THE DEACYLATION OF RAT PLATELET PHOSPHOLIPIDS DURING THROMBIN-INDUCED AGGREGATION STUDIED BY A FLUORESCENCE METHOD

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1. Introduction

Evidence supporting the roles played by phospholipase A_2 or phospholipase C and diglyceride-lipase in the release of arachidonic acid have been reviewed in [1]. The precise mechanism, however, remains open to controversy. There is general agreement that thromboxanes are formed from arachidonic acid released from the membrane phospholipids in thrombin-stimulated platelets. Furthermore, apparently selective phospholipase A_2 inhibitors such as mepacrine, bromophenacylbromide and CB 874 inhibit aggregation in human, rabbit and guinea-pig platelets [2,3]. The exclusive action of these phospholipase A_2 inhibitors nevertheless remains questionable since they act also as amphiphilic membrane-active agents [1]. The release of arachidonic acid by the phospholipase C pathway is an alternative to the direct action of phospholipase A_2 and is supported by the apparent accumulation of diglycerides during platelet activation [4,5].

A sensitive assay of phospholipase A_2 activity in venom of *Crotalus adamanteus* has been developed using the fluorescent substrate 2-parinoyl-lecithin [6]. The deacylation of the lecithin was monitored by the release and binding of the free parinaric acid to albumin recorded as an increase in fluorescence polarization (P) as the physical environment of the fluorophore changed. The sensitivity of the assay (~1 nmol/min) and its adaptability to provide continuous measurement of phospholipid deacylation allows us to study the phospholipase reaction associated with platelet aggregation.

The incorporation of parinaric acid into phospholipids has been reported in *Escherichia coli* [7] and its use as a fluorescent probe of the physical state of the membrane lipid domain described. We have succeeded in incorporating parinaric acid into the phospholipids of platelet membranes and we describe the use of these labelled preparations to provide information about the mechanism of deacylation in the platelet aggregation reaction.

2. Materials and methods

2.1. *Incorporation of all-*trans *parinaric acid into the platelet lipids*

Fresh, citrated platelet-rich plasma was incubated at 37°C under a CO_2-rich atmosphere with 10 μl/ml of all-*trans* parinaric acid, 9,11,13,15-octadecatetraenoic acid in the all-*trans* configuration (Molecular Probes Inc., Texas), in ethanol (30 mM). The incubation medium was shaken gently at intervals during 2–3 h incubation. The thrombin-induced aggregation of labelled platelets was challenged with control platelets incubated for the same time without parinaric acid. No difference was detectable when parinaric acid remained at <0.4–0.5 mM. The presence of aspirin (1 mM) did not alter the extent of incorporation of parinaric acid into membrane phospholipids. The lipids of the labelled platelets were extracted [8] and purified by thin-layer chromatography [9] and assayed for their parinoyl moiety by fluorescence measurements.

Oxidation of the highly unsaturated fluorescent parinaric acid was minimised by including 100 ppm (w/v) butylhydroxytoluene in all solvents and handling extracts in an inert atmosphere. Some parinaric acid (11–17%) was incorporated into the triglyceride fraction, (13–18%) into phosphatidylethanolamines, (54–76%) into phosphatidylcholines and (0–11%)

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into phosphatidylserine + phosphatidylinositol. The hydrolysis of a sample of the phosphatidylcholine fraction by *Crotalus adamanteus* phospholipase A_2 [10] showed that 68% of the fluorescent fatty acid was located at carbon atom 2 of the glycerol moiety.

2.2. *Fluorescence measurements*

The device for measuring fluorescence polarization (P) has been described in [6]. To examine the platelet system the incubation medium was continuously stirred by a magnetic bar in the cuvette which was siliconized each day. The fluorescence background (albumin-rich Tyrode buffer + unlabelled platelets), light scattering and an instrumental correction factor G [11] were taken into account in the calculation of the fluorescence polarization. Photobleaching of the probe was reduced by using low illumination. Fluorescence lifetime measurements were performed with a single-photon counting device (Applied Photophysic-Ortec).

2.3. *Incubation conditions for assay of released parinaric acid and aggregometry*

The fluorescent labelled platelets were extemporaneously washed out from the excess of unincorporated parinaric acid by dilution and centrifugation in a large volume of 1% albumin-rich Tyrode buffer. The washed platelets were gently resuspended in 0.5 ml albumin-rich Tyrode and further diluted with albumin-rich Tyrode containing 0.33 mM $CaCl_2$. The suspension was stirred at 37°C in a thermostatted cuvette in the spectrofluorometer. After equilibration for 3–5 min the platelets were activated by addition of thrombin (81 NIH-U/mg, Roche, Switzerland). The intensity of fluorescence at 420 nm polarized parallel ($I_{||}$) and perpendicular ($I_{\perp}$) to the polarized excitation beam at 320 nm were recorded continuously. The aggregometric tests were performed in the same way and the scattered light (in contrast to light transmission as measured by most aggregometers) was recorded by setting both of the monochromators of the spectrofluorometer at 600 nm.

