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POSSIBLE INVOLVEMENT OF DIRECT STIMULATION OF PROTEIN KINASE C BY UNSATURATED FATTY ACIDS IN PLATELET ACTIVATION

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Abstract—Arachidonate and other unsaturated fatty acids stimulated platelet protein kinase C in a dose-dependent manner (5–50 μg/ml), when the activity was assayed with either isolated substrates or the platelet cytosol. When human platelets were stimulated by arachidonate, two types of platelet activation were observed. Platelet activation induced by a low level of arachidonate (0.1–5 μg/ml) was inhibited by aspirin, but activation induced by a high level of arachidonate (10–50 μg/ml) was not. These activations were associated with the phosphorylation of 40K and 20K proteins. Other unsaturated fatty acids (10–50 μg/ml) also induced platelet aggregation which was not inhibited by aspirin. Arachidic acid and methyl arachidonate, which did not stimulate protein kinase C, also did not induce platelet responses. Although a low level of arachidonate (0.45 μg/ml) induced the rapid and transient formation of [^{3}H]-1,2-diacylglycerol and [^{32}P]phosphatidate in intact platelets prelabeled with [^{3}H]arachidonate or [^{32}P]P_i, unsaturated fatty acids at a high concentration (50 μg/ml) did not stimulate phospholipase C. Incubation of fura 2 loaded platelets with a high level of unsaturated fatty acids evoked a rise in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) but this $[Ca^{2+}]_i$ elevation alone was not associated with platelet activation. These results suggest that a high level of unsaturated fatty acids induces platelet activation, without phospholipase C stimulation, and that the ability of unsaturated fatty acid to directly activate protein kinase C may contribute toward the activation of platelets by a high level of unsaturated fatty acid.

It is generally accepted that exogenous arachidonate can induce irreversible aggregation and secretion after bioconversion to prostaglandin endoperoxides and thromboxane A_2 [1–6]. However, Hashimoto *et al.* [7] found that this arachidonate-induced activation of platelets can be separated into two types, using different concentrations of arachidonate: a low level of arachidonate (<20 μM) induced aggregation which is inhibited by aspirin, and a high level of arachidonate (>30 μM) induced aggregation not blocked by aspirin. Arachidonate induces phosphorylation of the 20K light chain of myosin and the 40K protein [8]. The 40K protein is phosphorylated by protein kinase C [9, 10], presumably activated by diacylglycerol from the enhanced turnover of inositol phospholipids by phospholipase C [11, 12]. The 20K light chain of myosin can be phosphorylated by both protein kinase C and the Ca^{2+}/calmodulin-dependent enzyme, myosin light chain kinase [13–15]. A low level of arachidonate has been shown to induce an indirect activation of phospholipase C, through the formation of cyclooxygenase metabolites [8]. Arachidonate and other unsaturated fatty acids directly activate protein kinase C, in a dose- and Ca^{2+}-dependent fashion, determined using crude or partially purified enzyme preparations [16, 17]. While arachidonate-induced platelet responses seem to be principally mediated by metabolites after a bioconversion, exogenous arachidonate may also exert a direct effect on the protein kinase C present in intact platelets, as is the case in leukocytes [16].

We have now examined whether the arachidonate-induced platelet activation is associated with the direct activation of protein kinase C by arachidonate. We obtained data suggesting that a high level of arachidonate (10–50 μg/ml) induces the phosphorylation events, without phospholipase C activation, and that the ability of unsaturated fatty acids, other than arachidonate, to stimulate protein kinase C may account for the activation of intact platelets by the fatty acids.

MATERIALS AND METHODS

Materials. Lipids were obtained from the following sources: phosphatidylserine, 1,2-diolein, *n*-eicosanoic acid, *cis*-11-eicosenoic acid, 11,14-eicosadienoic acid, 8,11,14-eicosatrienoic acid, arachidonate and methyl arachidonate were from Supelco (Bellefonte, PA, U.S.A.). These lipids were dissolved in chloroform (50 mg/ml). After removing the chloroform with a stream of N_2 gas in ice, the residue was suspended in 20 mM Tris–HCl (pH 7.5) by sonication for 3–5 min at 0° and then used in the experiments. Isotopes were purchased from the following sources: carrier-free [^{32}P]orthophosphate (40–60 mCi/ml), Japan Atomic Energy Research Institute (Ibaragi, Japan); [^{14}C]serotonin (5-hydroxy[side chain-2-^{14}C]tryptamine creatinine sulfate, approx. 50 Ci/mmol), Amersham International

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plc. (Buckinghamshire, England); [γ-^{32}P]ATP (1000–3000 Ci/mmol) and [^{3}H]arachidonate (60–100 Ci/mmol), NEN Research Products (Boston, MA, U.S.A.). 1-(5-Isoquinoline-sulfonyl)-2-methylpiperazine (H-7*) was purchased from Seikagaku Kogyo Ltd. (Tokyo, Japan) and staurosporine was a gift from the Kyowa Hakko Co., Ltd. (Tokyo, Japan). Protein kinase C was prepared to apparent homogeneity (>95%) from fresh human platelets, as described [18]. A mixture of the 20K and 17K myosin light chains from turkey gizzards was prepared according to Perrie and Perry [19] and was separated from contaminating calmodulin by DEAE-Sephacel chromatography [20]. Histone H-1 was purchased from Boehringer-Mannheim (Indianapolis, IN, U.S.A.). Fura 2-AM was obtained from Dojindo Laboratories (Kumamoto, Japan).

Enzyme assay. Protein kinase C was assayed in a solution (200 μl) containing 40 mM Tris–HCl, pH 7.5, 5 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (1000–2000 cpm/pmol), 0.2 mM $CaCl_2$, 50 μg/ml of phosphatidylserine or various amounts of fatty acid, and an appropriate amount of substrate protein, as indicated. Other conditions are given in the figure legends. The assay was initiated by the addition of ATP following 2- to 3-min preincubation periods at 25°, and then incubation was carried out for various periods of time and was terminated by pipetting 40 μl of the reaction mixture onto Whatman No. 3MM filter paper discs. These discs were then washed, as described by Corbin and Reimann [21]. Enzyme activities were linear as a function of incubation time, in all the assays.

