Diacylglycerol lipase activity in human platelet intracellular and surface membranes

Some kinetic properties and fatty acid specificity

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Received 22 October 1984

Diacyl glycerol lipase activity has been examined of intracellular and surface membranes isolated from human blood platelets by free flow electrophoresis Enzyme activity is present on both membranes but is activated at different substrate concentrations (K_m 14 μM and 140 μM for intracellular and surface membrane, respectively) Both enzyme activities are stimulated by EGTA and GSH, and inhibited by added Ca^{2+} The specificity of the intracellular membrane enzyme has been investigated using a range of diacylglycerol substrates differing only in their '2' position fatty acid Arachidonic acid is clearly the preferred '2' position moiety with activities towards eicosatrienoic, linoleic, oleic and palmitic acid-containing substrates, all substantially lower

Diacylglycerol lipase *Human platelet membrane* *Fatty acid specificity*

1. INTRODUCTION

Two pathways have been proposed which result in the liberation of arachidonic acid, the major precursor for prostanoid synthesis during platelet activation. One pathway involves phospholipase A_2 acting principally upon phosphatidyl choline (but perhaps also phosphatidyl ethanolamine) to release the arachidonic acid from *sn*-2 of the phospholipid [1,2] whilst the other pathway requires the sequential action of a phosphatidyl inositol-specific phospholipase C and diacylglycerol lipase [3–5].

For a more complete understanding of the relative importance of these two pathways in the platelet, and particularly the extent of their contribution to arachidonate release during activation, more information is required about the subcellular localisation and kinetic properties of the enzymes. Recently, by using a free flow electrophoresis technique to prepare highly purified human platelet membrane subfractions representing surface and intracellular membranes, we were able to report that phospholipase A_2 and diacylglycerol lipase are predominantly associated with membrane elements characterised as of intracellular origin [6]. Since the enzyme for the conversion of the released arachidonic acid into endoperoxides and thromboxanes also have a similar intracellular membrane association [7] it appears that the enzymatic modification of phospholipids and the synthesis of prostanoids from the liberated fatty acids can be regarded as intracellular membrane processes rather than taking place at the surface membrane level.

We present here studies on the enzyme(s) concerned with diacyl glycerol hydrolysis present in platelet intracellular and surface membranes. We report on the effects of calcium, EGTA and reduced glutathione on the membrane activities and the fatty acid specificity of the intracellular membrane lipase.

Published by Elsevier Science Publishers B V (Biomedical Division)
00145793/85/$3 30

2. MATERIALS AND METHODS

The diacyl glycerols with different labelled '2' position fatty acids were prepared from labelled phosphatidyl cholines supplied by New England Nuclear. All contained palmitate in position '1' and the '2' position fatty acids were [^{14}C]arachidonate, [^{14}C]eicosatrienoate – 8.11.14., [^{14}C]linoleate, [^{14}C]oleate and [^{14}C]palmitate. The specific activities were approx. 60 mCi/mol. The 1-stearyl-2-[^{14}C]arachidonylphosphatidylcholine (60 mCi/mol) was supplied by Amersham International and the corresponding unlabelled phosphatidyl choline was obtained from Avanti Polar Lipids (Birmingham, AL). All other reagents and chemicals were supplied by Sigma and were of analytical grade quality unless otherwise stated.

2.1. *Membrane preparation*

Platelets were isolated from fresh buffy coats supplied by the Blood Transfusion Service Laboratories (Tooting, London SW17 0RB). The procedure for the isolation of the platelets was based upon that described in [8]. For the treatment with neuraminidase at the whole cell level before fractionation the platelets were suspended (approx. 10^{10} cells/ml) in a buffer containing 10 mM Hepes, 150 mM NaCl, 3.0 mM EDTA, 4.0 mM potassium chloride (pH 6.2) and incubated at 37°C for 20 min with neuraminidase (Sigma Type X) at 0.05 units/ml. The platelets were then washed at room temperature in a buffer containing 0.15 M NaCl, 4 mM KCl, 3 mM EDTA and 10 mM Hepes (pH 7.2) at room temperature, and then resuspended in a buffer containing 0.34 M sorbitol and 10 mM Hepes (pH 7.2) at 4°C. Leupeptin was added to a concentration of 25 μg/ml (50 μM) and the platelet suspension was sonicated for 10 s. After removal of the homogenate by centrifugation at 1200 × g, for 15 min at 4°C, the cells were again resuspended in sonication buffer containing leupeptin and further sonicated for 10 s. The supernatant homogenates were combined and layered onto a linear sorbitol density gradient (1.0–3.5 M) buffered with 10 mM Hepes (pH 7.2). This was centrifuged at 42000 × g for 90 min to obtain a mixed membrane fraction which was located in the low density region (1–1.5 M) and discretely separated from a granule fraction which formed a zone in the higher density region (2.5–3.0 M). The membrane fraction containing components of both surface and intracellular membranes was recovered as a pellet by centrifugation at 100000 × g for 60 min and resuspended in 0.4 M sorbitol buffered to pH 7.2 with 10 mM triethanolamine. This membrane suspension was separated into surface and intracellular membranes using high voltage free flow electrophoresis (Bender Hobein VAP5 instrument) as in [8]. The apparatus was operated at 1200 V, 130 mA and at a buffer flow rate of 2 ml/min. Peaks representing surface and intracellular membranes were separately pooled and centrifuged at 100000 × g, 60 min to recover the membranes.

