Vol. 154, No. 1, 1988 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
July 15, 1988 Pages 380-386

FURA-2 USED AS A PROBE TO SHOW ELEVATED INTRACELLULAR FREE CALCIUM IN PLATELETS OF DAHL-SENSITIVE RATS FED A HIGH SALT DIET

Sudesh Vasdev[1], Peter Thompson[2], Chris Triggle[2], Peter Fernandez[1], Peter Bolli[1], and V.S. Ananthanarayanan[3]

Disciplines of Medicine[1], Basic Sciences[2] and Biochemistry[3] Memorial University, St. John's, Newfoundland , Canada

Received June 7, 1988

Elevated intracellular free calcium concentration $[Ca^{2+}]_i$ in vascular smooth muscle cells has been implicated in the pathophysiology of hypertension. Platelet $[Ca^{2+}]_i$ was measured using the fluorescent indicator, Fura-2, in Dahl sensitive (DS) and resistant (DR) rats given high (8% NaCl) and low (0.4% NaCl) salt diets, as well as in the spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) rats. The aim of this study was to show whether $[Ca^{2+}]_i$ is elevated in salt induced hypertension. Platelet $[Ca^{2+}]_i$ and systolic blood pressure (SBP) were higher ($p < 0.001$) in DS rats given a high than low salt diets. In contrast, no changes in platelet $[Ca^{2+}]_i$ and SBP were observed in DR rats. In SHR, platelet $[Ca^{2+}]_i$ and SBP were higher ($p < 0.001$) than in the WKY rats. Platelet $[Ca^{2+}]_i$ correlated with SBP in all groups of rats ($r=0.929$; $p < 0.001$, $n=38$). The parallel increase in SBP and $[Ca^{2+}]_i$ in the DS high salt rats and the SHR suggests that an increased $[Ca^{2+}]_i$ is involved in the pathophysiology of hypertension in the two models which differ with respect to the pathogenesis of their hypertension. This increase in $[Ca^{2+}]_i$ therefore seems to reflect an abnormality in $[Ca^{2+}]_i$ handling in hypertension regardless of its cause.

An increase in the concentration of intracellular free cytosolic calcium $[Ca^{2+}]_i$ in vascular smooth muscle initiates contraction (1) which in turn determines the degree of tension (2). Although the resting levels of $[Ca^{2+}]_i$ have not been measured in vascular smooth muscle cells from hypertensive patients or hypertensive animal models, it is reportedly increased in platelets of patients with essential hypertension (3,4,5,6) and seems to relate directly to the level of systolic blood pressure (SBP) (3,4,5). Furthermore, antihypertensive treatment lowers $[Ca^{2+}]_i$ in proportion to the fall in SBP (3,4). An increase in $[Ca^{2+}]_i$ in platelets could reflect similar changes in vascular smooth muscle cells, which share many common intracellular features with platelets, including a calcium-

0006-291X/88 $1.50

dependent contraction-coupling process (7). Dahl salt-sensitive (DS) rats develop severe hypertension on a high salt diet but remain normotensive for a variable time while eating a low salt diet, whereas Dahl salt-resistant (DR) rats remain remarkably normotensive on either diet (8). To investigate whether alterations in $[Ca^{2+}]_i$ occur in the DS rats fed a high salt diet, we have measured the $[Ca^{2+}]_i$ concentration in platelets of DS and DR rats placed on high and low salt diets. For comparison $[Ca^{2+}]_i$ concentrations were also measured in platelets from spontaneously hypertensive (SHR) as well as their normotensive control Wistar-Kyoto rats (WKY).

METHODS

Up to the age of 6 weeks, all Dahl rats were fed a low salt diet (0.4% NaCl). At age 6 weeks, 16 Dahl salt sensitive (DS) and an equal number of Dahl resistant (DR) rats were separated into two groups to receive for 4 weeks either high (8% NaCl) or low (0.4% NaCl) salt diets along with unrestricted access to tap water. For comparison, 6 age matched SHR (Okamoto-Aoki Strain), and 6 normotensive WKY rats of similar body weight (Purchased from Charles River, Quebec, P.Q.) were used in the study.

