Increased Basal and Thrombin-Induced Free Calcium in Platelets of Essential Hypertensive Patients

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SUMMARY Intracellular free calcium, \([\text{Ca}^{2+}]_i\), was studied in platelets of essential hypertensive subjects and normotensive controls under basal conditions and after stimulation with epinephrine, norepinephrine, angiotensin II, ouabain, and thrombin, using the fluorescent calcium indicator quin 2. Basal \([\text{Ca}^{2+}]_i\) was significantly higher in hypertensive subjects (n = 32) than in normotensive controls (n = 30; 167.4 ± 5.0 vs 143.2 ± 3.1 nmol/L; p < 0.001). Epinephrine, norepinephrine, angiotensin II, and ouabain had no effect on platelet calcium, whereas thrombin induced a dose-dependent increase in \([\text{Ca}^{2+}]_i\) in both the presence and absence of extracellular calcium. This \([\text{Ca}^{2+}]_i\) increase in the presence of extracellular calcium, which depends mainly on calcium influx, was significantly higher (p < 0.05) in platelets of hypertensive subjects at all thrombin concentrations (ranging from 0.025-0.1 U/ml), while the \([\text{Ca}^{2+}]_i\) increase in the absence of extracellular calcium, which depends only on release from intracellular stores, was similar in hypertensive subjects and controls. These results suggest that, in essential hypertension, there is not only increased platelet resting \([\text{Ca}^{2+}]_i\), but also an increase in agonist-mediated calcium influx, which appears to indicate a cell membrane abnormality in the platelets of subjects with essential hypertension. (Hypertension 9: 230-235, 1987)

KEY WORDS • essential hypertension • intracellular calcium • thrombin • platelets • quin 2 • calcium influx • epinephrine • norepinephrine • ouabain • angiotensin II

The importance of calcium ions in the pathogenesis of arterial hypertension has long been recognized (for a review, see Reference 1). Increased free cytosolic calcium concentration, \([\text{Ca}^{2+}]_i\), is related to an increase in active tension of smooth muscle cells and is therefore responsible for an increase in arteriolar resistance. \(^2\) \([\text{Ca}^{2+}]_i\) is also important for the functional state of other cells involved in blood pressure regulation (e.g., aldosterone-secreting adrenal cells, renin-secreting juxtaglomerular cells, and adrenergic terminations). \(^4\) For these reasons \([\text{Ca}^{2+}]_i\), conceivably may play a pivotal role in blood pressure regulation and may be altered in functional derangements responsible for the development of arterial hypertension.

Some attempts have been made to measure free calcium directly within the cell by exploiting ion-sensitive microelectrodes. \(^5\) \(^6\) but substantial progress has been made only recently using a new technique based on trapping the fluorescent \([\text{Ca}^{2+}]_i\), indicator quin 2. \(^7\) \(^8\) This method possesses the substantial advantage of operating on undisrupted cells.

To date, \([\text{Ca}^{2+}]_i\), has been measured by the quin 2 technique in platelets \(^9\) \(^10\) and in lymphomonocytes and neutrophils \(^11\) \(^12\) of normotensive and hypertensive humans or rats. Although white blood cell studies have
yielded contradictory results, Erne et al. and Bruschi et al., found significant increases in platelet basal $[Ca^{2+}]$, in hypertensive patients. In the present study, we attempted to measure platelet $[Ca^{2+}]$, in hypertensive patients under basal conditions and after stimulation induced by the agonists epinephrine, norepinephrine, angiotensin II, and thrombin.

**Materials and Methods**

**Subjects**

Sixty-two subjects were studied under basal conditions: 32 subjects with uncomplicated essential hypertension and 30 normotensive control subjects matched for age and sex. Platelet stimulation studies were performed in 22 subjects from the total study population, namely, 11 hypertensive subjects and 11 controls.

The 32 hypertensive subjects were selected on the basis of a family history of hypertension and an absence of any form of secondary hypertension and any form of metabolic or renal disease. They consisted of 20 men and 12 women, ranging in age from 25 to 63 years (mean age, 44 ± 9 [SD] years), with systolic blood pressure of 155 mm Hg or higher and diastolic blood pressure of 105 mm Hg or higher (World Health Organization class I-II).