2.2. *Preparation of diacylglycerols*

[^{14}C]Diacylglycerols of defined fatty acid composition were obtained by treating the above range of phosphatidylcholines with a commercial preparation of phospholipase C (Sigma, *C. perfringens*) and extraction with diethyl ether. The labelled diacylglycerols were further purified on thin-layer chromatography (TLC) with hexane-diethyl ether-acetic acid (60:40:1) at 4°C and re-extracted from the TLC plates with diethyl ether. The amounts recovered were estimated by their radioactivity.

Diacyl glycerol lipase assays were carried out as described earlier [6]. The incubation mixtures contained 100 mM NaCl, 50 mM Hepes (pH 7.0), approx. 100 μg membrane protein and different concentrations of diacylglycerol (as stipulated in legends) dissolved in 5 μl acetone. Incubations were carried out for 5 min at 37°C and terminated by the addition of 3 ml chloroform-methanol-hexane (25/28/20) followed by 1 ml 0.05 M KOH-K_2CO_3 (pH 10) to partition the released fatty acids into the aqueous phase [5,9]. The amount of fatty acids released was measured by liquid scintillation counting.

3. RESULTS

The density gradient fractionation and free flow electrophoresis procedure used in these investigations produces 2 well separated membrane subfractions representing surface (SM) and intracellular (IM) membranes. The separation depends upon a short exposure of the intact platelets to neuraminidase before sonication and fractionation

which, by removal of the enzyme-labile sialic acid moieties, reduces the surface membrane vesicles to a lower electronegativity than the intracellular membrane vesicles. The difference in electrophoretic mobility between the two membranes is sufficient to produce fairly discrete peaks in the electrophoresis chamber which allows fraction pooling across the peaks with minimal cross contamination. Preliminary studies in which diacylglycerol hydrolysis was measured in mixed membrane fractions, taken directly from the sorbitol density gradients prepared from sonicates of neuraminidase-treated and untreated cells, showed that the enzyme treatment had no effect upon the lipase activity. In the density gradients the lipase activity was exclusively confined to the low density region containing the mixed membranes with no significant activity detectable in either the cytosol or granule fractions (not shown).

Fig.1 shows the distribution of the activities towards diacylglycerol measured at low substrate concentrations (17 μM) and the effects of using the protease inhibitor leupeptin during the initial sonication of whole platelets. We confirm our earlier findings [6] that diacylglycerol hydrolysis is

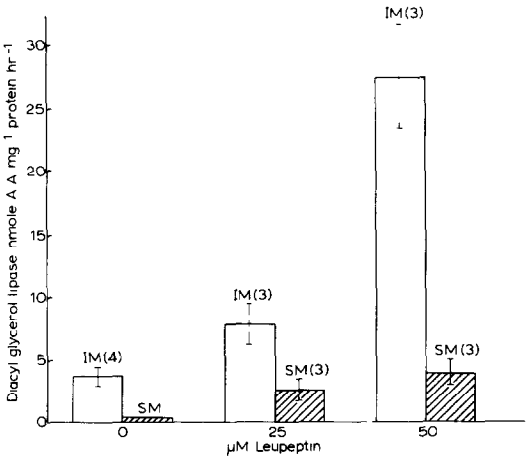

Fig 1. Effect of leupeptin added during the whole platelet sonication procedure on the diacylglycerol activities of human platelet surface (SM) and intracellular (IM) membranes. The diacylglycerol substrate was 1-stearoyl-2-arachidonyl-*sn*-glycerol at 17 μM and assays were performed as described in section 2 without added Ca^{2+}, EGTA or GSH in the assay medium. Figures in parentheses indicate the number of membrane preparations studied.

primarily associated with intracellular membranes. At this concentration of substrate the surface membranes show approximately one fifth of the specific activity displayed by the intracellular membranes. Also shown is the effect of the proteolytic inhibitor leupeptin. Membranes prepared from homogenates containing 25 and 50 μM leupeptin showed a 2- and 7-fold increase in enzyme activity when compared with control preparations. A similar enhancement was found for the surface membranes but the specific activities were of course correspondingly lower. Leupeptin added in the assay mixture showed no effect on lipase activity.

