Effects of Acetyl Glyceril Ether Phosphorylcholine on Human Platelet Function in Vitro

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AGPC (PAF), at $1.9 \times 10^{-8}$ M or higher, induced concentration-dependent aggregation and release in human platelet-rich plasma. Comparative studies with arachidonate, collagen, ionophore, and ADP suggested that AGPC was a strong stimulus for platelet aggregation and probably a moderate agonist for release, as well as a relatively weak inducer of TXA$_2$ production. The initial phase of AGPC-induced aggregation was independent of ADP release and TXA$_2$ formation, since it was not inhibited by ASA, apyrase, or CP/CPK. Whereas irreversible aggregation occurred in parallel and were inhibited by both apyrase and ASA. Washed human platelets did not respond to exogenous AGPC in the absence of ADP and did not appear to generate significant quantities of AGPC upon stimulation with thrombin or ionophore.

ACETYL GLYCERYL ether phosphorylcholine (AGPC, 1-O-hexadecyl/octadecyl-2-acetyl-sn-glyceryl-3-phosphorylcholine) is a lipid mediator of allergic and inflammatory reactions that is capable of inducing aggregation and release in rabbit platelets, and also possesses antihypertensive properties. Prior to establishment of its structure, AGPC was known as platelet-activating factor (PAF). In this investigation the response of human platelets to exogenous chemically pure AGPC, as well as production of AGPC by stimulated platelets, was studied.

MATERIALS AND METHODS

Blood was collected by gravity flow into ACD (USP Formula A, 0.15 ml/ml blood) or 3.2% sodium citrate (1:9). Platelet-rich plasma (PRP) was prepared by centrifugation at 225 g for 15 min (10°C). In general, aggregation responses were somewhat more prominent when citrate was the anticoagulant. Platelet counts (10$^9$ platelets/ml. After preincubation with ASA, the cells were then incubated with stirring for 20 min (or, on one occasion, for 10 min). The cells were then pelleted by centrifugation at 2000 g for 10 min at 10°C. Supernatants were stored at 20°C or methanol-extracted. Thrombin-stimulated platelet pellets were resuspended to a volume of 5–15 ml in saline:buffer (1:3) and extracted separately.

For extraction, the supernatant was reduced in volume to 5–15 ml by lyophilization and added dropwise to 95 volumes of absolute methanol. The mixture was stirred at room temperature for 30 min. Precipitated protein was removed by centrifugation at 2000 g for 20 min at 20°C. Chloroform (0.95 volumes) and deionized water (0.8 volumes) were added to the methanol extract (1 volume). After vigorous mixing the phases were separated in a separatory funnel and finally dissolved in chloroform:methanol (9:1).

Platelet Aggregation Studies

PRP (0.4 ml) was added to siliconized aggregometer cuvettes, which were then flushed with 5% CO$_2$-air and capped. PRP was preincubated with or without inhibitors at 37°C for 2 or 5 min. PGE$_1$ was added 1 min before the stimulus. Final concentrations of inhibitors: aspirin (ASA), 1 mM; creatine phosphate (CP), 5 mM; creatine phosphokinase (CPK), 20 U/0.5 ml; apyrase, 40 U/0.5 ml; PGE$_1$, 10$^{-6}$ M. Cuvette volumes were adjusted to 0.5 ml with buffer. Release reactions were stopped 4 min after stimulation by placing cuvettes in ice. Platelets were then removed by centrifugation (4°C; 15,000 rpm; 3 min). Aliquots (50 µl) of each supernatant were taken for scintillation counting in Aquasol II. PRP was fully responsive to AGPC stimulation for a period of 2–3 hr following preparation.

Washed platelet suspensions were prepared as previously described. For aggregometry a total of 10$^9$ platelets were diluted to a final concentration of 200,000/cu mm in buffer.

