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EFFECT OF TICLOPIDINE ON ARACHIDONIC ACID METABOLISM IN PLATELET PHOSPHOLIPIDS *IN VITRO*

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Abstract—In the presence of human platelets prelabelled with [^{14}C]arachidonic acid (AA), ticlopidine (10^{-4}–10^{-3} M) induces a release of radioactive AA essentially from phosphatidylcholine. This effect is more limited with [^{3}H]-8,11,14-eicosatrienoic acid. Phospholipid hydrolysis occurs immediately after addition of the drug, is dose-dependent and is accompanied by cell lysis. If platelets are previously lysed by sonication, further incubation at 37° leads to a deacylation of phosphatidylcholine, part of the released AA being reincorporated into phosphatidylethanolamine. Under these conditions, ticlopidine effect on phosphatidylcholine disappears and only a slight inhibition of AA incorporation into phosphatidylethanolamine is observed. On the other hand, upon incubation of non-labelled platelets with [^{14}C]-AA, ticlopidine impairs the incorporation of AA into all platelet phospholipids under conditions which are no longer lytic for the cells. Half maximum effect is observed at 10^{-5} M and $5 \cdot 10^{-5}$ M for phosphatidylcholine and phosphatidylinositol, respectively. It is thus concluded that ticlopidine inhibits the acylation reactions responsible for AA entry into glycerophospholipids. This effect is insufficient for inducing extensive phospholipid hydrolysis unless cell lysis is obtained. However, it might be responsible for the release of trace amounts of AA in intact cells. These results are discussed in relation to the recent finding that ticlopidine promotes an increased synthesis by platelets of prostaglandins D_2 and E_1 (M. Lagarde *et al.*, *Prostagl. Med.* **2**, 433–449, 1979), which might be involved in the antiaggregating effect of the drug.

Ticlopidine is a new inhibitor of platelet aggregation [1], whose mechanism of action remains presently unknown. Among various results reported on biochemical effects of the drug on platelets, one can notice the lack of inhibition of cyclo-oxygenase [2, 3], a slight decrease of endoperoxide conversion into thromboxane B_2 [3] and some modifications of adenylate cyclase [4] and of ADP-binding [5].

Several studies have given evidence for a possible involvement of phospholipases in platelet activation [6, 7]. Two biochemical pathways have been described, the first one involving a phospholipase A_2 [8, 9], the second one being supported by a phosphatidylinositol-specific phospholipase C [10, 11] coupled to a diglyceride lipase [12, 13]. Both pathways achieve the release of arachidonic acid (AA) from platelet phospholipids, which is a prerequisite for endoperoxide, thromboxane and lipoperoxide biosynthesis [14]. Furthermore, phospholipase A_2 could be involved in the production of 1-alkyl-*sn*-glycero-3-phosphorylcholine, the putative precursor of platelet activating factor (PAF-acether) [15–17], whereas the phospholipase C pathway leads to the formation of phosphatidic and lysophosphatidic acids [12], which are able to activate platelets through calcium mobilization [18, 19].

On a practical point of view, both pathways have been investigated by following the release of AA from platelet phospholipids after preliminary labelling with radioactive AA [14, 20, 21]. Such a methodology allows a global approach of AA metabolism, although it does not discriminate between phospholipase A_2 and phospholipase C/diglyceride lipase. The same experimental model was used in the present study, in order to investigate the possible effects of ticlopidine on platelet phospholipases.

MATERIALS AND METHODS

Materials. Ticlopidine, 5-(2-chlorobenzyl)-4,5,6,7-tetrahydrothieno (3,2-C) pyridine hydrochloride, was supplied by Parcor, Toulouse, France. 1-[^{14}C]arachidonic acid (55 mCi/mmole) and 8,9,11,12,14,15(n) [^{3}H]eicosa-8,11,14-trienoic acid (130 Ci/mmole) were purchased from the Radiochemical Centre, Amersham, England. The latter one was diluted with cold 8,11,14-trienoic acid (Sigma, Saint Louis, Mo, USA) to the same specific radioactivity as arachidonic acid. Bovine thrombin and 5,8,11,14-eicosatetraynoic acid were obtained from Hoffmann La Roche, Basel, Switzerland. Bovine serum albumin was from the Centre Régional de Transfusion Sanguine, Toulouse, France. All the glassware used for platelet isolation was siliconized with Sigmacote (Sigma).

