PLATELET α-GRANULE release is an aspect of platelet activation that occurs more readily than dense granule secretion in response to weak agonists, but the mechanisms leading to membrane fusion and secretion of the two granule types have generally been assumed to be similar. Dense granule secretion is required for homotypic (platelet-platelet) interactions, whereas α-granules appear to be central to platelet trafficking with other cell types (heterotypic interactions). P-selectin, expressed after fusion of the α-granule with the platelet surface membrane, mediates activated platelet-leukocyte adhesion. In addition, there is growing interest in the role of α-granule contents (eg, transforming growth factor-β [TGF-β] and platelet-derived growth factor [PDGF]⩾3) in the modulation of vascular injury and repair mechanisms. In particular, the failure of aspirin and other cyclooxygenase inhibitors to prevent restenosis of atherosclerotic lesions after angioplasty4,5 and the emerging importance of aspirin-independent thromboregulation6 raises questions regarding the dependency of α-granule release on products of arachidonate metabolism. We have studied the effects of inhibition of platelet cyclooxygenase, lipoxygenase, and Na+/H+ exchange on the secretion of platelet α-granules in response to adenosine diphosphate (ADP), using the detection of P-selectin–positive platelets as an index of α-granule release and the binding of leukocytes and P-selectin–positive platelets as a marker for P-selectin function.

MATERIALS AND METHODS

Materials. Amiloride, bovine thrombin, and acetylsalicylic acid (ASA) were obtained from Sigma (St Louis, MO); diluents for these materials were dimethyl sulfoxide (DMSO), phosphate-buffered saline (PBS), and ethanol (ETOH), respectively. The platelet lipooxygenase inhibitor 5,8,11-eicosatriynoic acid (ETI) was from Calbiochem (San Diego, CA) and was diluted in ETOH as recommended by the manufacturer. Platelet agonists included epinephrine HCI (Parke-Davis, Morris Plains, NJ) and adenosine diphosphate (ADP, Sigma), both diluted in PBS, and arachidonic acid (BioData, Hatboro, PA), dissolved in distilled water. Controls for all of these reagents in each experiment consisted of the appropriate final concentration of diluent without agonist, eg, 0.1% DMSO for amiloride and 0.1% ETOH for ASA and ETI.

Antibodies. All monoclonal antibodies (MoAbs) were used as purified whole IgG1. The MoAb 1E3 (donated by Dr K. Ault, Maine Medical Center Research Institute, South Portland, ME) is specific for P-selectin (GMP-140/PADGEM, CD62). P29 (AMAC, Westbrook, ME) recognizes glycoprotein IIb/IIIa (GPIIb/IIIa). Anti-CD45 (HLE; Becton-Dickinson Immunocytometry Systems, San Jose, CA) recognizes a CD45 isofrom present on neutrophils, monocytes, and lymphocytes, but not erythroid cells or platelets.

Aggregometry. Whole blood from normal volunteers on no medications was drawn into sodium citrate at 0.38% final concentration. Platelet-rich plasma (PRP) and platelet-free plasma were prepared in the standard fashion, and the platelet count in the PRP was adjusted to 2.5 × 10⁸/mL. Standard platelet aggregometry was performed at 37°C using a dual sample DP-247E aggregometer (Sienco, Morrison, CO). To determine that agonists were active, PRP samples were stimulated with agonists to show complete platelet aggregation. For in vitro ASA inhibition experiments, PRP was incubated with ASA (500 μmol/L to 5 mmol/L) or diluent at 37°C for 60 minutes (unless otherwise indicated) before the addition of agonist. The adequacy of aspirin-treatment at the above doses and continued function of the diluent-treated PRP was ensured by aggregometry of both PRP samples with arachidonic acid at a final concentration of 0.5 and 1 mg/mL. These arachidonic acid experiments showed normal primary and secondary aggregation in the diluent-treated PRP and complete inhibition of aggregation in the aspirin-treated sample.