Endogenous phosphorylation of the cytosol of human platelets. Human platelets were collected by sedimentation of platelet-rich plasma at 2000 *g* for 10 min. All other remaining procedures were performed at 4°. The platelet pellet (5–10 g) was washed twice by resuspension in 4 vol. of 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5 mM EDTA and 1 g/L dextrose, and centrifugation was carried out at 2000 *g* for 10 min. The final platelet pellet was suspended in 2–3 vol. of 40 mM Tris–HCl, pH 7.5, 2.5 mM dithiothreitol, 2 mM Na_2EDTA, 2 mM EGTA, 0.4 mM phenylmethylsulfonyl fluoride and 100 mg/L leupeptin, and then homogenized with a ground glass homogenizer in ice. The platelet homogenate was centrifuged at 100,000 *g* for 60 min, and the resulting supernatant fraction was the source of the cytosol of platelets. Endogenous phosphorylation of the platelet cytosol was assayed in the solution containing 40 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 10 μM [γ-^{32}P]ATP, the platelet cytosol (150 μg/tube), 50 μg/ml phosphatidylserine or arachidonate and 200 μM $CaCl_2$ or 2 mM EGTA in a total volume of 0.2 ml. The reaction was performed by the addition of the platelet cytosol and terminated by the addition of 50 μl of five times concentrated Laemmli sample buffer [22] and boiling. The sample was subjected to SDS–polyacrylamide slab gel electrophoresis.

Preparation of platelet suspensions. Venous blood was freshly drawn from healthy donors who had not received any medication during the previous 4 weeks. Washed platelets were prepared by the method of Schmidt and Rasmussen [23], using the erythrocytes as a supporting cushion. Platelets were finally resuspended in a modified Tyrode–Hepes buffer that contained a final concentration of 140 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.1% dextrose, 3.75 mM NaH_2PO_4 and 15 mM Hepes, pH 7.5 (suspension buffer).

Measurement of platelet aggregation and serotonin release. The washed platelet suspension (5×10^8/ml) was incubated for 30 min at 37° with [^{14}C]serotonin (0.1 μCi/ml platelet suspension). Under these conditions, >85% of the radioactivity was taken up in the platelets. Aggregation of the [^{14}C]serotonin-labeled platelets was monitored photometrically at 37°, using a Rikadenki 2 Channel Aggregometer (RAM 21) as described [24]. [^{14}C]Serotonin released from platelets was expressed as a percentage of the total uptake. Aggregation responses were quantified as the maximum extent of aggregation, calculated by the maximum change in light transmission, and expressed as a percentage, taking the difference between light transmission for the platelet suspension and the suspension buffer as a value of 100%. Percent inhibition of aggregation by the drug was calculated by dividing the percent aggregation by that observed in the control run, then multiplying by 100. All concentrations given refer to final ones.

Measurement of [^{3}H]-1,2-diacylglycerol, [^{32}P]phosphatidate and protein phosphorylation in intact platelets. A 0.5-ml sample of platelet suspension (5×10^8/ml) prelabeled with [^{3}H]arachidonate was placed into aggregometer tubes and exposed to the fatty acids. Incubations were stopped by adding 4 vol. of chloroform/methanol (1:2) for lipid extraction [25]. [^{3}H]-1,2-Diacylglycerol and [^{3}H]arachidonate were separated on thin-layer chromatography [26] and localized by co-chromatography with unlabeled standards, visualized by iodine vapor, and measured by liquid scintillation counts.

The platelet suspension (0.5 ml) prelabeled with [^{32}P]orthophosphate was stirred in the aggregometer at 37° and was stimulated with the fatty acid. At the various times indicated, the reaction was terminated by adding 1/5 vol. of five times concentrated Laemmli sample buffer [22] and the mixture was incubated at 100° for 3 min. The sample was subjected to SDS-polyacrylamide gel electrophoresis. For the measurement of [^{32}P]phosphatidate, incubations were stopped by adding 4 vol. of chloroform/methanol (1:2) for lipid extraction [25], and the phosphatidate was separated by thin-layer chromatography [27]. [^{32}P]Phosphatidate was localized by radioautography, and radioactivity was measured by liquid scintillation counting.

Measurement of $[Ca^{2+}]_i$ in platelets with fura 2. Fura 2-AM (1 μM) was loaded into washed platelets for 30 min at 37°, and the cells were washed again to remove the dye. Platelets loaded with fura 2 were resuspended in Hepes buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM Hepes, pH 7.4, 5 mM

* Abbreviations: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; H-7, 1-(5-isoquinoline-sulfonyl)-2-methylpiperazine; $[Ca^{2+}]_i$, intracellular free Ca^{2+} concentration; EGTA, ethyleneglycolbis(amino-ethylether)-tetra-acetate; and SDS, sodium dodecyl sulfate.

glucose). Concentration of the loaded fura 2 was calculated to be 20–30 μM. Fluorescence was measured at 340 and 380 nm excitation and 550 nm emission, using a Hitachi F-4000 fluorescence spectrometer, according to the method of Tsien *et al.* [28] and Grynkiewicz *et al.* [29]. Aliquots (2 ml) of fura 2 loaded platelet suspensions (1×10^8/ml) were transferred to quartz cuvettes, maintained at 37° in a circulating water bath, continually stirred with a small magnetic stirrer, and stimulated by fatty acid. In all experiments, the fluorescence of untreated and unloaded cells was used for subtraction of autofluorescence. To minimize the time-dependent effects on platelet responsiveness and leakage of fura 2, experiments were designed to be completed within 1 hr. $[Ca^{2+}]_i$ in the figures represents the fluorescence ratio, obtained by dividing the fluorescence values at 340 nm by those at 380 nm. This ratio changes in the same direction as $[Ca^{2+}]_i$ [28].

SDS–Polyacrylamide gel electrophoresis. Aliquots containing 50–60 μg of protein were electrophoresed through 0.1% SDS–15% polyacrylamide gel, using the Laemmli buffer [22]. Gels were then stained with Coomassie brilliant blue, dried, and subjected to radioautography, using Kodak X-Omat AR film with an intensifying screen at −80°. The estimation of radioactivity of 20K and 40K proteins was done by cutting out specific areas of the gels, which were then placed in scintillation vials and heated for 2 hr in 30% hydrogen peroxide. The scintillation fluids were then added, and the radioactivity was determined.

RESULTS

Effects of arachidonate and its analogs on platelet protein kinase C activity. Table 1 shows the ability of saturated or unsaturated fatty acids with 20 carbon atoms, such as *n*-eicosanoic acid (arachidic acid, C20), *cis*-11-eicosenoic acid (C20:1), 11,14-eicosadienoic acid (C20:2), 8,11,14-eicosatrienoic acid (C20:3), arachidonate (C20:4) and methyl arachidonate (methyl C20:4), to activate platelet protein kinase C, using both histone H-1 and isolated light chains of smooth muscle myosin as substrates. Figure 1 shows the effect of increasing concentrations of representative fatty acids on protein kinase C activity. Unsaturated fatty acids (2–20 μg/ml) stimulate the phosphorylation of either myosin light chains or histone H-1 as phosphatidylserine stimulates this phosphorylation. Various fatty acids differed in their abilities to stimulate protein kinase C. In each case,

Table 1. Effects of a variety of fatty acids on the phosphorylation of histone H-1 or isolated myosin light chains by platelet protein kinase C in the presence or absence of Ca^{2+}

