Localization of cyclo-oxygenase and thromboxane synthetase in human platelet intracellular membranes

Frank CAREY,* Suzanne MENASHI† and Neville CRAWFORD†‡

*I.C.I. Pharmaceuticals Division, Mereside, Alderley Park, Macclesfield, Cheshire SK10 4TG, U.K. and

†Department of Biochemistry, Royal College of Surgeons of England, 35/43 Lincoln's Inn Fields,
London WC2A 3PN, U.K.

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Platelet mixed membrane fractions can be separated into discrete vesicle subpopulations of surface and intracellular origin. Intracellular membrane vesicles are the predominant site of phospholipid-modifying enzymes that liberate arachidonic acid. We report the selective enrichment in intracellular membranes of cyclo-oxygenase and thromboxane synthetase activities. Surface membrane fractions show no such enrichment. These results suggest that a sequence of activities leading to the biosynthesis of thromboxane from arachidonate is associated with the intracellular membrane elements known as dense tubular membranes.

The mobilization of arachidonic acid from platelet phospholipids and its conversion to prostanoids and related mediators may be obligatory events in platelet activation and release. Smith & Willis (1970) were the first to show that platelet aggregation was accompanied by release of prostaglandins E_2 and $F_{2\alpha}$. Subsequently, intermediates in prostaglandin biosynthesis, the prostaglandin endoperoxides, were identified (Hamberg & Samuelsson, 1973; Nugteren & Hazelhof, 1973) and their transformation to a labile substance, thromboxane A_2 , by an enzyme of microsomal origin in platelets has been described by Hamberg *et al.* (1975) and Needleman *et al.* (1976). A number of groups have shown that cyclo-oxygenase and thromboxane synthetase activities are associated with platelet particulate fractions (Hamberg *et al.*, 1975; Hammerström & Falardeau, 1977; Ho *et al.*, 1976; Yoshimoto *et al.*, 1977).

We recently described a procedure for the differential isolation of human platelet surface and intracellular membranes from a mixed membrane fraction by using high voltage free flow electrophoresis (Menashi *et al.*, 1981). These two membrane subpopulations were vesicular, and clearly of different cellular origin on the basis of the discrete localization of surface markers and enzyme profiles. Moreover, the surface and intracellular membranes differed markedly in lipid and polypeptide composition and in microviscosity as measured by fluorescent depolarization. A further report (Lagarde *et al.*, 1981) revealed that the intracellular membranes were the predominant site of activity of the two major enzymes involved in the liberation of

arachidonic acid from phospholipids, phospholipase A_2 and diacylglycerol lipase.

In the present paper we present evidence for the enrichment in the platelet intracellular membranes of the two enzymes essential for the biosynthesis of thromboxane from arachidonic acid. These findings, together with the earlier lipase results of Lagarde *et al.* (1981), suggest that the sequence of enzymes involved in the mobilization of arachidonic acid from membrane phospholipid and its subsequent conversion to endoperoxides and to thromboxane may be functionally and structurally coupled on an intracellular membrane complex commonly referred to by platelet electron microscopists as the dense tubular membrane system.

Experimental

Isolation of platelet surface and intracellular membranes

The procedure for the isolation and characterisation of human platelet surface and intracellular membranes has been described in full elsewhere (Menashi *et al.*, 1981). Briefly, a granule and mixed membrane fraction was prepared from sonicated platelets by using sorbitol density gradient centrifugation. High voltage free flow electrophoresis was then used to separate three membrane subfractions on a charge-dependent basis from the mixed membrane fraction taken from the low density zone of the gradient. This zone was free of granule contaminants. The separated membrane subfractions were characterized by their enrichment of surface membrane marker, *Lens culinaris* ^{125}I -labelled lectin

‡ To whom reprint requests should be addressed.

