Distribution of Phospholipids, Fatty Acids, and Platelet Factor 3 Activity Among Subcellular Fractions of Human Platelets

By M. Johan Broekman, Robert I. Handin, Arie Derksen, and Phin Cohen

As compared with other methods, our recently reported method for subcellular fractionation of human platelets improves the separation of mitochondria, alpha granules, and lysosomal enzyme activities. The relative purity of these fractions has led us to undertake the present study to compare the subcellular distribution of phospholipids, fatty acids, and platelet factor 3 (clot-promoting) activity. Two findings pertaining to distribution of phospholipids were entirely new. (1) In the alpha granule zone, plasmalogen phosphatidyl ethanolamine peaked at the expense of diacyl phosphatidyl ethanolamine. (2) The fatty acid composition of the membrane lysophosphatidyl choline suggested that it may have been formed by the action of platelet phospholipase A₁ activity. The fatty acids of the membranes showed a markedly asymmetrical distribution in noncholine versus choline phospholipids. The latter held 94%, 72%, and 85%, respectively, of the total content of 16:0, 18:1, and 18:2 fatty acids, whereas 55% of the 18:0, 72% of 20:4, and 67% of higher polyenoic acids other than 20:4 were esterified to the noncholine group. The most important new information related to clot-promoting activity, which, on the basis of protein content, was highest in the membrane fractions, but on the basis of phospholipid content in the nonmembranous fractions. The discussion centers on possible explanations for this novel finding.

PLATELETS participate in hemostasis by forming aggregates at the site of vascular injury and by providing a source of phospholipoprotein to promote clotting. Phospholipid and calcium are both necessary for the optimal activation of factor X by factors IXa and VIII₁,² and for the activation of prothrombin by factors Xa and V.³,⁴ In model systems in vitro, phospholipids whose micelles are negatively charged or whose fatty acids are both unsaturated are more active in promoting coagulation; however, the relevance of these data to in vivo processes is not established. The clot-promoting properties of platelets have been called platelet factor 3 (PF-3). The mechanism by which platelets make clot-promoting activity available is not well understood; however, its appearance is closely associated in vitro with the release reaction.⁵ PF-3 is not a unique property of platelet phospholipids, since other blood cells, as well as cells or subcellular fractions of diverse origin, can also furnish clot-promoting activity.
After platelet aggregation and the release reaction, the clot-promoting activity that is made available could reside in plasma membranes or intracellular organelles, or both. Subcellular fractionation of platelets should provide a method to examine various platelet constituents for their clot-promoting activity. Marcus et al. and Day et al. have shown that, in addition to the membranes, various subcellular fractions of platelets contain PF-3 activity. However, in those studies the resolution of the granular fraction into its components was incomplete. Recently we have reported a method for subcellular fractionation of human platelets that separates alpha granule and mitochondrial fractions in addition to microsomal and lysosomal enzyme activities. The availability of more purified platelet fractions has promoted the present study, which compares the distributions of phospholipids, fatty acids, and PF-3 (clot-promoting) activity among subcellular fractions of human platelets.

MATERIALS AND METHODS

Platelet procurement and fractionation. Washed platelets were derived from a plastic pack system (Fenwal Laboratories, Deerfield, Ill.) and homogenized by a modification of the nitrogen decompression technique in that each batch of platelets was subjected to three decompressions instead of one. Ultracentrifugation was for 90 min at 217,500 g

Phospholipid and fatty acid composition studies. Previously reported methods were used to extract, separate, localize, and quantify phospholipids. The Rf of bis-(monoacylglycerol) phosphate was determined with an authentic sample that was a gift of Dr. J. F. Wherrett, Toronto, Ont., Canada. After transesterification of phospholipids, fatty acid methyl esters were separated and quantified as recently described.

PF-3 activity. The method of Spaet and Cintron was used to assay each platelet fraction at four concentrations: undiluted, and diluted 1:2, 1:5, 1:10, in 0.15 M NaCl to give protein concentrations, respectively, of 45, 22.5, 9.0, and 4.5 µg per assay. The concentration of Russell’s viper venom (RVV) was adjusted so that the clotting time with no added platelet material was 25 sec and with an excess of platelet material was never shorter than 7 sec. Storage of subcellular fractions for several days at 4°C or –20°C did not change their clot-promoting activities.

Chemicals. All chemicals were of reagent grade. Methanol and chloroform were redistilled before use. Enzyme substrates and RVV were obtained from Sigma Chemical Co. (St. Louis, Mo.).

