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ACTIVATION OF PROTEIN KINASE C BY *cis*- AND *trans*-OCTADECADIENOIC ACIDS IN INTACT HUMAN PLATELETS AND ITS POTENTIATION BY DIACYLGLYCEROL

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Octadecadienoic acids (linoleic acid and linolelaidic acid) and the diacylglycerol, 1-oleoyl-2-acetyl-*rac*-glycerol (OAG) concentration-dependently induced activation of gel-filtered human platelets, i.e. aggregation and phosphorylation of 20 kDa and 47 kDa peptides. In contrast, octadecenoic acids (oleic and elaidic acid) and octadecanoic (stearic) acid were inactive. Octadecadienoic acid-induced platelet activation was suppressed by the protein kinase C inhibitor, polymyxin B, but not by the cyclooxygenase inhibitor, indomethacin. OAG-induced activation was potentiated by octadecadienoic acids present at non-stimulatory concentrations. Our data suggest that octadecadienoic acids and diacylglycerol synergistically induce platelet activation *via* protein kinase C. Furthermore, linolelaidic acid may provide a useful experimental tool to study fatty acid regulation of protein kinase C in intact cells.

In human platelets, thrombin induces polyphosphoinositide degradation to inositolphosphates and diacylglycerol as well as the release of AA from phospholipids (1,2). Diacylglycerol activates protein kinase C, which phosphorylates specific proteins, the phosphorylation of which preceeds aggregation and granule release (3,4). Various synthetic diacylglycerols, e.g. OAG, substitute for endogenous diacylglycerol to activate protein kinase C (5,6). *Cis*-

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Abbreviations:
AA, arachidonic acid; LA-*cis*, linoleic acid; LA-*trans*, linolelaidic acid; OAG, 1-oleoyl-2-acetyl-*rac*-glycerol.

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unsaturated fatty acids, such as AA and oleic acid (*cis*-9-octadecenoic acid), but not its geometrical isomer, elaidic acid (*trans*-9-octadecenoic acid) have been reported to be activators of protein kinase C *in vitro* and to induce phosphorylation of proteins and cellular activation (7-11). In addition, we recently demonstrated that *trans*-9-*trans*-12-octadecadienoic acid (LA-*trans*), the geometrical isomer of *cis*-9-*cis*-12-octadecadienoic acid (LA-*cis*) activates purified protein kinase C *in vitro*. Furthermore, fatty acid-induced enzyme activation was potentiated by diacylglycerol, indicating that fatty acids and diacylglycerol may synergistically be involved in hormonal stimulation of protein kinase C (12,13). In this study we demonstrate that octadecadienoic acids induce platelet activation, which is potentiated by OAG. Evidence is presented that these effects are mediated *via* protein kinase C.

MATERIALS AND METHODS

Fatty acids (99% pure), OAG (97% pure), indomethacin, polymyxin B and thrombin were obtained from Sigma Chemie (Taufkirchen, F.R.G.). Sepharose 2B-Cl was purchased from Pharmacia (Freiburg, F.R.G.) and carrier-free $^{32}P_i$ was from Amersham-Buchler (Braunschweig, F.R.G.). All other chemicals were of analytical degree. Stock solutions of AA (6 mM), LA-*cis* and LA-*trans* (20-80 mM) were prepared in 100% ethanol under nitrogen, protected from light and stored at -20°C. OAG (6 mM), indomethacin (5 mM), stearic and elaidic acid (80 mM) were dissolved in dimethyl sulfoxide.

Citrated blood was obtained by venous puncture from healthy male volunteers who had taken no drugs for at least 3 weeks. Platelet-rich plasma was prepared by centrifugation for 15 min at 60 x g. Platelets were separated from plasma by gel filtration using a sepharose 2B-Cl column equilibrated at room temperature with a buffer consisting of 150 mM NaCl, 5.5 mM glucose, 30 µM bovine serum albumin and 15 mM Tris-HCl, pH 7.4 (14,15). Platelet concentration in pooled peak fractions of the eluate was determined in a Coulter Counter (Coulter Electronics, Krefeld, F.R.G.).

Platelets (2 x 10^8/ml) were supplemented with 0.7 mM of $CaCl_2$. Platelet aggregation was measured by turbidometry (16). All assays contained 0.5% ethanol (v/v) and 0.5% dimethyl sulfoxide (v/v), which were without effect on platelet aggregation. Platelets were incubated for 2 min in the absence or presence of inhibitors prior to addition of stimuli. Aggregation experiments were carried out at 37°C under constant stirring of cells with 10^3 rpm using a Braun aggregometer (Braun, Melsungen, F.R.G.).