	Protein kinase C activity (pmol/min)			
	Histone H-1		Myosin light chains	
	Ca^{2+}	EGTA	Ca^{2+}	EGTA
None	2.48	2.29	7.91	1.81
Phosphatidylserine	94.42	8.51	52.51	4.03
C20	2.44	2.16	4.42	2.03
C20:1	27.65	26.78	111.30	19.30
C20:2	45.99	26.37	99.24	6.76
C20:3	48.72	23.26	46.16	4.68
C20:4	26.26	26.19	45.32	2.66
Methyl C20:4	2.94	2.35	9.14	2.48

Effects of 50 μg/ml of each of the fatty acids on platelet protein kinase C (15 nM) were measured in the presence of 100 μM $CaCl_2$ or 2 mM EGTA, using histone H-1 (0.4 mg/ml) or mixed myosin light chains (0.8 mg/ml) as the substrate, as described under Materials and Methods. Values are the average of duplicate determinations, in a single experiment. Similar results were obtained in two additional experiments. Key: C20, *n*-eicosanoic acid; C20:1, *cis*-11-eicosenoic acid; C20:2, 11,14-eicosadienoic acid; C20:3, 8,11,14-eicosatrienoic acid; C20:4, 5,8,11,14-eicosatetraenoic acid (arachidonate); and methyl C20:4, methyl arachidonate.

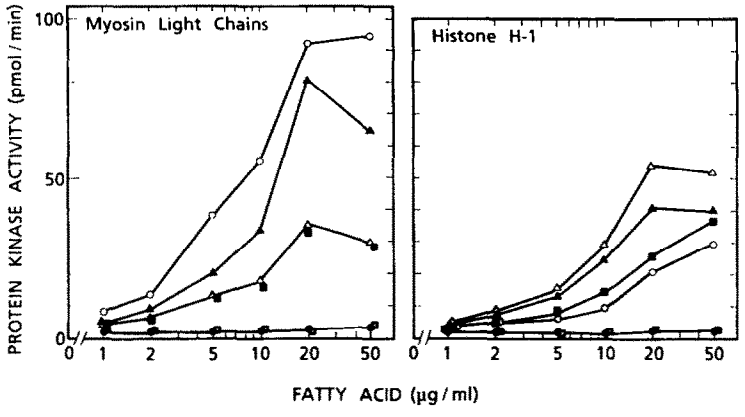
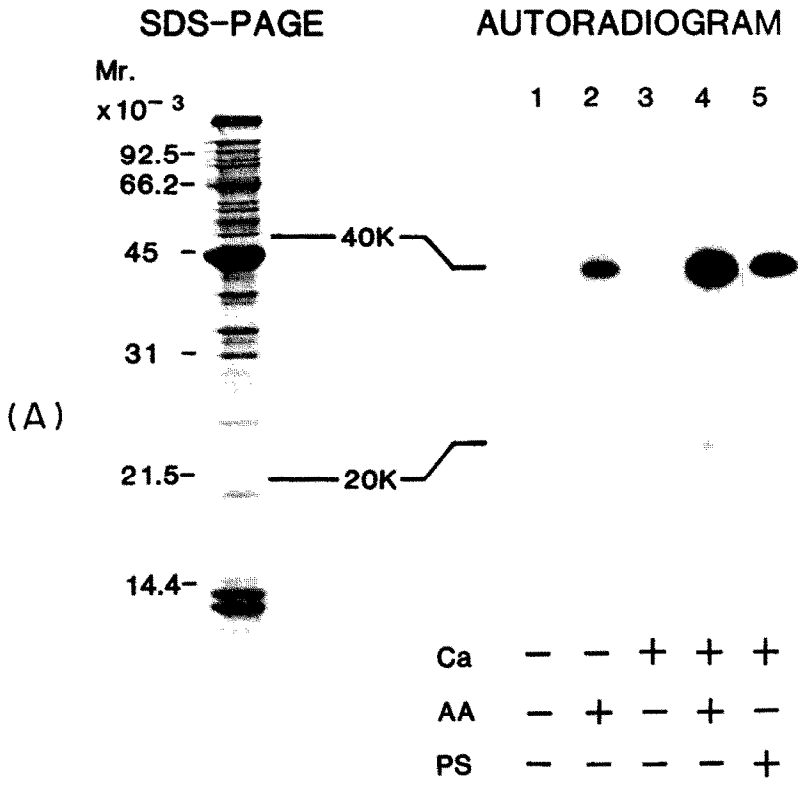


Fig. 1. Effects of a variety of fatty acids with 20 carbon atoms on the phosphorylation of isolated myosin light chains or histone H-1 by platelet protein kinase C. Protein kinase C (15 nM) was assayed at 25° in 20 mM Tris–HCl, pH 7.5, 50 μM [γ-^{32}P]ATP, 5 mM $MgCl_2$, 100 μM $CaCl_2$, histone H-1 (0.4 mg/ml) or mixed myosin light chains (0.8 mg/ml), and various amounts of each fatty acid including *n*-eicosanoic acid (●), *cis*-11-eicosenoic acid (○), 11,14-eicosadienoic acid (▲), 8,11,14-eicosatrienoic acid (△), arachidonate (■) and methyl arachidonate (□).



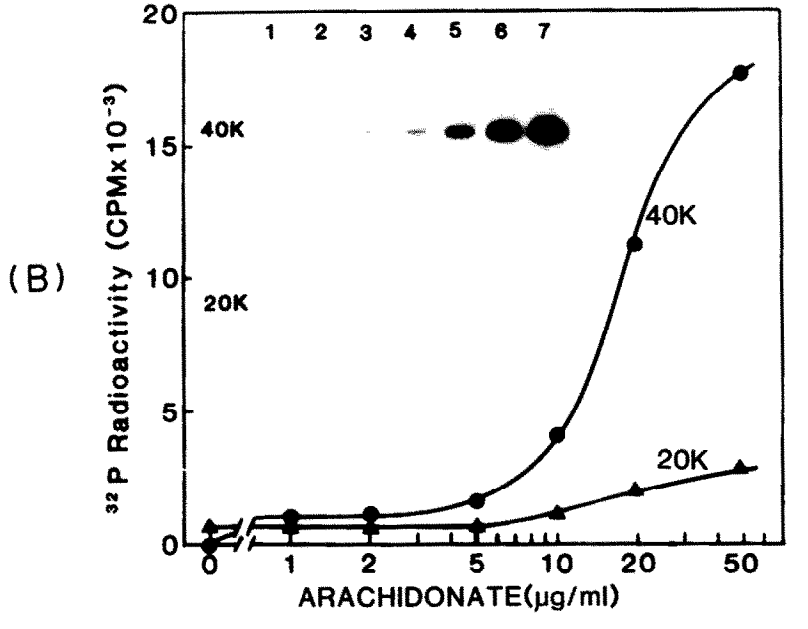


Fig. 2. Effect of arachidonate on the endogenous phosphorylation of the cytosol of human platelets. (A) Ca^{2+}/arachidonate-dependent phosphorylation of 20K and 40K proteins. (B) Dose-dependence of arachidonate on the phosphorylation of 40K (●) and 20K (▲) proteins in the presence of 200 μM Ca^{2+}. The cytosol fraction of platelet homogenate was incubated in the presence (+) or absence (−) of 200 μM Ca^{2+}, 50 μg/ml phosphatidylserine (PS) and 50 μg/ml arachidonate (AA), as described under Materials and Methods. Protein phosphorylation was analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), followed by autoradiography. Lanes in Fig. 2B indicate autoradiograms showing phosphorylation in the presence of arachidonate, using the following concentrations (μg/ml): lane 1, 0; lane 2, 1; lane 3, 2; lane 4, 5; lane 5, 10; lane 6, 20; and lane 7, 50.