Body weight and SBP were measured weekly, the latter using the tail cuff method (Model 5A Amplifier, ITTC Life Science Instruments, Woodland Hills, CA). Each tail cuff blood pressure was the average of 4 individual measurements.

Measurements of $[Ca^{2+}]_i$ in platelets:

At age 10 weeks rats were lightly anaesthetized by intraperitoneal pentobarbital (Nembutal sodium; 10 mg/100 g body weight). After thoracic cage resection, 8 to 10 ml of blood was drawn into a vacutainer tube containing 0.07 ml of 15% EDTA by intracardiac puncture. Platelet-rich plasma was prepared by centrifugation at 120 x g for 20 minutes at room temperature. Platelets were isolated by gel filtration on a sepharose CL-2B column (8 X 200 mm) and equilibrated with platelet buffer elution medium containing 10 mM HEPES, 145 mM sodium chloride, 5 mM potassium chloride, 1 mM magnesium sulfate, 0.5 mM sodium phosphate, 6 mM glucose (pH 7.4 at 37°C). Platelets were collected at a concentration of approximately 10^6 to 10^7 cells per ml into a plastic tube. To measure $[Ca^{2+}]_i$, platelets were incubated for 30-45 minutes with 1 μM Fura-2AM (Molecular Probes Inc., Eugene, OR, USA) in calcium - poor medium containing platelet buffer in a shaking water bath at 37°C. After incubation, extracellular Fura-2AM was removed by passage through the sepharose column, and the external calcium was restored by addition of 1 mM calcium chloride to the medium. Platelets were post-incubated at 37°C for at least 30 minutes and the fluorescence excitation spectrum was scanned from 300-420 nm with emission wavelength fixed at 510 nm, using a SHIMADZU Model RF540 spectrofluorometer. All measurements were done in duplicate and the mean value was used for statistical calculation.

The standard procedure (9) for calculating $[Ca^{2+}]_i$ from dual wavelength measurements of Fura-2 was used which involves the following equation: $[Ca^{2+}]_i = Kd \cdot [(R-R_{min})/(R_{max}-R)] \cdot \beta$, whereby R is the ratio of fluorescence of the sample at 340 and 380 nm; R_{max} and R_{min} the ratios for Fura-2 free acid at the same wavelengths in the presence of saturating Ca^{2+} and in nominally

zero Ca^{2+}, respectively. β is the ratio of fluorescence of Fura-2 at 380 nm in zero and saturating Ca^{2+} and Kd is the dissociation constant of Fura-2 for Ca^{2+}, assumed to be 224 nM at 37°C. R_{max} was determined by rupturing the cells with Triton X-100 (0.5%) along with adding a saturating amount of calcium. R_{min} was determined by adding Triton X-100 (0.5%) and EGTA (10 mmol/l) and by increasing the pH to 8.3. Correction for autofluorescence was made by subtracting the fluorescence of unloaded cells from an equal density of cells loaded with Fura-2 to obtain a fluorescent signal that was solely representative of intracellular Fura-2. Platelets were counted using a Coulter counter (Model S-PLUS IV) and results of $[Ca^{2+}]_i$ are given as nmol/l.

Statistical Analysis

All data are expressed as mean values $\pm$ SD. Statistical analysis of results was performed by Student's t-test (unpaired).

RESULTS

The coefficient of variation by the method of duplicates for the measurement of $[Ca^{2+}]_i$ was 8.3% (n=15). A typical excitation spectrum of rat platelets loaded with Fura-2 is shown in Fig. 1.