To further confirm inhibition of platelet-dense granule release by in vitro incubation with ASA, endogenous platelet serotonin content was measured in samples of PRP after aggregometry as previ
ously described. In brief, PRP incubated with ASA (500 μmol/L final concentration) or diluent was examined with aggregometry as above using as agonist 5 μmol/L ADP. The entire contents of the aggregometry tube were aspirated onto a filter, and the filter was examined for serotonin content by high performance liquid chromatography (HPLC). All samples were incubated with 10 μmol/L fluoxetine to block reuptake of serotonin. Comparison of serotonin content in equal volumes of aspirin- and diluent-treated unaggregated PRP yielded the percentage of serotonin release. Each experiment was performed four times.

**Platelet P-selectin expression in PRP.** In preliminary experiments to determine the optimal time course of ADP stimulation, fresh untreated PRP was mixed with agonist or diluent by brief (1 second) vortexing and incubated at 37°C at time 0. Timed samples were then removed at 5, 10, and 15 minutes and immediately added to paraformaldehyde (1% final concentration); these samples were fixed for 60 minutes at 4°C before washing and labeling with antibody to determine P-selectin expression, as previously described.

To determine P-selectin expression after a variable duration of ASA incubation, PRP was treated with ASA (500 μmol/L final) or diluent at time 0 and placed at 37°C. At 15, 30, and 60 minutes, samples were removed for baseline levels of P-selectin and for stimulation with 3 doses of ADP to determine agonist-induced P-selectin expression. All other in vitro ASA experiments used PRP incubated with ASA or diluent for 60 minutes at 37°C before the addition of other inhibitors or agonists.

To determine if platelet lipoxigenase has a significant role in P-selectin expression, ETI (5 to 20 μmol/L) or control diluent was incubated with fresh PRP for 15 minutes at 37°C before the addition of ADP; in separate experiments, PRP from donors who had ingested ASA (see below) were incubated with ETI or diluent before ADP stimulation to determine if overproduction of 12-Hydroxyeicosatetraenoic acid (12-HETE) in cyclooxygenase-inhibited platelets had an effect on P-selectin expression. For examination of the role of Na+/H+ exchange in ADP-induced P-selectin expression, fresh PRP (no ASA treatment) was incubated with amiloride or HCl (the latter titrated the PRP to a pH of 6.8) before agonist. Samples were fixed after agonist and then washed into normal pH buffer before antibody labeling. Amiloride activity and pH effect on P-selectin expression were confirmed by inhibition of aggregometry of fresh PRP to ADP.

For thrombin stimulation experiments, PRP was gel-filtered using Sepharose 2B (Pharmacia, Piscataway, NJ) as previously described. Gel-filtered platelets were adjusted to a count of 1.5 x 10^9/mL, incubated with 500 μmol/L ASA or diluent for 60 minutes at 37°C, and then stimulated with 0.01 U/mL thrombin for 5 minutes at 37°C. Samples were then fixed in 1% (final) paraformaldehyde as above, washed, and examined for P-selectin expression.

**β-Thromboglobulin (βTG) measurements in PRP.** The percentage of βTG release was measured as previously described. In brief, PRP incubated for 60 minutes with ASA or diluent was stimulated with 5 μmol/L ADP as above. Timed samples were then fixed for P-selectin expression or drawn immediately into cold stabilizing buffer and subsequently centrifuged for platelet-free supernatant to determine βTG in the release. Identical volumes of PRP were completely lysed with Triton X-100 (Sigma) to determine total βTG content. Released βTG was then expressed as a percentage of total content. Each experiment was performed in duplicate.

**Leukocyte-platelet adhesion in whole blood.** Citrated whole blood was incubated with 500 μmol/L ASA or diluent for 60 minutes at 37°C. Whole-blood samples were then stimulated with epinephrine (1 μmol/L) for 10 minutes at 37°C, followed by ADP (5 μmol/L) at 37°C as previously described; timed whole-blood samples were then removed directly into 1% paraformaldehyde. Samples were fixed for 60 minutes, washed, and examined for platelet P-selectin expression and the percentage of leukocyte-platelet conjugates.