Fig. 1. Distribution of protein (○), ^{125}I -labelled lectin (▲) and NADH:cytochrome *c* reductase (●) in the electrophoretically separated membrane subfractions

Pooled fractions N_I (intracellular membranes) and N_{II} and N_{III} (surface membranes) were taken as indicated by the horizontal bars for the enzyme assays.

(applied to the platelets at the whole cell level), adenylate cyclase and NADH:cytochrome *c* reductase activities, cholesterol and phospholipid composition, morphological criteria and their disparate polypeptide profiles characterised by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis. Two of the membrane subfractions were identified as of surface origin (N_{II} and N_{III}) and the third represented platelet intracellular membranes (N_I); see Fig. 1. The full phospholipid analyses and the fatty acid composition of the phospholipid classes present in the surface and intracellular membranes have now also been reported (Lagarde *et al.*, 1982).

Assay procedures

The measurement of cyclo-oxygenase activities in all the platelet subcellular fractions was performed by a modification of the procedure of Ho *et al.* (1976). Platelet subcellular fractions were incubated in 50 mM-Tris/HCl, pH 7.4, at 25°C in the presence of 10 μM -[1- ^{14}C]arachidonic acid. Reactions were stopped by acidification with 0.1 M-citric acid to pH 3.5 and the radioactive metabolites were extracted into ethyl acetate, separated by t.l.c. and quantified by scintillation counting. Radioactive prostanoids were identified by co-migration with authentic standards. A metabolite that co-migrated on t.l.c. with authentic prostaglandin D_2 , and which represented <10% of polar arachidonate metabolites, was not included in the measurements of the cyclo-oxygenase because this had associated with it a variable contaminant known to be a product of the lipoxygenase pathway. In addition thromboxane B_2 was identified by inhibition of its synthesis with indomethacin and 1-nonylimidazole

and prostaglandins E_2 and $\text{F}_{2\alpha}$ by the inhibition of their synthesis with indomethacin and elevation with the selective thromboxane synthetase inhibitor 1-nonylimidazole. Reactions were linear up to 2 min and activities were determined as initial velocities under conditions where <15% of substrate was utilized. The cyclo-oxygenase activity was expressed as pmol of (thromboxane B_2 + prostaglandins E_2 and $\text{F}_{2\alpha}$)/min per mg of protein calculated by reference to the specific activity of the [1- ^{14}C]arachidonic acid. Thromboxane synthetase activity was measured in two ways in the platelet subcellular fractions. In the first procedure, 10 μM -arachidonic acid was present, as described earlier, and the thromboxane B_2 produced was determined by radioimmunoassay. In the second procedure, the prostaglandin endoperoxide (prostaglandin H_2) was prepared by the procedure of Ubatuba & Moncada (1977) and thromboxane synthetase activity was determined with 10 μM -prostaglandin H_2 as substrate and again radioimmunoassay of the thromboxane B_2 produced. Reactions were linear for up to 1 min and activities are initial velocities.

Results and discussion

The distribution and enrichment of cyclo-oxygenase and thromboxane synthetase in human platelet homogenates, subcellular fractions prepared by density gradient centrifugation (mixed membranes and granules) and in the membrane subfractions prepared by free flow electrophoresis (N_I , intracellular membranes; N_{II} and N_{III} , surface membranes) are shown in Table 1 and Fig. 2. The distribution of cyclo-oxygenase was characterized

Table 1. *Distribution of cyclo-oxygenase in human platelet subcellular fractions*

The specific activities shown for the four different platelet preparations are expressed as means $\pm$ s.d. for four determinations, with the exception of platelet preparation 2 in which the data for the subcellular fractions are the mean of two determinations. Relative specific activities with respect to homogenate values are given in parentheses for each preparation. N.D., not detected.