Platelets (5 x 10^8/ml) were labelled with $^{32}P_i$ (0.5 mCi/ml) for 60 min at 37°C. Labelled platelets were gel-filtered a second time yielding a final concentration of 3.5 x 10^8/ml. After equilibration with 0.7 mM of $CaCl_2$, platelets were incubated with stimuli for various times under the same conditions as in the aggregation experiments. The reactions were terminated by addition of one half the volume of a stop solution consisting of 9% SDS (w/v), 27% gylcerol (v/v), 0.015% brome phenol blue (v/v), 300 mM dithiothreitol and 190 mM Tris-HCl, pH 6.8. After boiling of samples for 5 min, aliquots corresponding to 4.6 x 10^6 platelet equivalents were analyzed by SDS-polyacrylamide gel electrophoresis (17). Gels were stained with Coomassie blue, destained, dried and subjected to autoradiography to analyze phosphoproteins.

In some experiments, dried gels were cut on the basis of autoradiographs, and the radioactivity in gel bands corresponding to 47 kDa peptides was determined by liquid scintillation counting.

RESULTS AND DISCUSSION

Original registrations of platelet aggregation are shown in Fig. 1. At concentrations above 200 μM, LA-*trans* induced aggregation. LA-*trans* (400 μM) led to maximal aggregation, which was complete after 2.5 min and was kinetically very similar to that induced by thrombin (data not shown). The protein kinase C inhibitor, polymyxin B (18), suppressed LA-*trans*-induced aggregation by 89%, whereas the cyclooxygenase inhibitor, indomethacin (19) was without effect. At concentrations between 3 and 30 μM, OAG also caused aggregation, which was more gradual than the response to LA-*trans* and reached its maximum after 8 min. The marginally stimulatory effect of OAG (3 μM) on platelet aggregation was increased to 5 times the initial value by 200 μM LA-*trans*. OAG (5 μM) plus LA-*trans* (200 μM) induced maximal aggregation as did 400 μM LA-*trans* or 30 μM OAG alone. Interestingly, the kinetics of synergistic platelet aggregation by LA-*trans* and OAG were similar to those of OAG alone.

Stearic and elaidic acid are no activators of protein kinase C (7,8,12, 13) and do not induce platelet activation (Table 1). Oleic acid failed to induce platelet aggregation, indicating that this fatty acid is an activator of protein kinase C *in vitro* (7,8,12,13) but not in intact platelets. Similar observations have been made for various diacylglycerols, which activate protein kinase C *in vitro* but not *in vivo* (5,6,20). LA-*cis* induced platelet aggregation to a similar extent as did LA-*trans* and synergistically enhanced OAG-triggered aggregation. In analogy to LA-*trans*, polymyxin B but not indomethacin inhibited the stimulatory effect of LA-*cis*. In contrast to the platelet aggregation induced by octadecadienoic acids, that induced by AA was completely suppressed by indomethacin. These data indicate that platelet activation by AA but not that by octadecadienoic acids, the latter being no substrates for cyclooxygenase (21), depends on metabolic conversion to aggregatory eicosanoids (22).

