Biochem. J. (1976) **158**, 283–287
Printed in Great Britain

Arachidonic Acid Release from Diacyl Phosphatidylethanolamine by Human Platelet Membranes

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(*Received* 4 *March* 1976)

At pH9.5 in the presence of 10mM-Ca^{2+}, human platelet membranes released 22% (167 of 785nmol) of arachidonic acid that was esterified to phospholipids. With the use of synthetic choline (dinonadecanoyl) and ethanolamine (diheptadecanoyl) phosphoglycerides as internal reference compounds, 115nmol of the released arachidonic acid was shown to be derived from endogenous breakdown of the phosphatidylethanolamine fraction. Further, the lysophosphatidylethanolamine that was released along with the arachidonic acid was shown virtually to lack fatty aldehydes and to contain a preponderance of fatty acids that have a preference for esterification at the 1-position of naturally occurring phosphatidylethanolamine of human platelets. These findings ruled out plasmalogen phosphatidylethanolamine as the source of the released arachidonic acid. We conclude that diacyl phosphatidylethanolamine was the principal source of arachidonic acid released by human platelet membranes under the conditions described.

Arachidonic acid-derived endoperoxides and thromboxanes are now recognized to be of great importance in the platelet aggregation and release reactions (Samuelsson *et al.*, 1975; Kolata, 1975). Virtually all of the arachidonic acid in platelet membranes is esterified, predominantly to phospholipids (Cohen & Derksen, 1969; Derksen & Cohen, 1975). A report from our laboratory described release of arachidonic acid from the phospholipid, as opposed to the neutral lipid, fraction of human platelet membrane preparations incubated at pH9.5 in the presence of 10mM-Ca^{2+} (Derksen & Cohen, 1975). The present study identifies diacyl phosphatidylethanolamine as the phospholipid that accounts for two-thirds of the liberated arachidonic acid under the conditions described previously.

Experimental

Washed platelets were prepared by differential centrifugation of fresh acid/citrate/dextrose-treated blood in a plastic pack system (Fenwal Laboratories, Deerfield, IL, U.S.A.) and resuspended in 1.15% (v/v) KCl to a protein concentration of 25mg/ml (Derksen & Cohen, 1973), before homogenization by the nitrogen-decompression technique (Broekman *et al.*, 1974). Subcellular fractionation was done at 5°C in Beckman rotors in a Beckman model L5-50 ultracentrifuge (Beckman Instruments, Palo Alto, CA, U.S.A.). Ultracentrifugations were for 85min at 163440*g* in an SW-40 rotor (r_{av}. 11.27cm) on a linear sucrose gradient (30–50%, w/w). The top soluble fraction (zone 1; see Broekman *et al.*, 1974) was discarded and the membranous layers (zones 2 and 3; Broekman *et al.*, 1974) were collected into 1.15% KCl and sedimented at 130000*g* for 40min in a 42.1 rotor (r_{av}. 6.9cm). The pellets were combined, then washed and resuspended in 1.15% KCl to a protein concentration of 2mg/ml, as determined by the biuret method (Gornall *et al.*, 1949). Incubations were begun immediately after isolation of the membranous preparation, and contained in 2.0ml: 200μmol of glycine buffer adjusted with HCl to a pH of 9.5; 20μmol of $CaCl_2$; 20mg of essential fatty acid-free bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.); 2.5mg of protein as membranes. Incubations were for 3h at 37°C in a Dubnoff shaker. Reactions were stopped by addition of 7ml of chloroform/methanol (2:5, v/v), in which were dissolved the following internal standards: 50mg of L-α-dinonadecanoyl phosphatidylcholine (custom synthesis; Supelco, Bellefonte, PA, U.S.A.); 50mg of L-α-diheptadecanoyl phosphatidylethanolamine (custom synthesis, Supelco); 10mg of heneicosanoic acid (Supelco).