stimulation of myosin light chain phosphorylation occurred in a Ca^{2+}-dependent manner. On the other hand, when protein kinase C was assayed with histone H-1, stimulation of protein kinase C activity by the unsaturated fatty acids was not Ca^{2+} dependent. Arachidonate was ineffective in activating calmodulin-dependent kinase myosin light chain kinase from platelets (data not shown). Arachidonate (50 μg/ml) had little effect on kinase activation in the presence of optimal amounts of phosphatidylserine (50 μg/ml). Arachidonate (50 μg/ml) plus diolein (up to 2 μg/ml) resulted in no further kinase activation than seen with arachidonate alone. The saturated fatty acid arachidic acid and methyl arachidonate (up to 50 μg/ml) had no effect on the protein kinase C activity. The major spots of the ^{32}P-labeled tryptic peptides from histone H-1 phosphorylated in the presence of arachidonate did not differ from those obtained from histone H-1 phosphorylated in the presence of phosphatidylserine (data not shown). Concentrations of calcium up to 100 μM stimulated the protein kinase C activity but higher concentrations of added calcium depressed the phosphotransferase activity, when myosin light chains were used as the substrate. In contrast to our findings, McPhail *et al.* [16] reported that unsaturated fatty acids stimulated the phosphorylation of histone, in a Ca^{2+}-dependent manner. Differences in experimental conditions may explain this discrepancy. In their study [16], detergent extracts from human neutrophils were used as the enzyme preparation, whereas we used a highly-purified protein kinase C from human platelets. Five to seven millimolar $MgCl_2$ was the optimal concentration for activation of platelet protein kinase C by arachidonate with both substrates. These results suggest that the unsaturated moiety of fatty acids may be important for the interaction with hydrophobic sites

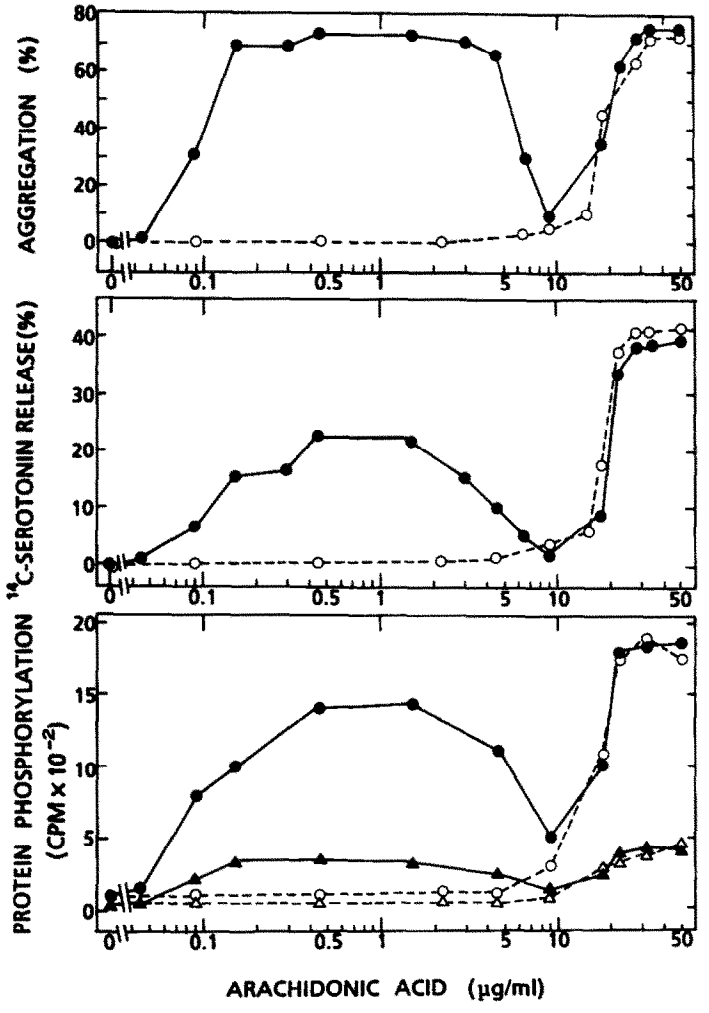


Fig. 3. Dose-dependent effects of arachidonate on platelet aggregation, serotonin release and phosphorylation of 40K (○, ●) and 20K (△, ▲) proteins of intact platelets in the presence (open symbols) or absence (closed symbols) of 0.1 mg/ml aspirin. After preincubation for 2 min, with or without 0.1 mg/ml aspirin, [^{14}C]serotonin-labeled platelet suspensions were stimulated with various concentrations of arachidonate. The phosphorylation of 40K and 20K proteins in arachidonate-stimulated platelets was analyzed, as described under Materials and Methods.

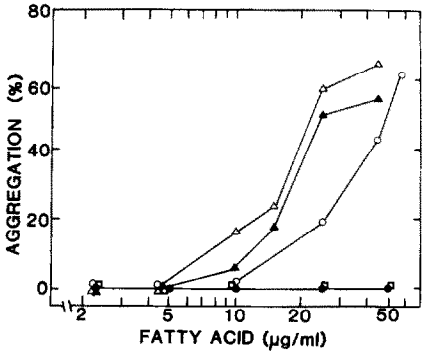


Fig. 4. Effects of various fatty acids on platelet aggregation. Platelet suspensions were stimulated with various concentrations of *n*-eicosanoic acid (●), *cis*-11-eicosenoic acid (○), 11,14-eicosadienoic acid (▲), 8,11,14-eicosatrienoic acid (△) and methyl arachidonate (□). Three separate experiments were performed, and data on one typical experiment are presented. None of the fatty acid-induced lactate dehydrogenase leaked from the platelets, at the concentrations tested.

on protein kinase C, allowing activation of the kinase.