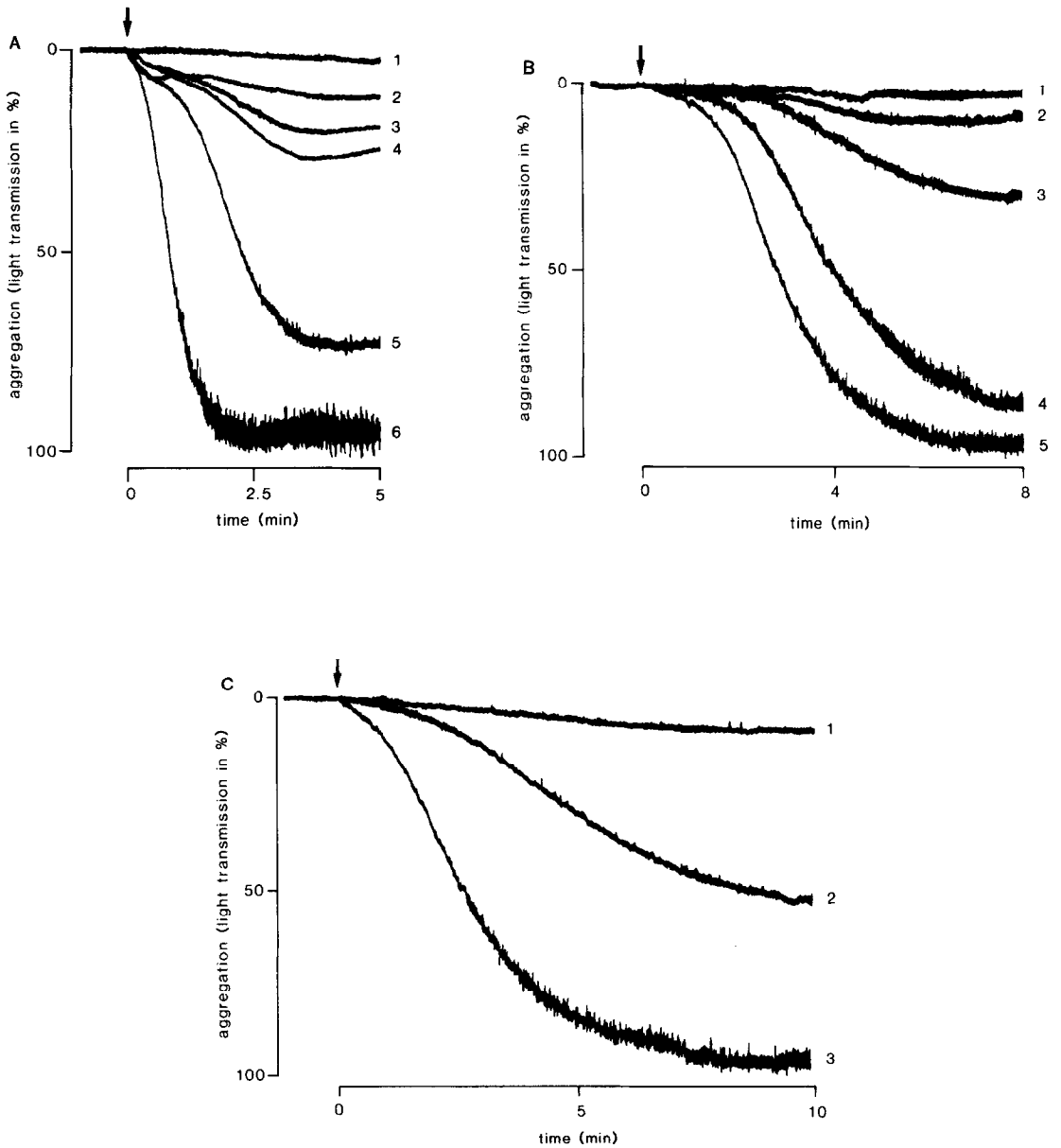

Fig. 1. Time course of platelet aggregation by linolelaidic acid, 1-oleoyl-2-acetyl-*rac*-glycerol and by linolelaidic acid plus 1-oleoyl-2-acetyl-*rac*-glycerol. Platelet aggregation was measured as described in MATERIALS AND METHODS. Arrows indicate the addition of stimuli. The extent of aggregation is referred to the aggregation induced by thrombin (0.25 U/ml), which amounted to 100%. Original registrations from one representative experiment are shown. Similar results were obtained in 6 independent experiments.

Panel A: 1, LA-*trans* 200 μM; 2, LA-*trans* 400 μM plus polymyxin B 100 μM; 3, LA-*trans* 250 μM; 4, LA-*trans* 300 μM; 5, LA-*trans* 350 μM; 6, LA-*trans* 400 μM; LA-*trans* 400 μM plus indomethacin 25 μM.

Panel B: 1, OAG 1 μM; 2, OAG 3 μM; 3, OAG 5 μM; 4, OAG 10 μM; 5, OAG 30 μM.

Panel C: 1, OAG 1 μM plus LA-*trans* 200 μM; 2, OAG 3 μM plus LA-*trans* 200 μM; 3, OAG 5 μM plus LA-*trans* 200 μM.

Table 1: Effects of various fatty acids on platelet aggregation

Addition	Platelet aggregation (Light transmission in %)
Stearic acid (400 μM)	0
Oleic acid (400 μM)	0
Elaidic acid (400 μM)	0
Linoleic acid (400 μM)	94 ± 6
Linoleic acid (400 μM) + polymyxin B (100 μM)	23 ± 17
Linoleic acid (400 μM) + indomethacin (25 μM)	95 ± 5
Linoleic acid (250 μM)	3 ± 2
OAG (5 μM)	16 ± 5
OAG (5 μM) + linoleic acid (250 μM)	78 ± 8
Arachidonic acid (30 μM)	84 ± 9
Arachidonic acid (30 μM) + indomethacin (25 μM)	0

Platelet aggregation was measured as described in MATERIALS AND METHODS. The extent of aggregation is referred to the aggregation induced by thrombin (0.25 U/ml), which amounted to 100%. Data are the mean ± S.E.M. of 3-5 independent experiments.