Most hypertensive subjects were previously untreated, and the remainder had discontinued any other form of drug therapy at least 2 weeks before the study began. Particular care was taken to ensure that those taking antiinflammatory and antiaggregating drugs were excluded. All subjects were on a normal, unrestricted diet. Blood samples for platelet studies and standard laboratory tests (creatinine, urea nitrogen, calcium, phosphate, electrolytes, plasma aldosterone, plasma renin activity) were obtained from subjects at 0800 after overnight fasting and recumbency.

$[Ca^{2+}]$, Measurement

Forty milliliters of blood was drawn by venous puncture with acid citrate dextrose (14 mM sodium citrate, 11.8 mM citric acid, and 18 mM dextrose), and platelet-rich plasma was prepared according to Hallam et al. Then heparin, 25 U/ml, was added to the platelet-rich plasma to prevent platelet activation.

Platelets were loaded by incubating the platelet-rich plasma for 30 minutes at 37°C with 15 mM quin 2 ace toxymethyl ester (quin 2 AM; Calbiochem Behring Diagnostics, La Jolla, CA, USA). After loading, the cells were centrifuged at 700 g for 20 minutes at room temperature and resuspended in a physiological saline solution containing 145 mM NaCl; 5 mM KCl; 10 mM 4-(2-hydroxyethyl)-1-piperazine-ethane sulfonic acid (HEPES; Boehringer Mannheim GmbH, West Germany); 0.5 mM NaHPO$_4$; 1 mM MgSO$_4$; 6 mM glucose; pH 7.4 at 37°C. The cells were counted in a Coulter counter (Model Fn; Coulter Electronics, Dunstable, Bedfordshire, England) and resuspended at 10$^8$ cells/ml. Platelets were exposed to calcium-free buffer for a short time (about 15 minutes). The external Ca$^{2+}$ concentration was then adjusted by adding 1 mM CaCl$_2$ for 30 minutes, a time sufficient to replenish intracellular stores, or 5 mM Na$_2$H$_2$EGTA (Sigma Chemical, St. Louis, MO, USA), as required by different experiments. After a short equilibration time (5 minutes), fluorescence was measured with a Perkin Elmer LS55 spectrofluorometer (Norwalk, CT, USA) in a thermostatted (37°C) quartz cuvette at a 339-nm excitation wavelength and a 492-nm emission wavelength.

The calibration of intracellular fluorescence as a function of $[Ca^{2+}]$, was performed essentially according to the method described by Tsien et al. In short, the intracellular calcium concentration was calculated by means of the equation $[Ca^{2+}]=115(F - F_{0} / F_{\text{max}} - F)$, where 115 represents the dissociation constant (in mM), $F$ is the fluorescence of the intact cell suspension, and $F_{\text{max}}$ is measured after cell disruption with Triton X 100 (Merck, Darmstadt, West Germany), final concentration 0.1%, by addition of 5 mM EGTA and adjustment of the pH to about 8.5 to make the effective $K_d$ for Ca EGTA 10$^{-9}$. $F_{\text{max}}$ can be obtained by exposing the quin 2 to a high Ca$^{2+}$ concentration (10 mmol/L) in order to saturate the indicator. Intracellular quin 2 was determined by comparing the fluorescence of a standard solution of quin 2 free acid in the presence of unloaded cells. In all experiments, samples from normotensive and hypertensive subjects were assayed in parallel. The intraassay variability was about 8%, and the day-to-day intrasubject variation was about 12%.

