Abnormal Calcium Handling By Platelets of Spontaneously Hypertensive Rats

Tetsuya Oshima, Eric W. Young, Richard D. Bukoski, and David A. McCarron

There is controversy as to whether platelet intracellular free calcium ([Ca\textsuperscript{2+}]\textsubscript{i}) is increased in spontaneously hypertensive rats (SHR) as compared with Wistar-Kyoto (WKY) rats. Discrepant results may be due to methodological problems including platelet activation during the collection process and leakage of intracellular dye used for [Ca\textsuperscript{2+}]\textsubscript{i} measurement. To provide further insight into this problem, [Ca\textsuperscript{2+}]\textsubscript{i} was estimated in fura-2–loaded platelets isolated from eight SHR and seven WKY rats at 12–14 weeks of age by using a two-syringe blood collection method and a correction method for fura-2 leakage. Basal [Ca\textsuperscript{2+}]\textsubscript{i} was higher in SHR than in WKY rats (61.6±5.6 vs. 54.0±3.9 nM, p<0.02). However, the difference disappeared when a correction for fura-2 leakage was not used (109.7±18.4 vs. 94.9±9.2 nM, p>0.1). Thus, differences in [Ca\textsuperscript{2+}]\textsubscript{i} between SHR and WKY rats may be obscured if dye leakage from platelets is not taken into account. Thrombin (0.1 units/ml) induced a rise in [Ca\textsuperscript{2+}]\textsubscript{i}, that was greater in SHR than WKY rats, both in the presence (491.4±31.6 vs. 377.5±21.7 nM, p<0.002) and absence (264.9±33.6 vs. 228.2±30.1 nM, p<0.05) of calcium in the media. These results indicate that thrombin-stimulated calcium influx as well as discharge of calcium from intracellular stores is increased in SHR platelets. Thus, under both basal and stimulated conditions, platelet calcium handling is abnormal in the SHR. (Hypertension 1990;15:606–611)

In the last decade, much attention has been given to the hypothesis that cellular calcium metabolism is abnormal in various forms of hypertension.\textsuperscript{1,2} Among the postulated defects are enhanced calcium influx across the cell membrane and reduced extrusion or sequestration of cytoplasmic calcium in association with elevated intracellular calcium ([Ca\textsuperscript{2+}]\textsubscript{i}). Because of the relative availability of platelets and similarity of calcium-dependent contractile processes shared by platelets and vascular smooth muscle cells,\textsuperscript{3} platelets have been used for analysis of the intracellular calcium metabolism.\textsuperscript{4} Since the introduction of calcium-sensitive fluorescent dyes such as quin 2, fura-2, and indo-1 to measure [Ca\textsuperscript{2+}]\textsubscript{i},\textsuperscript{5} there have been several reports on the resting level of [Ca\textsuperscript{2+}]\textsubscript{i} in platelets from patients with essential hypertension\textsuperscript{6–8} and from spontaneously hypertensive rats (SHR)\textsuperscript{9–14} in comparison with their controls. For SHR, there is disagreement as to whether platelet [Ca\textsuperscript{2+}]\textsubscript{i} is increased. Some investigators have found increased [Ca\textsuperscript{2+}]\textsubscript{i} in SHR,\textsuperscript{9–11} whereas others have found no difference between SHR and normotensive Wistar-Kyoto (WKY) rats.\textsuperscript{12–14} In the stroke-prone SHR (SHRSP), decreased [Ca\textsuperscript{2+}]\textsubscript{i} was reported.\textsuperscript{15} We hypothesized that methodological problems such as the leakage of fluorescent dye from cells, calcium buffering capacity of the dye, and inadvertent activation of platelets during isolation from blood, could result in inaccurate [Ca\textsuperscript{2+}]\textsubscript{i} estimates. In the present study, our first objective was to examine whether [Ca\textsuperscript{2+}]\textsubscript{i} is increased in platelets from SHR when these methodological issues were addressed.