Preparation of platelet suspension prelabelled with radioactive fatty acids. Blood was drawn from human volunteer donors using acid/citrate/dextrose as an anticoagulant [22]. Platelets were isolated and washed at room temperature according to Ardlie *et al.* [23]. Briefly, the platelet-rich plasma was obtained by centrifugation of the blood at 150 *g* for 15 min. Platelets were pelleted at 1000 *g* for 15 min and resuspended in a Tyrode buffer (pH 6.5) containing 2 mM $MgCl_2$, 5.5 mM glucose, 0.25 mM EGTA and 0.35% (w/v) bovine serum albumin. Final concentration of cells was $5 \cdot 10^{8}$/ml. The suspension was added to tubes containing radioactive fatty

acid(s) dissolved in ethanol so as to obtain a final concentration of 10 nCi/ml, the volume of ethanol not exceeding 5 μl/ml of suspension. After incubation for 60 min at 37°, the platelets were washed once in the same buffer and finally suspended in a Tyrode buffer (pH 7.35) containing 1 mM $MgCl_2$, 2.5 mM $CaCl_2$, 11 mM glucose and lacking albumin, unless otherwise stated. Final concentration was adjusted to 10^9 cells/ml.

Preparation of platelet lysates prelabelled with radioactive fatty acids. A platelet suspension prepared as described above was kept in an ice bath and lysed by sonication (3 times 15 sec) using an MSE sonifier at maximum output.

Incubation of radioactive platelet suspensions or lysates with ticlopidine. Appropriate volumes of platelet suspensions were added to tubes containing eicosatetraynoic acid so as to obtain a final concentration of 40 μM, the ethanol volume not exceeding 10 μl/ml suspension. After a preincubation of 10 min at 37°, the suspension was then additionned with appropriate volumes of a 10^{-2}M ticlopidine solution in 0.15 M NaCl or with the same volumes of NaCl for the controls. Incubations were then performed at 37° under shaking for various times. It was verified that, despite the acidic pH of the 10^{-2}M ticlopidine solution, the pH of the Tyrode buffer was not modified until final concentrations of 10^{-3}M.

Incubation of non-labelled platelet suspensions with 1-[^{14}C]-*AA.* The platelet suspension was prepared by the Ardlie's method as described above, the incubation step with radioactive fatty acid being omitted. Incubations were started by adding appropriate volumes of the suspension ($5 \cdot 10^8$ cells/ml) to tubes containing [^{14}C]-AA (80 nCi/ml, final concentration) dissolved in ethanol (4 μl/ml) and various volumes of 10^{-2} ticlopidine as described above.

Lipid analysis. The incubations were terminated by adding 90 mM EDTA (pH 7.4) dissolved in 0.15 M NaCl (50 μl/ml) and lipids were immediately extracted according to Bligh and Dyer (24). This procedure enabled a 95 per cent extraction of free fatty acids, as checked in control experiments with radioactive AA. Phospholipids were separated by two-dimensional thin-layer chromatography on Silicagel F 254-coated plates (Merck), 0.25 mm thickness, as described elsewhere [25]. In this system, fatty acids were not resolved from other neutral lipids, which were located at the front of both solvents. Non-esterified fatty acids represented over 90% of the total radioactivity present in this spot (see Results). The separation of neutral lipids was performed by monodimensional thin-layer chromatography according to Derksen and Cohen [26]. In this case, all the phospholipids were concentrated at the origin. The various lipid spots were detected by exposure to iodine vapor and were directly scraped into scintillation vials containing 10 ml Instafluor (Packard). Radioactivity was determined by liquid scintillation counting with an Intertechnique liquid scintillation spectrometer (model 4000) with automatic quenching correction.

Determination of cell lysis. Cell lysis was determined by measuring lactate dehydrogenase as previously described [27], using the method of Wroblewski and La Due [28].

