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Hypertension Does Not Affect Intracellular Calcium Uptake in Human Platelets

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The relationship between the Ca^{2+} transport of platelet endoplasmic reticulum and hypertension was analyzed in 17 untreated patients exhibiting various degrees of hypertension. Each patient underwent a 24-h recording of ambulatory blood pressure. Platelets from patients were permeabilized with saponin and the rate of ATP-driven thapsigargin-sensitive Ca^{2+} uptake determined using the fluorescent Ca^{2+} indicator fluo-3. No relationship between blood pressure (systolic, diastolic, day, night) and the rate of Ca^{2+} uptake into the sacroplasmic reticulum of platelets was found. A weak but insignificant correlation between Ca^{2+} uptake and the heart rate was noted. Therefore, the increase in cytosolic Ca^{2+} of platelets in hypertension may not be due to changes of the activity of Ca^{2+} uptake into the sacroplasmic reticulum. Am J Hypertens 1996;9:136–142

KEY WORDS: **Platelets, calcium transport, hypertension.**

Despite extensive research, the relationship of calcium and arterial hypertension remains unclear. Intracellular Ca^{2+} is a major determinant of basal myogenic tone of vascular muscle. Therefore, an increase of cytosolic Ca^{2+} in arterial smooth muscle leads to an increase in peripheral resistance characteristic for hypertension. The systemic hormonal signals associated with hypertension influence endothelial cell, vascular muscle cells and blood cells. Erne et al[1] have documented an elevation of platelet cytosolic free Ca^{2+} in hypertension and a close correlation of the Ca^{2+} levels in platelets and blood pressure in untreated hypertensive patients. Furthermore, both platelet Ca^{2+} and vasoconstriction were normalized by antihypertensive treatment,[1,2] indicating a close relationship between platelet Ca^{2+} dynamics and circulating hormonal factors. Cytosolic Ca^{2+} overload has been described in a variety of cells in both hypertension[3–5] and pressure-induced cardiac dysfunction.[6]

Whereas the relationship of platelet cytosolic Ca^{2+} and vasoconstriction has been clearly established, the molecular basis of elevated Ca^{2+} levels remains unclear. The rapid rise in cytosolic Ca^{2+} observed upon exposure of platelets to humoral factors from hypertensives[7,8] indicates that systemic hormonal factors influence platelet cytosolic Ca^{2+} and that the changes in platelet cytosolic Ca^{2+} are effected by a rapid mechanism, ie, Ca^{2+} influx across the plasma membrane or intracellular Ca^{2+} mobilization. The reversal of Ca^{2+} levels by means of Ca^{2+} antagonists has led to the hypothesis that Ca^{2+} influx is responsible for elevated platelet Ca^{2+}.[1,9] However, the enhanced hormone-induced elevation of platelet cytosolic Ca^{2+} is not corrected by antihypertensive therapy,[10] demonstrating that the fundamental defect in Ca^{2+} dynamics has not been restored. Moreover, voltage-operated Ca^{2+} channels do not mediate agonist-evoked Ca^{2+} entry in platelets.[11] Besides voltage-operated Ca^{2+} channels, a variety of other mechanisms contribute to the Ca^{2+} dynamics of platelets[11,12]: agonist-mediated Ca^{2+} in-

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flux; ligand-gated Ca^{2+} channels; Ca^{2+}-ATPase pumps present in both the plasma membrane and membrane of endoplasmic reticulum[13,14]; Na^+/Ca^{2+} exchanger[15]; inositol 1,4,5-trisphosphate-induced Ca^{2+} release from intracellular Ca^{2+} stores; and a series of cytosolic and endoplasmic reticulum Ca^{2+} binding proteins. Resink et al[16] have documented a reduction of the calmodulin-stimulated Ca^{2+}-ATPase activity and an increase in basal Ca^{2+}-ATPase activity in platelets from hypertensive compared to normal subjects, suggesting a reduction in plasma membrane Ca^{2+} extrusion activity and an activation of the sarcoplasmic reticulum Ca^{2+} pump in hypertension. Other studies also have described a decrease in Ca^{2+}-ATPase activity in hypertension.[17,18] In the present study we have determined the rate of Ca^{2+} uptake in permeabilized platelets from 17 untreated subjects with various degrees of hypertension in order to test if alteration of the sarcoplasmic reticulum Ca^{2+}-ATPase pump activity contributes to elevated platelet cytosolic Ca^{2+} in hypertension.