**In vivo aspirin treatment.** Experiments in which whole blood or PRP were incubated with aspirin in vitro were confirmed in identical experiments using blood obtained from volunteers before aspirin ingestion and after two doses of 650 mg of aspirin (aspirin taken 8 hours and 1 hour before phlebotomy). PRP was made from the sample obtained before aspirin ingestion and immediately tested by aggregometry to confirm complete aggregation to all agonists, including arachidonic acid (0.5 and 1.0 mg/mL); the PRP sample obtained after two doses of aspirin was similarly tested by aggregometry and showed complete absence of aggregation to arachidonic acid and absent second wave to ADP. Both PRP samples (albeit on separate days) were then subjected to stimulation by ADP, as well as ETI/diluent incubation before ADP. Whole-blood samples at the same time points were stimulated with epinephrine/ADP.

**Flow cytometry.** In both PRP and whole-blood experiments, the determination of the percentage of platelets expressing P-selectin (P-selectin–positive platelets) and the mean fluorescence of platelet P-selectin was performed as previously described, using biotin-1E3 and phycoerythrin-streptavidin. In brief, leukocytes were excluded with a combination of size and platelet-specific marker (anti-GPlb/IIa) gating. A threshold for P-selectin positivity was set using an irrelevant isotype-specific MoAb. Mean P-selectin fluorescence for the entire platelet population was expressed in arbitrary fluorescence units. The determination of the percentage of neutrophil-platelet and monocyte-platelet conjugates in whole blood after epinephrine/ADP was performed as previously described. In Brief, neutrophils and monocytes were acquired using a combination of size and the specific leukocyte marker anti-CD45, thus excluding unbound platelets. Using size and right-angle scatter, the neutrophils and monocytes were analyzed separately for platelet-marker (GPlb/IIa) fluorescence (again using a threshold set with an irrelevant isotype-specific MoAb). Leukocytes with positive platelet marker fluorescence were expressed as the percentage of leukocyte-platelet conjugates (relative to the total number of leukocytes).

**RESULTS**

**Platelet aggregometry and serotonin release.** In vitro incubation of PRP with 500 μmol/L aspirin for 60 minutes consistently resulted in complete inhibition of platelet aggregation to arachidonic acid at 0.5 and 1.0 mg/mL final concentration; by contrast, the diluent-treated (ETOH) PRP showed normal primary and secondary wave aggregation to the same arachidonic acid doses, thus serving as a positive control. Incubation of PRP with 500 μmol/L aspirin for 60 minutes consistently produced complete inhibition of second-wave aggregation in response to 2 to 10 μmol/L ADP (Fig 1A), compared with diluent-treated PRP at the same dose of ADP (Fig 1B). As expected, the primary wave of ADP-induced aggregation was unaffected by aspirin treatment. The only effect of increasing doses of ADP on aspirin-treated PRP was to prolong the disaggregation phase of the primary wave.

To confirm that inhibition of ADP-induced second-wave aggregation by aspirin correlated with inhibition of dense granule release, platelet aggregation was combined with measurement of platelet serotonin content as detailed in
AS A AND α-GRANULE RELEASE

Materials and Methods. Total platelet serotonin content preagonist was 41.0 ± 2.1 ng/mL. Stimulation of diluent-treated PRP with 5 μmol/L ADP (Fig 1B) resulted in second-wave aggregation and 55.4% ± 9.8% serotonin release (22.7 ± 3.8 ng/mL); aspirin-treated PRP showed no second wave to 5 μmol/L ADP (Fig 1A), and serotonin release was only 3.2% ± 3.6% (1.2 ± 0.9 ng/mL). As noted in Materials and Methods, the serotonin release assay was performed in the presence of fluoxetine.

