Evidence for a Role for Na\(^+\)–H\(^+\) Exchange in Activation of Human Platelets by PAF

J. David Sweatt, Mindy S. Schwartzberg, Marshall Frazer, E.J. Cragoe Jr., Ian A. Blair, Peter W. Reed, and Lee E. Limbird

We have found previously that inhibitors of Na\(^+\)–H\(^+\) exchange block platelet arachidonic acid release and subsequent secondary aggregation and serotonin release in response to epinephrine, ADP, and thrombin (0.004 U/ml). The present study demonstrates that the addition of ethylisopropylamiloride, an inhibitor of Na\(^+\)–H\(^+\) exchange, leads to an inhibition of platelet activating factor–induced serotonin release and thromboxane B\(_2\) production in human platelets in citalrated plasma. In addition, platelet activating factor–induced platelet secretion is blocked by the cyclooxygenase inhibitor indo- methacin or the thromboxane antagonist SQ 29548, indicating that arachidonic acid mobilization and metabolism is required for platelet activating factor to elicit platelet activation. Our interpretation of the present findings is that platelet activating factor–induced secretion of dense granules from the human platelet requires the production of cyclooxygenase metabolites from arachidonic acid and that Na\(^+\)–H\(^+\) exchange plays an important, albeit not exclusive, role in mobilization of arachidonic acid in response to platelet activating factor. (Circulation Research 1987;61(suppl II):II-6–II-11)

Platelet activating factor (PAF, AGEPC, PAF-acether) is known to be a potent stimulus for platelet aggregation and serotonin release in many species. PAF has been shown to cause platelet arachidonic acid release,\(^1,4\) Ca\(^{2+}\) mobilization,\(^2,3\) and phosphatidylinositol hydrolysis.\(^4,5\) However, some controversy exists regarding whether PAF requires arachidonic acid mobilization to elicit platelet aggregation and secretion.\(^3,5,10\) Furthermore, the biochemical pathways and physiologic control mechanisms PAF uses to elicit platelet arachidonic acid release are also unclear.

PAF elicits inhibition of platelet adenylate cyclase in a manner regulated by Na\(^+\) and guanine nucleotides.\(^1,2\) and, therefore, is similar to the so-called platelet weak agonists, epinephrine and ADP.\(^2,3,14\) And like epinephrine and ADP, PAF may have a dependency on arachidonic acid release to elicit platelet activation.\(^12,13\) Recently, we reported that epinephrine and ADP, as well as low concentrations of thrombin (<0.04 U/ml), require an intact Na\(^+\)–H\(^+\) exchange mechanism to elicit platelet arachidonic acid mobilization, full aggregation, and serotonin release.\(^16,18\) Given the similarities between PAF and epinephrine, ADP, or low thrombin concentrations as platelet stimuli, the hypothesis that inhibitors of Na\(^+\)–H\(^+\) exchange would affect the ability of PAF to elicit platelet activation was tested.

In this study, we report that PAF-induced serotonin secretion and thromboxane production is inhibited by the addition of analogues of amiloride selective for the inhibition of Na\(^+\)–H\(^+\) exchange. Thus, PAF-induced stimulation of human platelets appears to be regulated, at least in part, via activation of Na\(^+\)–H\(^+\) exchange. Furthermore, addition of SQ 29548 (1 μM), a thromboxane antagonist,\(^19\) also leads to a blockade of PAF-induced platelet secretion and thromboxane B\(_2\) production, which suggests that PAF may cause mobilization of a small initial pool of arachidonic acid that leads to mobilization of a larger quantity of arachidonic acid through a positive feedback cycle.

Materials and Methods

\([\text{H}]\) Serotonin binoxalate was obtained from New England Nuclear (Boston, Mass.). All other materials were from sources as described previously.\(^16\)

Platelets in platelet-rich plasma were used, except where noted, for all studies. Plasma was obtained from aspirin-free male donors, using one-tenth volume acid-citrate dextrose (ACD in a millimolar concentration of dextrose 140, Na\(^+\) citrate 74.8, citric acid 41.6, pH 6.5) as the anticoagulant.

