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ACCUMULATION OF DIACYLGLYCEROL INDUCED IN PLATELETS BY EXOGENOUS FATTY ACIDS

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SUMMARY: Free fatty acids added in ethanol to human platelets prelabelled with [^{14}C]arachidonate induce an accumulation of radioactive diacylglycerol. Unsaturated fatty acids are ten times more potent than palmitate. Ethanol alone does not alter the distribution of radioactivity. Increasing the concentration of arachidonate leads to increased diacylglycerol formation. The fatty acid effect is independent of thrombin, which itself causes a relatively small change in diacylglycerol levels. Neither the labelled triacylglycerol nor the labelled free fatty acid appears to be the source of the diacylglycerol formed which may arise from the activation of phosphatidylinositol phosphodiesterase.

The release of arachidonate from membrane phosphoglycerides is one of the essential reactions resulting in platelet aggregation by thrombin and other agents (1). It has previously been held that the arachidonate is released by a phospholipase A_2. Phospholipase A_2 activity has been isolated from human platelets (2), but as yet there has been no evidence for a phospholipase A_2 that specifically releases arachidonate from phospholipids (3). More recently, evidence has been presented supporting the idea that the initial reaction involves phospholipase C acting upon phosphatidylinositol (PI) to form diacylglycerol with subsequent release of arachidonate from the diacylglycerol by diacylglycerol lipase (4,5). Although diacylglycerol could not be detected in the preparations of platelet fractions of Trugnan et al. (2), these workers did not discount the earlier suggestion of Chap (6) that phospholipase C and diacylglycerol lipase operating together could form arachidonate. We have now observed that free fatty acids, particularly those containing double bonds, cause the accumulation of radioactive diacylglycerol in platelets that have previously been incubated to incorporate radioactive arachidonate.

METHODS

Materials: [1-^{14}C]arachidonate (specific activity 56 mCi/mmole) was purchased from The Radiochemical Centre, Amersham. Arachidonate, linoleate, palmitate, oleate and bovine serum albumin Fraction V were purchased from Sigma Chemical Company Ltd., London. Siliconized glassware was used throughout.

Abbreviation: PI = phosphatidylinositol

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Preparation of Platelets: 100 ml blood were collected in syringes containing 1 mM EDTA from normal volunteers, and immediately transferred to siliconized tubes containing 0.38% trisodium citrate. The blood was centrifuged at 200 g for 15 min. The platelet rich plasma was used to prepare the platelets according to the method of Bills et al. (7). Platelets were finally suspended in buffered saline containing 5 mM D-glucose, 134 mM NaCl, 15 mM Tris-HCl pH 7.4, and 1.0% bovine serum albumin at a cell concentration of 3-4 x 10^8/ml. All operations were carried out at 4°C.

[^{14}C]Arachidonate Labelling of Platelets: The labelled fatty acid was blown to dryness under a stream of oxygen free nitrogen and made up to 1 μCi/ml in buffered saline containing 1% albumin. 20 μl of this was added per ml platelet suspension. Incubations were for 60 min at 37°C. The platelets were then cooled to 4°C and 1 mM ice cold EDTA was added. After centrifugation of the platelet suspension at 2000 g for 20 min at 4°C, the pellet was washed with buffered saline containing EDTA but without albumin. The platelets were finally suspended in saline buffer without EDTA or albumin at a cell concentration of 5-7 x 10^8/ml. Protein content was assayed according to the method of Lowry et al. (8).

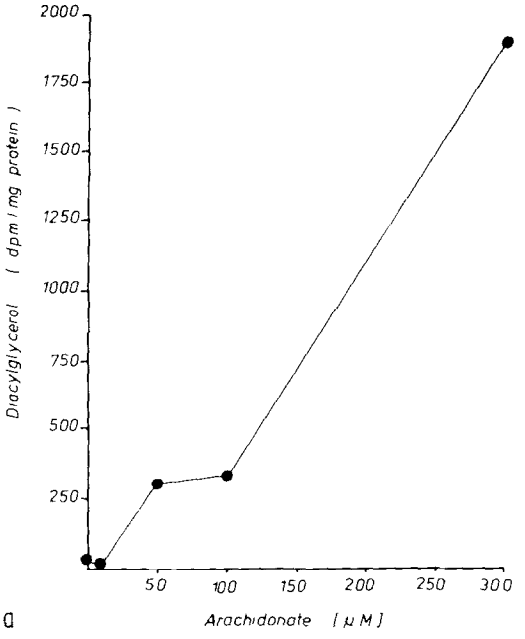
Incubation with Exogenous Fatty Acids: 3 ml of the prelabelled platelet suspensions were incubated for 5 min at 37°C, with or without fatty acids dissolved in 40 μl ethanol. Arachidonate was added between 10 μM and 300 μM. Palmitate, oleate and linoleate were added at a concentration of 300 μM.