Platelet expression of P-selectin. Preliminary experiments were performed to identify the time course of peak P-selectin expression in response to ADP; the percentage of P-selectin-positive platelets was found to plateau at 5 minutes after addition of agonist. The percentage of P-selectin-positive platelets in response to 5 μmol/L ADP (expressed as a percentage of the 5-minute value) at 10 and 15 minutes after ADP were 98% ± 9% and 96% ± 10%, respectively (mean ± SD for 5 experiments). The mean P-selectin fluorescence similarly peaked at 5 minutes. Platelet P-selectin response to 2 μmol/L and 10 μmol/L ADP likewise peaked at the 5-minute time point. Therefore, P-selectin expression in all ADP agonist experiments was subsequently measured 5 minutes after the addition of ADP.

Table 1 examines P-selectin expression during time of aspirin incubation. PRP was incubated with aspirin or diluent for 15, 30, and 60 minutes; aliquots were stimulated with 2, 5, and 10 μmol/L ADP for 5 minutes. As shown in Table 1, incubation with aspirin for up to 60 minutes resulted in no inhibition of the ADP-induced increase in P-selectin–positive platelets when compared with ADP stimulation of diluent-treated controls (P > .05 by paired t-test).

The mean P-selectin expression per platelet also did not show inhibition by aspirin treatment. The baseline percentage of platelets expressing P-selectin in the PRP before addition of ASA or diluent was 3.8% ± 1.3% with a mean P-selectin fluorescence of 4.1 ± 2.6. After 60 minutes of incubation in ASA, the baseline percentage of P-selectin–positive platelets and the mean P-selectin fluorescence were 5.6% ± 2.3% and 3.3 ± 2.2, respectively, while the diluent-treated sample had a baseline value of 5.4% ± 1.7% and 3.5 ± 2.3, respectively (mean ± SD for 5 experiments). Thus, incubation with ASA or diluent did not produce artifactual platelet activation to a degree that might obscure inhibition of P-selectin release to agonist. Figure 2 shows representative contour graphs of PRP incubated with aspirin or diluent, then stimulated with buffer, ADP 5 μmol/L, or thrombin 0.01 U/mL. Aspirin treatment did not inhibit the increase in the percentage of P-selectin–positive platelets to ADP and thrombin, nor did aspirin affect the mean P-selectin fluorescence per platelet after agonist addition.

The increase in P-selectin after addition of ADP was accompanied by formation of a small number of microaggregates. The percentage of platelets in microaggregate form and the size of the microaggregates did not differ between the aspirin-treated and control samples. The uniformity of platelet “size” and percentage of microaggregates formed in the aspirin-treated and control samples ensures that greater α-granule release in the control sample was not masked by aggregation of the activated platelets. This was confirmed by analyzing platelet P-selectin using single-platelet forward-scatter gates: with this single-platelet gate, there was no difference in the percentage of P-selectin–positive platelets or the mean P-selectin fluorescence between control and aspirin-treated samples. In addition, to determine whether α-granule release had a higher threshold for inhibition than dense granule release, the in vitro dose of aspirin was increased as high as 5 mmol/L; no inhibition of P-selectin induction on ADP-stimulated platelets was noted. Similar results were obtained after volunteer subjects took 650 mg of aspirin 8 hours and 1 hour before phlebotomy. Second-wave aggregometry was completely inhibited to 2 to 10 μmol/L ADP, whereas ADP-induced platelet P-selectin expression in PRP was unaffected compared with samples drawn before aspirin ingestion (data not shown). As noted in Materials and Methods, adequate aspirin treatment was confirmed by arachidonic acid aggregometry before and after aspirin ingestion.

Examination of βTG release. To further confirm loss of inhibition of α-granule release by ASA, βTG release was measured as a percentage of total platelet content of βTG. Samples were assayed 5 and 10 minutes after ADP (5 μmol/L final); βTG release at these time points in the aspirin-treated sample were 98% ± 17% and 110% ± 29% of the diluent-treated sample, respectively.