In order to ascertain that octadecadienoic acids induce platelet activation *via* protein kinase C, protein phosphorylation in intact platelets was studied (Fig. 2). In agreement with previous reports, we found that thrombin caused phosphorylation of a 47 kDa peptide, which is the major substrate of protein kinase C in intact platelets, and of a 20 kDa peptide corresponding to myosin light chain, which is phosphorylated by protein kinase C and by myosin light chain kinase (1,4,5,23). OAG and octadecadienoic acids at concentrations inducing maximal aggregation caused strong phosphorylation of the 47 kDa pep-

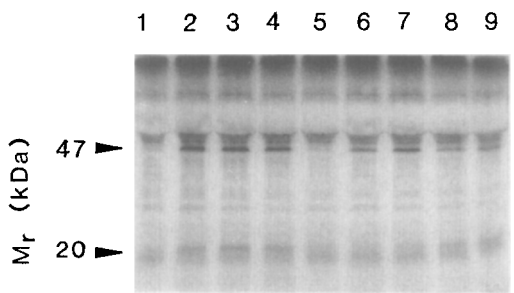

Fig. 2. Effect of thrombin, 1-oleoyl-2-acetyl-*rac*-glycerol and octadecadienoic acids on phosphorylation of 20 kDa and 47 kDa peptides in intact platelets. Gel-filtered platelets prelabelled with $^{32}P_i$ were incubated for 2 min with various stimuli. Proteins were analyzed as described in MATERIALS AND METHODS. Lane 1, no addition; Lane 2, OAG 30 μM; Lane 3, thrombin 0.25 U/ml; Lane 4, LA-*trans* 400 μM; Lane 5, LA-*trans* 200 μM; Lane 6, OAG 5 μM; Lane 7, LA-*trans* 200 μM plus OAG 5 μM; Lane 8, LA-*trans* 400 μM plus polymyxin B 100 μM; Lane 9, LA-*cis* 400 μM.

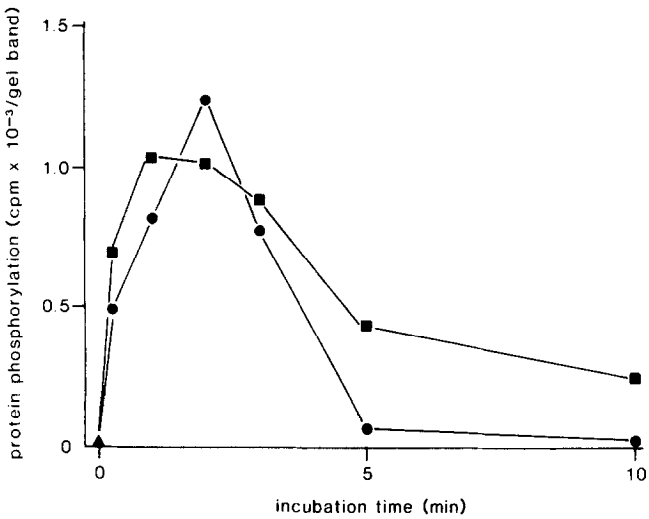

Fig. 3. Time course of phosphorylation of a 47 kDa peptide in human platelets by 1-oleoyl-2-acetyl-*rac*-glycerol and by linolelaidic acid. Gel-filtered platelets prelabelled with $^{32}P_i$ were incubated with 30 μM OAG (■) or 400 μM LA-*trans* (●) for various times. Proteins were analyzed as described in MATERIALS AND METHODS.

tide and less marked phosphorylation of the 20 kDa peptide, whereas elaidic acid was inactive (data not shown). LA-*trans* at a non-aggregatory concentration (200 μM) did not induce protein phosphorylation but significantly enhanced phosphorylation of the 47 kDa peptide by 5 μM OAG. As was the case with aggregation, polymyxin B strongly diminished protein phosphorylation by LA-*trans*. Phosphorylation of the 47 kDa peptide by OAG and LA-*trans* was time-dependent and reached maxima 1 and 2 min after stimulus addition, respectively (Fig. 3). Thereafter, the 47 kDa peptide was rapidly dephosphorylated. Ten minutes after stimulus addition, phosphorylation of the 47 kDa peptide induced by LA-*trans*, but not that induced by OAG, completely disappeared.

In conclusion, we present evidence that octadecadienoic acids activate protein kinase C in intact platelets, the activation of which is synergistic with that induced by diacylglycerol. Distinct differences in the kinetics of platelet aggregation and of protein phosphorylation were observed between fatty acids and diacylglycerol, indicating that these lipids are no equivalent activators of protein kinase C. The results presented support our previous suggestion that fatty acids and diacylglycerol may synergistically be involved in hormonal activation of protein kinase C (12,13).

Octadecadienoic acids are not metabolized *via* cyclooxygenase (21) and, in contrast to LA-*cis*, LA-*trans* does not affect platelet function by increasing membrane fluidity (24). Therefore, LA-*trans* may provide a useful experimental tool to study fatty acid regulation of protein kinase C in intact cellular systems.

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