesium concentration, Mg_i, was determined according to the formula:

$$Mg_i = K_d(MgATP) \times (\phi^{-1} - 1) \quad (9)$$

where K_d (MgATP) is the apparent dissociation constant for the reaction MgATP = Mg²⁺ + ATP, = 0.4×10^{-5} M under physiological conditions at 37°C and at pH 7.2; and ϕ = ATP_{free}/ATP_{total} as determined from the chemical shift differences of the α and β phosphoryl group resonances of ATP in the ³¹P-NMR spectrum.

 pH_i . Measurement of the chemical shift difference of the 3- and 2phosphoryl resonances of 2,3-diphosphoglycerate (2,3 DPG) on the ³¹P-NMR spectra at known different absolute pH levels, allows for a determination of pH_i in experimental samples (10). Packed red blood cells (RBCs) were lysed by repeated freezing and thawing, and aliquots of the hemolysate were titrated to pH 7.0–7.4, using HCl or NaOH. ³¹P-NMR spectra were obtained at each pH value, and the chemical shift difference of the 3- and 2-phosphoryl resonances of 2,3 DPG were plotted against the pH value at which that spectrum was obtained. This titration curve was linear within the pH range tested and was used to determine the pH_i of unknowns (3).

¹⁹ F-NMR analysis of Ca_i. Ca_i was determined in RBCs by a modification of the method of Murphy et al. (11), using the fluorinated, membrane-permeant Ca chelator, QUIN-MF-AM.

10 ml of heparinized blood was spun at 10,000 rpm for 10 min, and the plasma was decanted. The packed cells were added to 100 ml Hanks' balanced salt solution buffer titrated to a pH of 7.4 with NaHCO₃. Cells were loaded with 20 μ M QUIN-MF-AM for 25 min at 37°C in a shaking water bath. Loaded cells were then spun at 10,000 rpm for 10 min, the supernatant medium was aspirated, and the cells were resuspended and incubated for 90 min in fresh buffer together with the originally decanted plasma fraction. Cells were then centrifuged, washed again with fresh buffer together with plasma, and recentrifuged and decanted into 10mm NMR tubes for analysis. ¹⁹F-NMR spectra were run on a spectrometer (Varian Analytical Instruments) operating at 470.4 MHz and 37°C. Free cytosolic calcium, Ca_i, was calculated according to the formula:

$Ca_i = K_{d,app} \times (Ca-QUIN-MF)/(QUIN-MF),$

where the $K_{d,app}$ of Ca-QUIN-MF was calculated at 126 nM in RBC hemolysates at 37°C, and where Ca-QUIN-MF/QUIN-MF was determined from the ratio of areas of the QUIN-MF and Ca-QUIN-MF resonance peaks on the ¹⁹F-NMR spectrum.

To approximate more closely the in situ cellular environment, we equilibrated calcium-bound and -free QUIN-MF in red cell hemolysates to determine the apparent dissociation constant, K_d , rather than in buffer solutions. The K_d thus determined, 126 nM, is lower than those for the fluorescent probes currently used to measure Ca_i and thus allows a greater sensitivity in distinguishing differences among the low Ca_i values we observed in red blood cells.

 ^{23}Na -NMR analysis of Na_i. The shift reagent, dysprosium trispolyphosphate $[Dy(PPPi)_2]^{7-}$ (5 mM) was mixed with 10 ml heparinized blood and then was centrifuged. ^{23}Na -NMR spectra were then obtained in packed cells as well as in the decanted plasma fraction to assess intracellular sodium, Na_i, and to normalize for Na in the extracellular space, according to the formula:

$$Na_i = [A_i/A_o \times S_o/(1 - S_o)] \times Na_o \quad (12)$$

where A_i , A_o are the integrated areas of NMR signals in the intracellular and extracellular space, respectively; S_o is the fractional extracellular space in the NMR window; and Na_o is the plasma Na concentration obtained by standard automated techniques.