Platelet agonist synergy. We confirmed a previous finding that preincubation of PRP with 1 μmol/L epinephrine causes an enhanced aggregation response to low doses of ADP. In unstimmed PRP, addition of 1 μmol/L epinephrine does not produce a significant increase in P-selectin expres-
Table 1. ADP Stimulation After Variable Aspirin Incubation

<table>
<thead>
<tr>
<th>Time (min)*</th>
<th>2 μmol/L ADP</th>
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<tr>
<td></td>
<td>ASA</td>
<td>Control</td>
<td>ASA</td>
</tr>
<tr>
<td>15</td>
<td>41 (11)</td>
<td>39 (9)</td>
<td>56 (10)</td>
</tr>
<tr>
<td>30</td>
<td>41 (12)</td>
<td>36 (11)</td>
<td>54 (12)</td>
</tr>
<tr>
<td>60</td>
<td>42 (8)</td>
<td>39 (8)</td>
<td>56 (6)</td>
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</tbody>
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Percentage of P-selectin-positive platelets

<table>
<thead>
<tr>
<th>Time (min)*</th>
<th>15</th>
<th>30</th>
<th>60</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>4.4 (1.1)</td>
<td>4.3 (1.3)</td>
<td>4.5 (1.6)</td>
</tr>
<tr>
<td>30</td>
<td>4.4 (1.7)</td>
<td>3.8 (1.3)</td>
<td>4.4 (1.4)</td>
</tr>
<tr>
<td>60</td>
<td>6.7 (2.7)</td>
<td>5.9 (1.7)</td>
<td>6.8 (2.1)</td>
</tr>
</tbody>
</table>

P-selectin fluorescence

* Duration of incubation with ASA, 500 μmol/L or diluent, followed by 5 minutes of ADP stimulation.

All values are the mean (SD) of five experiments.

Aspirin treatment of PRP did not affect the synergistic increase in P-selectin-positive platelets to epinephrine and ADP.

Effect of lipoygenase inhibition on P-selectin expression. To determine whether increased production of lipoygenase products such as 12-HETE in aspirin-treated platelets after ADP or thrombin, PRP was incubated with aspirin (A, B, and C) or diluent (D, E, and F) for 60 minutes and at 37°C, then stimulated for 5 minutes with buffer (A and D), ADP 5 μmol/L (B and E), and thrombin 0.01 U/mL (C and F). The x-axis is forward scatter (FSC), a measure of cell size; the y-axis (FL2) is P-selectin fluorescence. The percentage of events in quadrant 2 (upper right) represent P-selectin-positive platelets. The percentage of P-selectin-positive platelets stimulated with buffer alone was 5% and 6% (A and D); after ADP 59% and 51% (B and E); and after thrombin 97% and 97% (C and F).
platelets affected α-granule release, PRP was obtained from donors after aspirin ingestion; the PRP was immediately incubated with ETI or diluent and then stimulated with 5 μmol/L and 10 μmol/L ADP. ETI incubation compared with control did not affect ADP-induced P-selectin expression; the percentage of P-selectin-positive platelets in ETI (20 μmol/L) was 102% ± 7% of the control for ADP 5 μmol/L and 110% ± 9% of the control for ADP 10 μmol/L (mean ± SD for 4 experiments). Mean P-selectin fluorescence after ADP was similarly not inhibited by ETI: 106% ± 13% and 120% ± 33% of control for ADP 5 μmol/L and 10 μmol/L, respectively (mean ± SD for 4 experiments). ETI incubated with PRP from nonaspirin-treated donors similarly showed no inhibition of ADP-induced P-selectin expression.

Effect of Na+/H+ transport on α-granule release. Na+/H+ exchange has been shown to be effectively blocked by increasing the extraplatelet [H+]. Acidification of PRP to a pH of 6.8 resulted in a 43% ± 5% inhibition (mean ± SD for four experiments) of P-selectin expression in response to 5 μmol/L ADP. Inhibition of Na+/H+ exchange with amiloride at doses of 0.05 mmol/L, 0.1 mmol/L, 0.5 mmol/L, and 1 mmol/L, also inhibited platelet expression of P-selectin in response to 5 μmol/L and 10 μmol/L ADP in a dose-response fashion (Fig 4). Inhibition appeared to be maximal at 0.5 mmol/L amiloride. Platelet aggregation in response to 5 μmol/L ADP was completely inhibited at a pH of 6.8, and amiloride-treated PRP also showed dose-dependent inhibition of ADP-induced aggregation in the same dose range of amiloride that inhibited ADP-induced P-selectin expression.