In the study of calcium handling in association with the pathophysiology of hypertension, the evaluation of [Ca\textsuperscript{2+}]\textsubscript{i} should be performed in stimulated cells involved in cell function rather than in the resting cells. Although the basal [Ca\textsuperscript{2+}]\textsubscript{i} level in platelets of SHR or SHRSP has been reported by several groups,\textsuperscript{9–15} the data on [Ca\textsuperscript{2+}]\textsubscript{i}, response to agonist is limited to two reports using quin 2.\textsuperscript{12,15} Quin 2 has several limitations including inhibition of the [Ca\textsuperscript{2+}]\textsubscript{i}, response to agonists. These problems are partly overcome by fura-2.\textsuperscript{16–18} Therefore, we have determined platelet [Ca\textsuperscript{2+}]\textsubscript{i}, with fura-2 in unstimulated conditions and in response to thrombin.

Methods

Male SHR and WKY rats were obtained from Charles River Breeding Laboratory (Wilmington,
weeks of age, and systolic blood pressure was determined by tail-cuff method to assure hypertension.

After light anesthesia was induced with ether, the abdominal cavity was opened and the aorta was cannulated with PE-90 tubing attached to a 21-gauge needle. We did not use cardiac puncture to collect blood because both platelets and clotting factors have been reported to be activated by this technique.\(^\text{19}\) In fact, we found clots in some samples drawn by cardiac puncture in a preliminary study. After drawing 0.5 ml blood into a 1 ml syringe, a second syringe containing 1 ml 3.8\% trisodium citrate was connected to the needle and 9 ml blood was slowly and steadily collected (two-syringe technique).\(^\text{19}\) None of our samples exhibited signs of red blood cell hemolysis or fibrin formation by visual inspection. Platelet rich plasma was then prepared by centrifugation at 300g for 8 minutes at room temperature. The centrifuge was allowed to coast to a stop without braking. Platelets were separated from plasma by gel filtration using a Sepharose 2B-CL (Pharmacia LKB Biotechnology Inc., Piscataway, New Jersey) column that had been equilibrated with an elution medium containing (mM) NaCl 145, KCl 5, MgSO\(_4\) 1, Na\(_2\)HPO\(_4\) 0.5, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) 10, and glucose 5 (pH 7.4). Washed platelets were incubated with 2 \mu M fura-2/acetoxymethylester (AM) (Molecular Probes, Eugene, Oregon) diluted from a 2 mM stock solution in dimethyl sulfoxide for 30 minutes at a platelet concentration of 5\times10\(^8\) cells/ml. After the cells were again gel-filtered to remove extracellular fura-2/AM, platelet counts were adjusted to 1\times10\(^8\) cells/ml, and CaCl\(_2\) was added to the cell suspension at a concentration of 1 mM.

For fluorescence measurements, 3 ml aliquots of platelet suspension were continually stirred by a magnetic stir bar in a quartz cuvette maintained at 37°C in a dual excitation wavelength fluorometer (CMZ system, SPEX Inds. Inc., Edison, New Jersey). The cells were alternately excited with ultraviolet light at 340 and 380 nm (16 Hz), and emission at 510 nm was detected with a photomultiplier tube connected to the SPEX DM1B data collection system. After fluorescence was recorded in the basal state, the [Ca\(^{2+}\)]\(_{\text{platelet}}\) response to 0.1 units/ml thrombin (Sigma Chemical Co., St. Louis, Missouri) was evaluated both in the presence of 1 mM calcium and in nominally calcium-free buffer (<10 nM) prepared by the addition of 10 mM ethylene glycol bis(\(\beta\)-aminoethyll ether) N,N',N'-tetraacetic acid (EGTA) at pH 7.4. These measurements were performed in duplicate. To minimize any time-dependent effect on platelet responsiveness and leakage of fura-2, platelets were kept at room temperature for no longer than 30 minutes, under which conditions platelet [Ca\(^{2+}\)]\(_{\text{platelet}}\) was stable. [Ca\(^{2+}\)]\(_{\text{platelet}}\) was estimated by using the equation\(^\text{16,18}\):

\[
[\text{Ca}^{2+}]_{\text{platelet}} = K_D \times (R - R_{\text{min}} - R_{\text{max}} - R) \times \beta
\]