For some studies, when the response of platelets suspended in Na\(^+\)-containing or Na\(^+\)-free buffer were compared, gel-filtered platelets were used. Gel-filtered platelets were prepared as described previously using N-methyl-d-glucamine as the Na\(^+\) substitute.\(^16\)

Platelet aggregation was measured as a change in light transmission using a Payton (Buffalo, N.Y.) aggregometer, and secretion was monitored by measuring the release of \([\text{H}]\) serotonin from the platelets, as described previously.\(^16\)

PAF was from Avanti Polar Lipids (Birmingham, Ala.), and preliminary experiments using fast atom bombardment mass spectrometry indicated that this standard was comprised mostly of saturated C\(_{16}\) alky chains in the sn-1 position of the PAF glycerol backbone (data not shown). However, small amounts of saturated C\(_{18}\) and unsaturated C\(_{15}\) and C\(_{17}\) were also
present in the sn-1 position in the PAF standard. Given
the heterogeneity (although slight) in the PAF stan-
dard, all data for PAF are reported in ng/ml instead of
molar concentrations.

Results

Effect of Ethylisopropylamiloride on PAF-Induced
Human Platelet Aggregation

To determine the effect of inhibition of Na\(^+\)-H\(^+\) exchange on PAF-induced platelet aggregation, the concentration-response curves for PAF-induced platelet aggregation in the presence or absence of 40 \(\mu\)M ethylisopropylamiloride (EIA) were determined. This agent has been shown previously to be a potent and selective inhibitor of Na\(^+\)-H\(^+\) exchange,\(^{25-22}\) and this concentration of EIA effectively blocks platelet arachidonic acid release and secretion in response to epinephrine and ADP.\(^{16}\) As shown in Figure 1, 40 \(\mu\)M EIA had no effect on the rate or extent of platelet aggregation in response to 200 ng/ml PAF. However, at lower PAF concentrations (e.g., 75 ng/ml), there was an effect of EIA to block full platelet aggregation. The observation that EIA did not affect platelet shape change or the initial rate of platelet aggregation in response to either 200 or 75 ng/ml PAF suggests that EIA does not interfere with the interaction of PAF with its specific receptor(s) on the human platelet. However, the observation that EIA does prevent the development of full aggregation in response to 75 ng/ml PAF suggests that EIA interferes with some part of the signaling process that results in full platelet activation at this concentration of agonist.

Effect of Ethylisopropylamiloride on PAF-Induced
Human Platelet Serotonin Release

To determine the effect of inhibition of Na\(^+\)-H\(^+\) exchange on PAF-induced platelet secretion, the concentration-response curves for PAF-induced platelet serotonin release in the presence or absence of 40 \(\mu\)M EIA were determined. As shown in Figure 2, EIA had a profound effect on platelet serotonin release at all concentrations of PAF tested. This finding is interesting in light of the fact that EIA had no effect on platelet aggregation in response to high concentrations of PAF (Figure 1). These data are consistent with the hypothesis that PAF-induced platelet serotonin release is mediated, at least in part, by a Na\(^+\)-H\(^+\) exchange mechanism. However, a certain degree of platelet serotonin release occurs in response to PAF even in the presence of EIA; this is in contrast to platelet activation by 10 \(\mu\)M epinephrine, where incubation of platelet-rich plasma with 40 \(\mu\)M EIA blocks all detectable epinephrine-provoked [\(^3\)H]serotonin release (data not shown). These data suggest the existence of another PAF-activated effector system that can elicit some platelet secretion independent of Na\(^+\)-H\(^+\) exchange.