**Effects of Platelet Stimulation on $[Ca^{2+}]$**

$[Ca^{2+}]$, was measured under basal conditions in 32 hypertensive patients and in 30 control subjects. In six normal subjects and six hypertensive subjects, we measured the platelet $[Ca^{2+}]$, response to epinephrine (Sigma), norepinephrine (Sigma), and angiotensin II (Sigma) over a concentration range of 10$^{-5}$ to 10$^{-9}$ mol/L. In 11 hypertensive subjects and in 11 controls, platelet $[Ca^{2+}]$, variations were measured in response to increasing concentrations (0.025, 0.05, 0.075, and 0.1 U/ml) of thrombin (Sigma) in the presence and absence of extracellular calcium. In each experiment, we considered only pairs of controls and hypertensive subjects who had reached approximately the same intracellular quin 2 concentration. In some experiments, a 30-minute incubation of platelets with 100 μM ouabain (Sigma) also was performed to assay the effect of the inhibition of Na$^+$, K$^+$-adenosine triphosphatase (ATPase) on platelet intracellular calcium. In parallel experiments, platelet shape change and aggregation in response to thrombin were monitored by a standard turbidimetric technique to assess the function of loaded platelets. In six hypertensive subjects and their matched controls, no significant differences in platelet shape change and aggregation were observed (data not shown).

All data are expressed as mean values ± SEM. The results were analyzed using Student's two-tailed $t$ test for grouped comparisons. Differences with a $p$ level less than 0.05 were regarded as significant.
Results

Platelet [Ca$^{2+}$], measurements during basal conditions (Figure 1) were significantly higher in hypertensive subjects than in control subjects (167 ± 5.0 vs 143.2 ± 3.1 nmol/L). No correlation was found between [Ca$^{2+}$], and systolic or diastolic blood pressure, nor between [Ca$^{2+}$], and plasma renin activity or plasma aldosterone.

Epinephrine, norepinephrine, and angiotensin II, assayed over the concentration range of 10$^{-8}$ to 10$^{-5}$ mol/L, had no effect on resting [Ca$^{2+}$], in either normal or hypertensive subjects, whereas stimulation with thrombin in the presence of extracellular calcium over the range of 0.05 to 0.1 U/ml resulted in a concentration-dependent increase in [Ca$^{2+}$], over basal levels (Figure 2) in both hypertensive subjects and normal subjects. In the presence of extracellular Ca$^{2+}$ (1 mmol/L), thrombin induces a marked, rapid increase in [Ca$^{2+}$]. This increase can reach levels of 1 μmol/L or more at higher thrombin concentrations and may be accounted for by calcium influx and by calcium release from intracellular stores. When the agonist is added in the presence of 5 mM EGTA in order to reduce extracellular calcium to negligible concentrations (approximately 10-20 nmol/L) and exclude Ca$^{2+}$ influx, the [Ca$^{2+}$], increase is much less, reaching levels of 200 to 400 nmol/L at the higher thrombin concentrations. Consequently, the [Ca$^{2+}$], increase clearly was due mainly to triggered influx and only to a lesser extent to release from intracellular stores.

In 11 hypertensive subjects and 11 control subjects, we measured the thrombin-induced platelet [Ca$^{2+}$], increase using the agonist in the 0.025 to 0.1 U/ml range. The [Ca$^{2+}$], increase was significantly higher in the hypertensive subjects than in the control subjects at all the concentrations tested (Figure 3). The response to the maximal thrombin concentration (0.1 U/ml), though consistently higher in the hypertensive subjects than in the controls, is not shown because the increase in [Ca$^{2+}$], was above 1 μmol/L, which is beyond the optimal sensitivity range (approximately 10–1000 nmol/L) of quin 2.

In the absence of extracellular calcium (see Figure 3), release from intracellular stores induced by thrombin at concentrations ranging from 0.05 to 0.5 U/ml was similar in hypertensive subjects and in control subjects. The intracellular quin 2 concentration was about the same (1.5-1.6 mmol/L) in the platelets of hypertensive subjects and control subjects in each paired experiment. The time required to return to baseline values after the transient [Ca$^{2+}$], peak was no different in hypertensive subjects from that observed in controls.

Finally, the 30-minute incubation of normal platelets with 100 μM ouabain had no effect either on resting [Ca$^{2+}$], or on the thrombin-stimulated increase in [Ca$^{2+}$].