Fig.2 shows the relationship between variation of substrate concentration (17–250 μM) and diacylglycerol lipase activity for the surface and intracellular membrane fractions. It is clear that the deacylation process associated with intracellular membranes shows higher specific activities at all substrate concentrations examined compared with the surface membranes. Indeed, diacylglycerol hydrolysis can only be substantially measured in surface membranes at concentrations of substrate greater than 60 μM and reached saturation at 100 μM. In contrast, substrate saturation of intracellular membranes was not reached at levels up to 250 μM diacylglycerol. Identical results were obtained with three other membrane preparations. In the inset to fig.2 double reciprocal plots for the two lipase activities are presented. Regression analysis gave apparent K_m values of around 14 μM for the intracellular membrane enzyme and 140 μM for the surface membrane enzyme.

We have examined the lipase activities in the two membrane fractions with respect to the presence of Ca^{2+}, EGTA and reduced glutathione (GSH) in the assay medium (table 1). These investigations reveal that Ca^{2+} dependence does not seem to be a feature of either diacylglycerol lipase activity. In fact, with the intracellular membrane enzyme, Ca^{2+} at 5 and 10 mM is inhibitory at the 17, 100 and 250 μM substrate levels. This inhibition by Ca^{2+} was also observed with the surface membrane enzyme activity, especially when measurements were carried out at high substrate concentrations (250 μM). In no case was complete inhibition of the enzyme demonstrated with added Ca^{2+}. On the other hand, EGTA has a stimulatory effect with both membrane fractions and the inclusion of 5 mM