Platelets as a Potential Source of AGPC

The procedure employed to prepare AGPC from washed platelets was basically that of Benveniste, Henson, and Cochran as modified by Pinckard, Farr, and Hanahan. Washed platelets derived from 4–10 U of freshly collected PRP were suspended in a mixture of 1 part normal saline to 3 parts buffer at an average concentration of 4 × 10$^6$ platelets/ml. After preincubation at 37°C for 15 min, 1.3 mM CaCl$_2$ was added, and the platelets were stimulated with 2.5 µM ionophore A23187 or human thrombin (1.3 U/10$^9$ platelets). They were then incubated with stirring for 20 min (or, on one occasion, for 10 min). The cells were then pelleted by centrifugation at 2000 g for 10 min at 10°C. Supernatants were stored at 20°C or methanol-extracted. Thrombin-stimulated platelet pellets were resuspended to a volume of 5–15 ml in saline:buffer (1:3) and extracted separately.

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The crude lipid extracts were purified by TLC using chloroform:methanol:water (65:35:6) as the solvent system. The separated lipids were briefly visualized with iodine. The area of the plates between lysolcithin and sphingomyelin standards, applied at the sides, and corresponding to AGEPC standard, was scraped and eluted. For aggregometry, the sample was taken up in buffer with vigorous vortexing after evaporation of solvents.

Materials

AGEPC and lyso-GEPC were kindly provided by Dr Donald J. Hanahan. These compounds were stored at -20°C in chloroform:methanol (9:1). Prior to use, aliquots were evaporated to dryness under nitrogen and suspended in buffer. Tris-Tyrode's albumin buffer: Tris, 15 mM; NaCl, 8.00 g/liter; KCl, 0.195 g/liter; MgCl₂·6H₂O, 0.213 g/liter; D-glucose, 1.00 g/liter; bovine serum albumin (Sigma, fatty acid free), 2.5 g/liter—adjusted to pH 7.4. Sources of all other reagents have been reported.

RESULTS

Studies With PRP

Addition of AGEPC to PRP resulted in a prompt concentration-dependent aggregation response, preceded by a shape change (Fig. 1). In PRP from 10 of 14 donors, irreversible aggregation took place with 7.6 x 10⁻³ M AGEPC or higher. In the remaining 4, the response was reversible. Some PRP samples demonstrated irreversible aggregation even at AGEPC levels of 3.8 x 10⁻³ M. However, aggregation induced by AGEPC concentrations below 7.6 x 10⁻⁸ M was usually monophasic. Although there were individual variations in dose–response curves at higher levels of AGEPC, 1.9 x 10⁻⁸ M induced a small reversible aggregation response in 11 of 12 plasmas tested (Fig. 1). AGEPC (7.6 x 10⁻⁵ M) was added to PRP from a patient with thrombasthenia (courtesy of Dr. Marjorie B. Zucker). A normal shape change occurred, followed by a very slight decrease in optical density, but no visible aggregation. A full aggregation response was obtained with the control PRP.

In all experiments, addition of AGEPC was preceded by 3 mM calcium. Controls in which no calcium was added resulted in suboptimal responses. When lyso-GEPC (3.8 x 10⁻⁵ M) was substituted for AGEPC, no aggregation occurred. AGEPC-induced aggregation was always preceded by a shape change, unlike the agglutination observed with ristocetin (1.5 mg/ml).

A synergistic effect between AGEPC and ADP was observed. When a suboptimal quantity of ADP for a given donor (0.5–2 μM) was added to PRP simultaneously with 1.9 x 10⁻⁸ M AGEPC, an aggregation response resulted that was greater than the sum of the individual responses as measured by maximal amplitude of the trace. This synergism was observed in each of three separate experiments, and also occurred with the platelets of a donor who had ingested aspirin.

