Evidence for a Role of Glycoprotein IIb-IIIa, Distinct From Its Ability to Support Aggregation, in Platelet Activation by Ionophores in the Presence of Extracellular Divalent Cations

By Bruce Lages and Harvey J. Weiss

Activation of blood platelets by most, if not all, agonists is closely associated with increases in the cytoplasmic Ca\(^{2+}\) concentration. Early evidence for this involvement of Ca\(^{2+}\) was obtained in part from studies demonstrating that ionophores, such as A23187 and ionomycin, which transport divalent cations across membranes, could activate platelets. The ability of the ionophores to induce secretion in the absence of aggregation in systems containing EDTA to remove extracellular divalent cations is not associated with any significant alteration of the ionophore-mediated [Ca\(^{2+}\)] increase, as measured in both aequorin-loaded GFP stimulated with A23187 and fura-2–loaded GFP stimulated with ionomycin. Incubation of normal GFP with either the monoclonal antibodies or the ligand binding site peptide RGDS in the presence of 1 mmol/L Ca\(^{2+}\) caused virtually complete inhibition of A23187–induced aggregation, measured as the loss of single platelets, but RGDS, in contrast to the antibodies, did not inhibit secretion or TxB\(_2\) formation. We conclude that platelet activation induced by ionophores in the presence, but not in the absence, of extracellular divalent cations involves a GP\(\text{IIb-IIIa}\)–dependent process that most likely involves a property of the ligand-occupied form of the complex distinct from its ability to support aggregation. This could represent another example of an aggregation-independent activity of the receptor-occupied state of the GP\(\text{IIb-IIIa}\) complex in signal transduction.

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formation, because A23187 induced similar extents of secretion and TXB$_2$ formation (Fig 1B). The ability of EDTA to restore secretion to normal levels was previously described, with ingestion of drugs during the 7- to 10-day period before study being denied. Normal subjects were hospital and laboratory personnel who were not on antiplatelet therapy and who denied ingestion of drugs during the 7- to 10-day period before study.

Statistics. Results are presented as mean values ± SEM for the number of subjects indicated. Statistical significance was determined by paired or unpaired t testing, as appropriate, with a P value of .05 or less considered significant.

RESULTS

A23187-induced responses in citrated PRP. This study was derived from initial observations of marked differences in A23187-induced activation of citrated PRP from normal subjects and from patients with Glanzmann's thrombasthenia, whose platelets are deficient in GPIIb-IIIa. Stimulation of citrated thrombasthenic PRP with 15 µmol/L A23187 resulted not only in the expected absence of aggregation, but also in an absence of secretion and TXB$_2$ formation, despite the known ability of A23187 to induce these responses independently of aggregation (Fig 1A). However, the inability of thrombasthenic platelets to aggregate did not appear to be responsible for the absence of secretion and TXB$_2$ formation, because A23187 induced similar extents of secretion and TXB$_2$ formation (measured in 1 patient) in thrombasthenic and normal PRP when 5 mmol/L EDTA was present (Fig 1B).

The ability of EDTA to restore secretion to normal levels in thrombasthenic PRP was not seen with other agonists. Secretion induced by collagen and arachidonate was also decreased in thrombasthenic PRP, compared with that in normal PRP, but was reduced even further in both cases by the addition of EDTA (Table 1).

To determine whether the absence of A23187-induced responses in thrombasthenic PRP could be attributed specifically to the absence of GPIIb-IIIa, we tested the effects of the complex-specific MoAb LJ-CP8 on A23187-induced responses in normal PRP. Incubation with Fab' fragments of CP8 for 2 minutes before the addition of A23187 produced responses identical to those in thrombasthenic PRP: secretion and TXB$_2$ formation were abolished in citrated PRP, but unaffected in PRP containing 5 mmol/L EDTA (Fig 2). Incubation with Fab' fragments of LJ-P10, which is also specific for an epitope on GPIIb-IIIa but inhibits neither fibrinogen binding nor aggregation, had no effect on these responses (Fig 2).