Discussion

We measured platelet [Ca$^{2+}$], in a group of hypertensive patients, under basal conditions as well as after stimulation, using the agonists epinephrine, nor-
platelet 

epinephrine, angiotensin II, and thrombin. Platelet 

[Ca$^{2+}$], values, as measured during basal conditions, showed a mean increase of 15% in essential hypertensive subjects as compared with normotensive controls. Despite the fairly substantial overlap, the difference was statistically significant ($p<0.001$). These results are in agreement with those obtained by Erne et al.$^9$ and Bruschi et al.$^{10}$ Our findings differed from those of Erne et al.$^9$ in that we were unable to detect any statistically significant correlation between platelet [Ca$^{2+}$], and arterial blood pressure. We do not believe, however, that failure to observe such a correlation detracts from the pathophysiological importance of the observed increase. In effect, blood pressure depends not only on arteriolar resistances but also on many other variables.

Various Ca$^{2+}$ handling abnormalities that may in some way account for an increase in [Ca$^{2+}$], have been described in various types of cells in experimental hypertension in rats as well as in human hypertension. The first of these is defective calcium binding to the inner cell membrane,$^{14,17}$ which may induce activation of potential-operated channels and an increase in calcium influx into the cell. Moreover, decreased calcium binding to vascular smooth muscle membranes has been associated with reduced membrane stabilization capacity, thus leading to an increase in membrane permeability to calcium and other ions.$^{15-17}$ Another abnormality described both in spontaneously hypertensive rats$^{18}$ and in the erythrocytes of patients with essential hypertension$^{19}$ consists in a reduced adenosine 5'-triphosphate (ATP)-dependent accumulation of calcium in cell membrane vesicles, indicating the existence of a reduced calmodulin-Ca$^{2+}$-ATPase interaction. This abnormality leads to a reduction in calcium extrusion across the cell membranes. Lastly, an additional mechanism that has been suggested to account for the increase in [Ca$^{2+}$], is that involving a hypothetical natriuretic factor, which is thought to act through the inhibition of Na$^+$,K$^+$-ATPase.$^{20,21}$

Our results show that norepinephrine and epinephrine, assayed over a broad range of concentrations, caused no appreciable increase in platelet [Ca$^{2+}$]. These findings are in agreement with recent data$^2$ showing that the addition of epinephrine alone to washed platelets has no effect on [Ca$^{2+}$].

Angiotensin II, again over a broad range of concentrations ($10^{-8}$ to $10^{-5}$ mol/L), had no effect on platelet [Ca$^{2+}$]. We find this phenomenon difficult to interpret, as the platelet membrane is known to possess receptor sites with a high affinity for angiotensin II.$^{23}$ Lastly, inhibition of Na$^+$,K$^+$-ATPase with ouabain produced no changes either in basal calcium values or in the thrombin-induced [Ca$^{2+}$], increase. Apparently, then, as other authors have already pointed out,$^{24}$ we can rule out the possibility that the Na$^+$-Ca$^{2+}$ exchange mechanism demonstrated by Daniel et al.$^{23}$ in muscle is operative in platelets.

The observation of an increase in resting [Ca$^{2+}$], in platelets incubated in a medium with physiological concentrations of extracellular calcium might suggest the existence of a membrane abnormality that facilitates calcium leakage into unstimulated platelets. This possibility has been considered by Bruschi et al.$^{27}$ who used a different methodological approach to study calcium influx into lymphocytes. These authors measured calcium influx in conditions of calcium pump inhibition both by vanadate and by ATP depletion by measuring quin 2 fluorescence. They were thus able to show that calcium influx into lymphocytes is ATP-dependent and is accelerated in the SHR. The increase in [Ca$^{2+}$], might also be related to the reduction in membrane binding sites observed by Postnov et al.$^{28}$ and Devynck et al.$^{14}$