RESULTS

Phospholipid Composition (Table 1)

Fifty-eight per cent of the total platelet phospholipid was recovered from the membranous fractions (zones 2 and 3). The remaining phospholipid was rather evenly distributed among the other particulate fractions. The ratio of phospholipid to protein in particulate fractions was highest in the membranes and lowest in zone 7, where alpha granules predominated overwhelmingly.

Choline phospholipids. PC decreased only in those zones where sphingomyelin was increased, a reciprocity between the major choline phospholipids which has previously been pointed out in comparisons of phospholipid composition of different cells of the same species or the same cell in different species.
### Table 1. Distribution of Phospholipid Phosphorus in Subcellular Fractions of Human Platelets

<table>
<thead>
<tr>
<th>Phospholipid Class</th>
<th>Zone 1</th>
<th>Zone 2</th>
<th>Zone 3</th>
<th>Zone 4</th>
<th>Zone 5</th>
<th>Zone 6</th>
<th>Zone 7</th>
<th>Zone 8</th>
<th>Zone 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>Choline PL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>37.8</td>
<td>41.8</td>
<td>39.5</td>
<td>38.6</td>
<td>39.3</td>
<td>38.4</td>
<td>31.6</td>
<td>31.8</td>
<td>34.5</td>
</tr>
<tr>
<td>SM</td>
<td>15.1</td>
<td>13.9</td>
<td>14.6</td>
<td>12.1</td>
<td>15.8</td>
<td>16.4</td>
<td>21.6</td>
<td>23.6</td>
<td>22.8</td>
</tr>
<tr>
<td>LPC</td>
<td>1.0</td>
<td>1.3</td>
<td>0.9</td>
<td>0.6</td>
<td>0.5</td>
<td>0.5</td>
<td>1.0</td>
<td>1.3</td>
<td>1.4</td>
</tr>
<tr>
<td>Ethanolamine PL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasmalogen PE</td>
<td>17.9</td>
<td>25.2</td>
<td>17.4</td>
<td>17.8</td>
<td>15.5</td>
<td>15.4</td>
<td>14.9</td>
<td>19.2</td>
<td>15.0</td>
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<tr>
<td>Diacyl PE</td>
<td>13.6</td>
<td>11.4</td>
<td>14.4</td>
<td>13.4</td>
<td>12.9</td>
<td>11.4</td>
<td>9.0</td>
<td>10.3</td>
<td>13.0</td>
</tr>
<tr>
<td>Plasmalogen PE/</td>
<td>1.32</td>
<td></td>
<td>1.53</td>
<td>1.13</td>
<td>1.16</td>
<td>1.19</td>
<td>1.70</td>
<td>2.13</td>
<td>1.46</td>
</tr>
<tr>
<td>Diacyl PE</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Other PL</td>
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<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PS</td>
<td>9.0</td>
<td>10.5</td>
<td>8.9</td>
<td>9.6</td>
<td>8.8</td>
<td>7.9</td>
<td>7.9</td>
<td>9.6</td>
<td>9.4</td>
</tr>
<tr>
<td>PI</td>
<td>3.6</td>
<td>4.6</td>
<td>4.4</td>
<td>4.6</td>
<td>3.8</td>
<td>3.5</td>
<td>3.8</td>
<td>2.8</td>
<td>4.6</td>
</tr>
<tr>
<td>DPI*</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.7</td>
<td>0.9</td>
<td>0.9</td>
<td>1.1</td>
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<tr>
<td>DPG</td>
<td>0.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.7</td>
<td>2.3</td>
<td>2.5</td>
<td>1.9</td>
</tr>
<tr>
<td>Origin</td>
<td>0.8</td>
<td>1.6</td>
<td>1.0</td>
<td>0.6</td>
<td>0.7</td>
<td>1.2</td>
<td>1.0</td>
<td>0.9</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Data shown for each phospholipid are the means of four analyses as per cent phospholipid phosphorus recovered from whole homogenate (H) and the nine subcellular fractions that can be obtained by the technique of Broekman et al.\(^\text{13}\) For preparation of platelet fractions, separation of phospholipids, and phospholipid analysis, see Materials and Methods.

For most abbreviations, see text. Other abbreviations: PL, phospholipid; SM, sphingomyelin; tr, trace (< 0.5%); --, not determined.

*The only member of the polyphosphoinositides that was found in more than trace amounts.

**P** shown as nm/mg and per cent of the total **P** phosphorus, 96% ± 5% of which was recovered from the gradient.

The abrupt change in the sphingomyelin content between the upper and lower halves of the gradient, as defined by zones 5 and 6, respectively, was most striking. Marcus et al.\(^\text{23}\) found a similar trend, but not of such sharpness or magnitude. By contrast, Kaulen and Gross,\(^\text{24}\) using EDTA-anticoagulated blood for platelet procurement, found the highest content of sphingomyelin in membranes.