Aspirin effect on P-selectin function. We have previously shown that stimulation of whole blood with epinephrine followed by ADP results in increased monocyte-platelet and polymorphonuclear leukocytes (PMN)-platelet adhesion that is P-selectin dependent. As shown in Table 2, aspirin incubation of whole blood followed by stimulation with epinephrine/ADP did not inhibit either P-selectin expression on unbound platelets, nor the increase in the percentage of monocytes binding platelets. The small increase in PMN-platelet binding after ADP stimulation, which is quantitatively lower in whole blood compared with monocyte-platelet binding, also was not inhibited by aspirin (data not shown).

**DISCUSSION**

Cellular homeostasis in the blood includes both homotypic (platelet-platelet) and heterotypic cell-platelet interactions. The ability of aspirin to inhibit platelet homotypic adhesion in response to weak agonists such as ADP has been well established. Platelet-heterotypic cell interactions have been examined for their role in the regulation of thrombosis, inflammation, and vasomotor tone. The α-granule is critical to a number of these heterotypic reactions. The α-granule contains platelet-derived growth factor and TGF-β, which have their primary effects on cells other than platelets. In addition, P-selectin, which is present on the internal membrane of the α-granule in the resting platelet, is expressed on the platelet surface after agonist-induced α-granule release. P-selectin belongs to the selectin family of adhesion molecules and mediates the binding of activated platelets to monocytes and PMN. In the present study, we examined the ability of aspirin to inhibit antigenic and functional platelet P-selectin expression after stimulation by ADP.

The adequacy of aspirin treatment was assessed by routine aggregometry, where incubation of PRP for 60 minutes with 500 μmol/L aspirin completely inhibited aggregation to 0.5 and 1 mg/mL arachidonic acid, as well as second-wave aggregation in response to 2 to 10 μmol/L ADP. In addition, serotonin release was found to be completely inhibited in ADP-stimulated platelets that had been incubated with aspirin. By contrast, the increase in P-selectin expression in response to ADP, both in the percentage of P-selectin-positive platelets and the mean P-selectin fluorescence was unchanged by in vitro or in vivo aspirin treatment. The low background P-selectin expression in unstimulated platelets after aspirin/diluent incubation ensured that baseline activation did not obscure any potential aspirin inhibition of P-selectin expression. Increasing the dose of aspirin to pharmacologic levels (5 mmol/L) did not uncover a greater threshold for inhibition of α-granule release.
The synergy between epinephrine and ADP that has been described for aggregometry was also found to be true for α-granule release. The percentage of P-selectin-positive platelets in response to low-dose ADP was doubled by preincubation of the platelets with a dose of epinephrine that by itself produced insignificant α-granule release. This synergy with epinephrine may be caused by an increase in the avidity of the ADP receptor for its ligand. Aspirin treatment did not inhibit the synergistic increase in α-granule release induced by epinephrine pretreatment.

Aspirin treatment of platelets also did not affect the levels of βTG released in response to ADP. Thus, secretion of α-granule contents, as well as surface markers of α-granule release, were found to be unchanged by aspirin incubation. As a technique for detection of α-granule release, βTG levels in our laboratory have proven to be a less sensitive marker than P-selectin expression, in part because of increased sensitivity of flow cytometric techniques for low numbers of activated platelets. Nonetheless, our finding confirms the ability of platelets to release α-granule contents despite inhibition of cyclooxygenase. The trend toward a higher percentage of P-selectin-positive platelets in aspirin-treated samples suggested that excess production of a lipoygenase product, such as 12-HETE, might promote α-granule release, overcoming any inhibitory effect of aspirin.