When human RBCs are analyzed repeatedly, the interassay variability of the above NMR-based ionic measurements for pH_i is ~ 0.0005 U and, for Mg_i, Na_i, and Ca_i, < 5%.

Table I. Basal Clinical and Laboratory Characteristics of Study Patients

	Salt-sensitive $(n = 9)$	Salt-insensitive $(n = 10)$
Age (y)	58±2	56±3
Sex (male/female)	7/2	5/5
Race (black/white)	2/7	0/10
Weight (body mass index)	27.9±2.1	25.5±1.9
Diastolic BP (mmHg)	153±8	156±8
Diastolic BP (mmHg)	101±3	102±2
Blood urea nitrogen (mg %)	15.9±1.1	17.5±1.6
Cr (mg %)	1.0 ± 0.06	1.0±0.07
U _{Ne} V (meq/d)	83±6	75±7
PRA (ng/ml per hour)	1.9±0.3	3.0±0.5*

* P < 0.05 vs. salt-sensitive.

Salt sensitivity was defined as a diastolic BP (DBP) on high salt > 5% greater versus the low salt diet. The effects of low and high salt diets, and placebo and nifedipine phases were compared for both salt-sensitive (SS) and -insensitive (SI) subjects by ANOVA in which salt sensitivity (SS vs SI) was a grouping factor, and the measurements on high and low salt, on placebo and nifedipine, were each considered separate treatments. Post-ANOVA *t* tests (unpaired, Bonferroni) were performed, adjusting the degrees of freedom appropriately. Paired *t* tests were performed to analyze the effects of salt and of nifedipine independently of how salt sensitivity was defined. Relationships between salt- and nifedipine-induced changes in pressure and intracellular ion levels were analyzed by linear regression analysis. All results are expressed as mean±SEM.

Results

Clinical and laboratory data. Basal clinical and laboratory values for the 19 patients for whom complete data were obtained are described in Table I. On the basis of diastolic pressure responses to high and low salt diets while on placebo tablets (see Methods), 9 patients (52 ± 2 y; 7 female, 2 male; 7 white, 2 black) were classified as SS, whereas 10 (56 ± 3 y; 5 male, 5 female; 10 white) were classified as SI. Significant differences in the data reported below remained unchanged whether or not the two black subjects were included in the analysis. Except for

elevated blood pressure, basal clinical and laboratory data were not outside normal limits and did not differ significantly among SS versus SI patient subgroups, with the exception of plasma renin activity, which was lower in subjects subsequently found to be SS versus SI (1.9 ± 0.3 vs 3.0 ± 0.5 ng/ml per hour, P< 0.05).

Urinary electrolyte excretion values are recorded in Table II. Steady state, 24-h average urinary sodium levels met the designated goal ranges in all the reported patients, although on the low salt diet, SS exhibited a lower UNaV than did SI individuals (P < 0.05). Urinary 24-h potassium excretion remained constant both on the two dietary salt periods and on placebo versus nifedipine treatment phases. Urinary calcium excretion was also higher in SI compared with SS subjects on the lowsalt diet and was higher on high versus low salt intakes both in SS and SI subjects on placebo and nifedipine. Additionally, nifedipine significantly increased average calcium excretion values in both SS and SI subjects (Table II). Urinary magnesium excretion was only modestly affected by differing dietary salt intake and although all levels tended toward higher values on high versus low salt, these differences were not consistently statistically significant. To ascertain whether we could successfully achieve and maintain patients on high and low salt-containing diets without concomitantly altering dietary levels of other minerals such as calcium and magnesium, we have in the past computer analyzed 3-d food records for nutrient and mineral content in patients undergoing the identical dietary salt protocol using the same dietitian personnel and have compared these values to the 24-h urinary excretion values for all the mineral species. Under the above conditions, it was found that urinary excretion values closely paralleled the food record data, further supporting the present data indicating that calcium and magnesium intake on the high and low salt diet phases were not significantly different.