3. Results

The release of parinaric acid from phospholipids of the platelet membrane can be monitored conveniently by a fluorescence hyperpolarization originating from a change in the probe environment from the platelet membrane to a tight hydrophobic binding site on the albumin. This effect is illustrated in fig.1 which shows a decrease in total fluorescence and a fluorescence hyperpolarization in proportion to the amount of defatted albumin added to a suspension of platelets containing exogenous parinaric acid. The fluorescence changes on binding of parinaric acid to the albumin are associated with a reduction in fluorescent lifetime from 11.6 ns in the platelet membrane to 3.8 ns when bound to albumin. Furthermore, binding of parinaric acid to albumin has been confirmed by an observed fluorescence energy transfer with a tryptophanoyl residue of the protein [12,13]. Studies of the time-course of binding of the free parinaric acid to the albumin showed that with addition of 0.25% (w/v) albumin representing a 2-fold excess of parinaric acid binding sites (calculated from [12]), binding equilibrium was achieved within 20 s. Additional hyperpo-

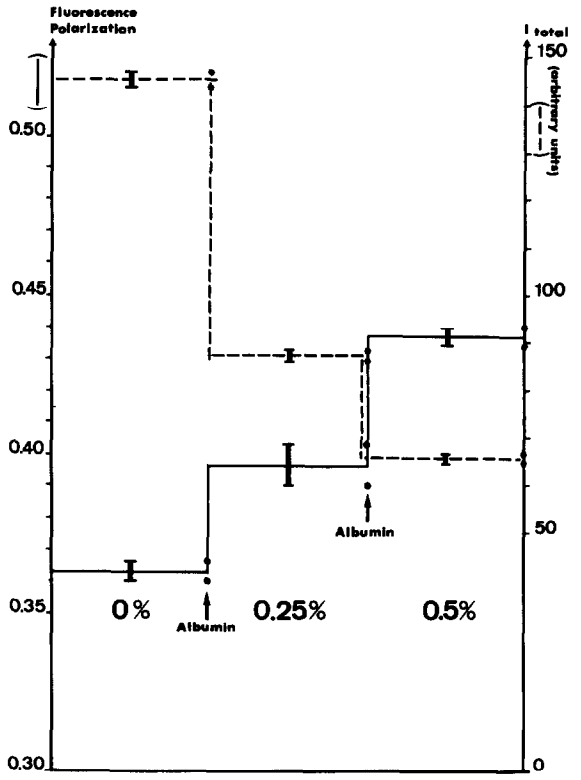

Fig.1. Hyperpolarization of fluorescence resulting in transfer of parinaric acid from platelet membranes to albumin-binding sites. Parinaric acid (60 nmol) was added to 10^9 platelets stirred in Tyrode (3 ml) at 37°C. The indicated amounts (w/v) of fatty acid free albumin are added and the fluorescence polarization and the total fluorescence intensity are determined. The bars indicate 1 SD.

larization to an apparent maximum value was observed on adding 0.5% (w/v) albumin to the suspension. Higher concentrations of albumin were avoided to maintain a relatively low fluorescence background and avoid effects on arachidonic metabolism [14] despite the possibility that some parinaric acid may remain bound to the membrane. Additional experiments were undertaken to examine the release of parinaric acid acylated from platelet membrane lipids by incubating labelled platelet preparations (preheated for 15 min at 100°C with 2 mM EDTA) in the presence of phospholipase A_2 from *Crotalus adamenteus* venom. There was a gradual increase in fluorescence polarization during incubation over 1 h in Tyrode solution (pH 8) containing 10 mM $CaCl_2$ and 1% (w/v) defatted albumin. The change in fluorescence after this period represented a 9% increase in the polarization value.