MATERIALS AND METHODS

Reagents Heparin, pentosan polysulfate, saponin, and hexokinase were obtained from Sigma (St. Louis, MO). Fluo-3 pentapotassium salt was from Molecular Probes (Eugene, OR). Fluo-3 was stored in 50 μL aliquots at a concentration of 1 mmol/L in dimethylsulfoxide at –70°C. Ionomycin was from Calbiochem (San Diego, CA), thapsigargin from LC Services Corporation. Aminoethyl Biogel P-2 was purchased from Bio-Rad (Richmond, CA), and Sepharose CL-2B from Pharmacia (Uppsala, Sweden).

Buffer Solutions All experiments were performed in 20 mmol/L HEPES buffer containing 155 mmol/L KCl, 5 mmol/L NaCl, 2 mmol/L $MgCl_2$, 2 mmol/L 1,4-dithioerythritol, and 0.3 mmol/L phenylmethanesulfonyl fluoride at pH 7.2, which was denoted buffer A. All solutions were depleted of Ca^{2+} by means of chromatography on EDTA-polyacrylamide as described previously.[19,20] Residual Ca^{2+} concentrations of solutions following treatment with EDTA-polyacrylamide were measured as described.[20]

Patients We analyzed platelets of 17 newly diagnosed hypertensive patients who were referred to the Division of Cardiology of the Kantonsspital Lucerne. None of the patients had received antihypertensive drugs before. Patients underwent a 12-lead electrocardiogram, a bicycle exercise test, and a 24-h recording of ambulatory blood pressure (HCR Blood Pressure, Disectronic, Burgdorf, Switzerland). Table 1 summarizes the clinical data and results of ambulatory 24-h blood pressure measurements.

Preparation of Platelets Platelets were prepared from platelet-rich plasma by gel filtration[21] using Sepharose CL-2B (Pharmacia) and adjusted to 108 cells/mL. Platelets were permeabilized by addition of 20 μg saponin/108 cells. Selective permeabilization was confirmed by measuring the release of fluo-3 from cells loaded with fluo-3 acetoxymethyl ester, and by monitoring the release of Ca^{2+} from internal membranes in permeabilized cells following addition of ionomycin. Platelets were used within 6 h of blood withdrawal.

TABLE 1. CLINICAL PARAMETERS OF THE SEVENTEEN PATIENTS SUBJECTED TO 24 H BLOOD PRESSURE DETERMINATION

Gender (men/women)	13/5
Age (years)	48 ± 13
Height (cm)	171 ± 10
Weight (kg)	75 ± 14
Office SBP (mm Hg)	169 ± 27
Office DBP (mm Hg)	111 ± 12
Day SBP (mm Hg)	148 ± 16
Day DBP (mm Hg)	97 ± 10
Night SBP (mm Hg)	136 ± 20
Night DBP (mm Hg)	87 ± 9
Day heart rate (beats/min)	76 ± 7
Night heart rate (beats/min)	64 ± 6

SBP, systolic blood pressure; DBP, diastolic blood pressure.

Measurement of Ca^{2+} Ca^{2+} concentrations were measured by means of the fluorescent Ca^{2+} indicator fluo-3. The indicator was added at a final concentration of 1 μmol/L to 1 mL of a suspension of platelets (108 cells/mL) placed into a 1-cm quartz cuvette. The suspension was continuously stirred using a magnetic stirrer. Fluorescence was measured by means of a Perkin-Elmer (Oakbrook, IL) 650-10S fluorimeter, interfaced via an analog/digital converter (PC-28, Instrumatic AG, Zurich, Switzerland) to a personal computer. Fluorescence of fluo-3 was excited at 505 nm and observed at 530 nm. ATP (1 mmol/L) and saponin (20 μg) were added to the cell suspension to activate Ca^{2+} uptake of intracellular Ca^{2+} stores. ATP at 1 mmol/L was found to saturate thapsigargin-sensitive Ca^{2+} uptake in platelets from many subjects of widely different degrees of hypertension (not shown).