RESULTS

Preliminary experiment. This experiment was devised in order to check the hypothesis that ticlopidine could inhibit the phospholipase A_2 activation triggered by thrombin. As shown in Table 1, thrombin induced a significant release of both fatty acids, but mainly AA, from platelet phospholipids. Surprisingly enough, ticlopidine alone produced a similar effect, the extent of hydrolysis being even greater in the latter case. However, thrombin- and ticlopidine-induced hydrolysis was not additive under these conditions and the percentage of hydrolysis obtained in the presence of the two agents was identical to that measured with ticlopidine alone. These results prompted us to study in more details the effects of

Table 1. Effects of thrombin and ticlopidine on the distribution of radioactive arachidonic and eicosatrienoic acids between the different lipid classes*

Conditions	Phospholipids (%)	Non esterified fatty acids (%)	Triglycerides (%)
Arachidonic acid			
Controls	98.3	0.7	0.9
Thrombin	86.1	13.0	1.0
Ticlopidine	76.1	22.8	1.1
Thrombin + ticlopidine	75.0	23.5	1.5
Eicosatrienoic acid			
Controls	94.4	0.7	4.8
Thrombin	92.0	3.2	4.8
Ticlopidine	89.3	5.9	4.9
Thrombin + ticlopidine	90.0	5.5	4.5

* Platelets were labelled with both [^{14}C]-AA and [^{3}H]eicosatrienoic acid and suspended in albumin-free Tyrode buffer (10^9 cells/ml). After a preincubation at 37° for 10 min with 40 μM eicosatetraynoic acid, ticlopidine (final concentration 10^{-3} M) and (or) thrombin (final concentration 5 U/ml) were added and incubation was performed for 5 min at 37°. The lipid extract [24] was then analysed by thin layer chromatography [26]. Results are expressed as the percentage of the total radioactivity recovered from the plates, which was identical in controls and assays.

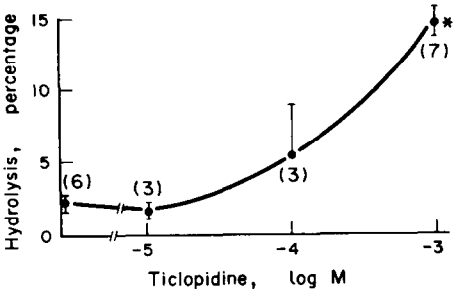

Fig. 1. Effect of increasing ticlopidine concentrations on AA release from platelet phospholipids. [^{14}C]-AA-prelabelled platelet suspensions (10^9 cells/ml) were incubated for 10 min at 37° with 40 μM eicostatetraynoic acid, followed by 30 min in the presence of ticlopidine. Phospholipid hydrolysis corresponds to the percentage of total lipid radioactivity present as free fatty acids upon thin-layer chromatography [26]. Results are mean ± S.E.M. In parenthesis is given the number of determinations. *Probability of significance according to Student's *t*-test, $P < 0.001$.

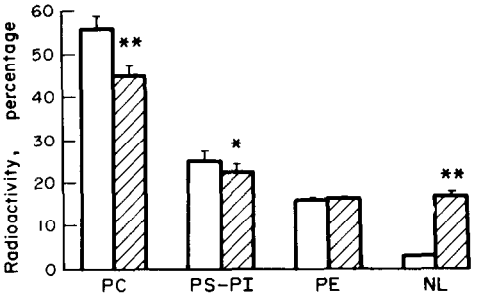

Fig. 3. Identification of phospholipids hydrolysed upon ticlopidine treatment of intact platelets. [^{14}C]-AA-prelabelled platelet suspensions (10^9 cells/ml) were preincubated for 10 min at 37° with 40 μM eicosatetraynoic acid in albumin-free Tyrode buffer. Ticlopidine (10^{-3} M, final concentration) was then added and incubation was prolonged for 30 min. Results (mean ± S.E.M., 4 experiments) are expressed as percentage of total lipid radioactivity recovered from the plates, which was identical in controls and assays. Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; NL (neutral lipids) refer to lipids from the solvent front. Open bars, no ticlopidine; hatched bars, 10^{-3} M ticlopidine. Probability of significance according to Student's paired *t*-test: *$P < 0.02$; **$P < 0.001$.

ticlopidine on phospholipid metabolism by using platelets which had been labelled with [^{14}C]-AA only.

Effect of ticlopidine on [^{14}C]-*AA-labelled platelets.* As depicted in Fig. 1, phospholipid hydrolysis was only detectable at ticloplidine concentrations higher than 10^{-5} M. The effect was dose dependent and appeared immediately after addition of the drug, but levelling off was observed after 15 min incubation (Fig. 2). However, determination of lactate dehydrogenase activity in the supernatants revealed that cell lysis strongly paralleled the phospholipid hydrolysis. The data of Fig. 3 clearly indicate that the AA released mainly originated from phosphatidylcholine, phosphatidylserine plus phosphatidylinositol being hydrolysed to a lower extent, whereas phosphatidylethanolamine remained unchanged.