DS rats on high salt diet developed hypertension and this was parallelled by an increase in platelet $[Ca^{2+}]_i$ as compared to the

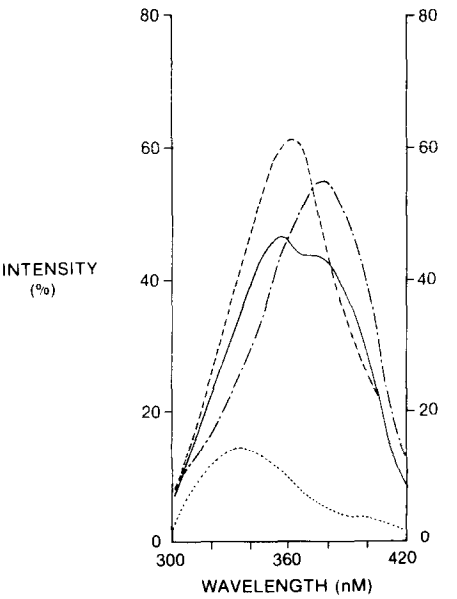

Fig. 1: Excitation spectra of platelets loaded with Fura-2, superimposed with excitation spectrum of the same sample of platelets in which intracellular Fura-2 has been equilibrated with Ca^{2+} saturating (Triton X-100) and Ca^{2+}-free solution (EGTA 10 mmol/l and pH 8.3). Cellular data were corrected for autofluorescence by substraction of the fluorescence of an equivalent density of unloaded platelets. Fluorescence excitation spectrum was collected from 300 to 420 nm; emission wavelength was fixed at 510 nm. ———, Sample; – – –, Saturating Ca^{2+}; — • — • — , Zero Ca^{2+}; ·····, Sample Blank.

Table 1: Cytosolic free calcium in platelets and systolic blood pressure in Dahl-resistant (DR) and Dahl-sensitive (DS) rats fed a low (0.4% NaCl) and high (8% NaCl) salt diet.

	LOW SALT	HIGH SALT
Cytosolic Calcium (nmol/l)		
DR	65 ± 9 (6)	81 ± 22 (7)
DS	98 ± 15 (7)	280 ± 38 (6)**
Systolic Blood Pressure (mmHg)		
DR	120 ± 4 (6)	125 ± 7 (7)
DS	144 ± 10 (7)	214 ± 16 (6)**

Values are mean ± SD; Number of animals in each group are given in parenthesis. ** represents significant difference ($p < 0.001$) from all other groups.

DS rats on low salt diet. In DR rats, high salt diet did not raise either SBP or $[Ca^{2+}]_i$ (Table 1). There was a direct correlation between platelet $[Ca^{2+}]_i$ concentrations and SBP in DS and DR rats on high and low salt diets ($r=0.939$; $p < 0.001$; $n=26$). In SHR, platelets $[Ca^{2+}]_i$ and SBP were higher as compared to WKY rats ($p < 0.001$; $n=6$ in each group) (Table 2). When the results of DS, DR, SHR and WKY rats were pooled the direct correlation between platelet $[Ca^{2+}]_i$ and SBP was maintained ($r=0.929$; $p < 0.001$, $n=38$) (Fig. 2). Body weight (mean ± SD) of DR and DS on low and high salt diet was 369 ± 19, 353 ± 20, 353 ± 6 and 329 ± 4, respectively. Body weight of DS rats on high salt diet was significantly lower ($p < 0.02$) than all other groups.

Table 2: Cytosolic free calcium in platelets, systolic blood pressure and body weight in spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) rats

	WKY	SHR	p
Cytosolic calcium in platelets (nmol/l)	70 ± 8	167 ± 38	< 0.001
Systolic blood pressure (mmHg)	119 ± 6	172 ± 16	< 0.001
Body weight (gm)	261 ± 11	267 ± 6	

Values are mean ± SD (n=6 in each group).