Effects of Different Analogues of Amiloride on
PAF-Induced Platelet Serotonin Release

We determined the EC\(_{50}\) values for a series of amiloride analogues in inhibiting PAF-induced platelet secretion to compare the ability of these compounds to inhibit PAF-induced platelet secretion with their selectivity in inhibiting Na\(^+\)-H\(^+\) exchange. As shown in Table 1, the order of potency for the amiloride analogues tested was methyl, methallylamiloride>ethylisopropylamiloride>dimethylamiloride. This order of potency for inhibition of PAF-induced secretion correlates well with the known order of potency for the agents in inhibiting Na\(^+\)-H\(^+\) exchange.\(^{20,21}\) Likewise, these data are in good agreement with our previous findings for these compounds as inhibitors of epinephrine-induced platelet arachidonic acid release and subsequent secretion of serotonin from platelet dense granules.\(^{16,18}\) These data are, therefore, consistent with the hypothesis that EIA is acting to inhibit

![Figure 1](http://circres.ahajournals.org/Downloaded from)

![Figure 2](http://circres.ahajournals.org/Downloaded from)
Effects of Ethylisopropylamiloride and SQ 29548 on PAF-Induced Human Platelet Thromboxane B₂ Production

The data presented thus far indicate that arachidonic acid mobilization is required for PAF to elicit platelet secretion. The addition of EIA also suppresses PAF-induced platelet secretion (Figure 2). Therefore, we sought to determine if EIA caused an inhibition of arachidonic acid mobilization in response to PAF, since we have found previously that inhibitors of Na⁺-H⁺ exchange block platelet arachidonic acid mobilization in response to epinephrine, ADP, and thrombin (0.004 U/ml).  

To test the hypothesis that EIA alters arachidonic acid mobilization in response to PAF, the effect of EIA on PAF-induced thromboxane B₂ production was confirmed in separate experiments using gas chromatography-mass spectrometry (GC-MS) to measure thromboxane B₂ (data not shown). The blockade of thromboxane B₂ production caused by EIA is probably attributable to a blockade of arachidonic acid mobilization since it has been found previously that EIA does not inhibit the conversion of exogenous arachidonic acid to thromboxane B₂ in the human platelet.  

The data in Table 2 demonstrate that the thromboxane antagonist SQ 29548 also blocks PAF-induced platelet thromboxane B₂ production. It is known that

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**Table 1. Effect of Amiloride Analogues on PAF-Stimulated Platelet Serotonin Release**

<table>
<thead>
<tr>
<th>Compound</th>
<th>EC₅₀ (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl, methallyl-amiloride</td>
<td>7 ± 1</td>
</tr>
<tr>
<td>Ethylisopropyl-amiloride</td>
<td>16 ± 2</td>
</tr>
<tr>
<td>Dimethyl-amiloride</td>
<td>51 ± 9</td>
</tr>
</tbody>
</table>

Platelet serotonin was assayed as described in "Materials and Methods" and Figure 1 legend. PAF was added at concentration of 75 ng/ml, and EC₅₀ values for inhibition of secretion were determined. Values are mean ± SEM for 3 experiments.

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**Figure 2. Effects of ethylisopropylamiloride (EIA) and SQ 29548 on PAF-induced platelet [³H]serotonin release.** Platelets in plasma were incubated with [³H]serotonin (126,000 cpm/ml) for 30 minutes at room temperature. Platelets were warmed (37°C) with stirring for 2 minutes before addition of 0.5 µM imipramine and the platelet stimulus. Serotonin release was measured after 5 minutes. Serotonin release was determined using 175-µl platelet aliquots and quantified as release of radioactivity into extraplatelet plasma. Percent specific release was calculated using the formula: (cpm released from control versus cpm released from stimulated platelets)/total platelet-associated cpm. Total platelet-associated radioactivity ranged from 7,000–11,000 cpm/175 µl aliquot. Platelet serotonin uptake ranged from 50–80% of added cpm. Results shown are the mean ± SEM from 3 or 4 experiments. The difference in [³H]serotonin release from control versus EIA- or SQ 29548-treated platelets is statistically significant at p<0.05 for all data where PAF was added as a final concentration 50 ng/ml.  