Ca^{2+} concentrations were calculated from the fluorescence signal using standard titrations of fluo-3 with Ca^{2+}. Binding of Ca^{2+} to fluo-3 was expressed as signal versus concentration of added Ca^{2+}, and evaluated as described.[20] From the dissociation constant (K^d) of the Ca^{2+}-fluo-3 complex, which was typically 900 nmol/L in buffer A, the fluorescence signal of Ca^{2+}-free indicator (f_F) and that of the Ca^{2+}-indicator complex (f_{FC}), the concentration of Ca^{2+} was calculated as follows:

$$[Ca^{2+}]_{free} = K_d \cdot (f_{obs} - f_F)/(f_{FC} - f_{obs}) \quad (1)$$

where f_{obs} represents the observed fluoresence signal. K_d was determined from titrations of fluo-3 with Ca^{2+} (final concentrations between 0 and 5 mmol/L; eval-

uation according to Eberhard and Erne[20]). f_F was determined after addition of EGTA (5 mmol/L), and f_{FC} by extrapolation of the concentration of added Ca^{2+} to infinity. Figure 1 illustrates the calibration of fluorescence. The Ca^{2+} flux due to the sarcoplasmic reticulum Ca^{2+}-ATPase pump, hence termed Ca^{2+} uptake, was determined from the difference of the overall Ca^{2+} flux before and after addition of thapsigargin (200 nmol/L), a specific inhibitor of the sarcoplasmic reticulum Ca^{2+}-ATPase.[22] In the presence of thapsigargin, a spontaneous Ca^{2+} flux out of the sarcoplasmic reticulum, the so-called leak, was observed and modelled by an exponential decay toward an equilibrium value (Figure 1B):

$$[Ca^{2+}] = a \cdot \exp(-k \cdot t) + [Ca^{2+}]_{eq} \quad (2)$$

where a and k are arbitrary constants, and $[Ca^{2+}]_{eq}$ the equilibrium concentration of Ca^{2+}. Thus, the rate of Ca^{2+} leak at a particular Ca^{2+} concentration is given by

$$d[Ca^{2+}]/dt = -k \cdot a \cdot \exp(-k \cdot t) = -k \cdot ([Ca^{2+}] - [Ca^{2+}]_{eq}) \quad (3)$$

The rate of Ca^{2+} uptake is the difference between the slope of the Ca^{2+} transient at a particular Ca^{2+} concentration before addition of thapsigargin (overall Ca^{2+} flux, Figure 1), and the rate of Ca^{2+} leak at that Ca^{2+} concentration (equation 3).

Data Analysis Data evaluation and nonlinear least-squares fits were performed using standard procedures.[23,24] Results are expressed as means ± SD. Individual Ca^{2+} uptake and leak rate determinations were averaged and SD values calculated if three or more individual values were measured. Linear least-squares regressions assuming uniform and unity SD values of the individual data points were used to analyze putative relationships between binding data and clinical parameters. Percentile points (P) were determined using the two-tailed t distribution.

RESULTS

The evaluation of fluorescence data and a typical Ca^{2+} transient used to determine Ca^{2+} uptake rates, i.e. the thapsigargin-sensitive Ca^{2+} flux, are shown in Figure

