Biochem. J. (1993) **290**, 849–856 (Printed in Great Britain)

Activation of phospholipase C and protein kinase C has little involvement in ADP-induced primary aggregation of human platelets: effects of diacylglycerols, the diacylglycerol kinase inhibitor R59022, staurosporine and okadaic acid

Marian A. PACKHAM,*§ Avinoam-Avyoav LIVNE,†‡ Deborah H. RUBEN* and Margaret L. RAND*
*Department of Biochemistry, University of Toronto, Toronto, Ontario, Canada, M5S 1A8
and †Department of Life Sciences, Ben-Gurion University of the Negev, Beer-sheva 84105, Israel

The primary phase of ADP-induced aggregation of human platelets does not involve appreciable formation of thromboxane A_2 or release of granule contents; lack of formation of inositol trisphosphate has also been noted. Because these responses of platelets to ADP differ so markedly from their responses to other aggregating agents, the roles in ADP-induced aggregation of diacylglycerol, protein kinase C, increases in cytosolic [Ca^{2+}], phosphorylation of pleckstrin (47 kDa) and phosphatases 1 and 2a were investigated. Washed human platelets, prelabelled with [^{14}C]5-hydroxytryptamine and suspended in Tyrode solution (2 mM Ca^{2+}, 1 mM Mg^{2+}), were used for comparisons between the aggregation induced by 2–4 μM ADP, in the presence of fibrinogen, and that induced by 0.05 units/ml thrombin. The diacylglycerol kinase inhibitor 6-{2-[(4-fluorophenyl)phenylmethylene]-1-piperidinylethyl}-7-methyl-5H-thiazolo[3,2-*a*]pyrimidin-5-one (R59022; 25 μM) had no, or only a slight, enhancing effect on ADP-induced aggregation, but potentiated thrombin-induced responses to a much greater extent. 1,2-Dihexanoyl-*sn*-glycerol or 1-oleoyl-2-acetyl-*sn*-glycerol (25 μM) added with or 30–90 s before ADP greatly potentiated aggregation without formation of thromboxane; staurosporine, an inhibitor of protein kinase C, reduced this potentiation. Staurosporine (25 nM) did not inhibit ADP-induced aggregation, although it strongly inhibited thrombin-induced aggregation and release of [^{14}C]5-hydroxytryptamine. All these observations indicate little or no dependence of primary ADP-induced aggregation on the formation of diacylglycerol or on the activation of protein kinase C. At 2–4 μM, ADP did not significantly increase the phosphorylation of pleckstrin (studied with platelets prelabelled with [^{32}P]orthophosphate), but 1,2-dihexanoyl-*sn*-glycerol-induced phosphorylation of pleckstrin was increased by ADP. Surprisingly, the diacylglycerols strongly inhibited the ADP-induced rise in cytosolic [Ca^{2+}] concurrently with potentiation of ADP-induced aggregation; thus the extent of primary aggregation is independent of the level to which cytosolic [Ca^{2+}] rises. Incubation of platelets with 1,2-dihexanoyl-*sn*-glycerol or 1-oleoyl-2-acetyl-*sn*-glycerol for several minutes reversed their potentiating effects on aggregation, and inhibition was observed. Incubation of platelets with okadaic acid, an inhibitor of phosphatases 1 and 2a, inhibited ADP- and thrombin-induced aggregation; although the reason for this effect is unknown, it is unlikely to involve inhibition of phospholipase C, since formation of diacylglycerol appears to have little involvement in the primary phase of ADP-induced aggregation.

