Abnormalities of Cytoplasmic Ca\(^{2+}\) in Platelets From Patients With Uremia

By J. Anthony Ware, Barbara A. Clark, Marianne Smith, and Edwin W. Salzman

Uremic patients have a hemorrhagic tendency, often associated with prolonged bleeding times and decreased platelet function in vitro. Whether these defects result from abnormalities in plasma factors affecting platelet activity, platelet surface receptors, intracellular platelet mediators, or other aspects of platelet behavior is unknown. To examine the possibility that the abnormality in platelet function may result from aberrations in Ca\(^{2+}\) homeostasis, blood was obtained from 29 patients with severe uremia. The platelets were washed, loaded with the Ca\(^{2+}\)-sensitive probe indo-1 and aequorin, gel-filtered, and resuspended in either plasma or buffer. Of the 29 patients, seven had template bleeding times prolonged to 11 minutes or more, but platelet aggregation in plasma was not consistently impaired in these patients. However, in aequorin-loaded platelets from the patients with long bleeding times, the highest elevation of cytoplasmic calcium ([Ca\(^{2+}\)]\(_i\)) in response to the Ca\(^{2+}\) ionophore A23187, arachidonate, adenosine diphosphate (ADP), or epinephrine was lower than that seen in platelets from both uremic patients with less prolonged bleeding times and normal volunteers. The reduced [Ca\(^{2+}\)]\(_i\) response was associated with decreased aggregation of gel-filtered platelets suspended in buffer. Suspending washed aequorin-loaded uremic platelets in normal plasma for 20 minutes did not reverse the decreased agonist-induced rise in [Ca\(^{2+}\)]\(_i\); platelets from a normal donor resuspended in uremic plasma aggregated and produced a normal increase in [Ca\(^{2+}\)]\(_i\) in response to agonists. We conclude that the platelet defect seen in some patients with uremia is associated with a decreased rise in platelet [Ca\(^{2+}\)]\(_i\) after stimulation and that this is a manifestation of an intrinsic platelet defect.© 1989 by Grune & Stratton, Inc.

Bleeding is a common and sometimes fatal event in uremic patients. Although the etiology of hemorrhage in these patients is multifactorial, the major cause is platelet dysfunction and impaired interactions between platelets and vessel walls. A direct correlation exists between prolongation of the template bleeding time and clinical bleeding in uremic patients. However, the pathogenesis of the uremic platelet defect is not entirely clear. Many abnormalities have been described, including impaired platelet adhesion, reduced thromboxane A\(_2\) synthesis, possibly due to defective cyclooxygenase, abnormal binding of von Willebrand factor (vWF) to platelet or subendothelial surfaces, a storage pool defect, and decreased platelet aggregation in response to exogenous agonists (although the latter finding has been disputed). Whether these abnormalities reflect the presence of a plasma-borne inhibitor, an abnormality of platelet membranes or receptors, or an intrinsic defect in intracellular regulation of platelet function is unknown.

An abnormality in the concentration of cytoplasmic calcium ([Ca\(^{2+}\)]\(_i\)) in resting or stimulated platelets could explain most of the manifestations of the uremic platelet defect, since many platelet functions require cytoplasmic or extrinsic Ca\(^{2+}\). Previous reports from our laboratory have shown a close correlation between increases in [Ca\(^{2+}\)]\(_i\) as measured by the photoprotein aequorin and the activation of normal platelets. These studies have shown that a rise in [Ca\(^{2+}\)]\(_i\) as indicated by aequorin precedes or coincides with the earliest evidence of platelet activation by all agonists and thus may reflect an aspect of Ca\(^{2+}\) homeostasis different from that shown by fluorescent Ca\(^{2+}\) probes such as quin2 or indo-1, which do not detect a [Ca\(^{2+}\)]\(_i\) rise in response to some agonists, including epinephrine. The purpose of the present study was to measure the [Ca\(^{2+}\)]\(_i\) as indicated by aequorin and indo-1 in resting and stimulated platelets from patients with uremia and to correlate the findings with the template bleeding time and with the aggregation of platelets suspended in plasma or buffer.

METHODS

Blood was obtained from 29 patients with chronic, stable uremia; 13 of the patients were undergoing long-term hemodialysis, and 16 had not undergone dialysis at the time of phlebotomy. The BUN ranged from 48 to 170 mg/dL (mean, 101 ± 32 SD) the serum creatinine ranged from 3.2 to 15.3 mg/dL (mean, 8.9 ± 2.7). Template bleeding times were obtained in 23 patients; in seven the bleeding times were prolonged to more than 11 minutes. No patient was receiving aspirin or any drug known to affect platelet function. Blood was obtained from the patients on long-term hemodialysis just before they were scheduled to undergo that treatment.