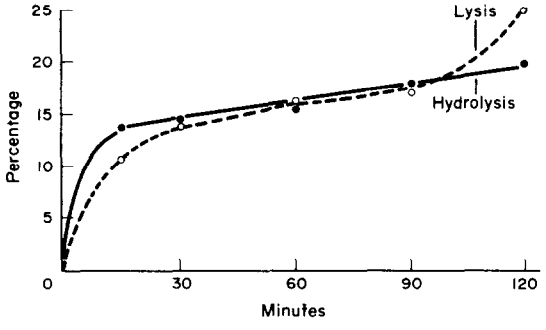

Fig. 2. Time-course of phospholipid hydrolysis and of cell lysis induced by ticlopidine. [^{14}C]-AA-prelabelled platelet suspensions in albumin-free Tyrode buffer (10^9 cells/ml) were preincubated for 10 min at 37° with 40 μM eicosatetraynoic acid. Ticlopidine (10^{-3} M, final concentration) was then added and incubation was prolonged for the indicated times. Phospholipid hydrolysis corresponds to the percentage of total lipid radioactivity present as free fatty acids upon thin-layer chromatography [26]. Cell lysis corresponds to the percentage of total platelet lactate dehydrogenase measured in the supernatants. Results are from a typical experiment selected from 7 different experiments. At 60 min, phospholipid hydrolysis and cell lysis were 14.9 ± 1.3% and 35.7 ± 15.7%, respectively (mean ± S.E.M. 3 experiments).

The same experiments as those depicted in Fig. 3 were then performed at a lower concentration of ticlopidine ($5 \cdot 10^{-5}$ M) with bovine serum albumin added to the medium. Under these conditions, the drug was no longer lytic towards platelets, and no significant hydrolysis could be detected for any phospholipid. For instance, the percentage of total radioactivity in the phosphatidylcholine was 45.0 ± 1.9 and 46.9 ± 2.0 (mean ± S.E.M., 4 experiments) in the absence and in the presence of ticlopidine, respectively.

Effect of ticlopidine on platelet lysates. The preceding results suggested a possible relationship between cell lysis and phospholipid hydrolysis induced by ticlopidine. In order to separate the action of the drug on the platelet membrane integrity from a possible direct effect on enzymes of phospholipid metabolism, the same experiments were then performed with platelets which had been previously lysed by sonication. Under these conditions, no hydrolysis could be detected, a very small but significant difference ($P < 0.05$ according to Student's paired *t*-test) being measured for phosphatidylethanolamine only: 18.9 ± 0.5 and 17.1 ± 0.8 per cent (mean ± S.E.M., 5 experiments) in the presence and in the absence of ticlopidine, respectively. However, the percentage of radioactivity in phosphatidylcholine from platelet lysates incubated for 30 min at 37° (47.8%) was lower than the percentage measured in non-lysed platelets incubated under the same conditions (56.0% from data in Fig. 3). This led us to investigate the behaviour of labelled platelet lysates during incubation at 37°. As shown in Table 2, 30 min incubation of lysed platelets was accompanied by a spontaneous release of AA from phosphatidylcholine, part of the radioactivity being

Table 2.Spontaneous release of AA from phospholipids of prelabelled platelet lysates*

Lipids	Before incubation	After incubation	P†
Phosphatidylcholine	54.4 ± 3.5	48.5 ± 2.9	<0.01
Phosphatidylserine + phosphatidylinositol	25.4 ± 2.5	25.8 ± 2.7	NS
Phosphatidylethanolamine	17.0 ± 1.0	18.9 ± 0.6	<0.02
Solvent front	3.2 ± 0.4	6.9 ± 0.4	<0.02

* Platelets were labelled with [^{14}C]-AA and lysed by sonication in Tyrode buffer lacking albumin (10^9 cells/ml, final concentration). Lipids were extracted immediately after lysis or after incubation for 30 min at 37°. Results (mean ± S.E.M., 4 experiments) correspond to percentage of the total radioactivity recovered from the plates, which was identical in controls and assays.