Effect of arachidonate on the endogenous phosphorylation of the cytosol of human platelets. Physiological stimuli induce the phosphorylation of the 20K light chain of myosin and the 40K protein, in association with the release reaction of serotonin [11, 12]. Although the identity and function of the 40K protein remain largely unknown, this phosphorylation is catalyzed by protein kinase C [11, 12]. The 20K light chain of myosin can be phosphorylated by both protein kinase C and the Ca^{2+}/calmodulin-dependent enzyme, myosin light chain kinase [14, 15]. We examined the effect of arachidonate on the endogenous phosphorylation of the platelet cytosol (100,000 *g* supernatant), containing protein kinase C and substrate sources. Ca^{2+} alone stimulated phosphorylation of the 20K protein. Phosphorylation of 40K protein *in vitro* was dependent on the concomitant presence of Ca^{2+} and phosphatidylserine (Fig. 2A). A similar stimulation of both 20K and 40K protein phosphorylation by arachidonate was observed, although stimulation of the 40K protein phosphorylation was more prominent. Increasing phosphorylation was seen with the addition of increasing amounts of arachidonate (5 μg/ml to 50 μg/ml), in the presence of Ca^{2+}, as shown in Fig. 2B.

Platelet activation and protein phosphorylation induced by arachidonate and its analogs. We used washed platelet suspensions in the absence of albumin. At concentrations from 0.1 to 4 μg/ml (a low level), arachidonate induced platelet aggregation and serotonin release dose dependently, while at concentrations from 4 to 10 μg/ml of arachidonate a dose-dependent decrease of both aggregation and secretion was observed (Fig. 3). This bell-shaped dose–response curve obtained with arachidonate has been noted by other investigators [1–6]. As shown in Fig. 3, the platelet responses induced by a low level of arachidonate were inhibited by 0.1 mg/ml aspirin, a compound which inhibits the cyclooxygenase [7, 8]. However, at higher concentrations (10–50 μg/ml: a high level), arachidonate again induced a dose-dependent aggregation and serotonin release, not suppressed by aspirin, in agreement with data in the literature [7]. A similar pattern of platelet responses was observed in all of these experiments, although there was some variation from experiment to experiment concerning precise concentrations of arachidonate at which particular responses occurred. No leakage of lactate dehydrogenase occurred during platelet aggregation and release induced by a high level of arachidonate. Other unsaturated fatty acids including C20:1, C20:2 and C20:3 induced an irreversible aggregation and serotonin release, dose-dependently, at concentrations from 5 to 50 μg/ml (Fig. 4). Aspirin had no effect on platelet responses induced by unsaturated fatty acids. The saturated analog C20 and methyl arachidonate, which did not stimulate platelet protein kinase C, did not induce platelet aggregation and secretion, at concentrations

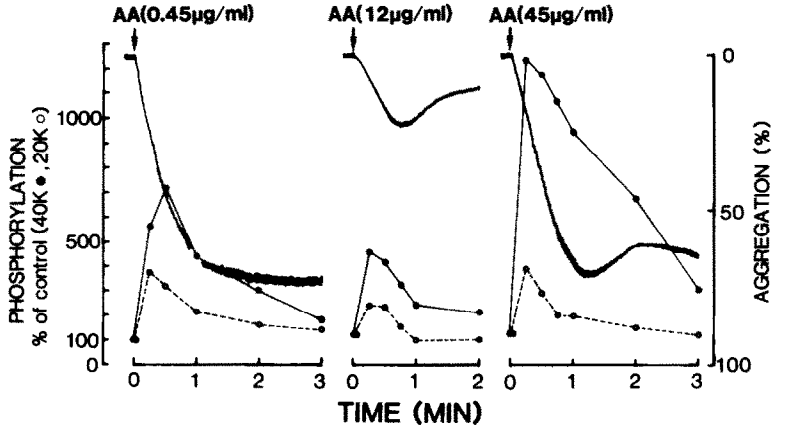


Fig. 5. Time course of phosphorylation of 40K (●) and 20K (○) proteins in intact platelets stimulated by arachidonate. The [^{32}P]P_i-labeled platelets were stimulated with three different concentrations of arachidonate (AA). Protein phosphorylation was analyzed, as described under Materials and Methods. Control values (100%) of 40K and 20K phosphorylation were 152 ± 20 and 62 ± 15 cpm respectively.

Table 2. Effects of inhibitors of protein kinase C on platelet aggregation induced by arachidonate (AA) or C20:2

Inhibitor	IC_{50} (μM)		
	AA (0.45 μg/ml)	AA (45 μg/ml)	C20:2 (45 μg/ml)
Aspirin	66	3000	1850
H-7	45	500	400
Staurosporine	0.009	0.81	0.35

The IC_{50} value was obtained visually from a plot of percent aggregation vs log concentration of each drug.

up to 50 μg/ml (Fig. 4).

Incubation of ^{32}P-labeled intact platelets with arachidonate resulted in a rapid increase in the phosphorylation of 20K and 40K proteins. Preceding the maximal secretion and aggregation, the maximal phosphorylation was observed approximately 15 sec after arachidonate addition, and after this time the phosphorylation gradually declined (Fig. 5). The level of phosphorylation of the 20K and 40K in Fig. 3 is the result of incubation of platelets with arachidonate for 15 sec. The arachidonate dose-response of phosphorylation of 20K and 40K proteins appeared to be similar to that of the platelet aggregation and serotonin release shown in Fig. 3. Aspirin completely inhibited phosphorylation of the 40K and 20K proteins as well as the platelet responses induced by a low level of arachidonate, whereas this agent did not inhibit these protein phosphorylations induced by a high level of arachidonate (Fig. 3). Other unsaturated fatty acids also induced the phosphorylation of 40K and 20K proteins at concentrations that caused platelet responses (data not shown). H-7 and staurosporine inhibit protein kinase C via a direct interaction on the catalytic site of the enzyme [30, 31]. The concentrations (IC_{50} values) of H-7 and staurosporine producing 50% inhibition of the platelet aggregation induced by arachidonate or C20:2 are summarized in Table 2. Both compounds inhibited platelet aggregation induced by not only a low level (0.45 μg/ml) of arachidonate but also by a high level (45 μg/ml) of arachidonate or C20:2, although the IC_{50} value of each drug was about ten