The LPC of the membranous zones, 2 and 3, had an unsaturated/saturated fatty acid ratio of approximately 0.2, and thus was predominantly of the 1-acyl configuration. By contrast, a value of 1 was reported for the same ratio in human plasma LPC\(^\text{25}\) reflecting an equal distribution of 1-acyl and 2-acyl derivatives. Thus, we suggest that the membrane LPC was not adsorbed from plasma but probably derived from endogenous phospholipase A\(_2\) activity.\(^\text{20}\)

**Ethanolamine phospholipids.** Platelets resemble brain\(^\text{26}\) and erythrocytes\(^\text{26}\) in their high content of plasmalogen PE. The latter peaked at the expense of diacyl PE, in zones 6 and 7, where alpha granules predominate heavily. The relationship, if any, between plasmalogen PE or sphingomyelin content and other constituents of alpha granules\(^\text{27}\) remains to be elucidated.

**Minor phospholipids.** The combined harvest of DPI from the homogenate as well as the fractions was less than one might have expected from our previous data on lipid extracts derived from intact platelets.\(^\text{15}\) This finding sug-
Table 2. Distribution of Fatty Acids (FA) Among the Phospholipids of Zone 2 (Membranes)

<table>
<thead>
<tr>
<th>Phospholipid</th>
<th>FA Types*</th>
<th>Total FA</th>
<th>U/S</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>16:0</td>
<td>18:0</td>
<td>18:1</td>
</tr>
<tr>
<td>Choline</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC</td>
<td>73.2</td>
<td>38.6</td>
<td>74.1</td>
</tr>
<tr>
<td>SM</td>
<td>19.8</td>
<td>5.5</td>
<td>0.7</td>
</tr>
<tr>
<td>LPC</td>
<td>1.2</td>
<td>1.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Noncholine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PPE</td>
<td>0.7</td>
<td>2.1</td>
<td>1.9</td>
</tr>
<tr>
<td>DPE</td>
<td>4.0</td>
<td>20.0</td>
<td>7.7</td>
</tr>
<tr>
<td>PS</td>
<td>0.4</td>
<td>22.4</td>
<td>15.5</td>
</tr>
<tr>
<td>PI</td>
<td>0.7</td>
<td>10.0</td>
<td>2.5</td>
</tr>
<tr>
<td>Total FA</td>
<td>20.1</td>
<td>16.2</td>
<td>16.2</td>
</tr>
</tbody>
</table>

On the left and top, respectively, are listed the human platelet's seven major phospholipids and seven major FA types. Given for each of the FA are the percentage content among (1) all phospholipids (vertical listing); (2) all FA that are esterified to all phospholipids (total FA, horizontal listing); and (3) all FA that are esterified to a given phospholipid (total FA, vertical listing). On the far right are given the unsaturated/saturated (U/S) FA ratios for the diacyl phospholipids. The raw data from which these results were derived will be sent upon request.

For preparation of membranous fractions, separation of phospholipids, and phospholipid and fatty acid analysis, see Materials and Methods.

For most abbreviations, see text. Other abbreviations: SM, sphingomyelin; PPE, plasmalogen PE; DPE, diacyl PE.

*FA are designated by the number of C atoms:number of double bonds.
‡16:1, 18:3, 20:0, 22:0, 22:1, 24:0, 24:1.

gested that the activity of endogenous phosphatase was enhanced by homogenization and fractionation.15,28

DPG peaked in fractions 5-7, where mitochondria were most abundant. Pertinently, this was the first phospholipid that qualified as a marker since Marinetti et al. showed its association with mitochondria.29 In studies with platelets Marcus et al. found an increase in DPG in a granular fraction that was a mixture of all nonmembranous particles.23 Because of the greater purity of our mitochondrial fractions, our data are the first to show clearly a marker role for DPG in platelets.

No bis-(monoacylglyceryl) phosphate, a putative lysosomal marker,19 was found in any subcellular fraction, even in concentrated samples.

Fatty Acid Composition

As previously shown for whole human erythrocytes30 and platelets,14,23 as well as platelet fractions obtained by the Teflon-pestle technique,23 a characteristic fatty acid predominance was associated with each phospholipid in all zones: PC (16:0, 18:1),* sphingomyelin (16:0, 20:0, 22:0, 24:0, 24:1), diacyl PE (18:0, 20:4), plasmalogen PE (20:4, higher polyenoic acids), PS (18:0, 20:4), PI (18:0, 20:4), and DPG (18:1, 18:2).