† P, probability of significance according to Student's paired *t*-test; NS, not significant.

accumulated in the phosphatidylethanolamine fraction. On the other hand, the same experiments performed with intact platelets did not reveal any difference in radioactivity distribution among phospholipids. For instance, the percentage of radioactivity in phosphatidylcholine was 54.9 ± 3.6 per cent and 54.4 ± 2.9 per cent at 0 and 30 min, respectively (mean ± S.E.M., 4 experiments).

When these results obtained on platelet lysates are considered together, one could thus conclude that ticlopidine effect on platelet lysates is not a stimulation of AA release from phosphatidylethanolamine, but an inhibition of AA reincorporation into the latter. Although the differences measured were rather low, these results could be indicative of a possible effect of ticlopidine on the processes of arachidonic acid incorporation into platelet phospholipids. This hypothesis was checked in the experiments described below.

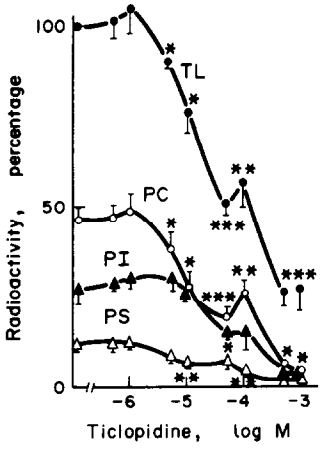

Fig. 4. Effect of various concentrations of ticlopidine on AA incorporation into platelet lipids. Platelets were suspended in Tyrode buffer containing albumin ($5 \cdot 10^8$ cells/ml) and incubated with [^{14}C]-AA (80 nCi/ml, final concentration) for 30 min at 37°. Results (mean ± S.E.M., 4 experiments) are expressed as percentage of the radioactivity present in total lipids from platelets incubated without ticlopidine. Abbreviations: TL, total lipids; PC, phosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine. The radioactivity in phosphatidylethanolamine and solvent front are not represented in the graph for more clarity. They corresponded to 9.4 ± 1.3% and 6.1 ± 1.1%, respectively, in control platelets (mean ± S.E.M.) Only phosphatidylethanolamine was inhibited, whereas lipids from the solvent front were not significantly modified. Probability of significance according to Student's paired *t*-test: *P < 0.05; **P < 0.01; ***P < 0.001.

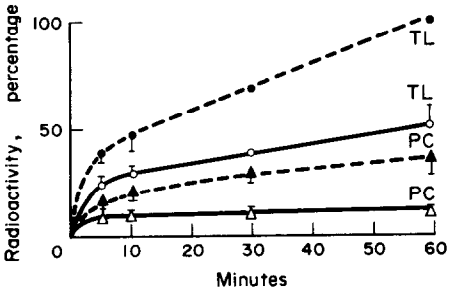

Fig. 5. Time-course of ticlopidine inhibition of AA incorporation into platelet lipids. Platelets were suspended in Tyrode buffer containing albumin ($5 \cdot 10^8$ cells/ml) and incubated with [^{14}C]-AA (80 nCi/ml, final concentration) for the appropriate times in the absence (----) or in the presence (————) of $5 \cdot 10^{-5}$ M ticlopidine. Results (mean ± S.D., 2 experiments) are expressed as percentage of the radioactivity present in total lipids from platelets incubated for 60 min without ticlopidine. Only total lipids (TL) and phosphatidylcholine (PC) are shown for more clarity.

Effect of ticlopidine on AA incorporation into platelet phospholipids. As shown in Figs. 4 and 5, the drug exerted a rapid and strong inhibition of AA entry into platelet phospholipids. It is noteworthy that all these experiments were conducted in the presence of bovine serum albumin, under which conditions a small lysis (9%) started at $5 \cdot 10^{-4}$ M ticlopidine only.

On the other hand, a significant effect on AA incorporation into total lipids and phosphatidylcholine was detected at $5 \cdot 10^{-6}$ M ticlopidine. A 50 per cent inhibition occured at $5 \cdot 10^{-5}$ M and 10^{-5} M for total lipids and phosphatidylcholine, respectively. Phosphatidylinositol appeared to be less sensitive to ticlopidine, since radioactivity remained unchanged at $5 \cdot 10^{-6}$ M, a significant effect being detected at $5 \cdot 10^{-5}$ M.