Lipids were extracted by the method of Bligh & Dyer (1959). Two t.l.c. systems were used. The first separated phospholipids on 0.5mm-thick silica gel H (Merck, Rahway, NJ, U.S.A.) impregnated with Na_2CO_3 (Guarnieri, 1975), with a developing solvent of chloroform/methanol (50:1, v/v), and in the same dimension, chloroform/methanol/acetic acid/water (100:60:16:7, by vol.). The second used 0.25mm-thick silica gel H and developing solvent of hexanes/diethyl ether/formic acid (550:450:7, by vol.) to separate non-esterified fatty acids from other neutral

lipids while keeping phospholipids at the origin (Derksen & Cohen, 1973). The separated lipids were detected by spraying with Rhodamine 6G, scraped, and methylated as described by Cohen & Derksen (1969). The methylated fatty acids, and, for phosphatidyl ethanolamine, the fatty aldehydes, were separated by g.l.c. and quantified as described by Derksen & Cohen (1975).

Results and Discussion

Some 58% of the phospholipids of human platelets are in the membranes (Broekman *et al.*, 1976). The phospholipids, in turn, contain 99% of the membranes' arachidonic acid (Derksen & Cohen, 1975), a fatty acid that shows a greater than 95% preference for esterification at the 2-position (van Golde, 1968). Thus release of arachidonic acid should be traceable to phospholipase activity. Previous studies from our laboratory showed that depletion of the arachidonic acid content of phospholipids accompanied its release (Derksen & Cohen, 1975). Under identical incubation conditions, the present work identifies phosphatidylethanolamine as the principal source of the released arachidonic acid.

Table 1 shows that in 3h at pH9.5 in the presence of 10mM-Ca^{2+}, a 2.5mg protein equivalent of platelet membranes released its five major fatty acids in the following amounts: palmitic acid, 58nmol; stearic acid, 64nmol; oleic acid, 36nmol; linoleic acid, 11nmol; arachidonic acid, 167nmol. Of the two most abundantly released fatty acids, 69% (115 of 167nmol) of the arachidonic acid, and 72% (46 of 64nmol) of the stearic acid came from the phosphatidylethanolamine fraction. This observation is notable because these fatty acids frequently pair in molecular species of all diacyl phospholipids, but are most abundant in the anionic group of phospholipids (ethanolamine, inositol and serine phosphoglycerides) of human platelets (Cohen & Derksen, 1969; Marcus *et al.*, 1969). The remainder of the released arachidonic acid and stearic acid, 28% (47nmol) and 25% (16nmol) respectively, was derived almost entirely from phosphatidylcholine as opposed to serine or inositol phosphoglycerides. By subtraction, only 3% of the released arachidonic acid (5 of 167nmol) and stearic acid (2 of 64nmol) originated in a combined fraction of inositol and serine phosphoglycerides. Of the three remaining fatty acids, 69% of the palmitic acid (39 of 58nmol), 58% of the oleic acid (21 of 36nmol) and 55% of the linoleic acid (6 of 11nmol) was released from phosphatidylcholine.

That palmitic acid was released in such relative abundance is attributable to the activity of a phospholipase A_1 whose pH optimum (pH8.5) is close to that for phospholipase A_2 activity (pH9.5) (Derksen & Cohen, 1975). If the incubations had been at pH8.5 instead of pH9.5, relatively more palmitic acid (as well as stearic acid and oleic acid) and less arachidonic

Table 1. *Release of five major fatty acids from endogenous phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of human platelet membranes*

Reaction mixtures containing 2.5mg of protein equivalents of human platelet membranes were incubated at 37°C for 3h at pH9.5 in the presence of 10mM-Ca^{2+}. Reactions were stopped by addition of chloroform/methanol (2:5, v/v), in which were dissolved the internal standards used to quantify release of non-esterified fatty acids from endogenous phosphatidylcholine and phosphatidylethanolamine (see Derksen & Cohen, 1975). Lipids were extracted as described by Bligh & Dyer (1959) and separated by two systems of t.l.c. as described in the Experimental section, before saponification and quantification by g.l.c. (Derksen & Cohen, 1975). In displaying the data it was assumed that within the phosphatidylethanolamine fraction all released non-esterified fatty acids were derived from diacyl phosphatidylethanolamine. For proof that plasmalogen phosphatidylethanolamine does not break down under the given incubation conditions, see Fig. 1. Results shown are the means of four experiments, each in triplicate. Abbreviations: $C_{16:0}$, palmitic acid; $C_{18:0}$, stearic acid; $C_{18:1}$, oleic acid; $C_{18:2}$, linoleic acid; $C_{20:4}$, arachidonic acid; DPE, diacyl phosphatidylethanolamine; PPE, plasmalogen phosphatidylethanolamine.

