LOCALISATION OF PHOSPHOLIPASE A_2 AND DIGLYCERIDE LIPASE ACTIVITIES IN HUMAN PLATELET INTRACELLULAR MEMBRANES

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1. Introduction

The liberation of arachidonic acid from endogenous phospholipids and its conversion to prostaglandins and related compounds may be critical events in platelet haemostatic behaviour since it is well established that prostaglandin production by platelets is increased during aggregation [1,2]. There are two enzymes known to be present in platelets, phospholipase A_2 and diglyceride lipase [3,4] which could be responsible for providing this fatty acid precursor by their action on suitable endogenous lipid substrates. These enzymes are involved in two separate pathways, phospholipase A_2 acting upon phosphatidylcholine and/or phosphatidylinositol and diglyceride lipase acting upon the 1,2-diacylglycerol produced by the specific action of phospholipase C on phosphatidylinositol [5,6]. However, knowledge about their subcellular localisation and the relative importance of these two enzymes in platelet function is very sparse indeed since most studies of their activities have been carried out either at the whole cell level or on platelet mixed membrane preparations containing elements of both surface and intracellular origin.

The resting platelet has essentially three major membrane systems:

1. A plasma membrane;
2. A surface connected open canalicular system (OCS) – This is an invaginated membrane domain which electron microscopy has revealed is clearly contiguous with the true surface exposed plasma membrane [7];
3. A dense tubular membrane system (DTS) which appears to be analogous to the endoplasmic reticulum of other cells and often seen in electron-micrographs closely juxtaposed to the OCS though not directly connected to it.

We have developed a procedure for the differential isolation of human platelet surface and intracellular membranes [8]. This is based upon the use of high-voltage free-flow electrophoresis to separate subfractions on a charge-dependent basis from a mixed platelet-membrane population. The separated intracellular and surface membrane vesicles have been characterized by a surface probe, marker enzymes and electron microscopy.

Here we report our studies of the localisation and basal activities of phospholipase A_2 and diglyceride lipase in these surface and intracellular membrane subfractions together with some comments about their possible respective roles in membrane phospholipid modification. Data on the action of thrombin on the diglyceride lipase is also presented.

2. Experimental

2.1. *Membrane preparation*

The surface and intracellular membrane fractions were prepared as in [8]. Briefly, fresh human platelets were washed and exposed to neuraminidase (0.03–0.05 U/ml containing 10^{10} platelets) to partly reduce the sialic acid content of the surface membrane and thus its electronegativity. The platelets were then lysed by ultrasonication and the homogenate separated into discrete mixed membrane and granule fractions on a buffered sorbitol density gradient (1–3.5 M containing 1 mM EDTA and 10 mM Hepes (pH 7.2)). The mixed membrane fraction which located in the low density region of the gra-

dient was removed and applied to the electrophoresis chamber of a Bender Hobein/MSE VAP5 apparatus run at a buffer flow rate of 2 ml/min and 1200 V, 130 mA. The peak of the most electronegative and the peak of the least electronegative membrane subpopulations, representing intracellular membranes and surface membranes, respectively, were separately pooled and stored for analysis at –4°C. When platelets were pretreated with thrombin (2 units/10^9 platelets) they were incubated for 5 min at 37°C just prior to sonication. The rest of the procedure was unchanged.

2.2. *Surface probes and marker enzymes*

For the labelling of the surface membrane the whole platelets were exposed to ^{125}I *Lens culinaris* lectin before sonication. This labelling was done exactly as in [8].

Antimycin-insensitive NADH-cytochrome *c* reductase (EC 1.6.2.1), the marker enzyme for the intracellular membranes was assayed as in [9]. The final reaction mixture contained an aliquot of membrane suspension, 80 mM potassium phosphate buffer (pH 7.0), 80 μM cytochrome *c*, 0.2 mM KCl, 10 μM antimycin (2 mg/ml in ethanol) and 0.85 mM NADH. The reaction was initiated by the addition of the NADH and followed for 5–10 min at 550 nm in a double-beam spectrophotometer.

2.3. *Lipid estimations*

Lipids were extracted from the membrane suspensions with chloroform:methanol (1:2, by vol.) exactly as in [10]. Cholesterol was determined as in [11] and phospholipid phosphorus as in [12]. Phospholipid was calculated by assuming 25 μg phospholipid/μg lipid phosphorus.