A23187-induced responses in GFP. To determine what factors might be responsible for the inhibition of A23187-induced responses seen in PRP in the absence but not in the presence of EDTA, the effects of the absence or blockade of Ib-IIIa were tested in GFP containing various additions, including 1 mmol/L Ca$^{2+}$ or Mg$^{2+}$ with or without fibrinogen, 1 mmol/L Mg$^{2+}$ or 1 mmol/L EDTA with fibrinogen, and fibrinogen or Ca, Mg-free buffer alone (Fig 3). In these studies, identical inhibitory patterns were obtained with LJ-CP8 IgG and Fab' fragments, and with MoAb M148 (IgG) and LJ-CP8. The results shown in Fig 3 are therefore combined data from experiments using LJ-CP8 and M148 IgG.

A23187-induced secretion was markedly reduced in thrombasthenic GFP and in normal GFP treated with antibody in all cases in which Ca$^{2+}$ or Mg$^{2+}$ was present. In contrast, when 1 mmol/L EDTA was present, secretion was unaltered by treatment with antibody, and occurred to a similar extent in thrombasthenic GFP. Secretion was also not altered significantly when Ca, Mg-free buffer alone was added (Fig 3, buffer), indicating that the absence of effect with EDTA was not caused by the EDTA itself.

When fibrinogen alone was added without divalent cations, the extent of secretion in normal GFP treated with antibody varied markedly. Although secretion was reduced on average in the 6 subjects tested (Fig 3, fib), this response

| Table 1. Effect of 5 mmol/L EDTA on $^{14}$C-SHT Secretion Induced by 20 µg/mL Collagen and 0.8 mmol/L Arachidonate in Normal Versus Thrombasthenic Citrated PRP |
|---------------------------------|-----------------|-----------------|
|                                | $^{14}$C-SHT Secretion (%) |
|                                | -EDTA            | +EDTA           |
| Collagen (20 µg/mL)            |                  |                 |
| Normal (n = 7)                 | 61 ± 5           | 11 ± 4          |
| Thrombasthenic (n = 3)         | 18 ± 3           | 4 ± 1           |
| Arachidonate (0.8 mmol/L)      |                  |                 |
| Normal (n = 7)                 | 41 ± 4           | 21 ± 7          |
| Thrombasthenic (n = 3)         | 18 ± 9           | 13 ± 1          |

Values shown are mean ± SEM percent secretion for the number of subjects shown, determined in samples taken from citrated PRP containing 1 µmol/L imipramine 5 minutes after addition of agonist.
Fig 2. Percent 14C-5HT secretion and TxB₂ formation (nmoles per 10¹¹ platelets) induced by 15 μmol/L A23187 in normal citrated PRP in the absence and presence of GPⅠb-Ⅱa complex-specific MoAbs. PRP was incubated with 0.15 mol/L saline or 26 μg/mL LJ-CP8 or LJ-P10 (Fab') for 2 minutes at 37°C before the addition of A23187 in the absence or presence of 5 mmol/L EDTA, and secretion and TxB₂ formation were measured in samples taken 5 minutes after stimulation. Values are the means ± SEM for three experiments.

was unaffected or only slightly reduced in 3 subjects, but virtually abolished in the 3 others. In contrast, the extent of secretion in each of the 3 thrombasthenic subjects was similar to that obtained in the presence of EDTA or buffer.

TxB₂ formation was more variable than secretion, but displayed a generally similar pattern. TxB₂ in normal controls was 442 ± 110 (n = 3) nmoles per 10¹¹ platelets with buffer added, 574 ± 74 (n = 4) with EDTA and fibrinogen added, and 387 ± 43 (n = 5) with Ca²⁺ and fibrinogen added (differences not significant). The amounts of TxB₂ formed in the presence of antibody were 92% ± 16% (buffer), 97% ± 2% (EDTA plus fibrinogen), and 6% ± 2% (Ca²⁺ plus EDTA) with fibrinogen added.

