CONCISE REPORT

Glycoprotein IIb-IIIa Complex and Ca\(^{2+}\) Influx Into Stimulated Platelets

By Michael J. Powling and Roger M. Hardisty

Changes in intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_i\)) in platelets stimulated with aggregating agents were measured with the fluorescent indicator dye quin 2. Ca\(^{2+}\) influx, but not intracellular mobilization, in response to adenosine diphosphate (ADP), platelet aggregating factor (PAF-acether), and sodium arachidonate was significantly inhibited by monoclonal antibodies against the glycoprotein (GP) IIb-IIIa complex; inhibition of thrombin-stimulated influx was inhibited to a lesser extent and reached statistical significance only at thrombin concentrations of 0.1 U/mL.

Cyttoplasmic Free Calcium, [Ca\(^{2+}\)]\(_i\), is an important intracellular mediator of secretory exocytosis in many cell types. In blood platelets, many different agonists, including thrombin, adenosine diphosphate (ADP), and platelet activating factor (1-O-hexadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine; PAF-acether), raise [Ca\(^{2+}\)]\(_i\), both by stimulating influx from the extracellular medium and through the discharge of Ca\(^{2+}\) from intracellular storage sites, predominantly the dense tubular system. There is increasing evidence that the latter effect is mediated by inositol triphosphate derived from the hydrolysis of phosphatidylinositol 4,5-bis-phosphate in the inner leaflet of the platelet membrane. The mechanisms controlling the net influx of extracellular Ca\(^{2+}\), however, are largely unknown.

Brass has recently produced evidence that the membrane glycoprotein (GP) IIb-IIIa complex, which carries both fibrinogen receptors and the major high-affinity binding site for Ca\(^{2+}\) on the unstimulated platelet surface, is involved in the control of Ca\(^{2+}\) transport across the plasma membrane of unstimulated platelets. We show here that monoclonal antibodies to the GP IIb-IIIa complex inhibit Ca\(^{2+}\) influx into platelets stimulated by ADP and other agonists. Such influx occurs normally in the platelets of patients with Glanzmann's thrombasthenia, however, so GP IIb-IIIa itself is evidently not directly involved in the transport mechanism. It may be that the antibodies inhibit Ca\(^{2+}\) influx by steric hindrance of a calcium channel closely adjacent to the GP IIb-IIIa complex.

MATERIALS AND METHODS

Normal and thrombasthenic human platelets were loaded with the fluorescent indicator dye quin 2 (Lancaster Synthesis, Morecambe, Lancashire, England), washed, and resuspended in Hepes buffer, pH 7.4 at 37°C as described by Hallam et al. Fluorescence was measured in an unstimulated system without added fibrinogen, in which little or no platelet aggregation occurred, in a Perkin-Elmer LS5 fluorescence spectrometer (Beaconsfield, Bucks, England) at 37°C, with an excitation of 395 nm and emission of 500 nm, and recorded on a Rikadenki chart recorder (Rikadenki, Inc., Osaka, Japan). [Ca\(^{2+}\)]\(_i\), values were derived from the fluorescence readings as described. Aggregation and adenosine triphosphate (ATP) secretion were determined in parallel on the same platelet samples to which human fibrinogen had been added to a concentration of 0.5 mg/mL and stirred at 900 rpm at 37°C in a Payton luminometer coupled to the same chart recorder. The effect on these platelet responses of the following monoclonal antibodies was determined:

M148, and B79.7, two antibodies directed against the GP IIb-IIIa complex; AP-1, against GP Ib\(_a\); W6.32, an HLA-class I monomorphic antibody; and 152, a mouse monoclonal antibody without activity against platelets. M148, AP-1, and 152 were used as ascitic fluid, and the other antibodies as purified IgG.

RESULTS

Normal platelets. In response to 10 μmol/L ADP, 20 nmol/L PAF-acether, or 20 μmol/L sodium arachidonate, [Ca\(^{2+}\)]\(_i\), rose from its resting level of about 60 nmol/L to 100–200 nmol/L in the presence of 2 mmol/L ethylene glycol tetra-acetic acid (EGTA) in the suspending medium (which reduced the extracellular [Ca\(^{2+}\)]\(_i\) to below 100 nmol/L) and from about 80 nmol/L to 600–1000 nmol/L in the presence of 1 mmol/L [Ca\(^{2+}\)]\(_o\) (Table 1). On the addition of human thrombin (0.5 U/mL) or A23187 (200 nmol/L), [Ca\(^{2+}\)]\(_i\), responses of about twice this magnitude were observed, both in the presence and the virtual absence of [Ca\(^{2+}\)]\(_o\) (Table 1). Preincubation of the platelets for one minute with the monoclonal antibody M148, at a concentration (5 μg/mL) that completely inhibited fibrinogen binding and aggregation, had no significant effect on the [Ca\(^{2+}\)]\(_i\), response to any agonist tested in the presence of EGTA, showing that the mobilization of Ca\(^{2+}\) from intracellular storage sites was unaffected. In the presence of 1 mmol/L [Ca\(^{2+}\)]\(_o\), however, the antibody resulted in a greater than 60% reduction in the [Ca\(^{2+}\)]\(_i\), responses to ADP, PAF-acether, and arachidonate, but had only a minor and statistically insignificant effect on the responses to thrombin and A23187 (Table 1). The effect of M148 on the secretion of ATP (Table 2) closely paralleled the [Ca\(^{2+}\)]\(_i\), response: secretion was completely abolished in response to ADP, reduced by about 75% in response to PAF, but reduced by only 25% in response to thrombin.