The time-course of release of parinaric acid was investigated in thrombin-activated platelets in the presence of different concentrations of defatted albumin. The results (fig.2) show a sigmoidal response to

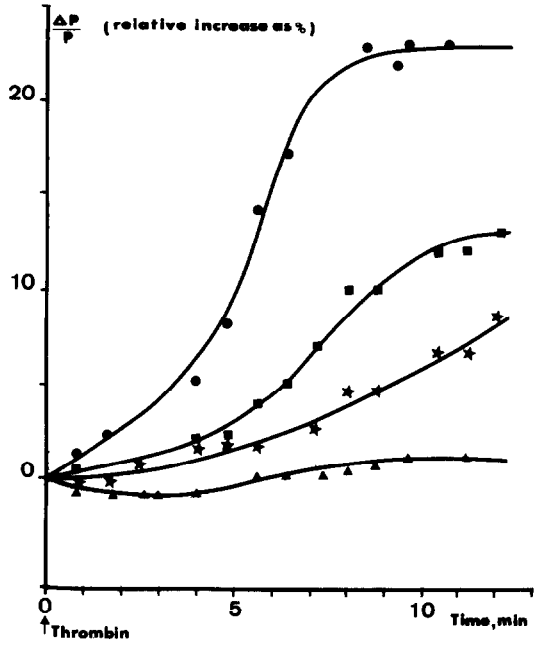

Fig.2. Influence of albumin level on kinetics of parinaric acid release. The fluorescence polarization is recorded as a function of time after the addition of thrombin to the fluorescence-labelled platelets suspension stirred in Tyrode buffer enriched with: 0.5% (•), 0.2% (■), 0.1% (★) or 0.0% albumin (▲).

stimulation by thrombin reaching an apparent maximum after 10 min when 0.5% or 0.2% (w/v) albumin is present in the suspending medium. The response to thrombin stimulation is not as clear when the albumin concentration is reduced to 0.1% (w/v) and cannot be detected in the absence of albumin. This is consistent with previous observations of the hydrolysis of 2-parinoyl-lecithin by phospholipase A_2 which could not be detected in the absence of albumin in the medium.

The concentration of platelets appears to influence the extent of the fluorescence hyperpolarization (fig.3). Platelet counts of the rats used in these experiments were $1.2–1.5 \times 10^6/mm^3$ and the platelet-rich plasma was diluted prior to assay. Fig.3 shows that with platelet concentrations in the range 850 000–400 000/mm^3 there are no significant changes in the response to thrombin. At the lowest concentration (286 000/mm^3) the response as observed by fluorescence hyperpolarization is considerably slower and reduced. Similar results were obtained with the aggregometric tests. Experiments with more diluted platelets suspensions (~200 000/mm^3) were undertaken but the reliability of the data was doubtful because of the low fluorescence signal and lack of sensitivity of the aggregometric test. Finally, the role of phospholipase A_2 activity in platelet response to thrombin was examined by observing the effect of the enzyme inhibitor bromophenacylbromide. A preincubation of platelets for 1 min in the presence of 0.64 mM or 0.16 mM causes an increase in response-time of aggregation after addition of thrombin (fig.4A) and of fluorescence polarization (fig.4B). The extent of fluorescence polarization is decreased by the inhibitor,

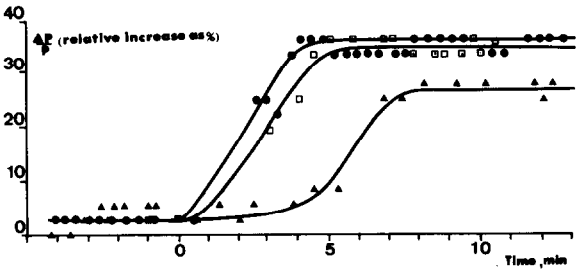

Fig.3. Influence of platelet count on hyperpolarization. The fluorescence polarization is recorded as a function of time after the addition of thrombin (0.33 U/ml) to various diluted platelet suspensions in 0.5% albumin-rich Tyrode (3.5 ml): (•) 855 000/mm^3; (□) 410 000/mm^3; (▲) 286 000/mm^3.

suggesting that less free parinaric acid is formed. It is noticeable that at the highest concentration used (0.64 mM) bromophenacylbromide blocks the release of the parinaric acid but that the platelet aggregation remains still possible.