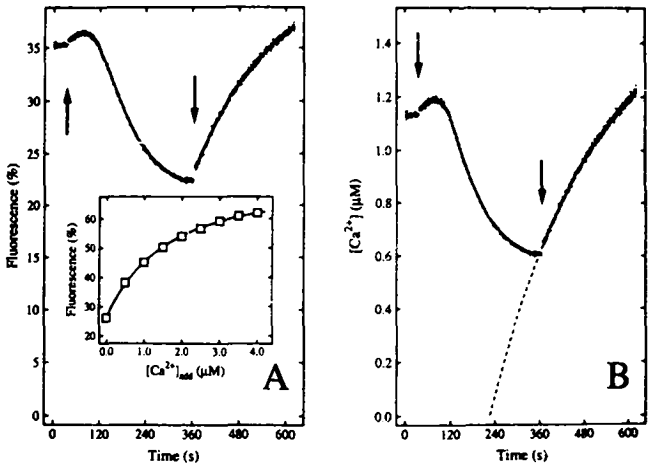

FIGURE 1. *Conversion of the fluorescence signal of fluo-3 (A) to Ca^{2+} concentrations (B). The arrows indicate (from left to right) the addition of saponin (20 mg/mL) and thapsigargin (200 nmol/L) to a suspension of platelets (108 cells/mL) containing ATP (1 mmol/L). The initial increase in fluorescence signal after addition of saponin is due to a decrease in turbidity upon permeabilization of the cells. The turbidity drops during the first 60 sec and remains stable thereafter for at least 15 min (not shown). The inset in A shows the correlation between fluorescence and added Ca^{2+} ($[Ca^{2+}]_{add}$, the total concentration of added Ca^{2+}) obtained in a separate experiment with an identical platelet suspension. The solid line represents the fitted binding curve (K_d = 723 nmol/L, f_F = 0.75%, f_{FC} = 73.25%; see Eberhard M, Erne P[20]). The Ca^{2+} concentration ($[Ca^{2+}]_{free}$ in equation 1) in B was calculated from the fluorescence signal in A according to equation 1. The dotted curve in B represents a monoexponential fit of the Ca^{2+} transient according to equation 2 (a = − 933 nmol/L, k = 0.0036 sec^{-1}, $[Ca^{2+}]_{eq}$ = 1599 nmol/L). Ca^{2+} concentrations are considerably underestimated at the start of the transient due to the turbidity change during permeabilization (see above).*

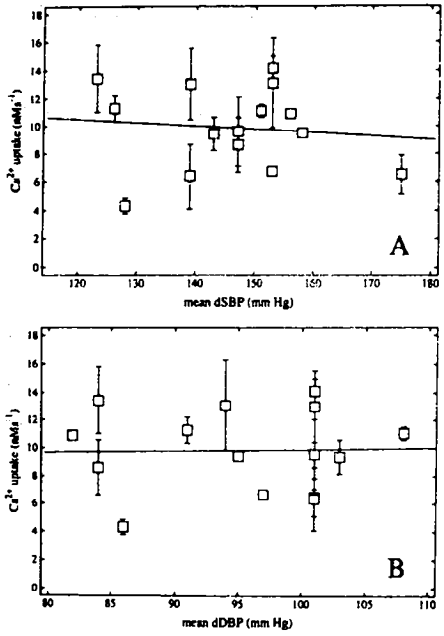

FIGURE 2. *Lack of correlation of Ca^{2+} uptake rates versus mean day systolic blood pressure (mean dSBP; A) and mean day diastolic blood pressure (mean dDBP). Lines were determined by linear regression (A: $r = -0.114$, $P > .5$; B: $r = 0.029$, $P > .5$).*

1. At a given Ca^{2+} concentration similar Ca^{2+} uptake rates were obtained when thapsigargin was added at various degrees of store loading (not shown). Since Ca^{2+} concentrations of suspensions of permeabilized platelet were around and above 1 mmol/L, and since the Ca^{2+} uptake rate did not significantly depend on the concentration of Ca^{2+} in the range between 0.8 and 1.5 mmol/L (not shown), Ca^{2+} uptake rates were evaluated at 1 mmol/L Ca^{2+} in the present study. To examine if spontaneous Ca^{2+} leak of the Ca^{2+} stores is linked to hypertension, the Ca^{2+} leak (Ca^{2+} flux in the presence of thapsigargin) was also evaluated at 1 mmol/L Ca^{2+}. In two out of the 17 patients examined the Ca^{2+} concentration of the platelet suspension after permeabilization was too high to allow the determination of Ca^{2+} uptake and leak rates at 1 mmol/L Ca^{2+}.

Figure 2 shows that Ca^{2+} uptake into sarcoplasmic reticulum is not related to average daytime blood pressure. Similar results are obtained with other blood pressure parameters (Table 1) and with Ca^{2+} leak from sarcoplasmic reticulum (Table 2). A weak correlation between both Ca^{2+} uptake and Ca^{2+} leak and heart rate was observed (Figure 3, Table 2), although these correlations are flawed by the substantial error of the values of Ca^{2+} uptake and leak. Furthermore, no correlation was found between Ca^{2+} uptake and age (Table 2). To corroborate the relationship between Ca^{2+} fluxes and blood pressure we analyzed a group of blood donors consisting of four normotensive and ten hypertensive subjects who did not undergo 24-h blood pressure determination (Table 3). No significant correlation between flux parameters and blood pressure was detected within this group (not shown). In addition, the differences in Ca^{2+} uptake and leak between the group of normotensives and the two hypertensive groups were statistically not significant (Table 3).