Plasma renin activity (PRA) values did not differ at either extreme of dietary salt intake among SS versus SI individuals. Similarly, both plasma renin activity and urinary aldosterone excretion values were significantly suppressed on high versus low salt intakes in both SS and SI subjects. Furthermore, chronic nifedipine treatment did not significantly change either PRA or urinary aldosterone excretion. These results and all subsequently reported data (see below) were unaffected by the order of the diets or drug treatments administered.

Blood pressure and intracellular ionic effects of salt. Table

 Table II. Urinary Electrolyte Excretion and Renin-Aldosterone System Responses to Dietary Salt

	Low salt				High salt			
	Salt sensitive		Salt insensitive		Salt sensitive		Salt insensitive	
	Placebo	Nifedipine	Placebo	Nifedipine	Placebo	Nifedipine	Placebo	Nifedipine
U _{Na} V (meq/d)	17±2	22±4	33±6*	33±5	295±26 [‡]	328±34 [‡]	286±36 [‡]	283±23‡
$U_{K}V \text{ (meq/d)}$	84±11	79±6	68±7	69±4	83±5	82±7	81±9	70±8
$U_{Ca}V (mg/d)$	90±11	118±19	141±16*	135±10	178±30 [‡]	222±33 [±]	222±18 [‡]	274±33 [‡]
U _{Mg} V (meq/d)	8.5±2.0	7.6±1.3	7.2±1.0	7.1±0.8	10.5 ± 1.4	8.3±12.1	8.3±0.7	8.5±0.8
U _{Creat} V (g/d)	1.7±0.1	1.8±0.2	1.7±0.1	1.5 ± 0.1	1.7±0.1	1.8±0.2	1.8 ± 0.1	1.5 ± 0.1
$U_{Aldo}V(\mu g/d)$	20±3	23±4	18±2	27±8	9±1‡	9±1‡	9±2‡	8±2‡
PRA (ng/ml per hour)	2.7±0.5	4.0±0.9	3.7±0.7	6.2±1.6	1.3±0.4 [‡]	2.0 ± 0.7	2.4±0.6 [§]	3.2±0.9 [±]

* Significant at P < 0.05 vs. ss. * Significant at P < 0.01 vs. low salt. Significant at P < 0.05 vs. low salt. Significant at P < 0.05 placebo.

Table III. Blood Pressure and Ionic Effects of Dietary Salt

	Low salt			High salt				
	Salt sensitive		Salt insensitive		Salt sensitive		Salt insensitive	
	Placebo	Nifedipine	Placebo	Nifedipine	Placebo	Nifedipine	Placebo	Nifedipine
Systolic BP (mmHg)	142±6	125±4*	155±6	137±5*‡	171±10 ^{\$}	143±8 [¶]	164±6	138±6 ¹
Diastolic BP (mmHg)	89±3	82±2 [¶]	$101 \pm 2^{\ddagger}$	92±3 ^{‡1}	103±3 ^{§¶}	87±2*	102±2	89±3*
Serum Na ⁺ (meq/liter)	139±0.6	139±0.5	138±0.8	138±0.6	139±0.6	140±0.6	139±0.9	140±0.8
Serum K ⁺ (meq/liter)	4.2 ± 0.05	4.3±0.09	4.2±0.1	4.2 ± 0.08	3.9±0.07 [∥]	4.2±0.1 [¶]	4.0±0.08	4.1 ± 0.1
Serum Ca _T (mg%)	9.3±0.1	9.5±0.1	9.5±0.2	9.9±0.4	9.4±0.1	9.7±0.1	9.4±0.2	9.9±0.2
Serum Mg _T (meq/liter)	1.90±0.04	2.0 ± 0.06	1.87±0.1	1.89±0.09	1.83 ± 0.04	1.95 ± 0.05	1.81 ± 0.07	1.80 ± 0.10
Serum CO ₂ (mg %)	23±2.0	25±1.2	22 ± 0.8	26±1.1 [¶]	25±0.5	25±0.8	$24 \pm 1.0^{\parallel}$	26±0.9
Na _i (meq/liter)	8.0±.15	9.4±1.6	5.4±1.0	6.6±0.1	10.8±2.1	10.4±0.7	6.1±0.3 [‡]	6.9±0.7
Ca _i (nM)	32.3±3.6	30.2 ± 1.2	32.7±4.9	22.0±6.1*	46.5±8.0 [∥]	31.4±5.1*	36.7±7.6	28.8 ± 5.2
$Mg_i(\mu M)$	207±21	199±8	196±12	206±17	143±16 [∥]	203±25	199±9 [‡]	205 ± 15
pHi	7.15±0.02	7.32±0.02 [¶]	7.23±0.01 [‡]	7.27±0.02 [¶]	7.10±0.01	7.27±0.03 [¶]	7.21±0.01 [‡]	7.25±0.01