Blood was collected into 1:10 volume of 0.150 mmol/L trisodium citrate and centrifuged at 2,200 × g for two minutes at 20°C to obtain platelet-rich plasma (PRP). Aliquots of PRP were stimulated with ADP, epinephrine, the Ca\(^{2+}\) ionophore A23187, or sodium arachidonate. The remaining portion of PRP was loaded with indo-1 as previously described or quin2 and fura-2, and then centrifuged at 430 × g for 15 minutes, washed with EGTA, and loaded with aequorin by a method previously reported by our laboratory. Platelets were suspended in modified Hepes-Tyrode's buffer containing 1 mmol/L Ca\(^{2+}\) and luminescence and aggregation were recorded simultaneously in the same sample with a modified-whole-blood Lumiaggregometer (Chronolog Corp, Havertown, PA). Luminescence signals were calibrated as previously described with use of calibration curve based on Mg\(^{2+}\) determinations in normal platelets. In some experiments, gel-filtered, aequorin-loaded platelets were resuspended in platelet-poor plasma, obtained by centrifuging PRP at 2,000 × g for 20 minutes. Room temperature before stimulation; results in these patients are considered separately from the remainder of the group. Platelets were
loaded with indo-1 as previously described, and were calibrated as described for quin2,15,16 with the KD of 250 nmol/L reported by Grynkiewicz et al.19 The excitation wavelength was 355 nm and the emission wavelength was 405 nm, with maximum and minimum frequency obtained by cell lysis in media containing Ca2+ or EGTA, respectively. MnCl2 was used in one sample for each patient to correct for dye leakage.19,20 All agonists were dissolved in buffer or saline.

The data are presented as mean ± SD and were analyzed by least-squares linear regression. Peak [Ca2+]i in platelets from the group of uremic patients with bleeding times longer than eleven minutes and in those from the uremic patients with less prolonged bleeding times was compared with use of Student's t-test. When patients are grouped according to bleeding time, the patients in whom this parameter was not measured are not included.

RESULTS

The patient groups described in Table 1 were divided into groups according to their bleeding time; a bleeding time of 11 minutes was chosen arbitrarily as the dividing point. There were no significant differences between these groups in age, weight, BUN, creatinine, mean systolic blood pressure taken at the time of venipuncture, platelet count, hematocrit, or use of drugs, including calcium antagonists (data not shown).

The results of the study are summarized in Fig 1 through 4. In general, the increase in [Ca2+]i after the addition of agonists was smaller in the platelets from the patients with uremia than in those from normal donors (Fig 1 is typical). Peak [Ca2+]i was reduced after the addition of receptor-mediated agonists such as ADP and epinephrine, as well as agonists that activate platelets without specific surface receptors, such as the Ca2+ ionophore A23187 and arachidonate. This reduction in [Ca2+]i (as measured by aequorin) in the platelets from uremic patients compared to that obtained in normal platelets was most remarkable in patients with longer bleeding times who tended to have lower peak [Ca2+]i in response to exogenous agonists than did patients with bleeding times of less than 11 minutes. This tendency was evident with all agonists but achieved statistical significance (in comparisons between uremic groups separated according to bleeding time) only in samples treated with Ca2+ ionophore A23187 and ADP (Fig 2). Thus, the patients with a severe uremic platelet defect, as defined by a prolonged bleeding time, had reduced [Ca2+]i mobilization compared with other uremic patients or normal volunteers.

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Table 1. Characteristics of the Uremic Patient Groups

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Patient Group (According to Bleeding Time)</th>
<th>&lt;11 Min</th>
<th>&gt;11 Min</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td>16</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>Age (yr)</td>
<td>63 ± 14</td>
<td>67 ± 12</td>
<td></td>
</tr>
<tr>
<td>Wt (lb)</td>
<td>140 ± 20</td>
<td>149 ± 62</td>
<td></td>
</tr>
<tr>
<td>Long-term dialysis (No. of patients)</td>
<td>7</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>BUN (mg/dL)</td>
<td>92 ± 27</td>
<td>102 ± 37</td>
<td></td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>8.5 ± 2.1</td>
<td>8.3 ± 1.4</td>
<td></td>
</tr>
<tr>
<td>Mean systolic BP (mm Hg)</td>
<td>150 ± 26</td>
<td>144 ± 30</td>
<td></td>
</tr>
<tr>
<td>Platelet count x 10^-3/µL</td>
<td>260 ± 100</td>
<td>210 ± 112</td>
<td></td>
</tr>
<tr>
<td>Hematocrit</td>
<td>27 ± 4</td>
<td>26 ± 1</td>
<td></td>
</tr>
</tbody>
</table>

* In six patients the bleeding time was not determined.