Fraction	Preparation ...	Specific activity (pmol of metabolites/min per mg of protein)				Mean relative specific activity
		1	2	3	4	
Gradient fractions						
Homogenate		148 ± 17 (1)	678 ± 40 (1)	480 ± 94 (1)	493 ± 12 (1)	1
Mixed membrane		487 ± 45 (3.29)	1321 (1.95)	608 ± 260 (1.27)	770 ± 1 (1.56)	2.02
Granule		N.D. (-)	N.D. (-)	44 ± 1 (0.09)	70 ± 3 (0.14)	0.12
Free flow electrophoresis fractions						
N _I		1229 ± 22 (8.3)	4263 (6.29)	1143 ± 97 (2.38)	1131 ± 16 (2.29)	4.82
N _{II}		336 ± 15 (2.27)	397 (0.59)	329 ± 27 (0.69)	546 ± 18 (1.11)	1.17
N _{III}		N.D. (-)	16 (0.02)	41 ± 2 (0.09)	90 ± 2 (0.18)	0.10

by measurement of the transformation of [$1\text{-}^{14}\text{C}$]arachidonic acid to prostaglandins E_2 , $\text{F}_{2\alpha}$ and thromboxane B_2 and thromboxane synthetase by the conversion of both arachidonic acid and prostaglandin endoperoxide to immunoreactive thromboxane B_2 .

In preliminary studies it was found that greater than 90% of these enzyme activities were sedimented by centrifugation of platelet homogenates at 100 000 g for 1 h. In measurements of the enzymes made on the major fractions taken from the sorbitol density gradient both the cyclo-oxygenase and the thromboxane synthetase showed enrichment with respect to homogenate activities in the low density mixed membrane fractions. In all granule fractions tested these enzymes were either below the limit of detection or substantially depleted with respect to homogenate activities. The data for the cyclo-oxygenase distribution are presented in Table 1. Although expressed as cyclo-oxygenase activities, thromboxane synthetase activities are included by implication since the procedure measured the utilization of [$1\text{-}^{14}\text{C}$]arachidonic acid. Table 1 also shows the distribution of the enzyme in membrane subfractions prepared by free flow electrophoresis from the gradient mixed membrane fraction. Here a clear differential distribution is evident, since consistently the intracellular membrane (N_I) gave the highest relative specific activities (mean 4.82) whereas the two surface membrane fractions N_{II} and N_{III} had mean relative specific activities of 1.17 and 0.1 respectively. For the experiments shown in Fig. 1 the mean ($\pm$ s.d.) value for homogenate thrombox-

ane synthetase activities from four different platelet preparations was 258 ± 50 pmol of thromboxane B_2 /min per mg of protein. The relative specific activities for all the subcellular fractions are given at the top of the histogram columns.

Independent evidence for the distribution of thromboxane synthetase was sought by two approaches. Firstly, the biosynthesis of thromboxane B_2 from arachidonic acid was investigated (Fig. 2a); the thromboxane B_2 formed was measured by radioimmunoassay. Under these conditions the mixed membrane fraction had a relative specific activity of 1.95 and the N_I fraction 5.87. Direct evidence for the enrichment of thromboxane synthetase in the N_I fraction was then obtained from the results of the experiment shown in Fig. 2(b). Here platelet subcellular fractions were incubated in the presence of the prostaglandin endoperoxide prostaglandin H_2 ; again the formation of thromboxane B_2 was measured by radioimmunoassay. In these experiments the mixed membrane fraction had a relative specific activity of 4.32 and the N_I membrane fraction 6.05. Thus, in all cases the profile of distribution of these enzymes was similar and confirmed the selective enrichment of both cyclo-oxygenase and thromboxane synthetase in the N_I membrane subfraction.