INTRODUCTION

ADP is a unique aggregating agent in a number of ways (Gachet and Cazenave, 1991). The primary phase of ADP-induced platelet aggregation is readily reversible and does not involve appreciable formation of thromboxane A_2 or the release of granule contents (Packham et al., 1989, 1992). In media with physiological concentrations of Ca^{2+}, ADP usually induces only the primary phase of aggregation of human platelets, regardless of its concentration (Packham et al., 1989). In a medium with a low concentration of ionized calcium ([Ca^{2+}]), ADP induces two phases of aggregation, but the second phase is blocked by inhibitors of cyclo-oxygenase, such as aspirin or indomethacin (Packham et al., 1989). Several investigators (Fisher et al., 1985; Sweatt et al., 1986; Vickers et al., 1990) have been unable to detect inositol trisphosphate formation during the primary phase of ADP-induced aggregation. It may be that ADP-induced activation of platelets is not dependent on stimulation–response coupling through the action of phospholipase C on phosphatidylinositol 4,5-bisphosphate, although this pathway is well established for other agonists such as thrombin (Kroll and Schafer, 1989; Siess, 1989). As Rink and Sage (1990) have noted, ADP causes a much more rapid increase in the concentration of intracellular Ca^{2+} than is caused by other aggregating agents, probably by a combination of rapid influx and mobilization of internal stores. Although Haslam and Lynham (1977) reported that myosin light chain (20 kDa) and pleckstrin (47 kDa) (Tyers et al., 1989) are not phosphorylated during the primary phase of ADP-induced aggregation of human platelets, we and other investigators (Kawahara et al., 1983; Daniel et al., 1986; Gerrard et al., 1987; Rand et al., 1991) have detected some phosphorylation of these proteins in response to concentrations of ADP that cause extensive aggregation. Pleckstrin is phosphorylated in resting platelets and phosphorylation of pleckstrin increases when platelets are exposed to aggregating agents (Lyons et al., 1975; Haslam and Lynham, 1977; Gerrard et al., 1987). Various

Abbreviations used: DHG, 1,2-dihexanoyl-*sn*-glycerol; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; R59022, 6-{2-[(4-fluorophenyl)phenylmethylene]-1-piperidinylethyl}-7-methyl-5H-thiazolo[3,2-*a*]pyrimidin-5-one; PMA, 4-phorbol 12-myristate 13-acetate; fura-2 AM, fura-2 acetoxymethyl ester; DMSO, dimethyl sulphoxide.

‡ Deceased.
§ To whom correspondence should be addressed.

diacylglycerols and tumour-promoting phorbol esters have also been shown to cause the phosphorylation of pleckstrin (Lapetina et al., 1985; Kajikawa et al., 1989).

To investigate further the reactions involved in the primary phase of ADP-induced aggregation, we have examined the effects of (a) a diacylglycerol kinase inhibitor, 6-{2-[(4-fluorophenyl)-phenylmethylene]-1-piperidinylethyl}-7-methyl-5H-thiazolo[3,2-*a*]pyrimidin-5-one (R59022) (de Chaffoy de Courcelles et al., 1985; Nunn and Watson, 1987), which prevents the phosphorylation of diacylglycerol to phosphatidic acid; (b) agents that activate protein kinase C, namely the cell-penetrating diacylglycerols 1,2-dihexanoyl-*sn*-glycerol (DHG) and 1-oleoyl-2-acetyl-*sn*-glycerol (OAG); (c) staurosporine, which inhibits PKC and (d) okadaic acid, which inhibits protein phosphatases 1 and 2a (Haystead et al., 1989; Lerea, 1991). Aggregation, release of dense granule contents, mobilization of Ca^{2+} and phosphorylation of pleckstrin were studied. In some experiments, the effects on thrombin-induced aggregation and release of dense granule contents were also examined for comparison with the effects on ADP-induced aggregation, because thrombin-induced responses have been much more thoroughly studied by other investigators.

EXPERIMENTAL

Materials

Materials were obtained from the following suppliers: ADP, human thrombin, indomethacin, imipramine, Hepes, DHG, OAG, prostaglandin E_1 and dimethyl sulphoxide (DMSO) from Sigma Chemical Co. (St. Louis, MO, U.S.A.); DHG was also obtained from Serdary Research Laboratories (London, ON, Canada), but the two preparations gave similar results; fibrinogen from Kabivitrum (Stockholm, Sweden) (dialysed against isotonic saline before use); albumin (fraction V) from ICN Immuno-Biologicals (Lisle, IL, U.S.A.); diacylglycerol kinase inhibitor I (R59022) and staurosporine from Calbiochem (La Jolla, CA, U.S.A.); okadaic acid from Upstate Biotechnology (Lake Placid, NY, U.S.A.); fura-2 acetoxymethyl ester (fura-2 AM) from Molecular Probes (Eugene, OR, U.S.A.); [^{14}C]5-hydroxytryptamine (serotonin), 3′-[^{14}C]creatinine sulphate (60 mCi/mmol) from Amersham Corp. (Oakville, ON, Canada); carrier-free [^{32}P]orthophosphoric acid from Dupont Canada (Mississauga, ON, Canada); radioimmunoassay kit for thromboxane B_2 measurements from NEN (Lachine, Quebec, Canada). Apyrase was prepared by the method of Molnar and Lorand (1961) and used as described elsewhere (Kinlough-Rathbone et al., 1983). DHG, OAG, R59022, staurosporine and okadaic acid were dissolved as concentrated solutions in DMSO and added in 1 μl volumes; the maximum amount of DMSO added to 1 ml of platelet-suspending medium was 3 μl; appropriate control samples containing DMSO were included so that comparisons were made at the same DMSO concentrations. In preliminary experiments we established that addition of as much as 5 μl of DMSO to 1 ml of platelet suspension did not affect the extent of primary ADP-induced aggregation; lengthening the incubation time with DMSO to 5 min before the addition of ADP also had no effect. The percentage of [^{14}C]5-hydroxytryptamine in the supernatant of samples to which only DMSO was added (mean $\pm$ S.E.M., 2.3 ± 0.3 %, $n = 9$) was subtracted before calculation (see below) of percentage release induced by additions of substances dissolved in DMSO. Additions of 1–3 μl of DMSO to 1 ml of platelet suspension did not have a significant effect on thrombin-induced release of [^{14}C]5-hydroxytryptamine. All concentrations are given as final concentrations after all additions to the platelet suspensions.

