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DIFFERENT ACYLATION AND ACCUMULATION IN FREE FORM OF ARACHIDONIC ACID OR ITS SODIUM SALT IN HUMAN PLATELETS

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ABSTRACT

Arachidonic acid uptake by platelets, studied by incubating platelet-rich plasma with increasing quantities of arachidonic acid, displays a maximal rate above 2.10^{-4} M plasma level. In free form, arachidonic acid is slightly accumulated by platelets while free linoleic acid accumulation increases proportionately to its plasma level above 3.10^{-4} M. Comparison of renewal rates of arachidonyl residues in main platelet lipids for arachidonic acid and for its sodium salt shows in the latter case a slower acylating process and a twice higher accumulation in free form thus a greater availability for thromboxane synthesis systems. This can explain why 0.5 mM arachidonate are sufficient to trigger aggregation while it isn't triggered by arachidonic acid below 1 mM plasma level.

INTRODUCTION

Platelet phospholipids (PL) mainly renew their fatty acid groups according to Land's pathway either by incorporating plasma free acids (1-6) or by exchanging whole lecithin molecules or their acyl groups with plasma (7-8). The uptake of free fatty acids varies with their plasma levels and more precisely with their molar ratio versus albumin (2, 3). Free arachidonic acid is more easily incorporated than linoleic acid (4-6). Furthermore, the trans formation of linoleic acid into arachidonic acid proceeds very slowly (1). This accounts for the greater quantity of arachidonic acid than linoleic acid found in human platelets (9, 10). Byproducts of cyclooxygenation

of arachidonic acid display an important role in platelet physiology : Prostaglandins D_2 and I_2 are inhibitors of platelet aggregation (11, 12) and thromboxan A_2 is the most potent in vitro aggregating factor known as of yet (13).

The regulation process of the pool of quickly releasable arachidonic acid appears to be critically important. In the present paper, we show that to the extent of its physiological variations, the free arachidonic acid plasma level regulates its own rate of incorporation in platelet phospholipids. We also show that human platelets display a very efficient mechanism which prevents free arachidonic acid but not linoleic acid accumulation.

Besides, authors studying platelet aggregation by arachidonic acid have used until now its sodium salt. We show here that the acid form doesn't trigger aggregation below a 1 mM plasma level. This difference in the behaviour of the platelets when in presence of a critical concentration of arachidonic acid or sodium arachidonate could be explained by their different incorporation capabilities in platelet lipids.

MATERIALS AND METHODS

Materials. 1-^{14}C linoleic acid (60 mCi/mmol) and 1-^{14}C arachidonic acid (55 mCi/mmol) were purchased from Amersham (Arlington Heights IL) at 98 % purity.Non labelled linoleic and arachidonic acids, and sodium salt of arachidonic acid were purchased from Sigma (St Louis MO). The Centre National de Transfusion Sanguine provided us with fresh blood collected from young male donors (20-30 years, A + group) who had not taken any drug within the previous week.

Platelet preparation. Blood samples were collected in an anticoagulant ACD solution according to Aster and Jandl (14) modified by Caen et al. (15). All experiments were carried out using either plastic vials or silicone-treated flasks. The platelet-rich plasma (PRP) was obtained after centrifugation at 400 g for 10 min. Platelets were pelleted at 1600 g for 10 min at 4°C. The pellet was then washed twice with 15 ml of 15 mM Tris buffer, pH 7.4 ; NaCl 0.15 M ; KCl, 5 mM and EDTA 1.5 mM. Platelets were counted after a 100 fold dilution in Malassez cell.

1-^{14}C arachidonate preparation. 1-^{14}C arachidonic acid (55 mCi/mmol) dis-

solved in 1 volume of ethanol was added to 9 volumes of a 9°/₀₀ saline solution containing 0.2 M Na_2CO_3, in a nitrogen atmosphere (0°C). The 0.1 M sodium arachidonate solution was then lyophilized. Resuspension was achieved with the help of a physiologic saline just before use, an then diluted in unlabelled sodium arachidonate to obtain an adequate specific activity. The preparation was controlled by thin-layer chromatography.

Lipid extraction and analysis. Platelet pellets were extracted by methanol-chloroform 2 : 1 (v/v). Plasma samples were extracted using Bligh and Dyer's technique (16) and lipid phosphorus was measured as described by Rouser et al. (17). The lipids were separated by chromatography and then assayed for radioactivity exactly as previously described (7).