As shown in Table 2 the platelet's fatty acids were asymmetrically distributed

*Fatty acids are designated by the number of C-atoms:number of double bonds.
Fig. 1. PF-3 activity in human platelet subcellular fractions. See Methods for details of platelet fractionation and PF-3 assay. On the ordinate is the Russell's viper venom clotting time in seconds; on the abscissa, the concentration of platelet protein in the PF-3 assay. At 9 μg, three groupings are apparent from greatest to least acceleration of clotting: 2–4; 5, 6, 8, 9, and homogenate; 1, 7.

among the choline versus the noncholine phospholipids of the membrane. The choline phospholipids held 94%, 72%, and 85%, respectively, of the total content of 16:0, 18:1, and 18:2. By contrast with 16:0, 55% of the 18:0 was found in noncholine phospholipids. Pertinently, 72% of 20:4, which is preferentially associated with 18:0 in molecular species of phospholipids of various origins, was esterified to the noncholine group. The latter also held 67% of the higher polyenoic acids other than 20:4. However, excluding plasmalogens in which higher polyenoics are disproportionately represented, the latter were more abundant in PC than the noncholine fractions. Also, considering only the diacyl species, the unsaturated/saturated fatty acid ratios of the noncholine phospholipids were considerably higher than that of PC.

Clot-promoting Activity (PF-3)

Figure 1 relates clot-promoting activity to protein content of the fractions; Table 3 to phospholipid phosphorus content.

Clot-promoting activity was tested at four concentrations of protein (Fig. 1). The results with the nine zones fell into three groupings which were most clearly delineated at 9 μg protein/assay. At this concentration, the clot-promoting activity of zones 1 and 7 was considerably diminished, whereas that of zones 2, 3, and 4 was much more difficult to attenuate. The clot-promoting activity of the remaining zones clustered about the values for whole homogenate.

Table 3 compares the clotting time with the phospholipid phosphorus content per assay of ten selected points in Fig. 1. The results with the nine zones fell into four groupings. At similar concentrations of phospholipid phosphorus, the membranous zones gave considerably longer RVV clotting times than zones.
Table 3. Relationship of Phospholipid Phosphorus Content and Clot-promoting Activity of Subcellular Fractions of Human Platelets

<table>
<thead>
<tr>
<th>Zone</th>
<th>nmoles Phospholipid Phosphorus/Assay</th>
<th>Russell’s Viper Venom Clotting Time (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>Membranes</td>
<td>2.21</td>
</tr>
<tr>
<td>3</td>
<td>Membranes</td>
<td>2.46</td>
</tr>
<tr>
<td>4</td>
<td>Mitochondria</td>
<td>2.60</td>
</tr>
<tr>
<td>5</td>
<td>Acid hydrolases</td>
<td>2.41</td>
</tr>
<tr>
<td>9</td>
<td>Debris</td>
<td>2.32</td>
</tr>
<tr>
<td>6</td>
<td>Mitochondria</td>
<td>1.67</td>
</tr>
<tr>
<td>7</td>
<td>Alpha granules</td>
<td>1.54</td>
</tr>
<tr>
<td>8</td>
<td>Alpha granules</td>
<td>1.63</td>
</tr>
<tr>
<td>1</td>
<td>Supernatant</td>
<td>0.30</td>
</tr>
<tr>
<td>H</td>
<td>Homogenate</td>
<td>1.49</td>
</tr>
</tbody>
</table>

Data were derived from Table 1 and selected points on Fig. 1.

*Numbers and characterizations of the nine subcellular fractions of human platelets that can be separated using our recently reported method. The zones are arranged in four groupings according to phospholipid phosphorus content.

DISCUSSION

Our data show that (1) there is a marked asymmetry in distribution of some phospholipids and fatty acids among subcellular fractions of human platelets, and (2) on the basis of the RVV time, clot-promoting activity of the fractions does not correlate with their phospholipid phosphorus content.

When the clot-promoting activity of the fractions was assayed at equivalent protein concentrations, the membranes were more active than the intracellular organelles (Fig. 1). This finding agrees with previous studies by Marcus et al. and Day et al. However, protein concentration may be an inappropriate benchmark for assessing clot-promoting activity by the RVV time, since the latter is considered to be dependent on phospholipid. Pertinently, the ratio of phospholipid to protein varies widely over the gradient, with membranes having the highest, and alpha granules the lowest ratio among the particulate fractions (Table 1). Yet, on the basis of phospholipid concentration, the alpha granules hold more clot-promoting activity than the membranes (Table 3). These data do not fit with the prevailing view that attributes most of the clot-promoting activity of platelets to their membranes. The findings cannot be accounted for by differences in phospholipid or fatty acid composition. Thus, it seems ap-
propriate to reconsider both the nature and availability of PF-3 activity in the whole platelet as well as its subcellular fractions.