With a view to clarifying the mechanism involved in
the increase in basal platelet \( [Ca^{2+}] \), we studied platelet activation with increasing doses of thrombin, a specific platelet agonist, in the absence and in the presence of extracellular calcium in both normotensive and hypertensive subjects. It should be stressed that the measurement of \( [Ca^{2+}] \), is not entirely independent of intracellular quin 2 concentrations, in that the dye increases the platelet calcium-buffering capacity, and thus the changes in \( [Ca^{2+}] \), induced by physiological stimuli may be attenuated. For this reason, we took particular care to consider only pairs of control subjects and hypertensive subjects with virtually identical loads of intracellular quin 2. We also attempted to study the two components of the thrombin-induced \( [Ca^{2+}] \), increase (i.e., influx and release from intracellular stores). In the presence of extracellular calcium, the increase in \( [Ca^{2+}] \), was mainly attributable to influx of the cation; in the presence of negligible amounts of extracellular calcium, only the intracellular mobilization component was responsible for the increase in \( [Ca^{2+}] \). Our data show that, whereas the release from intracellular stores was the same in the two groups of subjects, receptor-operated calcium influx was increased in hypertensive patients.

Thrombin has been shown to cause an increased influx of \(^{45}\text{Ca} \) into platelets, raising the permeability of the cell membrane. The thrombin-induced influx would not appear to be inhibited by verapamil at concentrations ranging from \( 10^{-8} \) to \( 10^{-5} \) mol/L, with the result that the existence of voltage-dependent calcium channels in platelets seems doubtful. Thus, the increased response of platelets to thrombin in hypertensive subjects could involve a receptor-operated mechanism leading to an increased transport of calcium across the cell membrane.

If the calcium extrusion mechanism were impaired in the platelets of hypertensive subjects, we would also expect to find, as compared with control subjects, a greater increase in \( [Ca^{2+}] \), dependent not only upon influx but also upon calcium release from intracellular stores. The return of the fluorescence toward basal values after the peak cannot easily be studied with quin 2 in thrombin-stimulated platelets in the presence of extracellular calcium because the \( Ca^{2+} \) concentration reached causes platelet aggregation and quenching of the fluorescence. In several cases, a more rapid return to basal values is observed in platelets in which a higher \( [Ca^{2+}] \), level is attained. We believe that this phenomenon is due to the greater \( [Ca^{2+}] \), concentrations reached, as the activity of the extrusion mechanism is known to be finely regulated by the \( [Ca^{2+}] \). Moreover, in the EGTA experiments, neither the \( [Ca^{2+}] \), peak nor the return to baseline values showed any very great differences in hypertensive subjects as compared with control subjects; thus, in this instance, extrusion mechanisms would not appear to be involved.

Although the possibility that an extrusion mechanism might be involved cannot be ruled out on the basis of our results, the increase in thrombin-stimulated calcium influx in platelets of subjects with essential hypertension seems more likely to be due to increased calcium transport across the cell membrane, according to Postnov et al., who demonstrated increased \(^{45}\text{Ca} \) uptake kinetics in platelets of SHR.

Platelets are regarded as a suitable model for the in vitro study of some aspects of vascular smooth muscle function. There are, in fact, many analogies between platelets and vascular smooth muscle cells. Both cell types contain a calcium-dependent contractile system and possess an adenylate cyclase-linked \( \alpha_2 \)-adrenergic-receptor system. Moreover, from a technical point of view, platelets represent an easily available model for in vitro studies.

However, the results of in vitro platelet studies can hardly be extrapolated in vivo to explain what may be happening in smooth muscle cells. What is more, platelets probably are not a good model for the study of changes induced by vasoactive agents capable of producing an increase in \( [Ca^{2+}] \), in smooth muscle cells.

Our data do not rule out the possibility that the abnormality detected could have been acquired in vivo by previous exposure to some unknown humoral factor(s). Nevertheless, this confirmation of an increased cytosolic calcium concentration and the demonstration of an increased calcium influx, when considered along with similar results obtained using different methodological approaches, focus attention on an extensive cell membrane abnormality (intrinsic or acquired) in arterial hypertension, that expresses itself in the form of impaired homeostasis of intracellular calcium.

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PLATELET INTRACELLULAR CALCIUM IN HYPERTENSION/Lecchi et al.

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