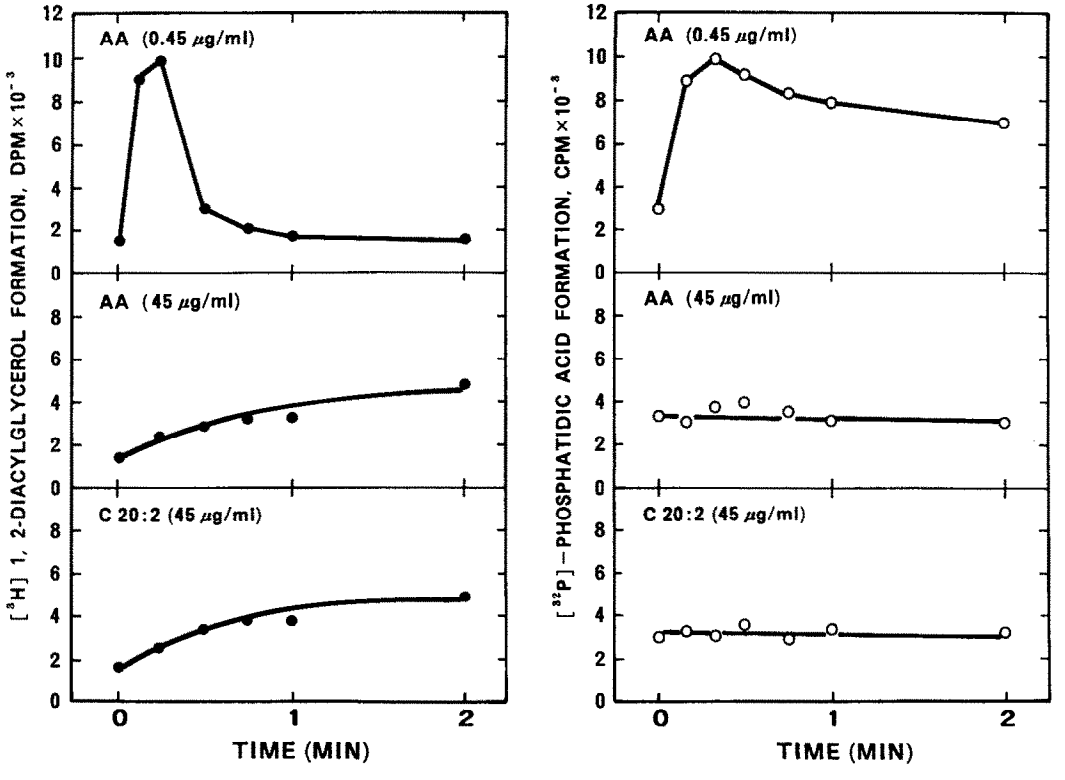


Fig. 6. Time course for the formation of [^{3}H]1,2-diacylglycerol and [^{32}P]phosphatidate in intact platelets stimulated by arachidonate or C20:2. The [^{3}H]arachidonate-labeled platelets for the measurement of [^{3}H]-1,2-diacylglycerol (●) or the [^{32}P]P_i-labeled platelets for the measurement of [^{32}P]phosphatidate (○) were stimulated with a low level (0.45 μg/ml) of arachidonate (AA) or a high level (45.0 μg/ml) of arachidonate or C20:2.

to ninety times lower in the aggregation induced by a low level than by a high level of arachidonate or C20:2.

Effects of unsaturated fatty acids on the activation of phospholipase C in intact platelets. The hydrolysis of inositol phospholipids by phospholipase C generates inositol phosphates and 1,2-diacylglycerol, and 1,2-diacylglycerol is phosphorylated rapidly to phosphatidate, which can be used to monitor phospholipase C activation [8]. As shown in Fig. 6, a low level of arachidonate (0.45 μg/ml) induced a rapid and transient formation of [^{3}H]-1,2-diacylglycerol and [^{32}P]phosphatidate. Pretreatment of platelets with aspirin (0.05 mg/ml) inhibited the formation of 1,2-diacylglycerol and phosphatidate. These data agree with those of Siess *et al.* [8]. However, at high concentrations (45 μg/ml) of arachidonate or C20:2, [^{3}H]-1,2-diacylglycerol and [^{32}P]-phosphatidate were not formed, although a high level of arachidonate or C20:2 did induce phosphorylation of the 20K and 40K proteins and activated the platelets. These data suggest that under certain conditions there is no phospholipase C activation and that the phosphorylation of 40K protein is independent of 1,2-diacylglycerol formation.

Changes in $[Ca^{2+}]_i$ *in response to unsaturated fatty acids.* Figure 7 shows Ca^{2+}-fura 2 fluorescence and aggregometer responses to arachidonate in the presence of 1 mM Ca^{2+} or 0.5 mM EGTA. $[Ca^{2+}]_i$ was represented by changes in the 340/380 nm fluor-

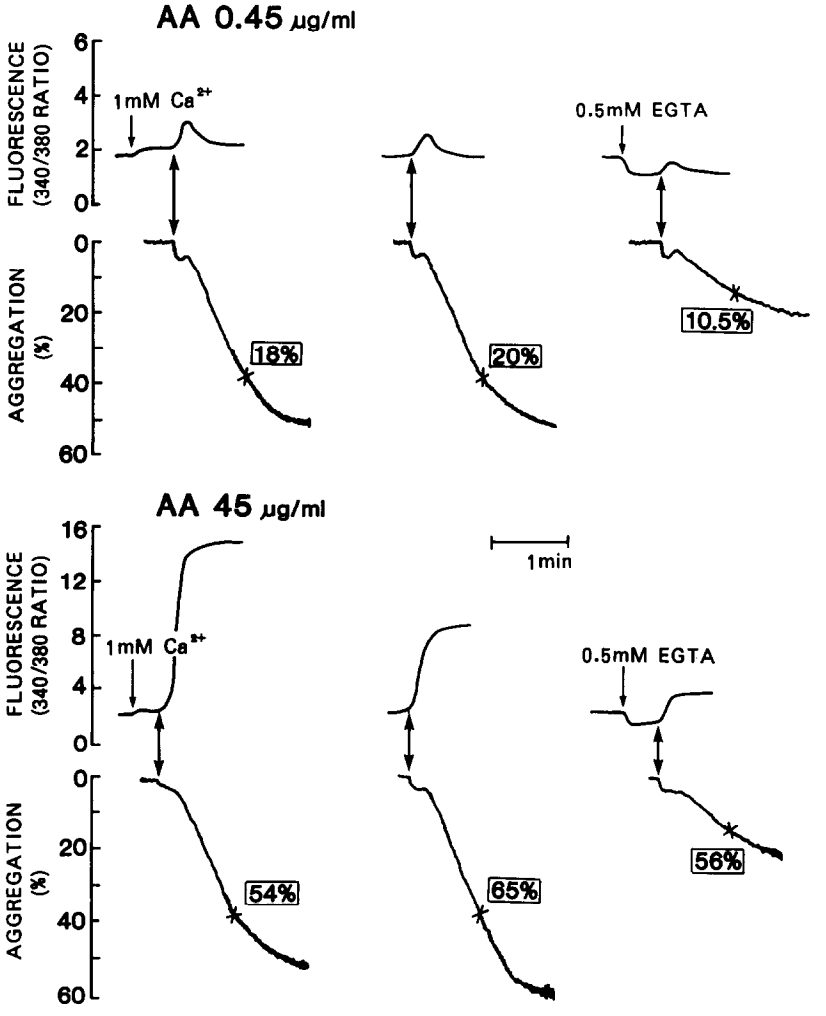


Fig. 7. Ca^{2+}-fura 2 fluorescence and platelet aggregation responses to arachidonate in the presence of 1 mM external Ca^{2+} or 0.5 mM EGTA. The upper trace of each set of two shows the ratio of fura 2 fluorescence (340/380 nm) as $[Ca^{2+}]_i$ responses, and the lower trace platelet aggregation in parallel with the fluorescence study in the same batch of cells. The 340/380 nm ratio changes in the same direction as $[Ca^{2+}]_i$ [28]. Platelets were loaded with [^{14}C]serotonin along with fura 2-AM. The cells were kept in nominally Ca^{2+}-free Hepes buffer throughout the study. $CaCl_2$ (1 mM) or EGTA (0.5 mM) was added 30 sec before the addition of arachidonate (AA) as indicated by the arrow. For aggregation studies, 370 μl of fura 2 loaded platelet suspension (3×10^8/ml) was used. Bidirectional arrows indicate the addition of arachidonate (upper sets: 0.45 μg/ml; lower sets: 45 μg/ml). Percents in the squares indicate serotonin release at 60 sec (×) after arachidonate addition. The figure shows representative traces from four similar experiments.