Fig 3. Effects of various additions on 14C-5HT secretion induced by 2 μmol/L A23187 in GFP from normal subjects (□), normal subjects incubated with anti-GPⅠb-Ⅱa MoAbs (○), and thrombasthenics (△). Additions were as follows: buffer, Ca, Mg-free Tyrode/HEPES; Ca, 1 mmol/L Ca²⁺, fib, 0.4 to 0.6 mg/mL DFP-treated fibrinogen; EDTA, 1 mmol/L EDTA, Mg, 1 mmol/L Mg²⁺. The combined results of studies using antibodies LJ-CP8 (26 to 100 μg/mL) and M148 (1:167 dilution from ascites) are shown. Secretion was measured 5 minutes after the addition of A23187. Bars represent means ± SEM for 6 normal subjects and 3 thrombasthenics.
fibrinogen) of the amounts formed in the absence of antibody. Similarly, TxB₂ formation in the 3 thrombasthenics was 461 ± 125 nmoles per 10⁹ platelets with buffer alone and 759 ± 37 with EDTA plus fibrinogen added, but only 55 ± 37 with Ca²⁺ and fibrinogen added.

Preliminary studies suggest that the inhibition of responses seen in the presence of Mg²⁺ may be caused, at least in part, by the presence of residual calcium. In some subjects, secretion and TxB₂ formation in GFP containing 1 mmol/L Mg²⁺ were inhibited by antibody to a lesser extent (37% ± 9% inhibition) when 0.1 mmol/L EDTA was present than when it was absent (87% ± 4% inhibition), thus suggesting a specific role for Ca²⁺.

The differential effects of the antibodies in the presence or absence of extracellular divalent cations were not caused by differences in the extents of cell lysis or in the extents of antibody binding to platelets under these conditions. Table 2 indicates that comparable extents of lysis (<5%) were obtained under all conditions studied in both the presence and absence of LJ-CP8. Table 2 also presents the relative extents of FITC-labeled LJ-CP8 binding to GFP, determined for the same incubation conditions as in Fig 3. The percentage of platelets positive for FITC-LJ-CP8 binding was identical under all conditions, indicating that the antibody bound to GPIIb-IIIa in all cases. The mean fluorescence intensities, which reflect the relative number of antibody molecules bound per platelet, were also similar for all conditions except for the one-third lower value obtained in the presence of EDTA. Thus, the number of antibody molecules interacting with Iib-IIIa was reduced significantly by EDTA, which has been shown previously to cause dissociation of the Iib-IIIa complex at 37°C,⁴⁻⁸ but was not altered by the absence vs presence of added divalent cations.

Specificity of the Iib-IIIa dependency for ionophore-induced responses. The dependence of secretion and TxB₂ formation on Iib-IIIa in GFP, as in PRP, was specific for stimulation by ionophore. As shown in Table 3, the extents of secretion induced by 0.1 U/mL thrombin and 0.2 μmol/L PMA were similar in thrombasthenic GFP and in normal GFP in the presence or absence of LJ-CP8 on addition of buffer, 1 mmol/L Ca²⁺, or 1 mmol/L EDTA. Thrombin-induced TxB₂ formation was also not altered significantly by the presence of LJ-CP8 or in thrombasthenic GFP. PMA did not induce significant amounts of TxB₂ formation in normal or thrombasthenic GFP.

A23187 dose-dependency of Iib-IIIa-mediated responses. Whether increasing concentrations of A23187 could overcome the Iib-IIIa dependency of platelet responses was examined in normal GFP in the presence of 1 mmol/L Ca²⁺. In response to 0.5, 1, and 2 μmol/L A23187, secretion in the presence of LJ-CP8 was reduced to 29%, 24%, and 28% (mean of 2 experiments), respectively, of that in the absence of antibody, whereas with 5 and 7.5 μmol/L A23187, secretion in the presence of antibody was 95% and 100% of that in its absence. However, cell lysis was less than 5% at 2 μmol/L or less of A23187, but increased to 15% and 21% at the two higher concentrations. Thus, within the limits of nonlytic concentrations of A23187, the Iib-IIIa dependency was not overcome by increasing ionophore concentrations.