where \(K_D\) represents the dissociation constant of fura-2 for calcium (224 nM), \(R\) is the ratio of fluorescence at excitation wavelengths 340 and 380 nm in intact cell suspension, \(R_{\text{max}}\) and \(R_{\text{min}}\) are the ratio of fluorescence at 340 and 380 nm under calcium-saturated and calcium-free conditions, respectively, and \(\beta\) is the quotient of 380 nm fluorescence of calcium-free and bound dye form. \(R_{\text{max}}\) was determined by lysing the cell with 50 \mu M digitonin in the presence of 1 mM calcium. \(R_{\text{min}}\) was then determined by the successive addition of 10 mM EGTA followed by adjustment of pH to 8.3 with 30 mM Tris. All values were corrected for autofluorescence by subtraction of fluorescence of unloaded platelets and test agents.

Correction for extracellular fura-2 leaked from platelets was carried out as originally described by Pollock and Rink\(^\text{18}\) with the following modification. EGTA (10 mM) was added to intact cell suspension at pH 7.4. Rapid initial drop (within 10 seconds) in the fluorescence signal at 340 nm after EGTA addition was considered to reflect the contribution by the extracellular dye as extracellular calcium was chelated. After lysis of the cells to estimate the total fura-2-associated fluorescence, the ratio of fluorescence change after EGTA at pH 7.4 in intact cell suspension to that in the total dye was regarded as the percentage of dye leaked from the cell. The fluorescent signal of external dye was then subtracted from the original signal. Similar estimates of dye leakage were obtained by this method (EGTA) and centrifugation (1,000g for 7 minutes) in which we calculated the ratio of supernatant (leaked) fura-2 fluorescence to the total fluorescence (data not shown). The time course of fura-2 leakage and [Ca\(^{2+}\)]\(_{\text{platelet}}\) at 37°C was examined in a separate group of six SHR and six WKY rats.

In a preliminary study, we examined the necessity of maintaining a temperature of 37°C after incubation for de-esterification of fura-2/AM. The extent of dye ester hydrolysis was evaluated by the range between \(R_{\text{max}}\) and \(R_{\text{min}}\). Under our experimental conditions, 10 minutes of incubation at 37°C increased these values; however, no further change was observed after 10 minutes. Therefore, each sample was warmed at 37°C for 10 minutes before fluorescence recording. Estimation of intracellular fura-2 concentration was made by the comparison of fluorescence signal at 340 nm in the presence of 1 mM calcium after cell lysis to that of known concentrations of fura-2.

All data are presented as mean±SD unless otherwise noted. Statistical comparisons were performed using the Mann-Whitney \(U\) test. Results were similar with the use of the unpaired Student's \(t\) test. Correlations between variables were tested by linear regression analysis. Statistical significance was defined as a \(p\) value of less than 0.05.

**Results**

Systolic blood pressure measured by tail-cuff method was higher in SHR than WKY rats (157±6
vs. 106±1 mm Hg, p<0.002). Body weight was lower in SHR than WKY rats (271±21 vs. 303±35 g, p<0.05). No difference was detected in platelet intracellular fura-2 concentration (105±22 vs. 113±13 μM), R_max (11.8±3.0 vs. 11.3±3.2), or R_min (0.74±0.08 vs. 0.74±0.06), indicating that platelets were loaded with the dye to a similar extent.

The time course of changes in fura-2 leakage and [Ca^{2+}]i at 37° C are shown in Figure 1. Extracellular leakage of fura-2 increased with time in both strains in a sigmoidal fashion. About half of the total dye leaked into the extracellular space after 20 minutes. There was no difference in fura-2 leakage between SHR and WKY rats. The corrected [Ca^{2+}]i was stable between 4 and 13 minutes but rose thereafter. This late increase in [Ca^{2+}]i may reflect gradual activation of platelets in the presence of external calcium at 37° C. Thus, reproducible measurements of [Ca^{2+}]i can be obtained up to at least 13 minutes when a correction is made for fura-2 leakage.