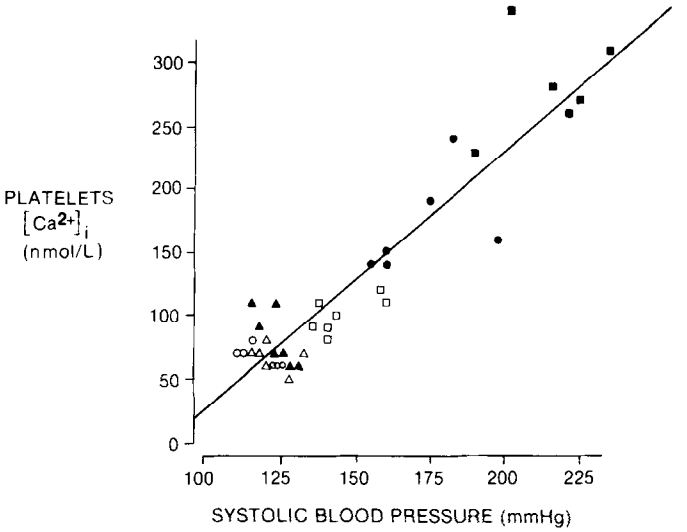

Fig. 2: Correlation between systolic blood pressure and cytosolic free calcium concentration in platelets of Dahl-sensitive rats on a low salt (□), a high salt (■), Dahl-resistant on a low salt (△) and a high salt (▲) diet. SHR (●) and WKY (○) were on a regular lab chow.

DISCUSSION

The results from the present study supports the hypothesis that a high salt diet leads to an increase in SBP in DS rats together with an increase in $[Ca^{2+}]_i$ while there was no significant change in either of these parameters in DR rats. The correlation between SBP and $[Ca^{2+}]_i$ supports their close functional relationship. Our results for platelet $[Ca^{2+}]_i$ are in agreement with those reported using Quin-2 in rats (5) and humans (3,4,5) which confirm a higher platelet $[Ca^{2+}]_i$ in association with hypertension (10).

Platelets were chosen for this study because besides being an easily available tissue, they may present abnormalities in calcium handling similar to those described in erythrocytes (11), adipocytes and vascular smooth muscle cells (12,13) of hypertensive man and rat. Fura-2 has several advantages over Quin-2 (9) for Fura-2 has a 30 fold greater quantum efficiency, a slightly higher K_d and spectral shift with a change from the bound to the unbound species. The lower buffering capacity of Fura-2 allows one to use more 'physiological' conditions for studying $[Ca^{2+}]_i$ in platelets. With Quin-2 the maximal fluorescence after cell lysis is a key variable for calculating $[Ca^{2+}]_i$ and cannot always be reliably determined but this problem

is greatly reduced with Fura-2 where quantification of $[Ca^{2+}]_i$ involves the ratio of two excitation wavelengths (9,14).

Increased dietary sodium has been shown to increase SBP in DS rats though the mechanisms involved are not fully elucidated. DS rats were shown to have increased production of a humoral 'hypertensinogenic' factor (15,16). One suggested hypothesis for this transferrable factor is the putative natriuretic hormone, or endogenous digitalis or ouabain-like factor which by inhibiting Na, K-ATPase, might act on peripheral resistance vessels to raise blood pressure (17-20). Na, K-ATPase inhibitory activity has been shown in the plasma of healthy, hypertensive and dialysis dependent renal subjects as well as in hypertensive animal models (20-24). Recently, it has been shown that plasma from patients with essential hypertension contains a substance that increases the cytosolic calcium concentration in platelets (25) and it has been suggested that a plasma factor that acts on platelets may do so on the vascular smooth muscle cell. Whatever the mechanisms by which salt induces hypertension, increased $[Ca^{2+}]_i$ appears to represent the common mediating factor (26). Whether the increased $[Ca^{2+}]_i$ represents a primary derangement in calcium handling at the vascular smooth muscle cell or is a secondary phenomenon is not yet known.

ACKNOWLEDGEMENTS

The authors wish to thank Ms. C. Sampson for technical assistance and Ms. Lynn Parsons for typing the manuscript. This study was supported by the Medical Research Council Grant MA-9811, the Canadian Heart Foundation, and Research and Development Grant #660179, Faculty of Medicine, Memorial University.