* Significant at P < 0.01 vs. placebo. [‡] Significant at P < 0.05 vs. SS. [§] Significant at P < 0.01 vs. LS. ^{||} Significant at P < 0.05 vs. LS. [§] Significant at P < 0.05 vs. placebo.

III reports the blood pressures in SS and SI subjects on each dietary salt intake on nifedipine versus placebo treatment. By definition, in SS on placebo tablets, blood pressure rose significantly on the high- versus low-salt diet (Δ systolic BP = 29±6 mmHg, P < 0.001; Δ DBP = 14±2 mmHg, P < 0.001). Conversely, in SI, no significant changes in diastolic pressures were observed (0±3 mmHg, P = NS), and systolic pressures rose only minimally (9±6 mmHg, P = NS) with salt loading.

Table III and Fig. 1 and 2 also demonstrate the intracellular ionic responses to salt loading. For the group as a whole, dietary salt loading elevated cytosolic free calcium, Ca_i (P < 0.05), and intracellular sodium, Na_i (P < 0.001), while it suppressed intracellular pH, pH_i (P < 0.01), and free magnesium, Mg_i (P < 0.05) (Fig. 1). These changes occurred predominantly among SS, not SI subjects (Fig. 2). Thus, Mg_i levels were significantly lower (P < 0.05) and Na_i levels significantly higher (P < 0.05) in SS versus SI subjects on high dietary salt intake. On both salt diets, pH_i was lower (P < 0.05) in SS versus SI individuals. Intracellular sodium rose significantly in all the subjects, but the magnitude of the rise was greater in SS versus SI individuals (P < 0.05; Fig. 2). For the group as a whole, when salt-induced changes in the ions were themselves compared, it was found that the changes in Ca_i were correlated



Figure 1. ¹⁹F-, ²³Na-, and ³¹P-NMR spectroscopic measurement of intracellular RBC cytosolic free calcium (Ca_i^f) , intracellular sodium (Na_i) , intracellular pH (pH_i) , and intracellular free magnesium (Mg_i^f) levels in unmedicated essential hypertensive subjects studied on low- (UNaV < 50 meq/d) and high- (UNaV > 200 meq/d) salt diets.

with the changes in Na_i (r = 0.504, P < 0.05), Mg_i (r = -0.513, P < 0.05), and pH_i (r = -0.378, P = NS).

Furthermore, for all subjects, independent of the definition of salt sensitivity, significant relationships were observed between the various intracellular ionic species and blood pressure. Significant, positive relationships were observed between both intracellular sodium and intracellular free calcium and the saltinduced change in diastolic pressure (Na_i: r = 0.747, P < 0.001; Ca_i: r = 0.726, P < 0.001). Additionally, the saltinduced change in pressure was closely and inversely related to the salt-induced change in free magnesium (r = -0.757, P < 0.001). Lastly, salt-induced changes in both systolic (r = -0.612, P < 0.001) and diastolic (r = -0.754, P < 0.001) (Fig. 3) pressures were inversely related to pH_i levels measured on the high salt diet and, for the systolic pressure change, to the basal pH_i on low salt (r = -0.857, P < 0.001). No significant relationships were observed between basal blood pressures or the effect of salt loading and basal urinary excretion values or changes in these values.