DISCUSSION

In the work reported herein, we have studied the effects of ticlopidine on AA metabolism in platelet

phospholipids. This experimental model was that devised by Bills *et al.* [14, 20], using platelet suspension previously labelled with radioactive fatty acids. Under such conditions, these authors observed that thrombin triggers the release of labelled AA from phosphatidylcholine and phosphatidylinositol and its further conversion into metabolites of cyclo-oxygenase/thromboxane synthetase and of lipoxygenase. In the present study, eicosatetraynoic acid was added to the incubation medium as an inhibitor for both cyclo-oxygenase and lipoxygenase. This allowed the measurement of AA release by detecting only the radioactivity of free fatty acids. The validity of such a method is shown in Table 1, since the phospholipid hydrolysis induced by thrombin is essentially similar to that reported by Bills *et al.* [14, 20].

Under these conditions, ticlopidine triggers phospholipid hydrolysis in a manner similar to thrombin, but only at concentrations which become lytic for the cells. Our results do not allow to identify the pathway (phospholipase A_2 or phospholipase C/diglyceride lipase) responsible for phospholipid hydrolysis. However, results from Fig. 3 indicate that AA release primarily occurs from phosphatidylcholine, which can be hydrolysed only by phospholipase A_2 [8–11]. This should lead to the appearance of 1-acyl-(*sn*)-glycero-3-phosphorylcholine (lysophosphatidylcholine), which could not be detected by the present methodology, the [^{14}C]-AA occupying only the 2-position of the phosphoglycerides.

A simple explanation for phospholipid hydrolysis would be the cell lysis induced by high concentrations of ticlopidine. In agreement with this view, incubation of platelet lysates is followed by a release of AA from phosphatidylcholine (Table 2), whereas ticlopidine effects are no longer apparent. However, part of the AA released from phosphatidylcholine in lysed platelets is reincorporated into phosphatidylethanolamine, probably plasmalogens, as first described by Rittenhouse-Simmons *et al.* [29, 30]. Under these conditions, ticlopidine suppresses such a reincorporation.

This result prompted us to look for a possible effect of ticlopidine on AA incorporation into phospholipids. As shown in Figs. 4 and 5, the drug is indeed able to inhibit AA esterification into platelet phospholipids at concentrations which are not lytic for the cells, i.e. under more physiological conditions. Noteworthy, this inhibition is not accompanied by an increase of free AA in the platelets, confirming the previous observation of Chambaz *et al.* [31], who showed that increasing the plasma concentration of AA over saturating levels required for acylation does not promote accumulation of free AA in the platelets.

The incorporation of AA into phospholipids as measured in the present system is the sum of various biochemical events: transmembranous uptake, activation through thiokinase into arachidonoyl-coenzyme A and acylation of an acceptor lysophospholipid through an acyltransferase [32]. So the global inhibition observed in this study could follow the blockade of any of these steps, which would merit attention for future studies. However, the first step can be easily eliminated as a target for ticlopidine, since a similar effect of the drug (inhibition of AA transfer from phosphatidylcholine to phosphatidylethanolamine) also occurs in lysed platelets. Furthermore, a decrease of acceptor lysophospholipids secondary to a phospholipase inhibition could also explain our results. However, such a hypothesis can be eliminated, since we have already shown that under lytic conditions ticlopidine promoted phospholipid hydrolysis. On the contrary, activation of a phospholipase A_2 induced by ticlopidine could also decrease the apparent incorporation of AA into platelet phospholipid, through an isotopic dilution of the exogenous radioactive precursor by endogenous cold AA. This is also highly improbable because we have shown that ticlopidine does not promote AA release from prelabelled platelets under these non-lytic conditions. But a specific effect of the drug on a non-labelled pool of AA cannot be excluded.

In conclusion, ticlopidine affects the metabolism of platelet phospholipids by inhibiting the incorporation of AA. Such an effect could be secondary to a blockade of thiokinase or acyltransferase(s), but further studies are still necessary to demonstrate it. The inhibition of AA incorporation is accompanied by a significant release of free AA from prelabelled platelet phospholipids only when cell lysis occurs. However, one cannot exclude the possibility of a small release of free AA, undetectable under our conditions of assay. This would fit with the previously reported increase of prostaglandin D_2 induced by ticlopidine [3], which could explain, at least partially, the antiaggregating effect of the drug.

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