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Figure 1: Platelet aggregation (top) and aequorin-indicated [Ca2+]i increase (bottom) in a uremic patient (right) and a normal donor (left) in response to either 5 or 25 µmol/L epinephrine (Epi). Fibrinogen (Fg), 400 µg/mL, was added to both samples. Aggregation and [Ca2+]i increase were recorded simultaneously in the same sample; the peak [Ca2+]i is indicated.

Figure 2: Peak [Ca2+]i as seen in gel-filtered, aequorin-loaded platelets taken from patients with uremia or normal donors and then stimulated by epinephrine, the Ca2+ ionophore A23187, arachidonate, and ADP. The bars represent the peak [Ca2+]i (mean ± SD) in platelet samples obtained from all uremic patients, normal donors, and uremic patients divided into groups according to bleeding time. The values in uremic patients with bleeding times (BT) shorter or longer than 11 minutes are compared statistically as shown by the brackets and P values. Although the mean peak [Ca2+]i in response to all agonists in patients with long bleeding times was lower than in patients with normal bleeding times, a comparison between groups separated according to bleeding times revealed a significant difference only in samples treated with A23187 or ADP.
peak [Ca\(^{2+}\)]\(_i\) nor decreased aggregation of platelets in plasma could be correlated with the level of BUN, creatinine, age, arterial blood pressure, or degree of anemia. However, the peak [Ca\(^{2+}\)]\(_i\) was closely associated with the extent of aggregation of washed gel-filtered platelets suspended in buffer. This relationship was statistically significant with arachidonate, A23187 (Fig 3), ADP, and epinephrine (\(P < .05\) and \(P < .025\) for the latter two agonists, respectively; data not shown).

To determine whether the decreased peak platelet [Ca\(^{2+}\)]\(_i\) in response to agonists was due to a factor present in uremic plasma, or, alternatively, represented an intrinsic platelet abnormality, aequorin-loaded platelets from uremic patients and from a normal volunteer were gel-filtered and then resuspended in either uremic or normal platelet-poor plasma, and aequorin luminescence and aggregation were examined following the addition of A23187 (Fig 4) and other agonists (data not shown). The luminescent signals obtained after stimulation could not be calibrated, since the influence of plasma on the Ca\(^{2+}\)-aequorin luminescence relationship has not been determined; however, it is apparent that the peak luminescence (and thus, presumably, [Ca\(^{2+}\)]\(_i\)) obtained with uremic platelets suspended in either uremic or normal plasma was reduced compared with that seen in normal platelets. In fact, samples of normal platelets suspended in uremic plasma demonstrated a higher peak luminescence than did the aliquots of the same platelet sample in autologous plasma. The platelet-poor plasma from both normal and uremic donors was itself only minimally luminescent, without differences between groups. The results suggest that the decreased stimulated [Ca\(^{2+}\)]\(_i\) seen in uremic platelets reflects an intrinsic platelet defect rather than the effect of brief exposure to an inhibitory plasma factor. This abnormality is not corrected by short incubations in normal plasma.

As we have previously noted,\(^{11,17}\) the fluorescent Ca\(^{2+}\) indicators such as quin2 or indo-1 may indicate the average [Ca\(^{2+}\)]\(_i\) in unstimulated platelets more accurately than does aequorin because of the latter’s logarithmic light response to Ca\(^{2+}\), which would tend to emphasize any localized elevations in [Ca\(^{2+}\)]\(_i\). Among uremic patients with bleeding times longer than 11 minutes, the resting [Ca\(^{2+}\)]\(_i\) in platelets suspended in medium containing Ca\(^{2+}\) was not significantly different from that found in platelets from patients with less prolonged bleeding times (173 ± 44 \(\times\) 151 ± 35 nmol/L, respectively). These values were similar to those seen in platelets from normal donors (150 ± 30 nmol/L). The addition of EGTA 3 mmol/L to the extracellular media reduced the resting [Ca\(^{2+}\)]\(_i\) in normal platelets to 105 ± 30 nmol/L, which was not significantly different from the value in platelets from uremic patients with bleeding times either greater than (110 ± 40 nmol/L) or less than (106 ± 27 nmol/L) 11 minutes. Thus, the uremic platelet defect was not accompanied by changes in the basal [Ca\(^{2+}\)]\(_i\) present in unstimulated platelets, as measured by indo-1.