	$C_{16:0}$		$C_{18:0}$		$C_{18:1}$		$C_{18:2}$		$C_{20:4}$			
	PC	PE	PC	PE	PC	PE	PC	PE	PC	PE	DPE	PPE
Esterified fatty acids in unincubated membranes (nmol)	401	57	209	149	329	41	120	23	204	411	197	214
Fatty acids released from incubated membranes (nmol)	58		64		36		11		167			
Fatty acids released from PC and PE (nmol)	39	18	16	46	21	13	6	5	47	115	115	0
Fatty acids released from sources other than PC or PE (nmol)	1		2		2		1		5			
$\frac{\text{Fatty acid released from a phospholipid}}{\text{Fatty acid esterified to that phospholidip}}$ (%)	10	31	8	31	7	31	5	23	22	28	58	0
$\frac{\text{Fatty acid released from PC or PE}}{\text{Fatty acid released from PC + PE}}$ (%)	67	32	26	74	63	36	53	47	28	72	72	0

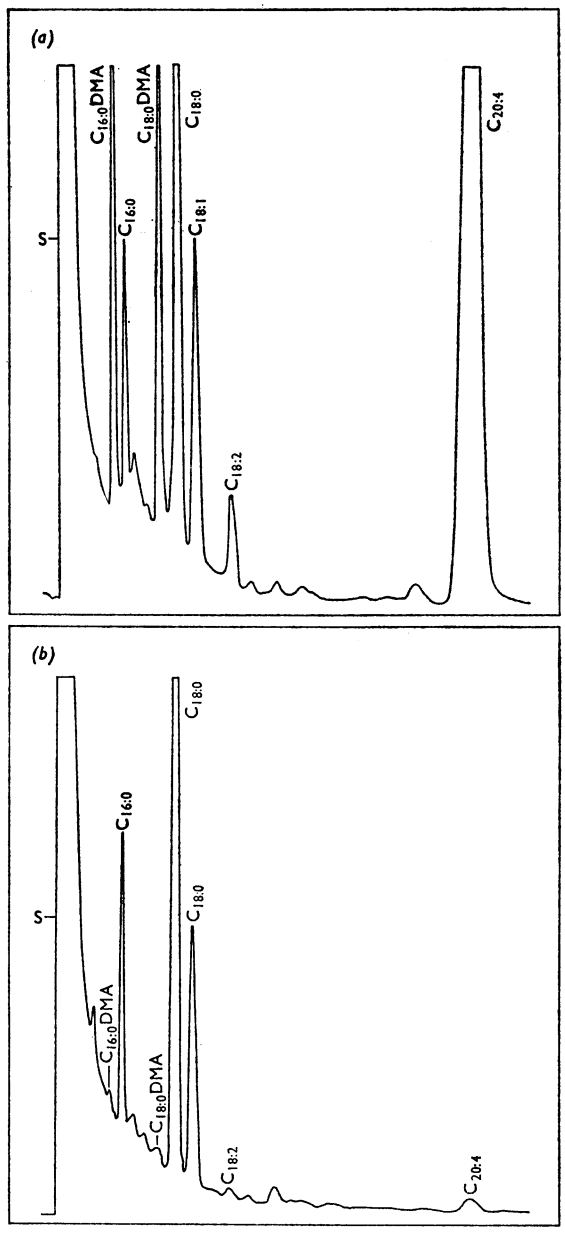

Fig. 1. *G.l.c. patterns of methylated fatty side chains of phosphatidylethanolamine (a) and lysophosphatidylethanolamine (b) fractions which were isolated by t.l.c. after incubation of 2.5 mg of protein equivalents of human platelet membranes at 37°C for 1 h at pH 9.5 in the presence of 10 mM-Ca^{2+}*

For other details, see the Experimental section. The phosphatidylethanolamine, comprised of nearly equal amounts of diacyl and plasmalogen forms, contained fatty aldehydes as well as a rich supply of arachidonic acid. The lysophosphatidylethanolamine contained an abundance of stearic acid, a paucity of arachidonic acid and virtually no fatty aldehydes (<0.5% of the total in phosphatidylethanolamine). This combination of findings proves that within the phosphatidylethanolamine fraction the phospholipase attack was directed towards the 2-position of the diacyl as opposed to the plasmalogen form of the lipid. Abbreviations: S, solvent front; DMA, dimethyl acetal derivative. For other abbreviations, see Table 1.

acid would have been released (Derksen & Cohen, 1975).