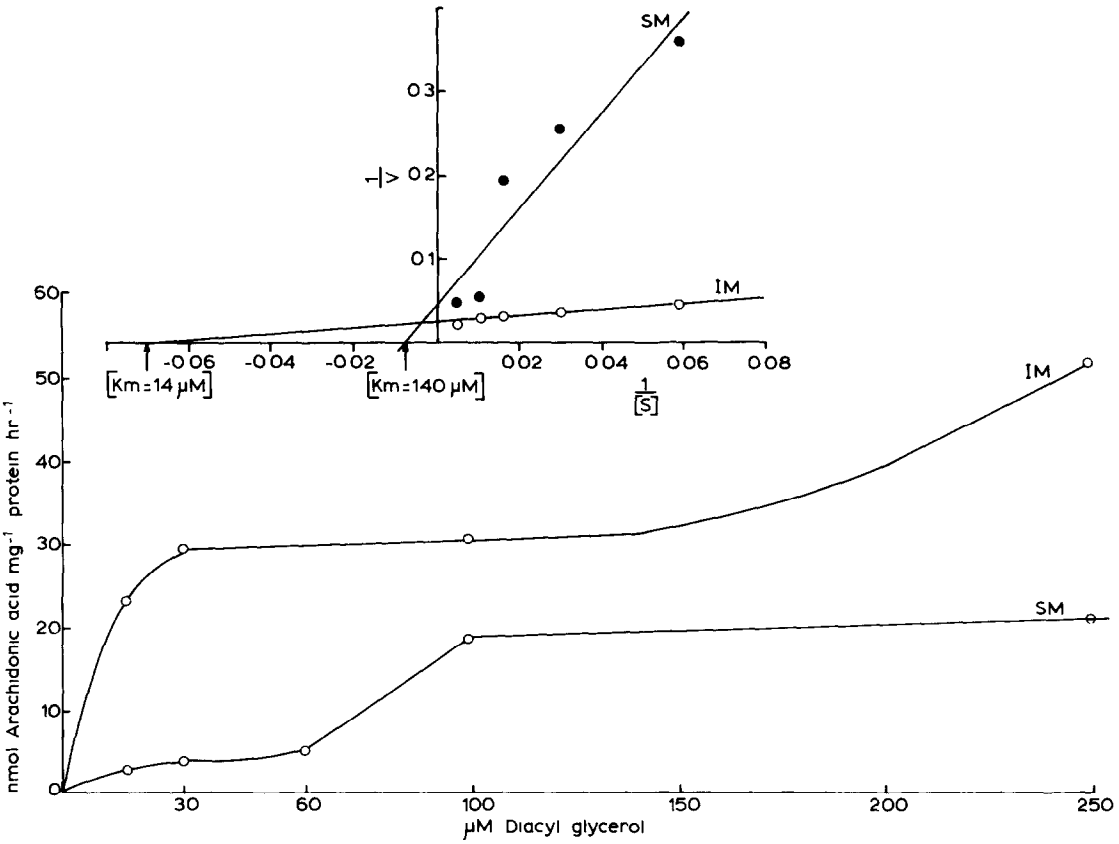

Fig.2 Showing change in enzyme activity with increasing substrate concentration in the range 17–250 μmol diacylglycerol. This substrate was prepared from 1-stearoyl-2-arachidonylphosphatidylcholine (see section 2) IM, intracellular membrane; SM, surface membrane. Inset shows a Lineweaver-Burk plot from the same experimental data The slopes [K_m/V_{max}] have been determined by linear regression. Identical data were obtained with three other membrane preparations

Table 1

Effect of Ca^{2+}, EGTA and GSH on the diacylglycerol lipase activities of the surface and intracellular membrane fractions measured at different concentrations of substrate

	Intracellular membrane			Surface membrane		
Substrate concentration	17 μM	100 μM	250 μM	17 μM	100 μM	250 μM
Control activities. (nmol·mg^{-1} protein h^{-1})	23 0	32 6	52 2	2.8	19.2	20 3
Control	100	100	100	100	100	100
EGTA, 5 mM	200	273	212	193	ND	200
Ca^{2+}, 5 mM	88	80	84	103	ND	56
Ca_a^{2+}, 10 mM	77	ND	84	92	ND	60
GSH, 10 mM	368	ND	142	350	ND	225

Values expressed as %. Assay conditions as described in section 2. ND, not determined

EGTA in assay mixtures containing 17, 100 and 250 μM diacylglycerol all gave activity values substantially higher (2–3-fold) than control activities.

In this context, trifluoperazine, an agent known to inhibit many Ca^{2+}/calmodulin-dependent enzymes and to inhibit human platelet phospholipase A_2 activities by as much as 80% (unpublished) was without effect on either lipase activity at concentrations of trifluoperazine as high as 100 μM. The inclusion of GSH in the assay medium appears to be advantageous at both low and high substrate concentrations with both the intracellular and surface membrane lipases (table 1). This stimulatory effect of GSH was most marked at the lower substrate concentration (17 μM) and resulted in a 3.7- and 3.5-fold enhancement of activity in the intracellular and surface membrane, respectively, over control values without added glutathione

Table 2 shows the data from experiments in which the specificity of the membrane enzyme was examined using a range of diacylglycerols with different ^{14}C-labelled fatty acids in position '2'. All diacylglycerols had palmitate at *sn*-1 and preliminary investigations revealed no difference in activities when 1-palmityl-2-arachidonyl diacylglycerol and 1-stearyl-2-arachidonyl diacylglycerol were compared with the same membrane preparation. It can be seen from this activity profile that arachidonic acid in the '2' position on the diacyl

Table 2

Fatty acid specificity of the platelet membrane diacylglycerol lipase using substrates with different '2' position fatty acids

Fatty acid in position '2'	Membrane[a] diacylglycerol lipase (nmol·mg^{-1}·h^{-1})
20:4 [5,8,11,14]	9 2 ± 3.8
20.3 [8,11,14]	4 8 ± 2 1
18.2 [9,12]	5.8 ± 2.3
18:1 [9]	3 7 ± 2 7
16:0	2 1 ± 0 2

[a] For these studies a mixed membrane fraction was used with a substrate level of 14 μM to measure essentially the intracellular membrane lipase activity

All the diacylglycerols contained palmitate at *sn*-1 and the results are mean values ± SD for 6 determinations

glycerol is the preferred substrate for this enzyme and the activities towards eicosatrienoic, linoleic, oleic and palmitic acids in this position are all consistently lower but with no clear rank order. These specificity studies were made using mixed membranes taken directly from the sorbitol density gradients. Hence, they show lower specific activities than those recorded for the purified intracellular membrane lipase. However, since a low substrate level (14 μM) was used throughout, the activities measured were essentially of intracellular membrane origin.