PAF-induced platelet function by inhibiting Na⁺-H⁺ exchange.

Effects of the Thromboxane Antagonist SQ 29548 on PAF-Induced Human Platelet Aggregation and Secretion

We have found previously that those platelet stimuli whose effects were sensitive to inhibition of Na⁺-H⁺ exchange were also sensitive to inhibition of the cyclo-oxygenase pathway of platelet activation, assessed either by inhibiting cyclooxygenase with indomethacin or aspirin⁹ or by adding a thromboxane antagonist, such as SQ 29548. Therefore, we determined whether PAF-induced platelet aggregation and secretion were blocked by inhibitors of the cyclooxygenase pathway of platelet activation. As shown in Figures 1A and 1B, treatment of platelets with the thromboxane antagonist SQ 29548 (1 µM) did not influence the shape change or initial rate of aggregation in response to PAF but did block PAF-induced irreversible aggregation at all concentrations of PAF tested. The effects of SQ 29548 on platelet aggregation and secretion (Figure 2) were mimicked by the addition of 25 µM indomethacin or by using plasma from donors who had been treated with aspirin (data not shown). These data suggest that arachidonic acid metabolites mediate the effects of PAF on human platelet irreversible aggregation. The effects of SQ 29548 on PAF-induced secondary aggregation were paralleled by a blockade of platelet secretion at all concentrations of PAF studied (Figure 2). Taken together, these data suggest that PAF-induced platelet irreversible aggregation and secretion are dependent on arachidonic acid release and metabolism. These data also are in agreement with previous reports using cyclooxygenase inhibitors to block PAF-induced platelet function.¹¹,²²
Table 2. Effect of Ethylisopropylamiloride and SQ 29548 on PAF-Induced Thromboxane B₂ Production

<table>
<thead>
<tr>
<th>Addition</th>
<th>Control 400 ng/ml</th>
<th>PAF 400 ng/ml</th>
<th>Control 75 ng/ml</th>
<th>PAF 75 ng/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>2.4 ± 1.1 (n=5)</td>
<td>15.0 ± 2.7 (n=5)</td>
<td>18.7 ± 3.6 (n=4)</td>
<td></td>
</tr>
<tr>
<td>Ethylisopropylamiloride (40 μM)</td>
<td>2.8 ± 1.4 (n=5)</td>
<td>5.0 ± 1.7 (n=5)</td>
<td>3.2 ± 1.3 (n=4)</td>
<td></td>
</tr>
<tr>
<td>SQ 29548 (1 μM)</td>
<td>5.2 ± 2.2 (n=3)</td>
<td>5.3 ± 2.8 (n=3)</td>
<td>2.4 (n=2)</td>
<td></td>
</tr>
</tbody>
</table>

Aliquots of platelet-rich plasma were taken 5 minutes after addition of vehicle or PAF and centrifuged for 15 seconds in a Beckman microfuge. Supernatant was assayed for thromboxane B₂ as described in "Materials and Methods." All data are reported as ng thromboxane B₂/ml plasma. Values are mean ± SEM for number of determinations indicated in parentheses. Platelet samples were stirred at 37° C in a Payton aggregometer, and each sample was allowed to warm for 2 minutes before addition of stimulus. In control experiments, the compound SQ 29548 was found not to interfere with the thromboxane B₂ radioimmunoassay. Apparent slight increase in thromboxane B₂ found in SQ 29548-treated control incubations is effect of higher plasma thromboxane B₂ levels in 3 donors used for these determinations.