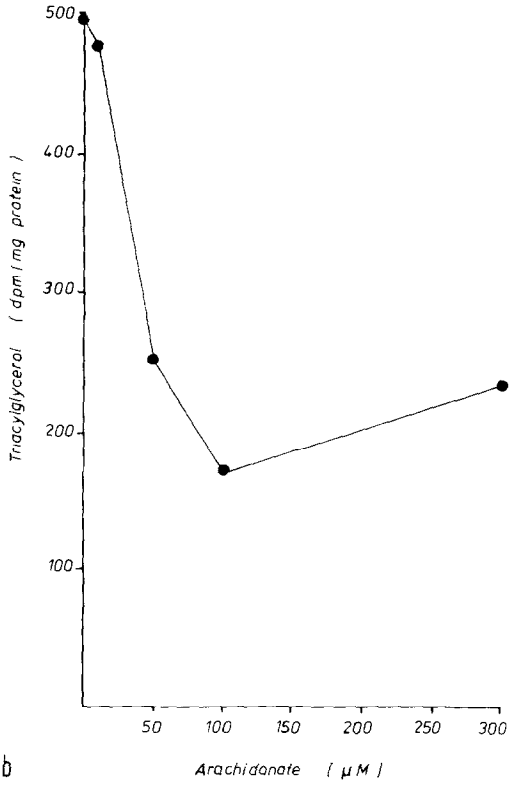
Thrombin Stimulation: To the above suspensions, either thrombin (5 U/ml) or an equivalent volume of saline was added. Incubations were continued for 5 min at 37°C after which the reaction was terminated by adding 0.8 ml 0.1 M EDTA.

Lipid Extraction and Fractionation: Total lipid extractions of the platelet suspensions were carried out according to the method of Bills et al. (9). Extracts were loaded onto columns containing 1 g silicic acid and eluted with 20 ml chloroform to give a neutral lipid fraction. The neutral lipids were separated on silica gel H thin layer chromatograms using petroleum ether/diethylether/acetic acid (90:10:1) or the upper phase of ethyl acetate/iso-octane/water (74:24:100) as a solvent system. Bands were visualised with iodine vapour and the plates were subjected to autoradiography to identify radioactive areas, which were scraped into vials and assayed in a scintillation counter.

RESULTS

The formation of radioactive diacylglycerol in prelabelled platelets, in the presence of increasing concentrations of exogenous non-radioactive fatty acid, is shown in Figure 1a. Low levels of acid in the region of 50 μM cause some diacylglycerol accumulation. Increasing the concentration of the fatty acid increases the incorporation of label into diacylglycerol. There is more than an 80 fold increase in diacylglycerol in the presence of 300 μM arachidonate compared with control cells in the absence of the acid. Although labelled triacylglycerol decreases in the presence of arachidonate up to a concentration of 100 μM (Figure 1b), this does not account for the continued increase of diacylglycerol at concentrations of fatty acid more than 100 μM, where the change in triacylglycerol is negligible. The radioactivity in the diacylglycerol fraction increases by 1600 dpm/mg protein as the concentration of non-radioactive fatty acid increases from 100 μM to 300 μM, whereas radioactivity in the triacylglycerol increases only by 60 dpm/mg protein