Blood pressure and intracellular ionic effects of nifedipine. Nifedipine significantly lowered blood pressure on both highand low-salt diets in all subjects and lowered pressure equally among SS and SI individuals on a given salt intake, as shown in Table III. For the group as a whole, however, diastolic pressure fell to a greater extent on the high salt diet (ΔDBP = -14 ± 2 vs -8 ± 2 mmHg, P < 0.05), and this corresponded to greater nifedipine-induced changes in Ca_i and Mg_i on high versus low salt diets (see below). This effect was especially true among SS individuals ($\Delta DBP = -16\pm 2$ mmHg on high salt vs -7 ± 2 mmHg on low salt, P < 0.05).

When compared with the ability of salt to elevate blood pressure, nifedipine significantly blunted the pressor effect of salt in SS individuals (Fig. 4). Thus, the diastolic pressure response to salt loading among SS subjects taking nifedipine was no longer statistically significant and, quantitatively, was reduced > 50% (Δ DBP on salt + nifedipine = 5±2 mmHg vs on salt + placebo = 14±2 mmHg; P < 0.05, nifedipine vs placebo) (Table IV). Hence, nifedipine decreased salt sensitivity in SS subjects.



Figure 2. Blood pressure and intracellular ionic responses to low and high dietary salt intakes in salt-sensitive vs -insensitive essential hypertensive subjects. *P < 0.05 vs lo salt; *P < 0.05 vs salt insensitive.

Nifedipine-induced changes in blood pressure were also associated with parallel changes in the various intracellular ion species measured. These calcium-antagonist-induced ionic changes were observed in all subjects and on each dietary salt intake, as shown in Fig. 5. For the group as a whole compared with placebo, nifedipine significantly lowered Ca_i (P < 0.05) on both low- and high-salt diets. This effect occurred to a greater extent on the high-salt diet ($\Delta Ca_i = -11.3 \pm 2.1 \text{ vs} - 6.6 \pm 1.7$ nM, P < 0.05), and is attributable to the fall in Ca_i among SS patients (see Table III), who conversely, on the low-salt diet did not display any significant change in Ca_i. Reciprocally, nifedipine elevated Mg_i levels, but only on the high-salt diet $(\Delta Mg_i = 32 \pm 9 \text{ vs } 2 \pm 6 \text{ mM}, P < 0.05)$. Conversely, nifedipine elevated Na_i levels only on the low-salt diet ($\Delta Na_i = 1.3 \pm 0.2$ vs 0.3 ± 0.2 meq/liter, P < 0.05). Intracellular pH was consistently elevated by nifedipine on both salt diets (Fig. 5). Furthermore, just as the ability of salt to raise pressure was related to baseline pH_i: the lower the pH_i, the greater the salt-induced pressor response (see above), the antihypertensive efficacy of nifedipine (ΔDBP) was also closely related to the baseline pH_i: the lower the pH_i, the greater the nifedipine-induced fall in blood pressure (r = 0.645, P < 0.001). Altogether then, with the exception of Na_i, nifedipine restored toward normal the deviations of blood pressure and of Ca_i, Mg_i, and pH_i characteristic



Figure 3. Inverse relation of salt-induced pressor responses to saltinduced intracellular pH levels in essential hypertensive subjects.

of the hypertensive state and induced by salt loading in SS hypertensive individuals.