PF-3 activity may be defined as a clot-promoting capacity that resides in all mammalian tissues and is universally unmasked by partial or total disruption of the cells. Intact cells keep their clot-promoting activity quiescent unless, as in the case with erythrocytes and platelets, the cells are exposed to hypotonic media\textsuperscript{10} or certain penetrating nonelectrolytes.\textsuperscript{10,32} Otherwise, erythrocytes must be lysed to show clot-promoting activity. Platelets are unique in that their clot-promoting activity is made available at a critical point in the enactment of the cell's physiologic role. PF-3 activation in vitro usually develops in parallel with platelet aggregation,\textsuperscript{33} but may depend upon the release reaction.\textsuperscript{9}

The source of the clot-promoting activity is not known. If it were simply a matter of furnishing phospholipid, the membranes, with 58\% of the total (Table 1), should play a dominant role. However, the concentration of phospholipid would appear to be less important than its availability. The intact, unstimulated platelet keeps its PF-3 reactive material hidden. The release reaction, in addition to other effects, makes PF-3 activity available. Whether this PF-3 activity derives from those subcellular constituents that are major participants in the release reaction (zones 4-9) or from a parallel reorientation of molecular components of the membrane (zone 2) remains to be determined. In this connection, it is useful to review certain aspects of PF-3 activity. Three considerations come to mind.

(1) The composition of the phospholipids themselves is important. With micelles and liposomes made from synthetic phospholipids, clot-promoting activity is enhanced as the negative charge\textsuperscript{5,8} or the unsaturation of the fatty acids\textsuperscript{4,7} increases. However, none of our subcellular fractions contained, on a percentage basis, an excess of negatively charged phospholipids or diunsaturated molecular species, as compared with other fractions.

(2) In the natural state the clot-promoting ability of phospholipids could be influenced by interactions with other membrane constituents. Pertinently, Niemetz and Marcus have recently demonstrated a role for platelets as clot promoters by showing that the procoagulant activity of leukocytes is enhanced in the presence of whole platelets.\textsuperscript{33} Possibly, our intact alpha granules presented a favorable surface for the clotting reactions of the PF-3 assay.

(3) We believe that more attention should be paid to the latency of PF-3 activity as related to newly developing concepts of membrane structure. Clearly PF-3 activity is normally not accessible to the plasma cofactors with which it reacts. It is therefore unlikely that the PF-3 reactive material is on the outer surface of the cell. In this context, recent information on the asymmetric distribution of membrane phospholipids is of interest. In human erythrocytes, the more thromboplastic, noncholine phospholipids are almost exclusively located on the inner side of the cell membrane\textsuperscript{34,36} where they would have no direct contact with the plasma. These noncholine phospholipids—diacyl PE, PS, PI—are clot promoting in several combinations,\textsuperscript{4,37} probably owing to the greater abundance of more negatively charged polar groups\textsuperscript{5,8} and diunsaturated molecular species\textsuperscript{5,7} in their mixed micelles. By contrast, the outer layer of the human erythrocyte\textsuperscript{34,36} contains the phospholipids, PC and sphingomyelin, that in various combinations, particularly those with the highest PC content, have
anticoagulant properties. A preliminary report indicates that the platelet’s PE is almost exclusively located on the inner surface of the membrane, providing a basis for the groupings in Table 2 that show the remarkably different fatty acid composition of the choline and noncholine phospholipids.

The foregoing seeks an explanation for the fact that the membranes are, on the basis of phospholipid phosphorus content, the least active of the particulate fractions (Table 3). In the same context, the alpha granules’ rather vigorous clot-promoting activity, in association with a relatively low phospholipid phosphorus content (Table 3), requires some explanation. It would appear that the PF-3 activity of intact alpha granules (see Fig. 5 of ref. 13) is more accessible to its reaction partners than the PF-3 activity of intact platelets is known to be. This observation suggests that, in addition to holding fibrinogen and PF-4 activity, alpha granules can also be important contributors to PF-3 activity.

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REFERENCES
DISTRIBUTION OF PHOSPHOLIPIDS


Distribution of phospholipids, fatty acids, and platelet factor 3 activity among subcellular fractions of human platelets

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