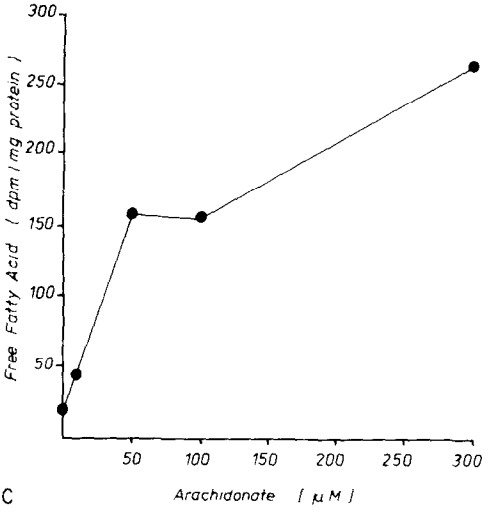


Fig. 1 Effect of exogenous arachidonate on the formation of (a) diacylglycerol, (b) triacylglycerol and (c) free fatty acid in human platelets. Cells were prelabelled with [^{14}C]arachidonate, washed and subsequently incubated with increasing concentrations of arachidonate for 5 min. Radioactive lipids were extracted and assayed as described in the methods section.

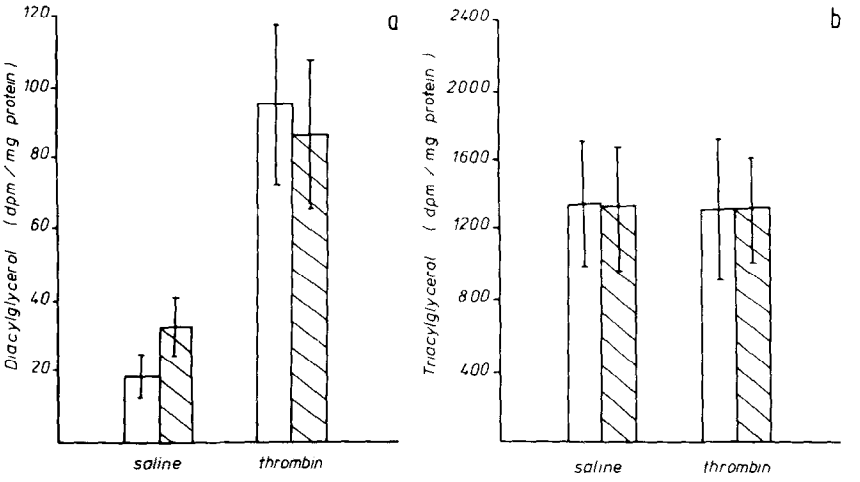


Fig. 2. Effect of thrombin on [^{14}C]arachidonate distribution in (a) diacylglycerol, (b) triacylglycerol of human platelets in the presence (hatched bars) or absence (open bars) of ethanol. Cells were prelabelled with [^{14}C]arachidonate, washed and finally incubated with or without thrombin for 5 min.

between the two concentrations. Figure 1c shows that as the concentration of non-radioactive fatty acid increases, the release of free [^{14}C]arachidonate follows a course that is similar to, but very much less pronounced than that of labelled diacylglycerol.

Figure 2 shows that ethanol alone had a negligible effect on diacylglycerol accumulation. Recently, it has been shown that ethanol in the concentration range of 33-300 mg/100 ml has no effect on [^{14}C]arachidonate incorporation into prostaglandins (10). In the absence of fatty acid, the steady state level of diacylglycerol is increased 5 fold when thrombin is added (Fig. 2a). There is no effect of thrombin on labelled triacylglycerol formation (Fig. 2b). It has been reported that thrombin stimulates a 30-fold transient increase in diacylglycerol which declines after 15 sec (4).

Further experiments investigated the effects of different fatty acids on labelled diacylglycerol, and triacylglycerol formation in the presence and absence of thrombin. Table I shows that oleate is almost ten times as potent as palmitate, in stimulating diacylglycerol formation. Both acids reduce incorporation of label into triacylglycerol to the same extent. Polyunsaturated fatty acids have a similar potency on diacylglycerol production to the monounsaturated fatty acid. Triacylglycerol levels are not significantly altered in the presence of the polyunsaturated fatty acids tested, although thrombin appears to inhibit formation of triacylglycerol when arachidonate is present; this may be due to a dilution effect of the non-radioactive acid.