Discussion

The mechanism by which increased dietary salt intake can elevate blood pressure in a significant proportion of the hyperten-



Figure 4. The effects of high and low dietary salt intakes on diastolic blood pressure in salt-sensitive hypertensive subjects studied on placebo vs nifedipine therapy. A significant salt-induced elevation in blood pressure (P < 0.05) occurred on placebo. On nifedipine, the difference in diastolic pressure on low vs high salt was no longer significant.

Table IV. Nifedipine Reverses Blood Pressure Effects of Salt Loading: BP (High Salt) – BP (Low Salt)

	Placebo	Nifedipine	Δ
SS $(n = 9)$			
Systolic BP	29±6	18±4	-11 ± 4
Diastolic BP	14±2	5±2*	-9±2
SI $(n = 10)$			
Systolic BP	9±6	1±5	-8 ± 5
Diastolic BP	0±3	-3 ± 3	-3 ± 3

All SS P < 0.05 vs SI. * P < 0.05 nifedipine vs placebo.

sive population remains undefined. Inadequate suppression of renin system or sympathetic nerve activity (13), and/or exaggerated stimulation of putative endogenous digoxin-like molecules (1, 14), of calcium-regulating hormones such as $1,25(OH)_2D(5, 15)$, PTH (16), and/or of the newly described parathyroid hypertensive factor (17), have all been invoked as possible etiologic factors. Ultimately, however, regardless of the extracellular mediators involved, a salt-induced alteration in cellular ion homeostasis has been presumed but not adequately demonstrated.

To better understand the clinical phenomenon of salt-sensitive hypertension directly at the cellular ionic level, we measured the blood pressure and intracellular ionic effects of dietary salt loading with and without co-administration of nifedipine in essential hypertensive subjects. We observed (a) a significant rise in intracellular sodium occurring in all subjects on high versus low dietary salt intakes, but exaggerated in salt-sensitive subjects; (b) salt loading in salt-sensitive, but not salt-insensitive individuals coordinately elevates cytosolic free calcium and suppresses intracellular pH and free magnesium levels. For all subjects, salt-induced elevations in blood pressure were closely related to the ability of salt loading to alter intracellular ion levels; and (c) that the ability of calcium channel blockade to reverse salt-induced changes in blood pressure is closely related to its ability to reverse the ionic effects of dietary salt loading, nifedipine reducing Ca_i and elevating Mg_i and pH_i levels.

Given the differences between RBCs and other cell populations, appropriate caution should be exercised in extending the results of this study to other tissues. Nevertheless, the close reciprocal relationships observed between salt- and nifedipineinduced changes in blood pressure and in RBC ion levels suggest that these ionic events in RBCs reflect similar ionic events in other tissues as well. Furthermore, since red cells lack intracellular organelles, these results support the concept that saltsensitive hypertension is both dependent on and mediated by cellular calcium accumulation from the extracellular space and by associated changes in intracellular Mg_i and pH. Moreover, these data provide a cellular ionic basis for understanding the previously observed enhanced hypotensive efficacy of calcium channel blockade in sodium volume forms of hypertensive disease (18-20). Lastly, of practical importance for research in this field, proper interpretation of intracellular ion measurements among various patient populations in the future will require concurrent assessment and/or control of dietary salt intake.

The use of NMR spectroscopy to measure intracellular min-



Figure 5. Blood pressure and intracellular Ca_i , Na_i , pH_i , and Mg_i levels (see Fig. 1 legend for symbols) on placebo vs nifedipine therapy on low- and high-salt diets for the essential hypertensive group as a whole.