The release of fatty acids was accompanied by an accumulation of lysophosphatidylethanolamine and, to a considerably lesser extent, lysophosphatidylcholine, as revealed on thin-layer chromatograms that were stained with Rhodamine 6G. G.l.c. analysis of the lysophosphatidylethanolamine (Fig. 1) showed that it contained (1) virtually no fatty aldehydes, (2) only a trace of arachidonic acid and (3) a preponderance of those fatty acids, particularly stearic acid, that share an overwhelming preference for esterification at the 1-position of diacyl phospholipids. Simultaneous accumulation of stearic acid and stearic acid-rich lysophosphatidylethanolamine was probably attributable to the fact that the pH of the reaction mixture (pH9.5) was far removed from the reported pH optimum (pH6.5) for lysophospholipase activity (see McMurray & Magee, 1972).

The paucity of fatty aldehydes in the liberated lysophosphatidylethanolamine demonstrates that the arachidonic acid was liberated from the diacyl as opposed to the plasmalogen fraction of the ethanolamine phosphoglycerides. Plasmalogens (phosphoglycerides in which the 1-position moiety is a long alkyl chain joined by an ether linkage) in human platelets are virtually confined to the ethanolamine phosphoglyceride fraction, comprising 52% of this fraction, and have the highest percentage of arachidonic acid at the 2-position of any phospholipid (Cohen & Derksen, 1969). That plasmalogens did not break down is important for two reasons.

First, available evidence shows that the 2-position fatty acids of plasmalogens are apparently less readily hydrolysed than those in diacyl phospholipids. For example, the testes of rats with essential fatty acid deficiency maintain nearly normal arachidonic acid content in their plasmalogen phosphatidylethanolamine, although eicosatrienoic acid extensively replaces arachidonic acid in most other phospholipids, including diacyl phosphatidylethanolamine (Blank *et al.*, 1973). In our experiments the plasmalogens proved equally unresponsive to events that markedly affected the diacyl compounds.

Second, there was no preferential release of arachidonic acid from the ethanolamine as compared with the choline phosphoglyceride fractions; each liberated approximately one-fourth of its arachidonic acid (Table 1). Because none of the released arachidonic acid originated in plasmalogens, it is apparent that, among diacyl phosphoglycerides, the ethanolamine form was a preferred substrate for the observed phospholipase activity. Thus the diacyl phosphatidylethanolamine, which comprises 15% of platelet membrane phospholipids and contains 25% of its arachidonic acid, accounted for 69% of the arachidonic acid that was released. These data allow assignment to diacyl phosphatidylethanolamine of a major role as provider of free arachidonic acid, and strongly suggest fulfilment of the role through the mediation of phospholipase A_2 activity. The degree of specificity of this enzyme needs to be resolved. Two possibilities come to mind.

The observed activity could be part of a large family of monospecific enzymes, each directed towards the 2-position of a given phospholipid or perhaps even a single molecular species of that phospholipid. There is a precedence in lipid biochemistry for the latter possibility, in that families of monospecific acyl synthetases (Pande & Mead, 1968) and acyltransferases (Lands & Merkle, 1963) have been described. However, there is as yet no evidence for analogous families of phospholipases.

On the other hand, the phospholipase A_2 activity may be group-specific, that is directed towards the 2-position of more than one phospholipid, as was shown with rat liver mitochondria both *in vivo* (Bjørnstad, 1966) and *in vitro* (Scherphof & van Deenen, 1965), where the phosphatidylethanolamines were found to be favoured, as opposed to exclusive, substrates for phospholipase A_2 activity. This possibility is supported by the data of Table 1, which document breakdown of phosphatidylcholine as well as diacyl phosphatidylethanolamine. That the latter liberated relatively more of its arachidonic acid (58%) than the former (22%) could, as suggested by Derksen & Cohen (1975), be secondary to anatomical sequestration of the enzyme on the inner membrane, where it would have greater access to the anionic phospholipids, including diacyl phosphatidylethanolamine, which other evidence suggests are also localized on the inner membrane (Bretscher, 1972; Verkley *et al.*, 1973).