Figure 2 shows the resting level of [Ca^{2+}]i in platelets from SHR and WKY rats before and after correction for extracellular fura-2. Although the difference between the groups was not statistically significant without correction (109.7±18.4 vs. 94.9±9.2 nM, p>0.1), corrected basal [Ca^{2+}]i was significantly higher in SHR than WKY rats (61.6±5.6 vs. 54.0±3.9 nM, p<0.02). As before (Figure 1), fura-2 leakage (the ratio of extracellular fura-2 to total fura-2) was not different between SHR and WKY rats (17.7±5.2% vs. 14.9±2.0%, p>0.2).

Thrombin (0.1 units/ml) evoked an increase in [Ca^{2+}]i, both in the presence and in the absence of extracellular calcium ([Ca^{2+}]o=1 mM and [Ca^{2+}]o <10 nM, respectively). Maximum [Ca^{2+}]i was observed within 6–9 seconds. Time to reach the peak was similar in both groups (SHR vs. WKY rats: 8.0±0.5 vs. 7.9±0.8 seconds in the presence of calcium; 7.4±1.2 vs. 7.6±0.9 seconds in the absence of calcium. Figure 3 shows peak [Ca^{2+}]i after thrombin in the presence (Figure 3A) and in the absence (Figure 3B) of calcium in buffer when a correction for leaked fura-2 was performed. In the presence of 1 mM external calcium, thrombin-induced [Ca^{2+}]i was significantly higher in SHR compared with WKY rats (491.4±31.6 vs. 377.5±21.7 nM, p<0.002), and there was no overlap in the distribution between groups. In the absence of external calcium, we found a small but significant difference in the peak [Ca^{2+}]i (SHR, 264.9±33.6 nM; WKY rats, 228.2±30.1 nM, p<0.05). Without correction for extracellular dye leakage, peak [Ca^{2+}]i in calcium-supplemented medium was still significantly different (SHR, 734.9±109.8 vs. WKY rats, 592.5±93.1 nM, p<0.02), whereas in calcium-free medium the difference disappeared (188.2±21.2 vs. 168.8±14.0 nM, p=0.08). The difference in thrombin-induced [Ca^{2+}]i, in the presence and absence of extracellular calcium was
Figure 4. Scatterplot showing correlation of basal intracellular calcium concentration ([Ca\(^{2+}\)]\(_i\)) with thrombin-induced [Ca\(^{2+}\)]\(_i\), in calcium-supplemented buffer (□) and calcium-depleted buffer (○) in spontaneously hypertensive rats (solid symbols) and Wistar-Kyoto (WKY) rats (open symbols) combined.

Interpretation as the thrombin-induced calcium influx across the cell membrane and was higher in SHR than in WKY rats (288.1±37.3 vs. 203.3±34.0 nM, p<0.05).

As shown in Figure 4, thrombin-stimulated [Ca\(^{2+}\)]\(_i\), was correlated with the resting level of [Ca\(^{2+}\)]\(_i\), in calcium-supplemented (r=0.684, p=0.005) and calcium-depleted buffer (r=0.633, p<0.02) in the combined groups.

Discussion

It has been hypothesized that a wide variety of tissues and cells share an abnormality of calcium handling in human and experimental hypertension. Because of ease of handling and ready availability, cellular calcium metabolism has frequently been assessed in circulating blood cells. Several investigators have reported values for basal [Ca\(^{2+}\)]\(_i\), in platelets and white blood cells from patients with essential hypertension in SHR in comparison with their respective normotensive controls. It might be suspected that in humans the comparative study of [Ca\(^{2+}\)]\(_i\), in blood cells has a disadvantage in interpretation of data because hypertensive patients have heterogeneity in several factors such as renin status, blood pressure level, age, and salt intake, each of which may influence intracellular cation characteristics and are difficult to control precisely. However, the human data, in fact, demonstrate consistently elevated [Ca\(^{2+}\)]\(_i\), in platelets from hypertensive subjects, whereas the status of platelet [Ca\(^{2+}\)]\(_i\), in SHR is controversial. It is possible that rat platelets may be more susceptible to methodological difficulties that mask the difference between SHR and WKY rats. For example, the effect on cell function of drawing 5-10 ml blood may be greater in small animals than in humans. Furthermore, the leakage of fura-2 from rat platelets seems to be greater in the present study than reported for human platelets.