SQ 29548 behaves as a thromboxane antagonist since this agent blocks the effects of the thromboxane mimetic compound U 44619 but does not block the conversion of arachidonate to thromboxane B₂. Thus, the observation that SQ 29548 blocks measurable thromboxane B₂ production must mean that a small, prior (but not measurable) pool of arachidonate is mobilized by PAF, is converted to thromboxanes, and subsequently evokes the release of a large pool of arachidonic acid that is readily measurable by radioimmunoassay after conversion to thromboxane B₂. Although the existence of a prior pool of mobilized arachidonic acid can be inferred to exist based on our findings that SQ 29548, indomethacin, or aspirin blocks measurable thromboxane B₂ appearance in response to PAF, this pool is not measurable using [³H]arachidonic acid release and may, like platelet activation by epinephrine or ADP, require negative ion GC-MS analysis to detect its occurrence. The ability of EIA to inhibit platelet thromboxane production (Table 2) could be attributable to either of two effects: 1) a blockade of the ability of active metabolites of arachidonic acid to elicit further arachidonate mobilization, or 2) a blockade of mobilization of the initial pool of arachidonic acid. It has been found previously that platelet aggregation, secretion, and arachidonic acid release elicited by the thromboxane mimetic compound U 46619 are not blocked by inhibitors of Na⁺-H⁺ exchange (Connolly et al. and unpublished observations). These data lead to the conclusion that the effect of EIA on PAF-induced platelet responses results from a blockade of arachidonic acid release, rather than from an inhibition of response to arachidonate metabolites, once produced. Thus, that inhibition of Na⁺-H⁺ exchange may block the ability of PAF to cause mobilization of a small, physiologically relevant pool of arachidonic acid is concluded. Mobilization of this pool may result in the mobilization of larger pools of arachidonic acid readily measurable by RIA for thromboxane B₂. Although not examined directly in this study, it is possible that PAF activation of human platelets results in the release or enhanced availability of ADP, which is known to activate human platelets via a pathway involving Na⁺-H⁺ exchange. If so, the contribution of Na⁺-H⁺ exchange to platelet activation in response to PAF may reflect, at least in part, the release of ADP or the potentiation of ADP effects.

Discussion

It has been found previously that inhibition of Na⁺-H⁺ exchange leads to an inhibition of platelet arachidonic acid release, secondary aggregation, and secretion in response to epinephrine, ADP, and low concen-

![Diagram of Proposed sequence of events occurring in PAF-stimulated human platelets.](https://example.com/diagram.png)
trations of thrombin. In the present study, it was found that inhibition of Na\textsuperscript{+}-H\textsuperscript{+} exchange suppresses, although it does not completely inhibit, PAF-induced platelet secretion. The involvement of Na\textsuperscript{+}-H\textsuperscript{+} exchange in platelet activation by PAF suggests that PAF shares a pathway for platelet activation analogous to that for the so-called weak agonists for platelets, epinephrine and ADP. Figure 3 provides a schematic diagram of our interpretation of the data obtained in the present study.

We also observed that PAF-induced platelet secretion and aggregation is inhibited by indomethacin or the thromboxane antagonist SQ 29548. These findings are in agreement with some previous studies\textsuperscript{3,4,15} but not with others.\textsuperscript{1,6,10} The present study was performed on human platelets in citrated plasma. Species differences or the presence of chelator in our experiment system may account for the difference between our results and those of other laboratories. Hopefully, future studies will permit a better understanding of the role of arachidonic acid metabolites in mediating PAF-induced platelet stimulation.