DISCUSSION

There is a general agreement that platelet cytosolic Ca^{2+} is increased in hypertension and that this process can be accomplished by rapid Ca^{2+}-regulating processes.[8] Both plasma membrane- and internal membrane-associated Ca^{2+} fluxes contribute to the observed changes in platelet cytosolic Ca^{2+}. Since the Ca^{2+}-ATPase of sarcoplasmic reticulum is believed to represent the major contribution to Ca^{2+} removal from the cytoplasm,[11,25] we have analyzed ATP-dependent

TABLE 2. CORRELATION OF Ca^{2+} UPTAKE AND LEAK WITH CLINICAL PARAMETERS

	Ca^{2+} Uptake			Ca^{2+} Leak		
Parameter	Slope	*P*	r	Slope	*P*	r
Age	0.083 ± 0.055	>.2	0.389	0.043 ± 0.029	>.1	0.383
Day SBP	−0.025 ± 0.060	>.5	−0.114	0.010 ± 0.031	>.5	0.093
Day DBP	0.011 ± 0.101	>.5	0.029	−0.003 ± 0.053	>.5	−0.015
Night SBP	0.019 ± 0.044	>.5	0.121	0.012 ± 0.023	>.5	0.141
Night DBP	0.130 ± 0.086	>.1	0.386	0.027 ± 0.048	>.5	0.155
Day heart rate	0.141 ± 0.113	>.2	0.327	0.105 ± 0.055	>.05	0.465
Night heart rate	0.260 ± 0.098	<.05	0.591	0.124 ± 0.054	<.05	0.541

15 hypertensive patients undergoing 24-h blood pressure determination (Table 1), for which Ca^{2+} uptake and Ca^{2+} leak values were determined (see Results).

SBP, systolic blood pressure; DBP, diastolic blood pressure.

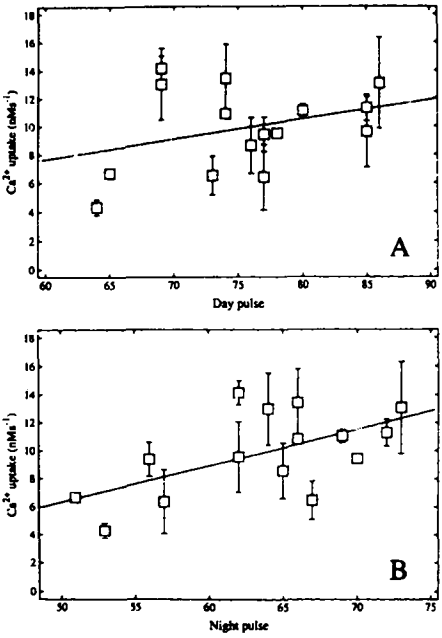

FIGURE 3. *Plot of Ca^{2+} uptake rates versus average heart rate during daytime (A) and nighttime (B). Lines were calcul..ed by linear regression (A: r = 0.141, P > .2; B: r = 0.260, P < .05).*

Ca^{2+} transport into sarcoplasmic reticulum in platelets from a series of patients exhibiting various degrees of hypertension. None of the patients received antihypertensive therapy prior to analysis. Thapsigargin, a specific inhibitor of all isoforms of internal membrane Ca^{2+}-ATPase[22,26], served to evaluate Ca^{2+}-ATPase transport activity and spontaneous Ca^{2+} leak of sarcoplasmic reticulum. No correlation of blood pressure with both Ca^{2+} uptake and Ca^{2+} leak and a weak but insignificant correlation of Ca^{2+} uptake with heart rate were found. Ca^{2+} uptake and Ca^{2+} leak did not significantly differ between hypertensive patients and a normotensive control group.