Platelet aggregometry. Platelet aggregation was studied using an Elvi-aggregometer. A volume of 0.25 ml PRP was added into the aggregometer cuvette and 5 µl of the aggregating agent were added after a steady base-line was obtained. Sodium salt was added after dissolution in the buffer, and arachidonic acid after dissolution in ethanol. Tests were carried out with arachidonate (20 : 4) in presence or in absence of 5 µl ethanol to rule out any effect of this solvant upon aggregation in our experiments.

RESULTS

Samples of PRP (8 ml) were incubated at 37°C for 1 hr with increasing quantities of fatty acids added in ethanol (0.1 ml). Mild agitation in plastic vials prevented aggregation, which was controlled by phase contrast microscopy. Platelets were immediately pelleted and washed as described under "Methods". The amount of incorporated arachidonic or linoleic acid was calculated by dividing the radioactivity of the different lipids by the specific activity of these acids in plasma.

Fig. 1 displays the rate of both total platelet uptake and incorporation of free arachidonic acid or linoleic acid when their plasma concentration increases. Linoleic acid uptake was found to be in direct proportion to its plasma level beyond 3.10^{-4} M due, to its accumulation in free form. On the contrary arachidonic acid incorporation is plateaued. The maximal rate of total arachidonic uptake is observed for plasma concentrations beyond 2.10^{-4} M. Accumulation of free arachidonic acid is negli-

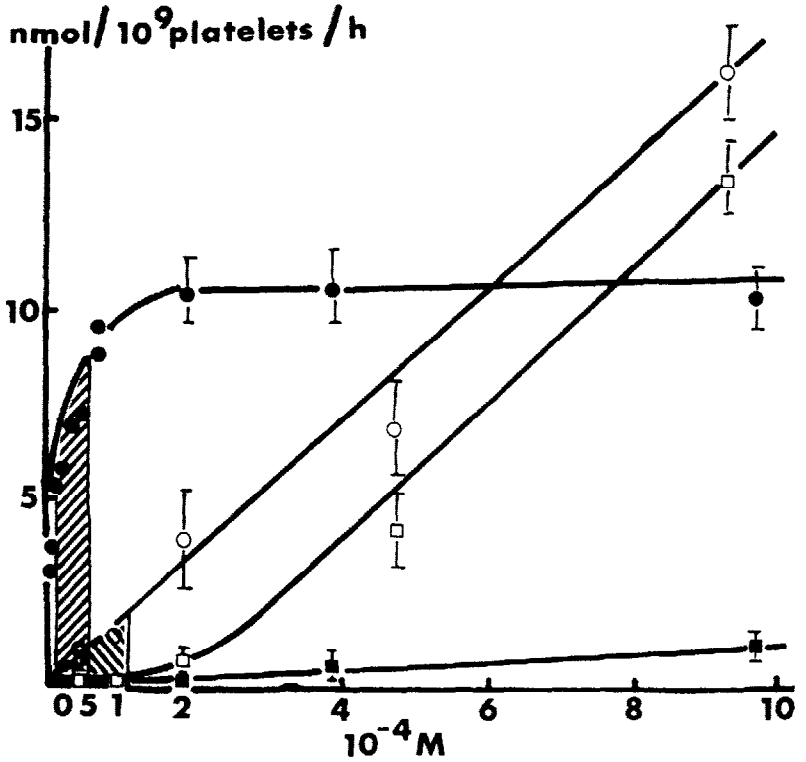

FIG. 1

Total incorporation and accumulation as free form of arachidonic and linoleic acids in platelets as a function of their plasma level as free form. For incubation procedures, see the text. Results are expressed in nmol per hr and per 10^9 platelets. Total uptake of arachidonic acid (● ●) and linoleic acid (O O). Accumulation as free form for arachidonic acid (■ ■) and linoleic acid (□ □). Physiological plasma concentrations are shadowed for each acid.

gible within its physiological concentrations in plasma (3-55 μM) and moreover remains very low far above these concentrations.