eral ions has received increasing attention (21). ³¹P-NMR spectra allow intracellular free magnesium and intracellular pH to be measured noninvasively and directly in packed cells and yields normal values for these ions in RBCs ($Mg_i = 220-250$ mM; $pH_i = 7.28 \pm 0.02$) and in other tissues comparable to or lower than other more invasive techniques (22, 23). Similarly, we measured intracellular free calcium in RBCs by using ¹⁹F-NMR spectroscopy, in which cells are loaded and then incubated with the flourinated EDTA-derived probe, QUIN-MF. Using this technique, basal levels of Ca. in RBCs of normotensive subjects, 20-30 nM, are in complete agreement with values obtained using other NMR (11, 24, 25) or fluorescent (26) probes. Lastly, the NMR measurement of Na_i is based on the addition of a cellular nonpermeant chemical shift reagent, $[Dy(PPP_i)_3]^{7-}$, which remains in the extracellular space and thus allows both intracellular and extracellular sodium to be clearly distinguished and quantitated. However, $\sim 15\%$ of the total intracellular sodium may be "NMR-invisible" and is presumed to represent membrane-bound and otherwise immobilized sodium. This property of measuring only "free" sodium, in addition to being able to precisely distinguish extracellular vis a vis intracellular sodium, may inadvertently help to explain why we were able to observe consistent increases in NMR Na_i in all subjects on high versus low dietary salt intakes when other careful studies have only been able to measure consistent increases in sodium transport pump activities (27) or have only seen changes when pressor responses were observed (28, 29).

This study complements and supports previous data suggesting that cellular calcium uptake from the extracellular space is critical to the pressor effects of salt loading. Thus, dietary salt-induced increases in blood pressure were closely and guantitatively related to salt-induced decreases in serum ionized calcium and to stimulation of 1,25(OH)₂D (5). We suggested that the fall in extracellular ionized calcium represented a reciprocal calcium transfer intracellularly mediated by factors such as 1,25(OH)₂D. Similarly, in normotensive dogs, while neither saline nor calcium infusions alone raised blood pressure, the combination of both saline and calcium was significantly pressor (30). Furthermore, pretreatment of these animals with a calcium channel antagonist prevented any significant pressor responses (30). These findings have recently been confirmed, salt-induced elevations of blood pressure being closely related to decreased serum ionized calcium and to a reciprocal increase in intracellular free calcium in both platelets and lymphocytes (31-33). We cannot distinguish in this study, however, the extent to which saltinduced changes in blood pressure and/or intracellular ions are due to intrinsic cellular defects of membrane transport, to the elaboration of circulating extracellular factors that alter cellular ion transport, or to a combination of both.

Indeed, each of the measured ion species may participate in the hypertensive process and in the pressor effects of salt. Mechanistically, calcium serves as part of the final common pathway mediating contraction in skeletal, cardiac, and vascular smooth muscle (34). The direct relation of free cytosolic calcium in platelets (35) and in RBCs (4) to the level of blood pressure suggests that this biochemical effect of calcium is both clinically relevant and reflected in a variety of tissues. Similarly, fasting RBC free magnesium levels are closely and inversely related to the blood pressure in normotensive and hypertensive subjects (2), and experimentally induced magnesium depletion increases smooth muscle contractility and can cause frank hypertension (36, 37). Furthermore, salt-induced hypertension has previously been associated with suppression of total RBC magnesium levels (30). Magnesium may exert its effects by virtue of its interactions with calcium and/or sodium. Thus, magnesium blunts the contractile effects of calcium in vascular smooth muscle (38), inhibits calcium binding to calmodulin (39), stimulates the rectifying calcium-dependent potassium channel (40-42), and stimulates Na⁺, K⁺ATPase activity (43). Suppressed intracellular free magnesium would therefore be expected to potentiate calcium-mediated contractions, exacerbate the resultant cellular depolarization, and cause cellular sodium accumulation, as has been demonstrated (44).