Ionophore-induced increase in cytoplasmic Ca²⁺ levels. To determine whether this Iib-IIIa dependence could be related to effects on the ionophore-mediated [Ca²⁺]ᵢ increase, secretion, TxB₂ formation, and [Ca²⁺]ᵢ levels were measured in platelets loaded with aequorin. The shapes of the aequorin peaks and the estimated relative increases in [Ca²⁺]ᵢ, produced by 2 μmol/L A23187 in the presence of 1 mmol/L Ca²⁺, were similar in normal platelets, normal platelets treated with LJ-CP8, and thrombasthenic platelets, despite the marked reductions in secretion and TxB₂ formation in the latter two cases (Fig 4). In addition, in fura-2-loaded normal platelets in the presence of 1 mmol/L Ca²⁺, the extent of ionomycin-induced secretion was also reduced by LJ-CP8 and M148 with no alteration in the [Ca²⁺]ᵢ increase (Fig 5).

Effects of RGDS on A23187-induced responses. We also compared the effects on A23187-induced responses of the ligand binding site peptide RGDS, which also blocks platelet aggregation, with those of the MoAbs. In distinct contrast to the antibodies, RGDS had no effect on the extent of secretion induced by A23187 in the presence of 1 mmol/L Ca²⁺, and even increased TxB₂ formation (P < .05, Table 4). However, RGDS was equally effective as LJ-CP8 and M148 in blocking aggregation, as measured by the loss of single platelets (Fig 6).

In some studies in which RGDS obtained from commercial sources was used, we did observe inhibition of secretion and Tx formation as well as of aggregation, similar to the

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**Table 2. Platelet Lysis and LJ-CP8 Binding in A23187-Stimulated GFP Measured in the Presence of the Additions Shown in Fig 3**

<table>
<thead>
<tr>
<th>Additions to GFP</th>
<th>Ca + fib</th>
<th>Ca</th>
<th>Mg + fib</th>
<th>Buffer</th>
<th>EDTA + fib</th>
<th>fib</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysis, as percentage of LDH in supernate</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>−LJ-CP8</td>
<td>4.8 ± 1.1</td>
<td>2.8 ± 0.2</td>
<td>2.5 ± 0.2</td>
<td>3.1 ± 0.2</td>
<td>3.2 ± 0.5</td>
<td>2.7 ± 0.5</td>
</tr>
<tr>
<td>+LJ-CP8</td>
<td>2.5 ± 0.6</td>
<td>2.8 ± 0.2</td>
<td>2.5 ± 0.2</td>
<td>3.1 ± 0.3</td>
<td>3.2 ± 0.5</td>
<td>2.7 ± 0.8</td>
</tr>
<tr>
<td>FITC-LJ-CP8 binding to GFP</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% Positive</td>
<td>86.3</td>
<td>85.5</td>
<td>85.9</td>
<td>95.1</td>
<td>86.1</td>
<td>81.8</td>
</tr>
<tr>
<td>mean fluorescence intensity</td>
<td>39.2</td>
<td>41.0</td>
<td>47.0</td>
<td>49.1</td>
<td>28.5</td>
<td>46.9</td>
</tr>
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</table>

GFP was stimulated by 2 μmol/L A23187 in the presence of the same additions as those described in Fig 3. Lysis was measured after 5 minutes of stimulation. Values shown are mean ± SEM for n = 5. FITC-LJ-CP8 binding was measured after 2 minutes of incubation at 37°C by flow cytometry as described in Materials and Methods. Percent positive is the percentage of total particles with fluorescence intensities above the upper edge of the autofluorescence peak (no antibody added), and the mean fluorescence intensities are the values obtained on 5,000 cells; autofluorescence was 1.7. The values given for both parameters are the means of duplicate measurements.

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Table 3. Thrombin and PMA-induced Responses in Normal GFP, Normal GFP Treated With LJ-CP8, and Thrombasthenic GFP in the Presence of Buffer, 1 mmol/L Ca²⁺, or 1 mmol/L EDTA