At thrombin concentrations of 0.1 U/mL and below ([Fig

For the Department of Haematology and Oncology, the Institute of Child Health and Hospital for Sick Children, London. Supported by a Medical Research Council grant no. G8224754SA. Submitted June 3, 1985; accepted June 24, 1985. Address reprint requests to Professor R.M. Hardisty, Hospital for Sick Children, Great Ormond St, London, WClN 3JH, England. © 1985 by Grune & Stratton, Inc. 0006-4971/85/6603-0042$00.00/0
entirely without effect on Ca\(^{2+}\) uptake in response to ADP. The platelets of the mother of one of the patients, a known heterozygote, showed [Ca\(^{2+}\)] responses to ADP in the presence of antibody, which was obtained in the experiments with the monoclonal antibody M148.

Table 1. Effect of Monoclonal Antibody M148 on Stimulated [Ca\(^{2+}\)] Responses in the Presence and Virtual Absence of Extracellular Ca\(^{2+}\)

<table>
<thead>
<tr>
<th>Agonist</th>
<th>Concentration</th>
<th>M148</th>
<th>n</th>
<th>[Ca(^{2+})] (nmol/L) (Geometric mean ± 1 SD)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADP</td>
<td>1 mmol/L</td>
<td>-</td>
<td>32</td>
<td>601 - 788 - 1,035</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>10 μmol/L</td>
<td>+</td>
<td>32</td>
<td>193 - 276 - 395</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;100 nmol/L</td>
<td>-</td>
<td>7</td>
<td>130 - 184 - 261</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>7</td>
<td>129 - 168 - 209</td>
<td></td>
</tr>
<tr>
<td>PAF</td>
<td>1 mmol/L</td>
<td>-</td>
<td>11</td>
<td>624 - 803 - 1,030</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>20 nmol/L</td>
<td>+</td>
<td>11</td>
<td>250 - 316 - 398</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;100 nmol/L</td>
<td>-</td>
<td>6</td>
<td>117 - 153 - 200</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>6</td>
<td>112 - 128 - 146</td>
<td></td>
</tr>
<tr>
<td>Na arachidonate</td>
<td>1 mmol/L</td>
<td>-</td>
<td>10</td>
<td>615 - 731 - 869</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td></td>
<td>20 μmol/L</td>
<td>+</td>
<td>10</td>
<td>178 - 265 - 394</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;100 nmol/L</td>
<td>-</td>
<td>6</td>
<td>104 - 143 - 198</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>6</td>
<td>95 - 130 - 177</td>
<td></td>
</tr>
<tr>
<td>Thrombin</td>
<td>1 mmol/L</td>
<td>-</td>
<td>11</td>
<td>656 - 1,200 - 2,576</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>0.5 U/mL</td>
<td>+</td>
<td>11</td>
<td>399 - 801 - 1,603</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;100 nmol/L</td>
<td>-</td>
<td>6</td>
<td>185 - 292 - 460</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>6</td>
<td>170 - 256 - 387</td>
<td></td>
</tr>
<tr>
<td>A23187</td>
<td>1 mmol/L</td>
<td>-</td>
<td>9</td>
<td>1,368 - 1,823 - 2,432</td>
<td>ns</td>
</tr>
<tr>
<td></td>
<td>200 nmol/L</td>
<td>+</td>
<td>9</td>
<td>708 - 1,170 - 1,932</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&lt;100 nmol/L</td>
<td>-</td>
<td>7</td>
<td>290 - 436 - 656</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>+</td>
<td>7</td>
<td>254 - 410 - 659</td>
<td></td>
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</tbody>
</table>

**Wilcoxon's signed ranks test, 2-tailed. ns, Not significant.

1. M148 reduced the mean [Ca\(^{2+}\)] response by about 40%—a significant effect, but less than that seen with concentrations of ADP, PAF, or arachidonate which produced similar responses in the absence of the antibody.

Exactly similar inhibition of the [Ca\(^{2+}\)] response to ADP was obtained in the experiments with the monoclonal antibody B79.7, which also binds to the GP IIb-IIIa complex, but no significant inhibition was induced by antibody AP-1 against GP Ib or by either of the control antibodies W6.32 and 152.