Fig. 2 shows arachidonic and linoleic incorporation rates in the major platelet phospholipids as being a function of their plasma level. Both fatty acids are mostly incorporated in phosphatidylcholine (PC) and phosphatidylinositol (PI) then in phosphatidylethanolamine (PE) and phosphatidylserine (PS). The ratio of maximum incorporation rate of arachidonic acid versus linoleic acid is close to 4 in PC and PE, 7 in PS and in PI.

Table I shows that arachidonyl turnover in PI occurs 5, 11 or 30 times

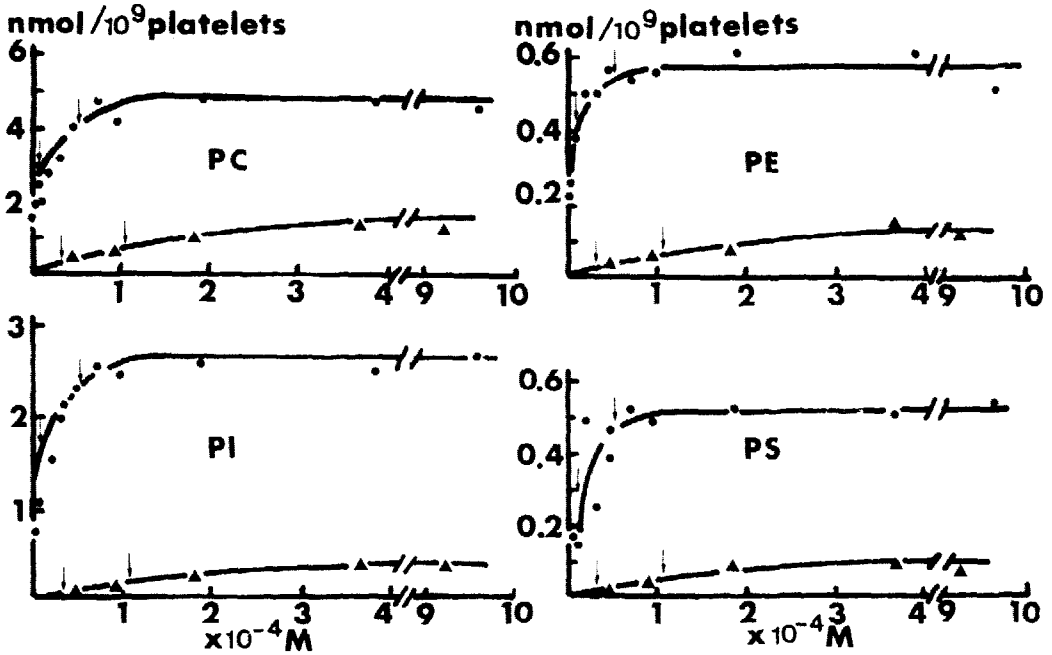

FIG. 2

Incorporation of arachidonic and linoleic acids in platelet phospholipids as a function of their plasma level as free form.
For incubation procedures, see the text. Results are expressed in nmol per hr and per 10^9 platelets. Arachidonic acid incorporation (● ●) linoleic acid incorporation (▲ ▲). Arrows delimite physiological plasma concentrations for each acid.

TABLE I

K'm and maximal turn over rate of platelet phospholipids for linoleic and arachidonic acid

PL	20 : 4		18 : 2	
	K'm[a]	V'm[b] (nmol/h/µmol)	K'm[a]	V'm[b] (nmol/h/µmol)
PC	4	43.6	150	11.8
PI	4	236.4	110	37.7
PS	11	22.3	355	3.1
PE	5	7.7	150	1.9

a) x 10^{-4} M for K'm values (apparent Km) : concentration of fatty acid which gives half of the maximal turn over rate.
b) V'm maximum turn over rate in nmol per hour per µmol of each phospholipid, calculated from data of fig. 2 and platelet phospholipids composition (9-10).

faster than in PC, PE or PS respectively, while for linoleic acid the turnover is 3, 10 or 17 times higher in PI than in PC, PE or PS. The apparent equilibrium constants (K'm) of these acylating systems are lower for arachidonic acid than for linoleic acid ; and for both fatty acids higher in PS than in PE, PC and PI.

In fig. 3 one can see that no aggregation was triggered by arachidonic acid below 1 mM although sodium arachidonate triggers irreversible aggregation at 0.5 mM. This difference was not due to a difference in the solvant used because the addition of 5 µl of ethanol was of no avail on the aggregation triggered by arachidonate.