Effects of Inhibitors

Apyrase or CP/CPK was utilized to remove extracellular ADP. Concentrations used were those that we found to completely inhibit aggregation induced by 8 μM ADP, which is the quantity found extracellularly when collagen is the stimulus. After preincubation with apynase or CP/CPK, PRP from 5 donors (6 experiments) that had aggregated strongly to AGEPC (7.6 x 10⁻⁸ M–7.6 x 10⁻⁵ M) invariably responded with aggregation curves that were greatly reduced in amplitude and completely reversible (Fig. 2). These reversible patterns, observed in the presence of either inhibitor, were virtually identical. When PRP was
preincubated with PGE$_1$ (10 $\mu M$) for 1 min, no shape change or aggregation response was observed with $3.8 \times 10^{-3} M$ AGEPC.

AGEPC-induced aggregation in the presence of 1 mM ASA was studied in PRP from 10 donors (14 experiments). The effect of ASA on irreversible aggregation varied from no apparent inhibition to partial reversal, and in 6 experiments, a completely reversible response occurred (Fig. 3). In individual donors, reversible aggregation occurred more readily at lower concentrations of AGEPC. Control ASA-treated platelets were totally unresponsive to 0.5 mM sodium arachidonate.

**Release of 5-HT, $\beta$TG, and PF-4 by AGEPC**

5-HT release was studied in PRP of three donors. A concentration of $3.8 \times 10^{-5} M$ AGEPC induced 31%, 27%, and 20% 5-HT secretion, respectively. Serotonin release induced by AGEPC was far less than that obtained with 5 $\mu g$ collagen (71%) or sodium arachidonate (64%). Secretion was always concentration-dependent. In the presence of ASA, 5-HT release by $3.8 \times 10^{-5} M$ AGEPC fell from 31% to 13% in one donor, and from 20% to 4% in another.

In three separate experiments, AGEPC-induced 5-HT release was measured in the presence of CP/CPK and apyrase. With the use of CP/CPK, release fell from 27% to 8% and from 22% to 13% with $7.6 \times 10^{-7} M$ AGEPC. With $3.8 \times 10^{-5} M$ AGEPC, release fell from 20% to 4%. The inhibitory effect of apyrase on 5-HT release induced by AGEPC paralleled that observed with CP/CPK. Thus, serotonin release was reduced from 27% to 5%, 22% to 11%, and 20% to 4%, respectively.

Release of the platelet secretory proteins $\beta$TG and PF-4 paralleled 5-HT in that it was concentration-dependent and inhibited by ASA. In contrast to 5-HT and $\beta$TG release, which were inhibited by both CP/CPK and apyrase, PF-4 secretion was not blocked by CP/CPK. This is of interest, since PF-4 release occurred in the absence of a secondary aggregation response (Fig. 2). Results of an experiment in which 5-HT, $\beta$TG, and PF-4 were measured and correlated with aggregation are shown in Table 1.

**AGEPC-Induced TXA$_2$ Formation in PRP**

In preliminary experiments neither an "$O_2$ burst" (3 donors) nor MDA formation$^{17}$ (1 donor) was detectable in AGEPC-stimulated PRP. These experiments were extended with the use of RIA and radio-TLC. Both assays indicated that AGEPC induced relatively low levels of TXA$_2$. For example, concentrations of AGEPC as high as $7.6 \times 10^{-5} M$ induced production of 2.0 ng TXB$_2$/ml above control values. In the presence of ASA, this quantity decreased to 0.1 ng/ml. In contrast, 0.5 mM arachidonate, collagen (5 $\mu g$/500 $\mu l$), and 6 $\mu M$ ionophore resulted in production of 195.7, 94.1, and 13.1 ng TXB$_2$/ml, respectively. ADP (8 $\mu M$) which, like AGEPC, does not induce an "$O_2$ burst," stimulated formation of 1.7 ng TXB$_2$/ml.

Results of radio-TLC experiments in a different donor paralleled those of the RIA studies. After arachidonate, collagen and ionophore stimulation, the TXB$_2$ area on the TLC plate represented 0.11%, 0.36%, and 0.15% of the total dpm recovered (after subtraction of control values). On the other hand, with $7.6 \times 10^{-5} M$ AGEPC as agonist, the TXB$_2$ area accounted for only 0.02% of the dpm. In the same experiment, no TXB$_2$ was measurable after addition of 8 $\mu M$ ADP to the PRP.