The present study demonstrates that PAF-induced arachidonic acid release is decreased when Na\textsuperscript{+}-H\textsuperscript{+} exchange is blocked, as assessed by the reduction in thromboxane B\textsubscript{2} production after EIA treatment. In studies not reported here, we also attempted to use reduction of extraplatelet Na\textsuperscript{+} concentrations or reduction of extraplatelet pH as additional interventions to block Na\textsuperscript{+}-H\textsuperscript{+} exchange. These manipulations were best accomplished by exchanging platelets into the media of interest via gel filtration. However, we found, as others have reported previously,\textsuperscript{1,24} that gel-filtered platelets rapidly lose their response to PAF. Thus, although our results following reduction of extraplatelet Na\textsuperscript{+} concentrations or extraplatelet pH were consistent with the hypothesis that a Na\textsuperscript{+}-H\textsuperscript{+} exchange pathway is involved in PAF-induced platelet activation, the short half-life (30 minutes) for PAF sensitivity of gel-filtered platelets suggests that observations made in these preparations should be interpreted with caution. Our results with analogues of amiloride on PAF-induced activation of platelets in platelet-rich plasma, however, are similar to our previous findings for epinephrine, ADP, and thrombin (0.004 U/ml) as platelet stimuli.\textsuperscript{16} These findings using diverse platelet stimuli suggest an intimate relationship between Na\textsuperscript{+}-H\textsuperscript{+} exchange and platelet arachidonic acid release. It has been hypothesized previously that the alkalization that results from receptor-accelerated Na\textsuperscript{+}-H\textsuperscript{+} exchange may increase the sensitivity of human platelet phospholipase A\textsubscript{2} activity to ambient Ca\textsuperscript{2+} concentrations.\textsuperscript{17,18}

One surprising finding in these studies was that the thromboxane antagonist SQ 29548 blocked irreversible (secondary) platelet aggregation to high concentrations of PAF, while inhibitors of Na\textsuperscript{+}-H\textsuperscript{+} exchange did not (Figures 1A and 2). This distinguishes the characteristics of PAF-induced platelet activation from epinephrine and ADP-induced activation since inhibition of Na\textsuperscript{+}-H\textsuperscript{+} exchange blocked both platelet secretion as well as irreversible aggregation elicited by all concentrations of epinephrine and ADP.\textsuperscript{16} Also, PAF is able to elicit some degree of platelet serotonin release, even in the presence of EIA (Figure 2), while it has been found previously that EIA leads to an essentially complete blockade of serotonin release in response to epinephrine and ADP.\textsuperscript{16} It is difficult to propose a biochemical model to explain these differences, given our present incomplete understanding of the biochemical effector systems used by PAF. However, one plausible explanation is that inhibition of Na\textsuperscript{+}-H\textsuperscript{+} exchange does not lead to a complete blockade of platelet arachidonic acid release in response to high PAF concentrations. This might result, for example, from the existence of multiple pathways for PAF-induced arachidonic acid release (see Figure 3) or from the existence of multiple subtypes of receptors for PAF\textsuperscript{23} linked to multiple effector mechanisms for arachidonic acid release. If platelet secretion and irreversible aggregation possess different sensitivities to arachidonic acid metabolites, then it would be predicted that under circumstances where arachidonic acid mobilization is partially blocked, differential effects on aggregation versus secretion would be noted. Nevertheless, the finding that SQ 29548 completely blocks PAF-induced platelet activation indicates that PAF-induced serotonin secretion is absolutely dependent on arachidonic acid release and metabolism. The finding that EIA only partially blocks PAF-induced serotonin release suggests that PAF may use two separable pathways to elicit full platelet activation. The schematic diagram in Figure 3 attempts to reflect the obligatory role of arachidonic acid metabolites in mediating PAF-induced platelet secretion and the important, albeit not exclusive, role of Na\textsuperscript{+}-H\textsuperscript{+} exchange in mobilization of arachidonic acid.

In conclusion, the data presented in this report suggest a role for Na\textsuperscript{+}-H\textsuperscript{+} exchange in regulating PAF-induced platelet arachidonic acid release and serotonin secretion. This finding is interesting in light of the fact that PAF has been shown to cause arachidonic acid mobilization not only in the platelet\textsuperscript{1} but also in rabbit iris smooth muscle,\textsuperscript{26} renal epithelial cells,\textsuperscript{27} fibroblasts,\textsuperscript{30} and neutrophils.\textsuperscript{31} What remains to be determined is how Na\textsuperscript{+}-H\textsuperscript{+} exchange might regulate PAF-induced phospholipase activation and the precise relation between PAF-induced phospholipase activation and the ensuing aggregation and secretion in the human platelet.

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Key Words • arachidonic acid • thromboxane B\(_2\) • phospholipase A\(_2\)
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