This study was carried out to test for effects of dye leakage and to minimize platelet activation during blood collection. We found a small but significant increase (14%) in basal [Ca\(^{2+}\)]\(_i\), in SHR. Our experiments highlight the importance of fura-2 leakage, which leads to overestimation of [Ca\(^{2+}\)]\(_i\), in the presence of extracellular calcium using a cell suspension system. When correction for extracellular fura-2 was not performed, basal [Ca\(^{2+}\)]\(_i\), was overestimated by 78% in SHR and by 76% in WKY rats. Our estimate of basal [Ca\(^{2+}\)]\(_i\), in platelets of WKY rats without this correction ranged within the previously reported values (Table 1). The significant difference in basal [Ca\(^{2+}\)]\(_i\), between SHR and WKY rats was no longer present when no correction was made for dye leakage.

Table 1. Basal Intracellular Calcium Concentration in Rat Platelets From the Previous Literature

<table>
<thead>
<tr>
<th>Reference</th>
<th>Dye</th>
<th>Blood collection</th>
<th>Correction for leaked dye</th>
<th>Age (wk)</th>
<th>SHR [Ca(^{2+})](_i), (nM)</th>
<th>WKY [Ca(^{2+})](_i), (nM)</th>
<th>Values are:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bruschi (9)</td>
<td>quin 2</td>
<td>not described</td>
<td>EGTA</td>
<td>4</td>
<td>130±8 (n=8)</td>
<td>124±8 (n=8)</td>
<td>mean±SEM</td>
</tr>
<tr>
<td>Murakawa (14)</td>
<td>quin 2</td>
<td>not described</td>
<td>none</td>
<td>8</td>
<td>146±9 (n=10)</td>
<td>103±6 (n=10)*</td>
<td>mean±SD</td>
</tr>
<tr>
<td>David-Dufilho (13)</td>
<td>quin 2</td>
<td>aorta</td>
<td>none</td>
<td>13</td>
<td>116±28 (n=7)</td>
<td>91±31 (n=7)*</td>
<td>mean±SD</td>
</tr>
<tr>
<td>Zimlichman (12)</td>
<td>quin 2</td>
<td>cardiac puncture</td>
<td>MnCl(_2)</td>
<td>12-14</td>
<td>115±15 (n=92)</td>
<td>116±21 (n=99)</td>
<td>mean±SEM</td>
</tr>
<tr>
<td>Baba (10)</td>
<td>quin 2</td>
<td>cardiac puncture</td>
<td>none</td>
<td>4</td>
<td>135±5 (n=10)</td>
<td>119±3 (n=10)*</td>
<td>mean±SEM</td>
</tr>
<tr>
<td>Vasdev (11)</td>
<td>fura-2</td>
<td>cardiac puncture</td>
<td>none</td>
<td>11</td>
<td>152±7 (n=11)</td>
<td>127±7 (n=12)*</td>
<td>mean±SEM</td>
</tr>
<tr>
<td>Umegaki (15)</td>
<td>quin 2</td>
<td>aorta</td>
<td>none</td>
<td>28</td>
<td>99±3 (n=10)</td>
<td>97±3 (n=12)*</td>
<td>mean±SD</td>
</tr>
<tr>
<td>Current study</td>
<td>fura-2</td>
<td>aorta</td>
<td>none</td>
<td>10</td>
<td>167±38 (n=6)</td>
<td>70±8 (n=6)*</td>
<td>mean±SD</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>EGTA</td>
<td>14</td>
<td>87±4 (n=15)</td>
<td>110±15 (n=16)*</td>
<td>mean±SD</td>
</tr>
</tbody>
</table>

[Ca\(^{2+}\)]\(_i\), intracellular calcium concentration; SHR, spontaneously hypertensive rats; WKY, normotensive Wistar-Kyoto rats.

*Significant difference between SHR and WKY rats.