<table>
<thead>
<tr>
<th>Additions to GFP</th>
<th>Buffer</th>
<th>1 mmol/L Ca²⁺</th>
<th>1 mmol/L EDTA</th>
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</thead>
<tbody>
<tr>
<td>Secretion, TxB₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thrombin (0.1 U/mL)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n = 3)</td>
<td>76 ± 2</td>
<td>557 ± 125</td>
<td>77 ± 1</td>
</tr>
<tr>
<td>Control + CP8 (n = 3)</td>
<td>75 ± 1</td>
<td>557 ± 111</td>
<td>78 ± 1</td>
</tr>
<tr>
<td>Thrombasthenic (n = 1)</td>
<td>73</td>
<td>420</td>
<td>76</td>
</tr>
</tbody>
</table>

PMA (0.2 μmol/L)

<table>
<thead>
<tr>
<th>Additions to GFP</th>
<th>Buffer</th>
<th>1 mmol/L Ca²⁺</th>
<th>1 mmol/L EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Secretion, TxB₂</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Controls (n = 3)</td>
<td>35 ± 3</td>
<td>—</td>
<td>46 ± 1</td>
</tr>
<tr>
<td>Controls + CP8 (n = 3)</td>
<td>36 ± 1</td>
<td>—</td>
<td>37 ± 1</td>
</tr>
<tr>
<td>Thrombasthenics (n = 3)</td>
<td>33 ± 1</td>
<td>—</td>
<td>31 ± 1</td>
</tr>
</tbody>
</table>

Secretion (percentage of total) and TxB₂ formation (nmoles per 10¹ⁱ platelets) are shown as means ± SEM for the number of subjects indicated. GFP was incubated with 58 μg/mL LJ-CP8 for 2 minutes at 37°C before the addition of agonist, and samples for secretion and TxB₂ taken 5 minutes after agonist addition. TxB₂ formation by PMA was not significant under any of the conditions tested.

effects of the MoAbs. However, when these RGDS preparations were chromatographed on Sephadex G-10, their ability to inhibit secretion was greatly reduced or eliminated completely, whereas their inhibitory activity towards aggregation was unaffected. Thus, it appears that some commercial RGDS preparations may contain substances, probably of higher molecular weight, that can alter their interactions with GPIIb-IIIa.

DISCUSSION

Using both GPIIb-IIIa–deficient platelets from thrombasthenic subjects and normal platelets in which GPIIb-IIIa was blocked by complex-specific inhibitory MoAbs, we have shown that the induction of secretion and TxB₂ formation by divalent cation ionophores exhibits a dependence on GPIIb-IIIa in the presence, but not in the absence, of extracellular divalent cations. This dependence appears to be specific for ionophore-induced activation, because it was not observed with thrombin or PMA, and appears to involve a property of the GPIIb-IIIa complex distinct from its ability to support aggregation.

The absence of A23187-stimulated secretion and TxB₂ formation in citrated PRP from thrombasthenics seen in these studies differs from a previous report of normal responses in thrombasthenic PRP. We believe this discrepancy may be caused by a difference in techniques used to stop the platelet responses. In the previous studies, EDTA alone instead of EDTA plus formaldehyde was used to stop activation, but it has been shown that, especially with ionophore, formaldehyde is necessary to prevent close cell contact-induced activation from occurring during centrifugation.

Although the dependence of the ionophore-induced responses on GPIIb-IIIa was observed only under conditions that favor aggregation, ie, in the presence of divalent cations,
induction of aggregation does not appear to be the mechanism by which IIb-IIIa mediates these responses. This study and numerous previous studies have shown that aggregation per se is not required for induction of secretion by ionophores, and our observations here that secretion and Tx formation were inhibited by the MoAbs, but not by RGDS, despite complete inhibition of aggregation by both substances, indicates that the inhibition of these responses did not result from the absence of aggregation. Thus, GPIIb-IIIa appears to mediate these responses via a property distinct from its ability to support aggregation.

This mediation may therefore represent another example of an activity of GPIIb-IIIa that is specific to the receptor-occupied form of the complex. Ligand binding to GPIIb-IIIa has been shown to expose new, antigenic sites on the complex that may be involved in subsequent activation events, including signal transduction. Several such ligand-induced binding sites (LIBS) have been identified, and antibodies raised against epitopes associated with these sites inhibit platelet adhesion to collagen and fibrin clot retraction. RGDS or RGD-containing peptides, as well as the macromolecular ligands, induce LIBS formation in IIb-IIIa, which is consistent with our finding that RGDS did not cause inhibition of ionophore-induced secretion, and even significantly increased Tx\(_\beta\) formation.