An earlier study has documented a substantial increase of platelet calmodulin-insensitive Ca^{2+}-ATPase activity in hypertension[16] indicating a stimulation of internal membrane Ca^{2+}-ATPase in hypertension. The discrepancy to our results may result from the different selection of hypertensive patients, the use of thapsigargin in the present study as opposed to calmodulin activation, and the determination of Ca^{2+} transport activities in permeabilized cells in the present experiments rather than Ca^{2+}-dependent ATPase activity in membrane preparations used in the study of Resink et al.[16] Takaya et al[17] found a decrease in maximal velocity and Michaelis constant of Ca^{2+}-ATPase in platelets from hypertensive patients. Since plasma membrane and internal membrane Ca^{2+}-ATPase activity was not discriminated, the respective contributions of the two Ca^{2+} pumps could not be evaluated in that study. In agreement with our results, no correlation of platelet sarcoplasmic reticulum Ca^{2+}-ATPase with blood pressure was documented, but a decrease of platelet plasma membrane Ca^{2+}-ATPase activity in hypertension was found recently.[26] Consistent with the decrease in calmodulin-sensitive Ca^{2+}-ATPase activity in hypertension,[16] these studies indicate that removal of Ca^{2+} across the plasma membrane is responsible for the well established rise of cytosolic Ca^{2+} in hypertension.[1,5]

Possible other mechanisms accounting for altered cytosolic Ca^{2+} in hypertension include increased agonist-induced Ca^{2+} influx,[10] activation of voltage-operated Ca^{2+} channels,[1] altered inositol 1,4,5-trisphosphate-induced Ca^{2+} efflux from internal Ca^{2+} stores,[26] and a decreased activity of Na^+/Ca^{2+} exchangers present in the platelet plasma membrane.[15,27] Whereas the density of voltage-gated Ca^{2+} channels in platelets is very low and plays only a minor role in the Ca^{2+} balance of platelets,[11] enhanced agonist-induced Ca^{2+} influx in platelets has been documented in patients with ischemic heart disease.[28] A

TABLE 3. COMPARISON OF Ca^{2+} UPTAKE AND LEAK BETWEEN HYPERTENSIVE PATIENTS AND NORMOTENSIVE SUBJECTS

Group	Normotensive (n = 4)	Hypertensive (n = 10)	Hypertensive* (n = 15)
SBP (mm Hg)	116 ± 8	137 ± 8	146 ± 14
DBP (mm Hg)	75 ± 6	90 ± 6	95 ± 8
Ca^{2+} uptake (nmol/L/sec)	8.02 ± 2.59	8.86 ± 2.55	9.85 ± 2.95
Ca^{2+} leak (nmol/L/sec)	2.89 ± 0.65	3.65 ± 1.24	3.38 ± 1.54

Normotensive defined as SBP (systolic blood pressure) ≤120 mm Hg, DBP (diastolic blood pressure) ≤80 mm Hg.

**This group comprises the 15 hypertensive patients undergoing 24-h blood pressure determination (Table 1), for which Ca^{2+} uptake and Ca^{2+} leak values were determined (see Results).*

decrease of platelet Na^+/K^+-ATPase activity in mild gestational hypertension has been suggested to contribute to increased cytosolic Ca^{2+} through the Na^+/Ca^{2+} exchanger.[29] However, Takaya et al[17] did not find altered platelet Na^+/K^+-ATPase activity in hypertension. Therefore, a decreased activity of the plasma membrane Ca^{2+}-ATPase and increased agonist-induced Ca^{2+} influx are likely to contribute to the increase in plateiet cytosolic Ca^{2+} in hypertension, whereas ATP-driven Ca^{2+} uptake into internal Ca^{2+} stores remains rather unchanged.

In the present study a weak relationship between Ca^{2+} uptake into internal membranes and heart rate was noted (Figure 3). Clearly, more patients and a wider range of heart rates will be needed to decide if this correlation is significant. A link between heart rate, which is controlled by the sympathetic and parasympathetic nervous system,[30,31] and Ca^{2+} dynamics of the platelet sarcoplasmic reticulum could be β-adrenergic receptors present in the heart and on platelets.[32,33] Whereas hypertension is usually associated with an increase in sympathetic activity,[34] its role in the Ca^{2+} balance in platelets remains obscure, although β-adrenergic receptors may affect Ca^{2+} pumping activity via phospholamban phosphorylation.[35]

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