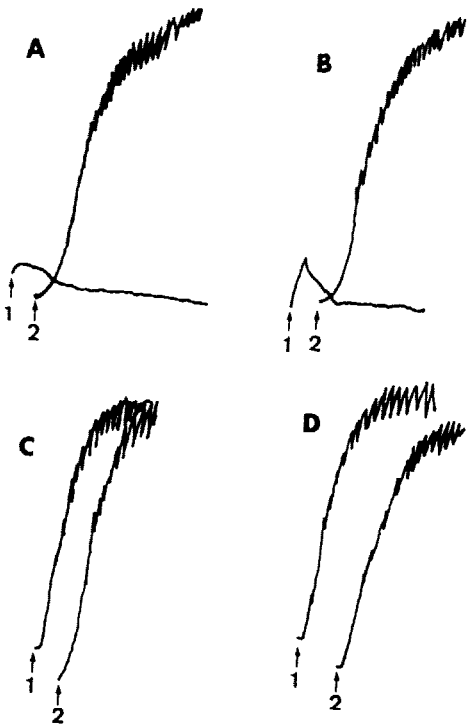

FIG. 3

Platelet aggregation triggered by arachidonic acid or arachidonate sodium salt.
Arachidonic acid is added in 5 µl ethanol, while arachidonate is added in 7 µl buffer. A : arachidonic acid 0.83 mM (1). Arachidonate 0.5 mM (2). B : arachidonic acid 0.9 mM (1). Arachidonate 0.5 mM (2). C : arachidonic acid 1 mM (1). Arachidonate 0.5 mM (2). D : arachidonate 0.5 mM without (1) or with 5 µl ethanol (2).

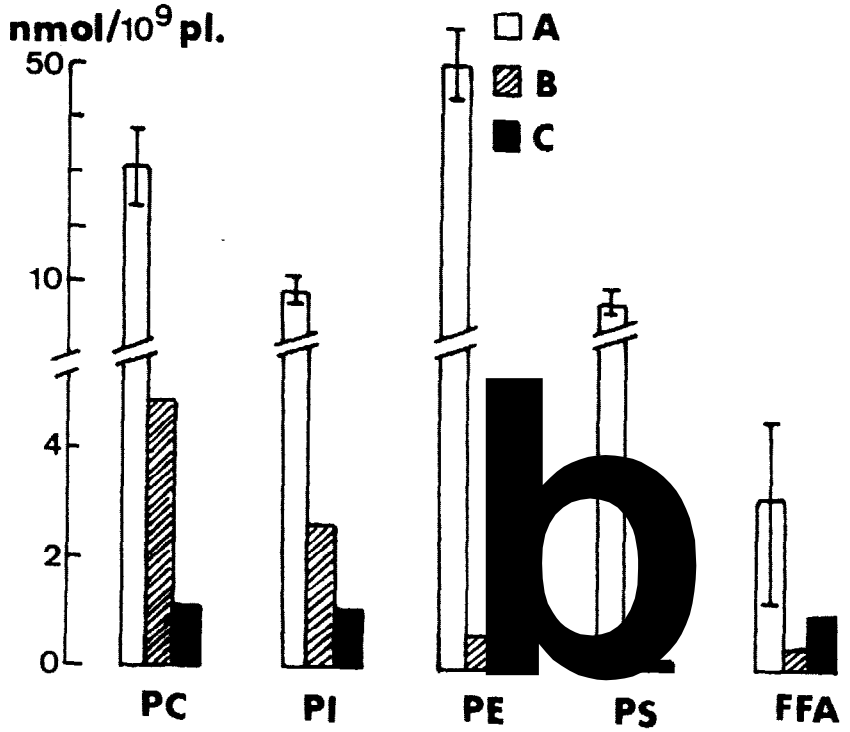

FIG. 4

Comparison of arachidonic acid renewal in platelet lipids from plasma free arachidonic acid or its sodium salt.
Samples of the same PRP incubated either with 300 µM arachidonic acid or 300 µM arachidonate sodium salt. In the first case exogenous unlabeled arachidonic acid and radioactive tracer were added in 0.1 ml ethanol to obtain this concentration. In the second case an aliquot of 0.1 ml saline solution of arachidonate (prepared as described under methods) was added to obtain 300 µM final concentration in plasma. For incubations procedures, see the text. Results are expressed in nmol per hr and 10^9 platelets. A : molecular specie. B : from arachidonic acid. C : from arachidonate sodium salt.