**Studies With Washed Platelets**

When AGEPC ($3.8 \times 10^{-5} M$) was added to washed platelets in the presence of fibrinogen (0.1 mg/ml) and calcium (3 mM), no aggregation response occurred. In contrast, this platelet preparation did respond to ADP in the presence of fibrinogen and calcium. When ADP and AGEPC were added simultaneously, aggregation took place that was greater than that elicited by ADP alone.

**AGEPC Generation by Stimulated Human Platelets**

Washed platelets prepared from 8 U of freshly collected PRP were pooled and stimulated with ionophore A23187 (2.5 $\mu M$) in the presence of calcium.
Table 1. AGEPC-Induced Secretion in Human PRP

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>5-HT (%)</th>
<th>βTG (%)</th>
<th>PF-4 (%)</th>
<th>Aggregation Response</th>
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<tbody>
<tr>
<td>AGEPC 3.8 x 10⁻⁸ M</td>
<td>31</td>
<td>46</td>
<td>37</td>
<td>+ + +</td>
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<tr>
<td>3.8 x 10⁻⁸ M + ASA</td>
<td>13</td>
<td>12</td>
<td>14</td>
<td>+ + +</td>
</tr>
<tr>
<td>7.6 x 10⁻⁷ M</td>
<td>22</td>
<td>34</td>
<td>28</td>
<td>+ + +</td>
</tr>
<tr>
<td>7.6 x 10⁻⁷ M + ASA</td>
<td>16</td>
<td>23</td>
<td>22</td>
<td>+ +</td>
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<tr>
<td>7.6 x 10⁻⁷ M + CP/CPK</td>
<td>13</td>
<td>19</td>
<td>27</td>
<td>Reversible</td>
</tr>
<tr>
<td>7.6 x 10⁻⁷ M + CP</td>
<td>22</td>
<td>39</td>
<td>26</td>
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<tr>
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<td>23</td>
<td>44</td>
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<tr>
<td>7.6 x 10⁻⁷ M + apyrase</td>
<td>11</td>
<td>13</td>
<td>8</td>
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<tr>
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<td>24</td>
<td>32</td>
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<td>+ + +</td>
</tr>
<tr>
<td>7.6 x 10⁻⁷ M</td>
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<tr>
<td>3.8 x 10⁻⁸ M</td>
<td>10</td>
<td>14</td>
<td>9</td>
<td>+ +</td>
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<tr>
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<td>1</td>
<td>2</td>
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<tr>
<td>Arachidonate</td>
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<td></td>
</tr>
<tr>
<td>0.5 mM</td>
<td>55</td>
<td>65</td>
<td>88</td>
<td>+ + +</td>
</tr>
<tr>
<td>0.5 mM + ASA</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Collagen</td>
<td></td>
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</tr>
<tr>
<td>5 µg/0.5 ml</td>
<td>70</td>
<td>51</td>
<td>46</td>
<td>+ + +</td>
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<tr>
<td>ADP</td>
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<td></td>
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<tr>
<td>4 µM</td>
<td>6</td>
<td>7</td>
<td>4</td>
<td>+</td>
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<tr>
<td>4 µM + ASA</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>+</td>
</tr>
</tbody>
</table>

*Percent values have been corrected for nonspecific release in unstimulated controls (606 and 353 ng/ml, respectively, for βTG and PF-4, and 71 cpm for 5-HT).
†100% release represents 8256 and 10,857 ng/ml, respectively, for βTG and PF-4, and 2198 cpm for 5-HT.
‡Aggregation responses were arbitrarily graded as follows: + monophasic with slow reversal; + + intermediate; + + + full response.