Table 1

Effect of Fatty Acids on Diacylglycerol and Triacylglycerol Formation in Prelabelled Human Platelets

		DIACYLGLYCEROL		TRIACYLGLYCEROL	
		Saline	Thrombin	Saline	Thrombin
Experiment A	Control	17±5	99±17	1893±356	1793±336
	Palmitate	122±49	213±64	1237±307	1620±339
	Oleate	1069±349	766±118	1336±199	1172±194
Experiment B	Control	21±9	93±34	813±142	879±127
	Linoleate	1105±361	997±275	926±136	915±151
	Arachidonate	929±292	1071±253	928±81	751±85

3 ml washed prelabelled platelet suspensions were incubated with or without 300 μM fatty acid in the presence or absence of thrombin for a total of 10 min as described under methods. Experiment A was carried out one week previously to experiment B, using the same volunteers for each experiment. Values are given as the mean dpm/mg protein ± S.E.M. of label incorporated into diacylglycerol and triacylglycerol.

DISCUSSION

The present results demonstrate that addition of exogenous free fatty acids to platelet preparations results in an accumulation of diacylglycerol which is independent of the presence of thrombin. The effect of the unsaturated fatty acids tested is in contrast to the lack of effect of palmitate. Free fatty acids have effects on a number of enzyme activities which may often be specific for saturated or unsaturated acids (11,12).

There appear to be several possible explanations of the effect of the fatty acids. The radioactive diacylglycerol could be formed as a result of the release of radioactive arachidonate from esterified sites in phospholipids or triacylglycerol, followed by its incorporation to diacylglycerol. This seems unlikely in view of the fact that no radioactivity accumulates in diacylglycerol during the pre-incubation with radioactive arachidonate. It is more likely that the diacylglycerol arises as a result of the action of a phosphodiesterase acting on PI. It has been shown that a rat brain PI-phosphodiesterase is stimulated by oleate and arachidonate (13).

Our results are consistent with the theory that diacylglycerol formation may be directly involved in providing substrate for prostaglandin synthesis. The release of arachidonate from platelet phospholipids is known to be inhibited by cAMP and to require Ca^{2+} for its full activity (1). It has been suggested that arachidonate release involves a PI-specific phospholipase

C, which is also inhibited by cAMP, followed by a diacylglycerol lipase (4,5), and that these enzymes require Ca^{2+} for optimal activity. Recently it has been shown that indomethacin at concentrations above those which inhibit cyclooxygenase can inhibit platelet diacylglycerol lipase only in the presence of thrombin. This leads to an accumulation of diacylglycerol (14). It is therefore possible that the unsaturated fatty acids are also inhibiting the diacylglycerol lipase. We have found (unpublished results) that prostaglandin synthesis is inhibited in the presence of fatty acid at concentrations that cause the accumulation of diacylglycerol. This could be a direct effect of the fatty acids themselves. Oleic, linoleic and γ-linolenic acids in the concentration range 0.9-3.6 mM, have been shown to inhibit prostaglandin synthesis in the sheep vesicular gland (15). However, it is also possible that the diacylglycerol which is formed could be responsible for this inhibition of prostaglandin synthesis.

There is a further possibility that the effect demonstrated here may be reflecting a physiological role of unsaturated fatty acids which may activate platelet PI-phosphodiesterase, after they have been released from phosphoglycerides by phospholipase A_2. Phospholipase activity depends on an increase in cytoplasmic levels of Ca^{2+} ions, promoted by ionophore A23187 or thrombin. Such an increase is independent of the extracellular levels of Ca^{2+} (16). A23187 and thrombin activate platelets, possibly by making the intracellular pools of Ca^{2+} more accessible to phospholipase A_2. Alternatively they could operate by activating the enzyme via a cAMP-dependent kinase (3), as a consequence of diacylglycerol formation. Unsaturated diacylglycerol lowers the concentration of Ca^{2+} and phospholipid required for a Ca^{2+}-activated phospholipid-dependent protein kinase in brain (17), and may have a similar action in platelets. This protein kinase can be activated without a net increase in intracellular Ca^{2+} as the unsaturated diacylglycerol increases the affinity of the enzyme for Ca^{2+}. In addition, diacylglycerol may be having an effect on phospholipase A_2 similar to that exerted on the protein kinase.

We have observed a similar accumulation of radioactive diacylglycerol in lymphocytes, pre-incubated with [^{14}C]arachidonate to label the membrane phospholipids, and then incubated for periods between 5 and 20 min with non-radioactive linoleate. Thus, it is possible that this effect of fatty acids will be found to be a general phenomenon that occurs with a variety of cell types. The role of diacylglycerol in the process of platelet activation remains to be determined.

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