**DISCUSSION**

In this study, platelets from uremic patients with prolonged bleeding times had less elevation of [Ca\(^{2+}\)]\(_i\) after stimulation than did platelets from normal donors or from...
uremic patients with less prolonged bleeding times. This reduction in peak [Ca\(^{2+}\)] did not require the presence of a plasma-borne inhibitor, as it persisted in gel-filtered platelets suspended in buffer; the defect was not corrected by brief incubation in normal plasma. The defect was correlated with reduced aggregation of washed gel-filtered platelets suspended in buffer. In this respect, the platelet abnormality seen in our study is similar to that seen in patients with attention-deficit disorder and bruising, whose platelets also aggregate normally when suspended in plasma but demonstrate reduced aggregation when separated from plasma.\(^{20}\)

This finding does not exclude the possibility that the reduction in peak [Ca\(^{2+}\)] was initiated by factors in the plasma, whose presence may not be required for the defect to persist or that may possibly require a longer period of incubation for induction of this effect. Thus, the results of this study would support a hypothesis that the proximate cause for the reduced aggregation of gel-filtered uremic platelets is the reduced agonist-induced peak [Ca\(^{2+}\)] as indicated by aequorin. The correlation between this abnormality and the prolongation of bleeding time, which is the best predictor of clinically important bleeding tendencies in uremic patients,\(^{1,3}\) suggests that this observation may be of clinical importance.

Measurement of the [Ca\(^{2+}\)] in unstimulated platelets with indo-1 that, like other fluorescent [Ca\(^{2+}\)] probes, might be expected to provide a more accurate representation of the average cytoplasmic [Ca\(^{2+}\)] in resting platelets than would the bioluminescent photoprotein aequorin.\(^{21,22}\) demonstrates that the [Ca\(^{2+}\)] in resting uremic platelets does not differ from that seen in normal platelets in either the presence or absence of extracellular Ca\(^{2+}\). This differs from the findings in patients with essential hypertension, in whom the [Ca\(^{2+}\)] in resting platelets, as determined by fluorescent indicators, is higher than in normotensive controls.\(^{21,22}\) The increase in [Ca\(^{2+}\)] in such patients appears to be due to a factor that can be transferred by incubating platelets in patients' plasma,\(^{22}\) in contrast to an intrinsic abnormality, as in platelets from uremic patients.

The findings of the present study are best explained by an alteration in the mechanisms required for intracellular [Ca\(^{2+}\)] mobilization or in Ca\(^{2+}\) entry from the extracellular space after platelet stimulation. The nature of these defects is not certain. Although exposure of the platelet surface to uremic plasma might be expected to alter agonist–receptor interactions, our findings of diminished [Ca\(^{2+}\)] mobilization and aggregation in response to the Ca\(^{2+}\) ionophore A23187, which does not require a surface receptor, suggest that the abnormalities in peak [Ca\(^{2+}\)] cannot be ascribed totally to this cause. The findings with A23187 also argue against a primary abnormality of cyclic adenosine monophosphate (AMP) in uremic platelets,\(^{23}\) since elevation of this nucleotide does not diminish the ionophore-stimulated rise in [Ca\(^{2+}\)].\(^{12,24}\)

Although the correlation of peak [Ca\(^{2+}\)] with bleeding time provides some evidence that the findings in this study are related to the hemorrhagic tendency in uremic patients, it is unlikely that reduced peak [Ca\(^{2+}\)] accounts for the entire hemostatic abnormality in these patients. Recent findings that prolongation of bleeding time in patients with renal failure is related to the degree of anemia\(^{25}\) and that its correction can be accomplished by transfusion of red cells\(^{25}\) or the administration of recombinant erythropoietin\(^{26}\) suggest that rheologic factors may be important in hemostasis in these patients. The effect of any of these therapeutic options on the ability of platelets to increase [Ca\(^{2+}\)] after the stimulus is unknown. Although it is not clear that abnormalities in the intracellular regulation of platelet function are the proximate cause of hemostatic abnormalities in these patients, the findings of this study suggest that an abnormality in the mobilization of [Ca\(^{2+}\)] by exogenous stimuli may contribute to the platelet defect associated with hemorrhage in uremic patients.

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