2.4. *Phospholipase A_2 assay*

2-[1-^{14}C]Arachidonyl-phosphatidylcholine (29 Ci/mol) was a gift provided by New England Nuclear (Boston, MA). We chose this substrate to measure phospholipase A_2 activity since this enzyme has been reported to be specific for arachidonic acid [13] and to discriminate this lipase from the phospholipase C diglyceride lipase pathway which is specific for phosphatidylinositol [5,6]. The assays were performed in 200 μl 100 mM Tris buffer (pH 9.0) containing 1 mM Ca^{2+}, 1.4×10^{-5} M substrate and 100 μg membrane protein. After 5 min incubation at 37°C the reaction was stopped by addition of 3 vol. ethanol. The ethanol–water extract was purified on thin-layer chromatography (TLC) with chloroform–methanol–water (63:27:4) as eluent. The spots containing arachidonic acid and remaining substrate were scraped off and counted in a liquid scintillation counter and the amount of arachidonic acid released determined.

2.5. *Diglyceride lipase assay*

2-[1-^{14}C]Arachidonyl-1,2-diacylglycerol (29 Ci/mol) was prepared from the labelled phosphatidylcholine by splitting it with a commercial preparation of phospholipase C (Sigma, *C. perfringens*). The labelled diglyceride obtained was extracted 3 times with diethylether and purified on TLC with hexane–diethylether–acetic acid (60:40:1) as eluent and the amount recovered determined by its radioactivity.

The enzyme assay was performed with this substrate under similar conditions to those described above for phospholipase A_2 except that the buffer used was: 50 mM Hepes (pH 7.0) containing 100 mM NaCl and 5 mM Ca^{2+}. The reaction was terminated by additions of chloroform–methanol–hexane and the radioactive arachidonate which partitioned in the aqueous phase as in [4,14] was counted by liquid scintillation.

3. Results

Table 1 shows the justification for referring to these membrane subfractions as surface and intracellular membranes. The iodinated lectin, to which the platelets were exposed at the whole cell level before fractionation, is ~5-fold enriched in the surface membrane fraction and significantly depleted in the intracellular membrane, with respect to both the mixed membrane and homogenate activities, implying almost exclusive localisation. In contrast the antimycin-insensitive NADH-cytochrome *c* reductase activity, a well recognised endoplasmic reticulum marker for other cells, is ~12-fold enriched in the intracellular membrane fraction and depleted in the surface membrane fraction. The lipid distribution figures also support this membrane classification and the cholesterol/phospholipid molar ratio of the surface membrane fraction is ~2.5-times higher than the ratio of these lipids in the intracellular membranes.

Table 2 shows that both the phospholipase A_2 activity and the diglyceride lipase activity are predominantly associated with intracellular membranes.

Table 1
Cholesterol and phospholipid content and enrichment values for lectin label and cytochrome *c* reductase in the homogenate, mixed membranes and surface and intracellular membrane fractions

	Homogenate	Mixed membranes	Surface membranes	Intracellular membranes
Cholesterol (μmol/mg protein)				
Mean ± SD (3)	0.181 ± 0.01	0.392 ± 0.05	0.691 ± 0.06	0.268 ± 0.03
Phospholipids (μmol/mg protein)				
Mean ± SD (3)	0.290 ± 0.02	0.746 ± 0.04	0.934 ± 0.06	0.920 ± 0.05
Cholesterol/P-lipid ratios				
Mean ± SD (3)	0.624 ± 0.03	0.525 ± 0.06	0.739 ± 0.05	0.291 ± 0.014
Enrichment ^{125}I *Lens culinaris*				
Mean ± SD (3)	1.0	3.30 ± 0	5.10 ± 0.52	0.57 ± 0.25
Enrichment NADH-cytochrome *c* reductase				
Mean ± SD (3)	1.0	3.16 ± 0.38	0.02 ± 0.02	12.47 ± 1.14

Enrichment values calculated with respect to homogenate from data expressed as ^{125}I cpm/mg protein for the lectin and nmol . min^{-1} . mg $protein^{-1}$ for the reductase activity. Number of preparations is in parentheses

On a mean basis (from 4 preparations) the phospholipase A_2 activity is >7-fold enriched in the intracellular membrane fraction and the diglyceride lipase activity 3–4-fold enriched in this fraction. There is no enrichment of either of these lipases in the surface membrane fraction and no significant activity of these enzymes in either the granule or the soluble-phase fractions taken from the sorbitol gradient at the pre-electrophoresis stage (not shown).