**Thrombasthenic platelets.** The platelets of three unrelated patients with Glanzmann's thrombasthenia, though they failed to aggregate in response to thrombin or ADP, gave completely normal [Ca\(^{2+}\)] responses to both agonists in the absence of M148. The platelets of two of these patients were also tested in the presence of the antibody, which was entirely without effect on Ca\(^{2+}\) uptake in response to ADP (Fig 2). The platelets of the mother of one of the patients, a known heterozygote, showed [Ca\(^{2+}\)] responses to ADP within the normal range, both in the presence and absence of the antibody (Fig 2). This patient and her mother had previously been found by Dr J.N. George (personal commu-

Table 2. Effect of Monoclonal Antibody M148 on ATP Secretion in the Presence of 1 mmol/L Extracellular Ca\(^{2+}\)

<table>
<thead>
<tr>
<th>Agonist</th>
<th>n</th>
<th>ATP Secretion (nmol/L per 10⁶ platelets)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>M148</td>
</tr>
<tr>
<td>ADP</td>
<td>9</td>
<td>0.88 ± 0.33</td>
</tr>
<tr>
<td>PAF</td>
<td>6</td>
<td>1.10 ± 0.55</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>1.69 ± 0.12</td>
</tr>
</tbody>
</table>

**DISCUSSION**

The two monoclonal antibodies to the GP IIb-IIIa complex that we have studied clearly inhibit the major part of the influx of Ca\(^{2+}\) into normal platelets stimulated by ADP, PAF-acether, or arachidonate, but have only a minor effect on Ca\(^{2+}\) influx into thrombin or A23187-stimulated platelets. That the slight reduction of Ca\(^{2+}\) influx seen in response to higher thrombin concentrations failed to reach statistical significance may have been partly due to the imprecision of [Ca\(^{2+}\)] measurements in the micromolar range by quin 2 fluorescence, but it is nevertheless notable that [Ca\(^{2+}\)], responses of the order of 600 to 1,000 nmol/L to lower

![Fig 1](https://www.bloodjournal.org/content/80/4/732/F1)

Fig 1. [Ca\(^{2+}\)] responses to various concentrations of thrombin in the presence (■) or absence (□) of monoclonal antibody M148. [Ca\(^{2+}\)], 1 mmol/L. n = 6–11. The bars represent 1 SEM.
concentrations of thrombin were less profoundly inhibited by M148 than similar responses to the three weaker agonists. Whatever the mechanism by which the antibodies inhibit Ca\(^{2+}\) influx, therefore, it would appear that thrombin also induces influx by an additional pathway. The inhibitory effect of the antibodies on Ca\(^{2+}\) influx did not depend on their inhibition of fibrinogen binding or aggregation since it was tested in an unstirred system without added fibrinogen. The best evidence against this possible explanation of the Ca\(^{2+}\)-blocking effect, however, comes from our findings in thrombasthenic platelets, which fail to bind fibrinogen or to aggregate, yet show a normal [Ca\(^{2+}\)], response to ADP and thrombin.

The attractive concept that the GP IIb-IIIa complex, which is the chief high-affinity binding site for Ca\(^{2+}\) on the platelet membrane, might itself be a calcium channel is also refuted by our observations of normal Ca\(^{2+}\) influx into stimulated thrombasthenic platelets, which are profoundly deficient in the complex. An alternative possibility, that the antibodies inhibit Ca\(^{2+}\) influx by steric hindrance of a calcium channel closely adjacent to the complex or by perturbation of the membrane in its vicinity, is supported by the absence of any such inhibitory effect on thrombasthenic platelets to which the antibodies do not bind. There are about twice as many GP IIb-IIIa complexes per platelet as GP Ila molecules, and it might therefore be argued that the Ca\(^{2+}\)-blocking effect of the antibodies to the former was a nonspecific one attributable solely to the larger number of their binding sites. The effect of M148 on the platelets of a thrombasthenic heterozygote with less than one quarter of the normal number of GP IIb molecules was within the normal range, however, and it therefore seems more likely that the blocking of the calcium channel depends on its close spatial relationship to the GP IIb-IIIa complexes in the membrane.

These findings are quite distinct from those of Brass, but do not conflict with them. He studied unstimulated platelets under steady-state conditions and found that the maximal rate of Ca\(^{2+}\) exchange was less than half normal, primarily because of a decrease in the rate of influx, both in thrombasthenic platelets and in normal platelets in which the GP IIb-IIIa complex had been dissociated by prior incubation with EGTA. It thus appears that an intact GP IIb-IIIa complex is required for maintenance of the relatively slow Ca\(^{2+}\) influx in unstimulated platelets, but not for the very rapid influx that results from stimulation with aggregating agents—though this may involve Ca\(^{2+}\) channels in close proximity to the complex. Brass and Shattil have shown that the additional Ca\(^{2+}\) binding sites exposed on the platelet membrane by stimulation with ADP, unlike those on unstimulated platelets, are present on thrombasthenic platelets, and therefore presumably distinct from GP IIb-IIIa: Might these new binding sites be related to the calcium influx that we have studied?

ACKNOWLEDGMENT

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REFERENCES


Glycoprotein IIb-IIIa complex and Ca2+ influx into stimulated platelets

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