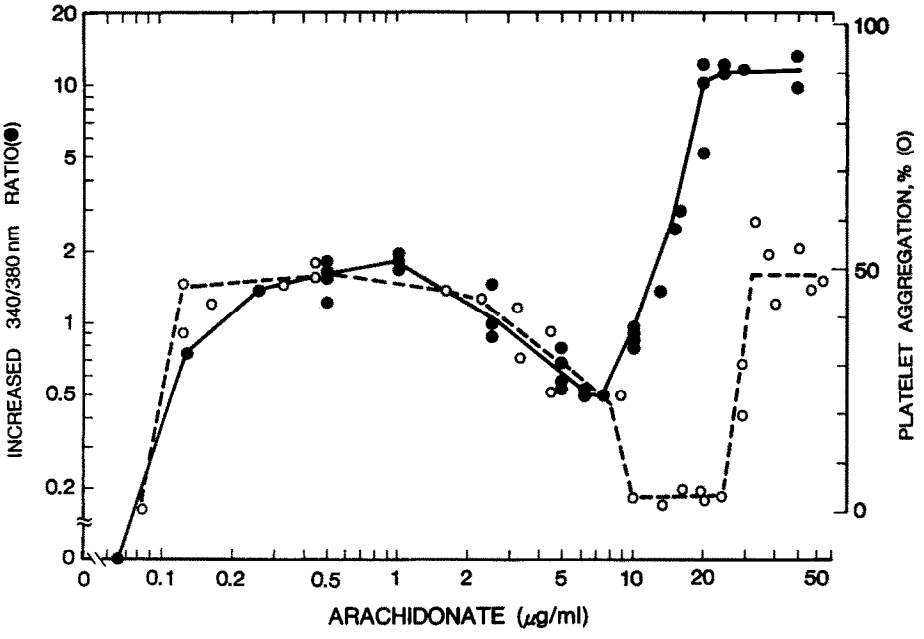


Fig. 8. Effects of various concentrations of arachidonate on platelet aggregation and the $[Ca^{2+}]_i$ change in fura 2 loaded platelets. Fura 2 loaded platelet suspensions were exposed to various concentrations of arachidonate, as indicated, in the presence of 1 mM external Ca^{2+}. Fluorescence and aggregation were monitored simultaneously, in parallel samples. The increased 340/380 nm ratio (●) was obtained by subtraction of the 340/380 nm ratio for resting platelets from the peak of the 340/380 nm ratio after the addition of arachidonate. Aggregation responses were quantified as the maximum extent of aggregation (○), as described in Materials and Methods. The data are pooled from five experiments using platelets from different donors.

escence ratio. On exposure to 0.45 μg/ml arachidonate, there was an immediate and slight increase in the 340/380 nm ratio. The increase in $[Ca^{2+}]_i$ reached a peak level within 15 sec, followed by a rapid decrease in the ratio of fluorescence. This transient elevation in $[Ca^{2+}]_i$ in the platelets was seen on exposure with a low level of arachidonate (0.125 to 5.0 μg/ml, Fig. 8). A full activating concentration of arachidonate (0.45 μg/ml) evoked an elevation in $[Ca^{2+}]_i$ from the resting level near 100 nM to 400 nM in the presence of 1 mM external Ca^{2+}. Although the peak value of the 340/380 nm ratio obtained in medium containing 0.5 mM EGTA was slightly less than that obtained in medium with or without 1 mM external Ca^{2+}, the data suggest that the rise in $[Ca^{2+}]_i$ after the addition of a low level of arachidonate was mainly due to mobilization of intracellular Ca^{2+}. In contrast, a high level of arachidonate (15.1 to 45 μg/ml) caused a rapid and massive increase in the 340/380 nm ratio, which was maintained for up to 2 min in 1 mM external Ca^{2+}. Arachidonate at 45 μg/ml raised $[Ca^{2+}]_i$ to about 4 μM and produced aggregation and the release of serotonin in the presence of 1 mM external Ca^{2+}. Although the aggregation induced by 45 μg/ml was inhibited in the presence of 0.5 mM EGTA, the release of serotonin was unchanged in the presence or absence of external Ca^{2+}. However, the peak value of $[Ca^{2+}]_i$ induced by 45 μg/ml of arachidonate in the presence of 0.5 mM EGTA was significantly lower than that achieved in the presence of 1 mM external Ca^{2+}. The peak value of $[Ca^{2+}]_i$ appeared to depend on the amount of external Ca^{2+}. If so, then the elevation in $[Ca^{2+}]_i$ after the addition of a high level of arachidonate is

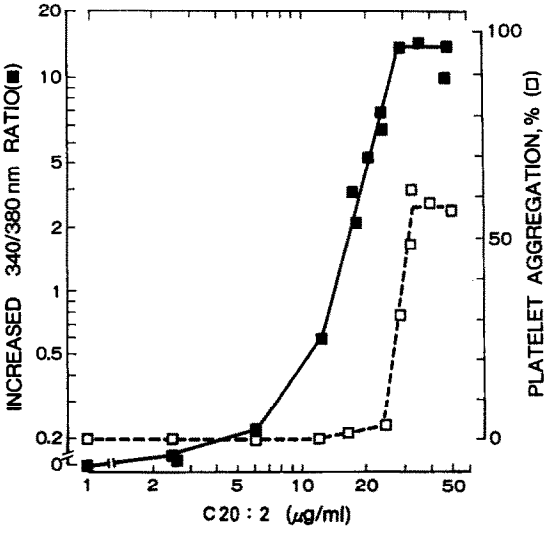


Fig. 9. Effects of various concentrations of C20:2 on platelet aggregation and the $[Ca^{2+}]_i$ change in fura 2 loaded platelets. Increased 340/380 nm ratio (■) was estimated, as described in the legend of Fig. 8. Aggregation responses were quantified as the maximum extent of aggregation (□), as described in Materials and Methods. The data are pooled from three experiments using platelets from different donors.

due mainly to the Ca^{2+} influx, and secretion is not associated with changes in $[Ca^{2+}]_i$. In the presence of 1 mM external Ca^{2+}, 19.0 μg/ml of arachidonate produced a similar rise in the 340/380 nm ratio but did not induce platelet aggregation (Fig. 8). Similar results were obtained with C20:2, as shown in Fig. 9. In the presence of 1 mM external Ca^{2+}, C20:2 also evoked an instantaneous, dose-dependent increase in $[Ca^{2+}]_i$, at concentrations from 2.5 to 49 μg/ml, and the pattern of elevation in the ratio of 340/380 nm resembled that produced by a high level of arachidonate (data not shown). Twenty-four micrograms per milliliter of C20:2 did lead to sufficient increases in $[Ca^{2+}]_i$, but aggregometer responses were not elicited. These results suggest that the elevation in $[Ca^{2+}]_i$ alone is not sufficient to induce platelet aggregation, in response to a high level of unsaturated fatty acid.