<table>
<thead>
<tr>
<th>Table 2. Whole-Blood Stimulation With Epinephrine and ADP</th>
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<tbody>
<tr>
<td>Time (min)</td>
</tr>
<tr>
<td>Preincubation</td>
</tr>
<tr>
<td>---</td>
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<td>0</td>
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<td>5</td>
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<td>10</td>
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<td>15</td>
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</table>

* After incubation with ASA or diluent for 60 minutes, samples were incubated with epinephrine 1 μmol/L at -10 minutes, followed by ADP 5 μmol/L at time 0. All values are the mean (SD) of five experiments.

However, experiments using the lipoygenase inhibitor ET failed to show any inhibition of P-selectin expression when used alone or in combination with aspirin, making this explanation unlikely.

To determine whether the function of P-selectin as an adhesion molecule was preserved in aspirin-treated platelets, we measured platelet-leukocyte adhesion in aspirin-treated whole blood. Our previous work has shown that stimulation of normal whole blood with 1 μmol/L epinephrine followed by 5 μmol/L ADP results in platelet activation and activated platelet adhesion to monocytes and PMN via P-selectin. Both in vivo and in vitro aspirin treatment did not change either the percentage of P-selectin-positive platelets in epinephrine/ADP-stimulated whole blood, nor the adhesion of these activated platelets to monocytes or PMN. Metabolic cooperation between activated platelets and PMN has previously been described for the production of such vasoactive compounds as leukotriene B₄ and C₄, dihydroxyecosatetraenoic acid (DiHete), and Lipoxin A₄ and B₄. These leukocyte-platelet interactions similarly are not inhibitable by aspirin or indomethacin, and in vivo work suggests that their formation may even be enhanced by cyclooxygenase inhibition. The role of platelet-leukocyte adhesion via P-selectin in this cooperativity is not known but might serve to maintain these cells in close proximity under conditions of high-shear stress.

Na⁺/H⁺ exchange is an early event in platelet activation that appears to be particularly important in platelet stimulation by weak agonists such as ADP or low doses of thrombin. Inhibition of Na⁺/H⁺ transport has been shown to be critical to activation of phospholipase A₂, to the liberation of arachidonic acid, to regulation of the phosphoinositide cycle, and to transformation of GPIIb/IIIa to a form competent for fibrinogen binding. Blockade of Na⁺/H⁺ exchange by extracellular acidification has previously been found to be effective in inhibiting activation of phospholipase C and phospholipase A₂ by ADP. We found that extracellular acidification to a pH of 6.8 similarly inhibited P-selectin expression by 43% in response to ADP stimula-
tion. Likewise, Na+/H+ transport can be inhibited by the diuretic amiloride, and this inhibition prevents platelet aggregation to ADP in a dose-dependent manner. Amiloride incubation of PRP similarly resulted in dose-dependent inhibition of P-selectin expression in response to ADP. The effects of extracellular acidification on platelet α-granule release may be important for the function of stored platelets for transfusion because there is growing evidence that P-selectin-positive platelets have a lower recovery after transfusion compared with unactivated platelets.

ADP-induced α-granule secretion and P-selectin expression in unstirred PRP proceed independently of arachidonic acid metabolites of both the lipoxygenase and cyclooxygenase pathways. However, α-granule secretion in response to ADP appears to require maintenance of the intracellular pH by the Na+/H+ transporter. Therefore, release of growth factors from the α-granule such as TGF-β and PDGF may be unaffected by cyclooxygenase inhibitors that block platelet-platelet interactions. In addition, as we have shown in this study, leukocyte-platelet interactions mediated by P-selectin are also independent of the metabolites of arachidonic acid. As the regulation of this pathway is further elucidated, it may prove possible to selectively inhibit α-granule secretion while preserving platelet aggregation.

ACKNOWLEDGMENTS

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Aspirin does not inhibit adenosine diphosphate-induced platelet alpha-granule release

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