(1.3 mM). The platelets aggregated, and the supernatant was lipid-extracted and purified by TLC. Material with an Rf value corresponding to the AGEPC standard induced a definite shape change and aggregation response in PRP. The amplitude of the aggregation response corresponded to that which we obtained with 3.8 x 10⁻⁸ M AGEPC and could be calculated to represent production of approximately 12.5 ng AGEPC by the 8 U of platelets. When thrombin (1.3 U/10⁶ platelets) was used as agonist for up to 10 U of platelets (four experiments), no aggregation response was elicited by TLC eluates originating from either the supernatant or pellet of these platelets.

DISCUSSION

The results indicate that AGEPC is a potent mediator of platelet aggregation in human PRP (Fig. 1). At concentrations as low as 3.8 x 10⁻⁸ M, irreversible aggregation could be obtained. The response of platelets from individual donors was variable and could not be correlated with those elicited by other stimuli. Thus, PRP responding optimally to collagen or arachidionate did not consistently do so with AGEPC. AGEPC also induced concentration-dependent release of 5-HT, βTG, and PF-4. Although AGEPC induced aggregation responses that were comparable to collagen and arachidionate, it was a less potent stimulus for secretion (Table 1) and for TXA₂ formation. Thus, in human PRP, AGEPC can tentatively be classified as a strong agonist for aggregation, a modest stimulus for secretion, and a relatively weak inducer of thromboxane formation. Several aspects of our results are similar to those reported by McManus and associates,¹⁸ who also observed calcium-dependent aggregation responses at comparable concentrations of AGEPC.

In some instances the aggregation curves obtained with AGEPC were biphasic in nature. This prompted experiments with antagonists of ADP-mediated secondary platelet aggregation.¹⁸,¹⁹ CP/CPK and apyrase eliminated the biphasic aggregation response and reduced the amplitude of the curve (Fig. 2). Although 5-HT and βTG release was inhibited by both apyrase and CP/CPK, PF-4 release was only inhibited by apyrase. This lack of inhibition of PF-4 release by CP/CPK in the setting of reversible aggregation was also reported by McManus et al.¹⁸ In addition to the apparent effect of released ADP on secondary AGEPC-induced aggregation and secretion, exogenous ADP exerted a synergistic effect with AGEPC.¹⁹ PGE₁, an agent that stimulates adenylate cyclase thereby elevating platelet cyclic AMP, completely inhibited shape change, primary and secondary aggrega-
gation, and 5-HT secretion inducible by AGEPC. Of the four inhibitors used, only PGE₁ abolished shape change and primary aggregation. An inhibitory effect of PGI₂ on aggregation but not on shape change was reported by McManus and associates. In contrast to its effect on rabbit platelets, we found that AGEPC is not a strong stimulus for thromboxane formation in human PRP. Thus, interpreting the extent of ASA inhibition on AGEPC-induced aggregation, when it did occur, is difficult. It is possible that the relatively small quantities of TXA₂ that do form act synergistically with AGEPC. Alternatively, the inhibitory effect of ASA on platelet aggregation is not as specific as previously suggested.

Although washed rabbit platelets are exceedingly sensitive to AGEPC, our results indicate that washed human platelets differ significantly in that responsiveness to pure AGEPC is lost as a consequence of washing. No aggregation was observed with AGEPC alone, but simultaneous addition of AGEPC plus ADP resulted in a synergistic effect. Lotner et al. reported that human platelets prepared by centrifugation over erythrocytes, but not other techniques, responded to PAF derived from human neutrophils. The synergistic effect we observed with ADP suggests that variability in results might be related to differences in the ADP content of individual platelet preparations.

Chignard and associates reported that ionophore-stimulated human platelets released PAF. In our experiments with ionophore-stimulated platelets, the material isolated from TLC plates also induced an aggregation response in PRP. However, it was calculated to represent only 12.5 ng/8 U of platelets. It is unlikely that this AGEPC originated from contaminating leukocytes, since examination of stained smears revealed the presence of only an occasional lymphocyte. With thrombin stimulation, no AGEPC activity was recoverable from up to 10 U of platelets. We concluded that although human platelets in PRP aggregate in response to exogenous AGEPC, the washed platelet is not a significant source of this unique lipid mediator of cellular responses.

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