DISCUSSION

This study was undertaken to examine whether the ability of unsaturated fatty acid to activate directly and regulate protein kinase C may play an important role in the cellular regulation of human platelets. Our results suggest that the mechanism of actions by a high level of arachidonate (10–50 μg/ml) on platelets differs from that seen with a low level of arachidonate (0.1 to 5 μg/ml), and that platelet activation induced by a high level of the unsaturated fatty acid, other than arachidonate, may be associated with a direct activation of protein kinase C. It was reported that platelet aggregation and secretion induced by a low level of arachidonate yield a bell-shaped dose–response curve, in parallel to the formation of phosphatidate and diacylglycerol (reflecting stimulation of phospholipase C) and the phosphorylation of 40K protein and 20K light chain of myosin [1–8]. Although phosphorylation of the 40K protein is nearly always associated with certain platelet functions in which protein kinase C has been implicated [11, 12], the precise mechanisms by which this enzyme contributes to the biological activity of platelets remain unknown. These responses are inhibited completely by the addition of aspirin or indomethacin [7, 8]. This activation induced by a low level of arachidonate is thought to be mediated principally by the metabolism of arachidonate to prostaglandin endoperoxides and thromboxane A_2 [7, 8]. Our present results support this hypothesis. The transient and slight elevation in $[Ca^{2+}]_i$, as was observed with a low level of arachidonate, was probably the result of stimulation of phospholipase C by the formation of thromboxane A_2.

A high level of arachidonate has been reported to induce platelet aggregation and secretion, independent not only of the cyclooxygenase products but also of the lipoxygenase products [7]. In addition, these thromboxane A_2-independent platelet responses could not be associated with the activation of phospholipase C, because there was no accumulation of 1,2-diacylglycerol and phosphatidate in intact platelets stimulated by a high level of arachidonate. Arachidonate and other unsaturated fatty acids at concentrations from 1.0 to 50 μg/ml can directly activate protein kinase C from platelets, when the activity is assayed with not only isolated substrates but also the platelet cytosol, as noted with neutrophiles [16]. This range of concentration is similar to that of a high level of arachidonate which induces platelet responses. Our data also suggest that the unsaturated moiety and carboxy group of fatty acids may be important for the interaction with the hydrophobic site on protein kinase C. A high level of arachidonate induced phosphorylation of 40K and 20K proteins in intact platelets, and these events appear to correlate with the responses of platelets. Different from those events induced by a low level of arachidonate, the phosphorylation was not inhibited by aspirin and occurred without the formation of 1,2-diacylglycerol and phosphatidate. Other unsaturated fatty acids directly stimulated protein kinase C and induced an irreversible aggregation, without activation of phospholipase C, whereas the saturated fatty acid and methyl arachidonate did not. H-7 and staurosporine, inhibitors of protein kinase C [30, 31], inhibited the platelet aggregation induced by unsaturated fatty acids, although we have no explanation as to why 10- to 90-fold higher concentrations of H-7 and staurosporine were required to inhibit the platelet aggregation by a high level of fatty acids. These results suggest that the mechanisms of activation involved by a high level of arachidonate could be the same as those by unsaturated fatty acids and that unsaturated fatty acids may activate platelets, at least in part, through a direct activation of protein kinase C.

It has been proposed that protein kinase C activation and Ca^{2+} mobilization (which may then activate Ca^{2+}/calmodulin-dependent processes) are both essential and that they act synergistically to elicit full physiological responses of platelets by natural agonists [12, 32]. The phorbol ester, 12-*O*-tetradecanoyl phorbol 13-acetate (TPA) has been reported to stimulate platelet responses by directly activating protein kinase C [33, 34]. TPA produces only a sluggish aggregation and secretion [15, 33] and does not produce any elevation in cytosolic Ca^{2+} concentration, determined using the fluorescent calcium probe quin 2 or fura 2 [35, 36]. In contrast, the incubation of fura 2 loaded platelets with a high level of unsaturated fatty acids induced a rapid and massive increase in $[Ca^{2+}]_i$ in the presence of 1 mM external Ca^{2+}. The pattern of $[Ca^{2+}]_i$ elevation evoked by a high level of unsaturated fatty acids was clearly different from that obtained with a low level of arachidonate. These data suggest that a high level of unsaturated fatty acids could elicit Ca^{2+} mobilization through the activation of mechanisms differing from those initiated by a low level of arachidonate. The peak value in $[Ca^{2+}]_i$ after the addition of a high level of arachidonate depended on the external concentration of Ca^{2+}, but the secretion of serotonin remained unchanged in the presence or absence of Ca^{2+}, thereby suggesting that the elevation in $[Ca^{2+}]_i$ itself is not associated with platelet secretion. Moreover, even in the presence of 1 mM external Ca^{2+}, there was a dissociation of the peak value in $[Ca^{2+}]_i$ from the aggregation response following exposure to a high level of the unsaturated fatty acid. These data suggest that the elevation in $[Ca^{2+}]_i$ alone may not be a trigger for

platelet activation, and that an excitatory intracellular signal, other than Ca^{2+} or diacylglycerol, may be involved in these platelet activations. We propose that platelet responses produced by unsaturated fatty acids may be evoked by the combined action of a direct stimulation of protein kinase C and Ca^{2+} mobilization.

Endogenous arachidonate is released from platelet phospholipids when platelets are stimulated with agents such as thrombin, collagen and platelet-activating factor. As much as 20 nmol/10^9 platelets are released by maximal stimulation [37–40]. Depending on the volume of distribution of the released arachidonate, a considerably high concentration could conceivably be achieved locally in the cells, and these local concentrations may well be within the range required for direct activation of protein kinase C. Therefore, under limited conditions in which there is no activation of phospholipase C, the generation of endogenous arachidonate may provide an additional stimulus for induction of protein kinase C activity due to a direct effect on the kinase in the activation of platelets.

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