Intracellular pH is also a critical control element in normal cellular function (23), and we have previously demonstrated lower intracellular pH levels in essential hypertensive versus normotensive human subjects, in rats with renin-dependent, 2K-1C Goldblatt hypertensive controls (3). Furthermore, maneuvers their normotensive controls (3). Furthermore, maneuvers that raised or lowered intracellular pH consistently changed blood pressure in the opposite direction: the higher the pH, the lower the blood pressure (3). These results have recently been confirmed, and increased total body acid production in hypertension, especially salt-sensitive forms of hypertension, has also been reported (45, 46). The mechanisms by which an altered pH_i might increase blood pressure remain undefined, but increases in cytosolic free calcium appear associated with intracellular acidification (47, 48).

Since salt-loading in this study resulted in coordinate changes in each of these mineral ion species, we cannot ascertain from these results alone which, if any, of these ions might be primarily responsible for mediating the pressor effects of salt and which might be only secondarily involved. Indeed, the multiple correlations we observed in the present study between the various intracellular ion species, blood pressure, and salt sensitivity (see Results) may also reflect similar close relationships among these ions themselves. Thus, we have found basal Ca_i and Na_i, as well as Mg_i and pH_i, to be directly related, while Ca_i and Mg_i were linked in an inverse manner (4). However, our recent work suggests that while elevation of cytosolic free calcium and/or suppression of free magnesium may be necessary, they may not be sufficient conditions for the emergence of elevated blood pressure and that intracellular pH may play a pivotal role in and/or be a marker for the hypertensive process. Thus, even in the absence of hypertension, subjects with obesity and/or non-insulin-dependent diabetes mellitus exhibited elevated Ca_i and suppressed Mg_i levels compared with their equally normotensive thin and nondiabetic controls (49). Only in the hypertensive subjects, whether thin, obese, diabetic, or not, were pH_i levels consistently lower (49). The importance of pH_i is further emphasized by the results of this present study. Not only were the pressor effects of dietary salt loading closely related to the basal pH_i, the lower the pH_i, the greater the pressor response, but the antihypertensive effects of nifedipine were equally closely related to the pH_i.

Lastly, this study provides a cellular ionic basis for the enhanced antihypertensive efficacy of calcium channel blockade previously noted in volume-dependent hypertensive states such as DOC-saline and Dahl salt-sensitive rat models and in essential hypertensive subjects with low plasma renin activity and/ or lower serum ionized calcium values (19, 20, 50). We have previously suggested that the lower average extracellular ionized calcium observed in the low renin state reflects a greater dependence of blood pressure on cytosolic calcium accumulation from the extracellular space vis a vis from internal stores (51), hence explaining their greater sensitivity to calcium channel blocking agents such as nifedipine (50). This present study provides additional evidence in favor of this hypothesis, by directly demonstrating in RBCs devoid of intracellular calcium stores, the linked blood pressure and cellular calcium elevating effects of salt loading and the reversal of these pressure and calcium effects by nifedipine. Furthermore, in the presence of nifedipine, dietary salt loading in salt-sensitive subjects no longer significantly elevated blood pressure; diastolic pressures did not rise out of the normal range (Table III). Hence, the enhanced clinical utility of calcium channel blocking agents in low renin and/or sodium volume-dependent forms of hypertension may derive from their ability to at least partially reverse salt-dependent cellular ionic changes. On the other hand, it is reasonable that nifedipine lowered pressure to at least some degree in all the subjects studied here, including salt-insensitive individuals, since a variety of mechanisms other than salt also contribute to cellular calcium uptake, the inhibition of which would lower average cytosolic free calcium levels and thus blood pressure.

Altogether, these results demonstrate physiologically similar cellular ionic mechanisms underlying the ability of dietary salt to exacerbate hypertension and of calcium channel blockade with nifedipine to ameliorate it, and emphasize the critical role of altered cellular ion metabolism in the hypertensive process. Regardless of mechanistic considerations, however, proper interpretation of intracellular mineral ion measurements in future studies may require a concurrent assessment of dietary salt intake. Using NMR spectroscopic techniques to measure intracellular ion levels in peripheral blood samples may also be useful in distinguishing clinically salt-sensitive subjects and is the focus of current studies.

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