A number of signal transduction events have been related to ligand binding to GPIIb-IIIa, and thus could be involved in the IIb-IIIa dependence seen here. These include stimulated Ca\(^{2+}\) fluxes, cyclic AMP regulation, Na\(^+\)-H\(^+\) exchange, cytoskeletal reorganization, the accumulation of 3-phosphorylated phosphatidylinositols, and the activation of phospholipid C\(_\alpha\), calpain, and tyrosine kinases and phosphatases. However, at least some of these events appear to be dependent on the induction of aggregation rather than on ligand binding to IIb-IIIa specifically, and most of them are inhibited by RGDS. Whereas activation of certain tyrosine kinases has been related specifically to ligand binding, this activation was observed only with multimeric ligands, and was also inhibited by RGDS. The GPIIb-IIIa dependency of the ionophore-induced responses thus differs from the IIb-IIIa dependence of most of these events, and could therefore reflect a new, as yet undefined, role of the ligand-occupied form of GPIIb-IIIa in mediating a process of signal transduction.

Results obtained in our, and previous, studies suggest several mechanisms that may account for the IIb-IIIa dependency of ionophore-induced activation in the presence of divalent cations.

We observed that blocking GPIIb-IIIa inhibited not only secretion, but Tx formation as well. Because ionophore-induced secretion may be Tx dependent, this finding raises the possibility that IIb-IIIa is required for the induction of secretion through its capacity to mediate Tx synthesis. We attempted to determine whether a IIb-IIIa requirement for secretion could be observed independently of Tx synthesis.

### Table 4. Effects of RGDS Peptide Versus W-CP8 on A23187-Induced Responses in GFP Containing 1 mmol/L Ca\(^{2+}\)

<table>
<thead>
<tr>
<th>Additions to GFP</th>
<th>CP8</th>
<th>RGDS</th>
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<tbody>
<tr>
<td></td>
<td>Buffer (78 μg/mL)</td>
<td>198 μg/mL</td>
</tr>
<tr>
<td>(^{14})C-5HT secretion (%)</td>
<td>61 ± 3</td>
<td>14 ± 6*</td>
</tr>
<tr>
<td>TxB(_\beta) formation (nmoles per 10(^{11}) platelets)</td>
<td>198 ± 44</td>
<td>28 ± 9*</td>
</tr>
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</table>

GFP containing 1 mmol/L Ca\(^{2+}\) was incubated at 37°C for 2 minutes with RGDS or LJ-CP8 before the addition of 2 mmol/L A23187. Samples for \(^{14}\)C-5HT and TxB\(_\beta\) formation were obtained 5 minutes after A23187 addition. Values shown are means ± SEM for n = 6.
* P < .05 versus buffer.

![Fig 5. Effects of antibodies LJ-CP8 (58 μg/mL) and M148 (1:100 dilution from ascites) on \([\text{Ca}^{2+}]_i\) and \(^{14}\)C-5HT secretion in fura-2-loaded GFP stimulated with 2 μmol/L ionomycin in the presence of 1 mmol/L Ca\(^{2+}\). GFP was loaded with fura-2 and \([\text{Ca}^{2+}]_i\) measured as described in Materials and Methods. The \([\text{Ca}^{2+}]_i\) versus time tracing in the absence of antibody is truncated at the point at which aggregation interfered with the fluorescence measurements. Extents of secretion obtained 5 minutes after stimulation are shown in the boxes next to each tracing.](image-url)
using aspirin- or indomethacin-treated platelets, but were unsuccessful because inhibition of cyclo-oxygenase reduced secretion to levels too low to observe any significant inhibition by the antibodies. However, other studies using A23187 and ionomycin11 in concentrations comparable with those in the present study have found secretion to be less sensitive to inhibition of Tx synthesis than observed in our studies, and in one study,6 no Tx dependence at all was found. Although the extent to which ionophore-induced secretion is dependent on Tx formation may therefore not yet be completely defined, it is clearly greater than that for the response induced by thrombin or PMA, for which no IIb-IIIa dependence was found. Secretion induced by 0.1 U/mL thrombin is completely unaffected by cyclo-oxygenase inhibition,23 and PMA-induced secretion occurs in the absence of significant amounts of TxB2 formation (Table 2).