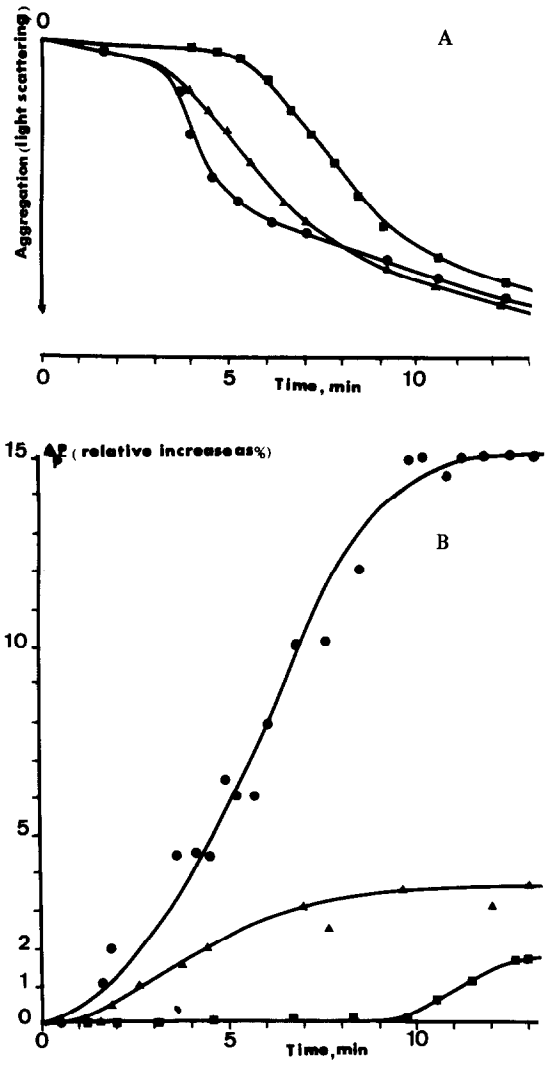

Fig.4. Influence of bromophenacylbromide on hyperpolarization and aggregation of platelets. (A) Fluorescence polarization is recorded after the addition of thrombin (0.33 U/ml) to the fluorescence-labelled platelets suspension in the absence (•) or presence of bromophenacylbromide added and stirred 1 min before the aggregating agent in the cuvette: (▲) 1.6×10^{-4} M; (■) 6.4×10^{-4} M. (B) The corresponding aggregometric curves are displayed under the same conditions. The light-scattering is recorded at 600 nm for the platelets suspension in the absence (•) or presence of bromophenacylbromide added as before: (▲) 1.6×10^{-4} M; (■) 6.4×10^{-4} M.

4. Discussion

It has been shown that activation of platelets results in the release of free fatty acids, and predominantly arachidonic acid from the glycerophosphatides of the platelet membrane. We have shown that the hyperpolarization of parinaric acid acylated to phospholipids of the membrane provides a useful method of monitoring processes associated with platelet activation. It is not possible, with the present method, however, to distinguish the two different pathways associated with fatty acid release from the membrane phospholipids. These are the activation of phospholipase A_2 which hydrolyses fatty acyl residues in the carbon 2 position of phosphatidylcholine and phosphatidylethanolamine [15,16] and secondly a pathway involving phospholipase C initially acting on phosphatidylinositol [4,5]. Then a subsequent action of a diglyceride-lipase [4] or of a phosphatidic acid specific phospholipase A_2 [17] achieves the release of arachidonic acid.

Some evidence suggests that the phospholipase A_2 pathway is the preferential route of parinaric acid release. Thus the amount of parinaric acid incorporated into phosphatidylcholine and phosphatidylethanolamine (67–94%) is considerably greater than that incorporated into phosphatidylinositol (0–11%) which is the specific substrate of the phospholipase C-type enzyme. More parinaric acid is released during platelet activation than could be accounted for by release from phosphatidylinositol alone. Deacylation from the carbon-2 position of phospholipids, however, cannot be established unequivocally from the present experiments. In the fluorescence labelling process 83–89% of the parinaric acid becomes incorporated into the membrane glycerophosphatides and of this, for example, 68% is located in carbon-2 position of lecithin and the remainder in carbon-1. Fluorescence hyperpolarization can also occur if 1-parinoyl-lysophosphatidylcholine is produced by the action of phospholipase A_2 since the lysophosphatide can bind strongly to albumin with consequent fluorescence changes [18].

Although the inhibition of parinaric acid release from stimulated platelets by bromophenacylbromide clearly points to an involvement of phospholipase A_2 in the platelet activation process its role in aggregation may not be as direct as is often assumed [17]. It may be, however, that there are some species differences in the processes of platelet activation and there is

some evidence that rat platelets have distinctive metabolic responses to the phospholipase A_2-released arachidonic acid [19]. A special activator factor of phospholipase A_2 in rat platelets has also been identified [20]. Another factor that could influence the mode of release of free fatty acid from the membrane is the concentration of thrombin used to trigger platelet aggregation (G. B., unpublished). We have found in preliminary experiments using 1-[^{14}C]arachidonic acid labelled platelet membrane phosphoglycerides a sharp increase in the radiolabelled diglyceride fraction of lipid extracts from platelets 30 s after stimulation with 0.8 U/ml of thrombin. This accumulation of diglyceride suggests a primary role for phospholipase C acting on arachidonate-containing phospholipids. It is our conclusion that both phospholipases A_2 and C are activated in rat platelets exposed to thrombin and that release of parinaric acid from fluorescence-labelled platelets takes place in a progressive manner in contrast to the rapid formation of diglyceride by the action of phospholipase C.

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