†Stroke-prone spontaneously hypertensive rats.
age, although the extent of dye leakage tended to be higher in SHR. Failure to correct for dye leakage gives much greater variation in individual data points for each strain, thus accounting for the lack of a statistically significant difference in [Ca\(^{2+}\)] between SHR and WKY rats. We also collected blood from the abdominal aorta using a two-syringe method instead of cardiac puncture to reduce possible stimuli for platelet activation. Our methodological changes may have contributed to lower coefficients of variation (9.1% in SHR and 7.1% in WKY rats) as compared with greater than 10% in previous reports (Table 1).

We also observed that the rise in platelet [Ca\(^{2+}\)], in response to thrombin was enhanced in SHR both in the presence and absence of extracellular calcium. As [Ca\(^{2+}\)], results from the balance among several calcium handling systems, careful interpretation of these data is required. The difference in thrombin-induced [Ca\(^{2+}\)] in the presence and absence of external calcium is considered to be the contribution of calcium influx to [Ca\(^{2+}\)], in thrombin-stimulated platelets. Because this difference was larger in SHR than in WKY rats, we conclude that calcium influx evoked by thrombin is increased in platelets of SHR. To our knowledge, this is the first report indicating enhancement of thrombin-induced calcium influx in platelets from SHR using a fluorescent calcium indicator. In consideration of reports of increased calcium influx in resistance vessels of SHR, our data support the suggestive evidence for similarity of calcium handling between platelets and vascular smooth muscle cells, although other reports show no difference in the resting level of [Ca\(^{2+}\)], in resistance arteries or single vascular smooth muscle cell.

When extracellular calcium is reduced to less than 10 nM (nominally calcium-free) by the addition of EGTA, thrombin induced a rise in [Ca\(^{2+}\)], presumably by discharging internal calcium stores such as the dense tubular system and plasma membrane rather than receptor-operated calcium influx. Because enhanced responsiveness of SHR platelets [Ca\(^{2+}\)], to thrombin was also observed in calcium-free medium, it is possible that thrombin-induced calcium release from intracellular stores may also be increased in SHR.

Our observation that the thrombin-induced rise of [Ca\(^{2+}\)], is increased in SHR was in contrast with the findings of Zimlichman et al. and Umegaki et al. using quin 2. However, it should be emphasized that quin 2 has a considerable limitation for the study of agonist-induced alternation in platelet [Ca\(^{2+}\)], in that quin 2–loaded platelets show a dose-dependent inhibition of stimulus-induced aggregation and secretion. Therefore, the previous failure to find a difference in the peak value to agonists between SHR and SHRSP and WKY rats may be in part due to the high calcium buffering effect of cytosolic quin 2.

The limited information about basal [Ca\(^{2+}\)], cannot explain fully a dysfunction of cellular calcium metabolism in relation to the pathophysiology of hypertension. It is not known how altered basal [Ca\(^{2+}\)], reflects calcium handling under stimulated conditions involved in cell function. As we found platelets of SHR to have a 30% increase in thrombin-induced [Ca\(^{2+}\)], and a 14% increase in basal [Ca\(^{2+}\)], the abnormality in calcium metabolism seems to be more pronounced under activated conditions. However, the finding that basal [Ca\(^{2+}\)], can predict thrombin-activated [Ca\(^{2+}\)], (Figure 4) suggests that the same mechanisms that are responsible for an elevated basal [Ca\(^{2+}\)], in SHR might be involved in the abnormal calcium handling by thrombin-stimulated platelets.

In summary, we have described an improved method for measurement of [Ca\(^{2+}\)], in fura-2–loaded platelets. The results indicate that basal [Ca\(^{2+}\)], is increased in fura-2–loaded platelets from SHR compared with those from WKY rats. In addition, the thrombin-induced rise of [Ca\(^{2+}\)], was significantly higher in SHR than in WKY rats, mainly due to an enhancement of agonist-induced calcium influx in SHR platelets. We suggest that the method described here can be used to examine agonist-induced calcium mobilization in rat platelets. The relation of calcium metabolism in platelets to vascular smooth muscle awaits further investigation.

Acknowledgments

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References


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