REFERENCES

1. Filo, R.S., Bohr, D.F. and Ruegg, J.C. (1965) Science 147, 1581-1583.
2. Bohr, D.F. (1963) Science 139, 597-599.
3. Erne, P., Bolli, P., Burgisser, E. and Buhler, F.R. (1984) N. Engl. J. Med. 310, 1084-1088.
4. Bolli, P., Erne, P., Hulthen, U.L., Ritz, R., Kiowski, W., Ji, B.H., Buhler, F.R. (1984) J. Cardiovasc. Pharmacol. 6, S996-S1001.
5. Bruschi, G., Bruschi, M.E., Caroppo, M., Orlandini, G., Spaggiari, M. and Cavatorta, A. (1985) Clin. Sci. 68, 179-184.
6. Hvarfner, A., Larsson, R., Morlin, C., Rastad, J., Wide, L., Akerstrom, G. and Ljunghall S (1988) J. Hypertens. 6, 71-77.

7. Niederman, R. and Pollard, T.D. (1975) J. Cell Biol. 67, 72-92.
8. Dahl, L.K., Heine, M. and Tassinari, L. (1962) J. Exp. Med. 115, 1173-1190.
9. Grynkiewicz, G., Poenie, M. and Tsien, R.Y. (1984) J. Biol. Chem. 260, 3440-3450.
10. Cooper, R.S., Shamsi, N., Katz, S. (1987) Hypertension 9, 224-229.
11. Postnov, Y.V., Orlov, S.N. (1980) In Intracellular Electrocytes and Arterial Hypertension (Georg, L.H., Ed.), pp. 144-151. Stuttgart, New York: Thieme Verlag.
12. Kwan, C.Y., Belbeck, L., Daniel, E.E. (1980) Mol. Pharmacol. 17, 137-140.
13. Devynck, M.A., Pernolle, M.G., Nunez, A.M., Meyer, P. (1981) Hypertension 3, 397-403.
14. Rao, G.H.R., Peller, J.D., White, J.H. (1985) Biochem. Biophys. Res. Commun. 132, 652-657.
15. Hirata, Y., Tobian, L., Simon, G. and Iwai, J. (1984) Hypertension 6, 709-716.
16. Tobian, L., Pumper, M., Johnson, S. and Iwai, J. (1979) Clin. Sci. 57, 345s-347s.
17. Blaustein, M.P. (1984) Hypertension 6, 445-453.
18. Haddy, F.J. and Pamnani, M.B. (1984) J. Cardiovasc. Pharmacol. 6, 561-574.
19. Buckalew, V.M., Jr. and Gruber, K.A. (1984) Ann. Rev. Physiol. 46, 343-358.
20. de Wardener, H.E. and Clarkson, E.M. (1985) Physiol. Rev. 65, 658-759.
21. Vasdev, S., Longerich, L., Johnson, E., Brent, D. and Gault, M.H. (1985) Res. Commun. Chem. Path. Pharmacol. 49, 387-???.
22. Vasdev, S., Longerich, L., Johnson, E., Brent, D. and Gault, M.H. (1986) In Cardiac Glycosides (E. Erdmann, K. Greeff, J.C. Skou, Eds.), pp. 157-164. Steinkopff Verlag Darmstadt.
23. Vasdev, S., Johnson E., Longerich, L., Prabhakaran, V.M. and Gault, M.H. (1986) Clin. Nephr. 27, 169-174.
24. Cloix, J.F., Crabos, M., Devynck, M.A., Elghozi, J.H., Henning, G., Kamal, L.A., Lacerda-Jacomini, L.C., Meyer, P., Pernollet, M.G., Rosenfeld, J.B. and De The, H. (1984) Adv. Nephr. 14, 161-171.
25. Lindner, A., Kenny, M. and Meacham, A.J. (1987) N. Engl. J. Med. 316, 509-513.
26. Sprenger, K.B.G. (1985) Clin. Physiol. Biochem. 3, 208-220.