Finally, our results and conclusions are at variance with a report by Bills *et al.* (1976), who labelled the phospholipids of whole platelets by incubation with [^{14}C]arachidonic acid and found that thrombin released the label mainly from the choline and inositol phosphoglycerides. Their studies were more physiologically appropriate than ours in that they used whole platelets instead of membranes, a pH of 7.4 as compared with a pH of 9.5, and thrombin as a natural stimulus for initiating arachidonic acid release. However, our method for measuring fatty acid release has distinct advantages. We measured release of arachidonic acid that was esterified under natural conditions *in vivo* as opposed to [^{14}C]arachidonic acid that was esterified *in vitro*. The distribution of the latter (see Table III, Bills *et al.*, 1976) apparently did not match its relative abundance in natural phospholipids (Cohen & Derksen, 1969; Marcus *et al.*, 1969). Thus the ethanolamine and serine phosphoglycerides in the platelet preparations of Bills *et al.* (1976) contained unnaturally low amounts of [^{14}C]arachidonic acid, and should not have been expected to release as much of this marker as the more heavily

labelled choline and inositol fractions. Further studies will be needed to resolve the conflicting results obtained by the two laboratories.

Platelet suspensions were derived from blood that was drawn from normal donors (Blood Bank, Massachusetts General Hospital, Boston, MA, U.S.A.) or from informed volunteers who underwent a two-unit plasmapheresis (Blood Bank, Peter Bent Brigham Hospital, Boston, MA, U.S.A.). Laura Varnum, R.N., performed the plasmapheresis. This work was supported by grants HL 13802 and 13584 from the Public Health Service and contract DADA 17-70-C-0083 from the Army.

References

Bills, T. K., Smith, J. B. & Silver, M. J. (1976) *Biochim. Biophys. Acta* **424**, 303–314

Bjørnstad, P. (1966) *J. Lipid Res.* **7**, 612–620

Blank, M. L., Wykle, R. L. & Snyder, F. (1973) *Biochim. Biophys. Acta* **316**, 28–34

Bligh, E. G. & Dyer, W. J. (1959) *Can. J. Biochem. Physiol.* **37**, 911–917

Bretscher, M. S. (1972) *J. Mol. Biol.* **71**, 523–528

Broekman, M. J., Westmoreland, N. P. & Cohen, P. (1974) *J. Cell Biol.* **60**, 507–519

Broekman, M. J., Handin, R. I., Derksen, A. & Cohen, P. (1976) *Blood* **47**, 963–971

Cohen, P. & Derksen, A. (1969) *Br. J. Haematol.* **17**, 359–371

Derksen, A. & Cohen, P. (1973) *J. Biol. Chem.* **248**, 7396–7403

Derksen, A. & Cohen, P. (1975) *J. Biol. Chem.* **250**, 9342–9347

Gornall, A. G., Bardawill, C. J. & David, M. M. (1949) *J. Biol. Chem.* **177**, 751–766

Guarnieri, M. (1975) *Lipids* **10**, 294–298

Kolata, G. B. (1975), *Science* **190**, 770–771, 812

Lands, W. E. M. & Merkle, I. (1963) *J. Biol. Chem.* **238**, 898–904

Marcus, A. J., Ullman, H. L. & Safier, L. B. (1969) *J. Lipid Res.* **10**, 108–114

McMurray, W. C. & Magee, W. L. (1972) *Annu. Rev. Biochem.* **41**, 129–160

Pande, S. V. & Mead, J. F. (1968) *J. Biol. Chem.* **243**, 352–361

Samuelsson, B., Granström, E., Gréen, K., Hamberg, M. & Hammarström, S. (1975) *Annu. Rev. Biochem.* **44**, 669–695

Scherphof, G. L. & van Deenen, L. L. M. (1965) *Biochim. Biophys. Acta* **98**, 204–206

van Golde, L. M. G. (1968) Ph.D. Thesis, University of Utrecht

Verkley, A. J., Zwaal, R. F. A., Roelofsen, B., Comfurius, P., Kastelijn, D. & van Deenen, L. L. M. (1973) *Biochim. Biophys. Acta* **323**, 178–193