Fig. 4 compares the arachidonic renewal rates in platelet lipids for free arachidonic acid or for its sodium salt, both at 300 µM, concentration for which incorporation has reached a plateau. Though renewal of the main phospholipids from sodium salt was lower than from the acid form, we found a higher renewal rate of arachidonic acid in free form when incubations were carried out with sodium arachidonate (1 nmol/10^9 platelets against 0.4 from the acid form).

DISCUSSION

Free linoleic acid accumulation in platelets increases proportionately to its plasma level above 3.10^{-4} M, which was already described by Spector et al. for palmitic, stearic, oleic and linoleic acid (3). On the contrary we show that arachidonic acid uptake by platelets displays a maximal rate above 2.10^{-4} M but varies sharply within free arachidonic acid physiological concentrations in plasma (fig. 1). In free form, arachidonic acid is slightly accumulated by platelets and it probably takes place in granules which are devoid of thromboxane synthetizing enzymes (18), which prevents occurrence of aggregation.

Maximal incorporation rates of arachidonic acid in platelet phospholipids are largely higher than linoleic acid ones (fig. 2). There is a discrepancy between these results and the usual relative amounts of both fatty acids in phospholipids of human platelets (9, 10). This could be easily explained by 1- the sharply changing rates of incorporation of these fatty acids within their own physiological plasma levels (arrowed in fig.2) and the different K'm values for the transacylation enzyme systems (Table I). 2- the different rates of phospholipid exchange between platelets and plasma according to their acyl group (7).

PI has the highest turnover of its acyl groups with arachidonic acid as well as with linoleic acid. The appearance of a very efficient acylating system in PI and PC for arachidonic acid corroborates the hypothesis already formulated (1, 7) that there exists a fast revolving reacylating-deacylating cycle.

When studying platelet aggregation by arachidonic acid, authors have until now used arachidonic acid as a sodium salt at concentrations between 0.5 and 0.7 mM (19, 20). For arachidonic acid levels up to 0.9 mM there is only a very slow accumulation in free form in platelets and no aggregation occurs (fig. 3) - thus no thromboxane synthesis occurs. This can be inferred from our laboratory results showing that platelets release large amounts of free arachidonic acid in plasma (results not yet published). Thus reacylating-deacylating cycle appears to play a major role in preventing the mechanism of aggregation in platelet basal state.

Yoshida and Aoki (21) recently showed that addition of albumin to washed platelets increases release of arachidonic acid and inhibits both collagen-induced aggregation and formation of MDA, a byproduct of cyclo-

oxygenase pathway. They concluded that there might be a threshold of arachidonic acid level beyond which albumin cannot trap the whole released arachidonic acid, the remainding becoming therefore efficiently available for the cyclooxygenase pathway.

This is emphasized by comparative renewal rates of arachidonic molecular species of the main platelet lipids for arachidonic acid and for its sodium salt (fig.4). This latter anionic form of arachidonic acid is intertwined into platelet membranes as to interact with phospholipids positively charged polar head groups (22). Furthermore this anionic form could not be activated as a CoA : thioester.

Acylation process was slown down when incubations were carried out with sodium arachidonate, which assesses that only a part of arachidonate is transformed in acid form in plasma or in membrane. This would agree with studies showing that the pK of fatty acids incorporated in phospholipidic structures is strongly shifted towards higher values (22). A lot of radioactivity is found in platelet FFA showing that part of the fatty acid has permeate the plasma membrane. However our technique does not allow to determine if arachidonate is there in anionic or protonated form. Further biophysical studies upon ionization equilibrium and motion of polyunsaturated fatty acids in depth of membranes are really necessary to elucidate these processes.

From fig. 4 it nevertheless can be seen that free arachidonic acid internal renewal concentration is twice higher when platelets are incubated with sodium arachidonate than when they are incubated with arachidonic acid at the same concentration. This will explain why in PRP 0.5 mM arachidonate are sufficient to trigger aggregation while it isn't triggered by arachidonic acid below 1 mM plasma level.

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