In addition, there are other activation mechanisms that are Tx-dependent when induced by ionophores, but are Tx-independent when induced by thrombin or PMA. One such mechanism is the activation of phospholipase C, with resulting formation of IP3 and diglyceride, the latter enhancing the activation of protein kinase C. These activation steps require thromboxane formation when induced by ionophores.10,11 In contrast, phospholipase C is activated by thrombin directly via a receptor-linked G-protein-dependent pathway32 and PMA activates protein kinase C directly.53 Our observations that secretion induced by both thrombin and PMA is independent of IIb-IIIa is thus consistent with the hypothesis stated above that the specificity of the IIb-IIIa requirement for activation by ionophores may be linked to the Tx dependency of this process.

The specificity of the GPIIb-IIIa dependence for activation in the presence of extracellular divalent cations suggests that there may be distinct mechanisms for ionophore-induced activation, depending on whether extracellular divalent cations, most likely Ca2+, are present or absent. The effect of the absence of these cations cannot be to alter the properties of GPIIb-IIIa to prevent inhibition by the antibodies, because the normal responses seen in thrombasthenic platelets indicates that GPIIb-IIIa is not required at all in the absence of divalent cations. The ionophores may therefore stimulate secretion and Tx formation by two distinct, and possibly mutually exclusive, mechanisms: a GPIIb-IIIa-dependent pathway that operates when extracellular Ca2+ is present, and a IIb-IIIa-independent pathway that operates in its absence.

Differences in the platelet responses to ionophore in the presence and absence of extracellular divalent cations suggest different mechanisms of activation have also been found previously. The studies of Holmsen and Dangelmaier,7 as well as our own,34 showed that secretion induced by A23187 was progressively reduced and delayed with increasing concentrations of extracellular divalent cations, including Ca2+, compared with the response in the presence of EDTA or EGTA, and both studies suggested that these differences

Fig 6. Comparison of the effects of MoAbs Lj-CP 8 and M148 versus RODS on 14C-SHT secretion and aggregation, as measured by single platelet counting, in normal GFP plus 1 mmol/L Ca2+ in response to 2 pmol/L A23187. Responses are shown in the absence (A, B) and presence (C, D) of Lj-CP 8 (58.2 μg/mL) or M148 (1:50 dilution) (A), and RODS (198 μg/mL) (B), each incubated for 2 minutes at 37°C with GFP before the addition of A23187. Aggregation is expressed as the percentage of initial single platelets remaining at each time point. The responses to the antibodies and to RODS were measured in separate experiments. Values are representative results from five similar experiments.
could be caused by the interaction of platelets with ionophore-divalent cation complexes versus free ionophore. In addition, White\(^4\) found that calpain was activated by A23187 in the presence, but not in the absence, of extracellular calcium, and noted that several platelet proteins were hydrolyzed during ionophore stimulation in the presence of calcium, which were not hydrolyzed when calcium was absent, or during stimulation with other agonists.

One event that occurs in the presence of extracellular cations and has been linked to GPIIb-IIIa is calcium influx. It is entirely possible that influx via membrane Ca\(^{2+}\) channels may occur in ionophore-stimulated platelets in addition to ionophore-mediated transport, and could even be the major pathway for external Ca\(^{2+}\) entry if the conclusion of the studies cited above,\(^7,^38\) that the platelet plasma membrane may be impermeable to ionophore-Ca\(^{2+}\) complexes, is correct. An involvement of GPIIb-IIIa in Ca\(^{2+}\) influx stimulated by other agonists has been reported in some studies,\(^19-21\) but refuted in another.\(^35\) GPIIb-IIIa incorporated into liposomes has been shown to support Ca\(^{2+}\) influx into the liposomes,\(^18\) and, of particular relevance to our results, this activity was inhibited by antibody M148 but not by RGDS.\(^36\)