In a study of the diglyceride lipase activity in platelets which had been pretreated with thrombin (2 units/10^9 cells) before fractionation (table 2) we were unable to demonstrate any significant difference in the activity of this enzyme between the control and thrombin-treated platelets.

4. Discussion

We report here that the basal activities of phospholipase A_2 and diglyceride lipase are predominantly located in the intracellular membranes of human platelets. Both enzyme activities are significantly enriched with respect to homogenate activity in this

Table 2
Phospholipase A_2 and diglyceride lipase activities in platelet homogenate, mixed membranes and surface and intracellular membrane fractions

Prep. no.	Phospholipase A_2 activity (nmol arachidonic acid released . h^{-1} . mg $protein^{-1}$)					Diglyceride lipase activity (nmol arachidonic released . h^{-1} . mg $protein^{-1}$)					
	1	2	3	4	Mean enrichment	Control platelets			Thrombin treated		
						1	2	Mean enrichment	1	2	Mean enrichment
Homogenate	2.4	0.8	2.1	2.9	–	5.4	5.4	–	3.4	4.1	–
	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)	(1.0)
Mixed	9.6	3.0	2.7	6.0	–	14.4	12.4	–	8.0	11.0	–
membranes	(4.0)	(3.8)	(1.3)	(2.1)	(3.1)	(2.7)	(2.3)	(2.5)	(2.4)	(2.7)	(2.6)
Surface	1.4	2.0	0.7	3.4	–	5.4	2.9	–	2.8	4.6	–
membranes	(0.6)	(2.5)	(0.3)	(1.2)	(1.2)	(1.0)	(0.5)	(0.8)	(0.8)	(1.1)	(1.0)
Intracellular	20.9	10.4	5.6	12.1	–	14.9	23.1	–	10.6	20.9	–
membranes	(8.7)	(13.0)	(2.7)	(4.2)	(7.2)	(2.8)	(4.3)	(3.6)	(3.1)	(5.1)	(4.1)

Enrichment values in parentheses have been calculated with respect to homogenate activities

membrane fraction which has been identified as of intracellular origin on the basis of the exclusive localisation of the marker enzyme NADH-cytochrome *c* reductase and the absence of a radioactive lectin label to which the platelets were exposed at the whole cell level.

To our knowledge the subcellular localisation of diglyceride lipase had not been investigated, but using zonal gradient centrifugation to subfractionate human platelet membranes others have failed to demonstrate a specific localisation of phospholipase A_2 [15]. These data substantiate our view that high-voltage free-flow electrophoresis has significant advantages over more conventional gradient procedures for the separation of human platelet membranes into subpopulations of different cellular origin.

The presence of these two lipases in platelet intracellular membranes, both concerned with the release of arachidonic acid, may be of considerable physiological significance since it has been reported that the internal dense tubular membrane system (DTS) of the platelet is the site of the prostaglandin synthetase enzyme complex [16]. Thus the release of arachidonic acid could be structurally closely associated with the enzymes involved in its oxidation. Although platelet membranes are low in diglyceride content, such an association is equally acceptable for the diglyceride lipase since the substrate for this enzyme could be provided by the action of phospholipase C which has been reported to be in the cytosol of platelets [17]. To fully understand the respective roles of phospholipase A_2 and diglyceride lipase and their functional importance in the production of prostaglandin precursor there are several problems requiring elucidation. In addition to more information about their regulation and kinetic data, knowledge is also required about the localisation and activity of diglyceride kinase since this enzyme could compete with diglyceride lipase for the diglyceride produced by phospholipase C.

Using a low concentration of thrombin (2 units/10^9 cells) a level, however, which is able to activate phospholipase C rather than phospholipase A_2 [18,19] we have been unable to demonstrate any significant change in diglyceride lipase activity after treatment of the intact cells with thrombin before membrane isolation. Higher activities however have been reported for this enzyme in homogenates from platelets exposed at the whole cell level to greater concentrations of thrombin [4 and this aspect certainly warrants more detailed investigation.

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