4. DISCUSSION

Bell et al. [5] were the first to demonstrate the presence of diacylglycerol lipase in human platelet microsomal fractions. They reported an apparent K_m of ≈300 μM and the activity was stimulated by Ca^{2+} and GSH. In contrast, Chau and Tai [10] using microsomes and 20 μM substrate, showed that the lipase was unaffected by Ca^{2+} or GSH. The latter workers also found using different isotopic labels at *sn*-1 and *sn*-2 that the release of the '2' position label involved the sequential action of diacylglycerol lipase acting at *sn*-1 first, followed by a monoacylglycerol lipase acting at *sn*-2. The rate-limiting step was found to be hydrolysis of the *sn*-1 fatty acid followed immediately by the removal of the *sn*-2 fatty acid. Confirmation of this sequential action has come from more recent reports [11,12] and from the initial observation of a monoacylglycerol lipase by Bry et al. [13].

Using well-characterised fractions representing surface and intracellular membranes of human platelets prepared by free flow electrophoresis we earlier reported [6] that release of arachidonic acid from *sn*-2 of diacylglycerol (thus measuring the combined actions of a diacylglycerol lipase and monoacylglycerol lipase) occurs primarily in intracellular membranes. This, coupled to the demonstration that the arachidonate metabolising enzyme (cyclooxygenase and thromboxane synthase) are also present in the intracellular membranes [7], indicates the close association or even tight coupling of enzymes associated with the release and metabolism of arachidonic acid. Here, again measuring release of arachidonic acid from 1-stearyl-2-arachidonyl *sn*-glycerol, we provide further evidence substantiating this view. We have

shown that the diacylglycerol deacylating activity of intracellular membranes is extremely active at all concentrations of diacylglycerol studied. It is optimally active in the absence of calcium (stimulated by 1 mM EGTA and partially inhibited by added calcium). The stimulatory effect of GSH suggests the presence of a sulphydryl group at the active site.

We also demonstrated here a diacylglycerol lipase activity in highly purified substrate membranes. Its properties appear to be similar to that described for the corresponding intracellular membrane enzyme(s) with respect to effects of Ca^{2+}, EGTA and GSH but only showed measurable activities at concentrations of substrate greater than 60 μM. Apparent K_m for the surface membrane enzyme was 10-times higher than the intracellular enzyme. Thus the physiological importance of the surface enzyme is questionable in the context of the likely levels of diacylglycerol present in resting and stimulated platelets. Prescott and Majerus [12] gave a value of 0.6 nmol/10^9 platelets and an identical value was recorded by Rittenhouse-Simmons [4]. As yet, we have not been able to determine whether the low- and high-K_m enzyme activities are different protein entities, but the disparity between the surface and intracellular membranes harvested by our free flow electrophoresis procedure with respect to polypeptide profiles, lipid composition and physical properties [8,14] might suggest that the kinetic differences may be due to differences in the character and composition of their bilayer microenvironments. A diacylglycerol lipase activity associated with plasma membranes obtained from platelets adsorbed onto polyethylene-imine bonded polyacrylamide beads followed by cell lysis has also been reported [15] although no information regarding levels of substrate used or comparison to intracellular membranes has been indicated.

The specificity data presented here for 5 different '2' position fatty acids are, we believe, the first comprehensive assessment of the *sn*-2 fatty acid specificity of the diacylglycerol deacylating activity of platelets. Previous reports using diglycerides of defined fatty acid composition have only compared arachidonate *vs* oleate as the position 2 fatty acid [12,16]. Our studies show a consistent preference for a '2' position arachidonyl moiety in the diacylglycerol substrate with all the other '2' position fatty acids examined showing approximately half or less activity. However, the rank order does not relate to the number of positions of unsaturated bonds since the activity shown towards 1-palmityl-2-linoleyldiacylglycerol was consistently higher than that shown towards 1-palmityl-2-eicosatrienoyldiacylglycerol. The lowest activities, however, were always those displayed towards 1,2-dipalmityldiacylglycerol. The combination of phospholipase C activity coupled to diacylglycerol hydrolysis is an important pathway contributing to arachidonic acid generation in human platelets following haemostatic activation. Our results, together with the recent demonstration that phospholipase C activation does not depend on cytoplasmic increases of calcium concentration [17,18], suggest that this whole pathway might be independent of changes in cell calcium levels. This contrasts with the release of arachidonic acid from phosphatidylcholine via phospholipase A_2 which is a well established calcium-dependent pathway.

ACKNOWLEDGEMENTS

We gratefully acknowledge the financial support of the British Heart Foundation and the Thrombosis Research Laboratories of Ciba-Geigy, Horsham, Sussex.

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