The effects of the antibodies and RGDS on the ionophore-induced responses that we observed are thus consistent with the putative role of GPIIb-IIIa in mediating Ca\(^{2+}\) influx. However, we also found that the overall increase in [Ca\(^{2+}\)], induced by the ionophores, as measured by both aequorin and fura-2, was not altered by the absence or blockade of IIb-IIIa, whereas a reduction in [Ca\(^{2+}\)] might be anticipated if inhibition of the ionophore-induced responses were caused by elimination of a IIb-IIIa-mediated Ca\(^{2+}\) influx. However, as discussed previously by Fox et al,\(^26\) it is possible that IIb-IIIa could mediate a very localized influx of Ca\(^{2+}\), changes in which might not alter the relatively large overall increase in [Ca\(^{2+}\)], that is reported by aequorin and fura-2. If such an influx were directly linked to subsequent activation events, then this could account for the specificity of the IIb-IIIa effects for the presence of extracellular cations. Furthermore, if the activation events linked to a IIb-IIIa-mediated Ca\(^{2+}\) influx were those leading to Tx formation, eg, activation of phospholipase A2, then, as discussed above, this could also account for the specificity of the IIb-IIIa effects for activation by ionophores.

These concepts of the pathways associated with the induction of secretion and TxB\(_2\) by ionophores in both the presence and absence of extracellular divalent cations, and our hypotheses of the sites at which GPIIb-IIIa could be involved, are presented schematically in Fig 7. When platelets interact with free ionophore in the absence of extracellular Ca\(^{2+}\), Tx formation, phospholipase C activation, and secretion are initiated solely by the release of internal Ca\(^{2+}\) stores and none of these events are influenced by GPIIb-IIIa. When extracellular Ca\(^{2+}\) is present, we presume that the ionophores elevate [Ca\(^{2+}\)], by a combination of direct transport of external Ca\(^{2+}\), Ca\(^{2+}\) influx via membrane channels, and possibly internal Ca\(^{2+}\) release as well. It is not clear whether the internal Ca\(^{2+}\) release induced by ionophores in the absence of extracellular cations occurs to the same extent, or at all, in the presence of external Ca\(^{2+}\) entry, because present methods for monitoring [Ca\(^{2+}\)], levels in platelets can not directly distinguish between Ca\(^{2+}\) derived from these two sources.

GPIIb-IIIa mediation of Tx formation and secretion, via a property of the ligand-occupied form of the integrin, is seen only when Ca\(^{2+}\) movement across the membrane occurs. The putative sites for GPIIb-IIIa involvement include Ca\(^{2+}\) influx, possibly via a channel that is specifically associated with IIb-IIIa, the events leading to Tx formation, or Tx-dependent phospholipase C activation. Further studies to explore these

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**Fig 7.** Schematic illustration of pathways of ionophore-induced activation that may be mediated by GPIIb-IIIa. In the absence of extracellular Ca\(^{2+}\), ionophores induce release of internal Ca\(^{2+}\) stores, which leads to Tx formation, phospholipase C activation, and secretion. None of these events require IIb-IIIa. In the presence of extracellular Ca\(^{2+}\), intracellular Ca\(^{2+}\) levels may be elevated by a combination of direct transport of Ca\(^{2+}\) across the plasma membrane by ionophore, Ca\(^{2+}\) influx via membrane channels, and possibly an altered extent of internal Ca\(^{2+}\) release. As outlined in the Discussion, the most likely sites for the involvement of IIb-IIIa under these conditions appear to be Ca\(^{2+}\) influx, Tx formation, and Tx-mediated phospholipase C activation.
relationships might provide new insights into both the signal transduction pathways that are directly influenced by Ca²⁺ influx or mobilization and the aggregation-dependent and -independent roles that GPIIb-IIIa may play in these pathways.

ACKNOWLEDGMENT

The authors gratefully acknowledge Milagros Arocho and Diane Sardini for expert technical assistance; Drs Zaverio Ruggeri, Roger Hardisty, Jacek Hawiger, and Kenneth Yamada for their generous donation of MoAbs and RGDS peptide; and Dr T. Kent Gartner for his advice on the purification of RGDS peptides.

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