The earlier reported data on the morphology, cholesterol:phospholipid ratio, marker enzyme and lectin binding properties of the N_{III} membrane fraction indicated that this N_{III} fraction was clearly of surface origin (Menashi *et al.*, 1981). This surface membrane fraction, which is also enriched in

Fig. 2. Distribution of thromboxane synthetase in human platelet subcellular fractions

(a) These data are derived from an experiment in which $10\ \mu\text{M}$ -arachidonic acid was used as substrate. The homogenate activity was 219 ± 6.7 pmol of thromboxane B_2 /min per mg of protein (mean $\pm$ s.d. for four determinations). Relative specific activities with respect to homogenate are shown above each column. HOM, homogenate; MM, mixed membrane fraction; GRAN, granule fraction as taken from the sorbitol gradients. N_I , N_II and N_III are respectively the intracellular membranes (N_I) and the two surface membrane domains prepared by free flow electrophoresis. N.D., not detected. (b) These results are derived from an experiment in which $10\ \mu\text{M}$ -prostaglandin H_2 was used as substrate. The homogenate was 258 ± 50 pmol of thromboxane B_2 /min per mg of protein (mean $\pm$ s.d., four determinations). N.D., not detected.

NADH:cytochrome *c* reductase, had the highest specific activity of cyclo-oxygenase and thromboxane synthetase.

Concerning the N_II membrane subfraction, in all preparations so far studied this fraction always contains a small but variable amount of cytochrome *c* reductase activity and in this study a similar small amount of the prostaglandin synthesizing enzymes. We are uncertain of the exact origin of these N_II membrane vesicles at the whole cell level, but their close similarity to the N_III surface membrane fraction has been earlier established on the basis of cholesterol:phospholipid ratios, polypeptide profiles, adenylate cyclase activity, presence of actin, sphingomyelin content, low fluidity, vulnerability to neuraminidase and lectin binding [Menashi *et al.*, 1981]. Simple contamination of this N_II fraction with N_I vesicles, which though containing the reductase and prostaglandin synthetase enzymes are clearly different in the above properties from N_II and N_III , is not supported by a comparison of enzyme ratios. A possible explanation is that N_II and N_III represent different domains with perhaps N_II representing invaginated areas of the surface; such areas are a feature of the inactivated platelet in electron micrographs. However, an equally plausible explanation could be that N_II and N_III have their origin in the surface membranes of different subpopulations in the circulating platelet pool. Since earlier studies revealed that N_II , unlike N_III , contained myosin, confirmed by its ATPase characteristics, in addition to the actin, such a subpopulation contributing N_II vesicles may have been slightly activated with actomyosin complex formation on the surface membrane. These views, however, must remain conjectural until such subpopulation heterogeneity can be examined with respect to membrane characteristics.

These findings support our view that both cyclo-oxygenase and thromboxane synthetase are predominantly, if not almost exclusively, located in human platelet intracellular membranes.

The role that prostaglandins, and thromboxane in particular, play in platelet activation and release still remains somewhat speculative, although a function for thromboxane A_2 as a mediator of Ca^{2+} release from the intracellular dense tubular membrane system has been postulated (Gerrard *et al.*, 1978). Some indirect evidence for the subcellular location of cyclo-oxygenase was provided by Gerrard *et al.* (1976) who demonstrated by histocytochemical means that a platelet peroxidase activity, with the properties of cyclo-oxygenase in tests with certain inhibitors, was associated with the dense tubular membrane system. Our findings confirm this biochemically and, taken together with those of Lagarde *et al.* (1981), indicate that an intracellular site is responsible for the deacylation of phospholipid

adenylate cyclase activity, has a relative specific activity for cyclo-oxygenase and thromboxane synthetase of approx. 0.1. In contrast the intracellular membranes (N_I), which are inaccessible to the lectin marker at the whole cell level and enriched in the endoplasmic reticulum enzyme antimycin-insensitive

by phospholipase A₂ and diacylglycerol by diacylglycerol lipase. In addition they show that the arachidonic acid released by these enzymes may be further metabolized by cyclo-oxygenase and thromboxane synthetase present in the same intracellular membranes. Further studies of the structure and function of these enzymes in intracellular membranes could be of considerable value in elucidating the role that prostaglandins play in platelet haemostatic response.

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