Preparation of platelets

Suspensions of washed platelets from human subjects were prepared as previously described (Kinlough-Rathbone et al., 1983). The subjects had not taken medication affecting platelet function for at least 2 weeks before the donation of blood. (Informed consent was obtained from each subject and the experiments were approved by the University of Toronto Human Subjects Review Committee.) The final suspending medium of the platelets was a Tyrode-based solution which contained 2 mM $CaCl_2$, 1mM $MgCl_2$, 137 mM NaCl, 2.68 mM KCl, 11.9 mM $NaHCO_3$, 0.42 mM NaH_2PO_4, 5.5 mM glucose, apyrase (2 μl/ml) and 0.35 % albumin (pH 7.35). Platelet counts for aggregation studies were adjusted to 500000/μl.

Measurement of release of dense granule contents

The platelets were labelled in the first washing solution with [^{14}C]5-hydroxytryptamine (1 μCi/10 ml of washing fluid) and release of [^{14}C]5-hydroxytryptamine was determined 3 min after the addition of an aggregating agent, as described previously (Greenberg et al., 1975). Imipramine (5 μM) was added before the other additions, to prevent the re-uptake of released 5-hydroxytryptamine. In experiments in which cyclo-oxygenase was inhibited, indomethacin (10 μM) was added to the platelet-suspending medium 5 min before any other additions.

Aggregation of platelets

Aggregation of platelets in 1 ml samples of platelet suspensions was recorded for 3 min at 37 °C using an aggregation module (Payton Associates, Scarborough, ON, Canada) at a stirring speed of 1100 rev.·min^{-1}. Samples for aggregation by ADP, DHG or OAG contained fibrinogen (400 μg/ml); fibrinogen was not added to samples for thrombin-induced aggregation. Low concentrations of ADP and thrombin were used to facilitate the observation of potentiating effects.

Measurement of protein phosphorylation

The platelets (5×10^9/ml) were labelled in a modified first washing solution (calcium-free, phosphate-free, Tyrode-based solution with 0.02 % EGTA, 0.35 % albumin, 5 mM Hepes, 10 μM prostaglandin E_1, pH 6.5) with 0.75 mCi [^{32}P]orthophosphate/ml of platelet suspension at 37 °C for 1 h.

For separation and measurement of labelling of [^{32}P]phosphoproteins after stimulation of the platelets with ADP and/or DHG, samples were added to Laemmli sample buffer (Laemmli, 1970) and were heated to 100 °C for 3 min. Platelet proteins (approx. 40 μg) were separated by SDS/PAGE with 11 % (w/v) acrylamide in the separating gel. The proteins in the gel were stained with Coomassie Brilliant Blue, the gels were dried and the ^{32}P-labelled proteins were located by autoradiography. The bands corresponding to pleckstrin were cut from the gels and their radioactivities were determined by liquid scintillation counting.

Measurement of cytosolic [Ca^{2+}]

Platelets in the first washing fluid were loaded with fura-2 AM by the method of Pollock et al. (1986). After the usual washing procedure, the platelets were resuspended in the final suspending medium, supplemented with 5 mM Hepes, at a platelet count of 200000/μl. Fluorescence measurements were made with an SPEX Fluorolog 2 Spectrophotometer (SPEX Industries, Metuchan, NJ, U.S.A.) with excitation at 339 nm and emission at 500 nm.

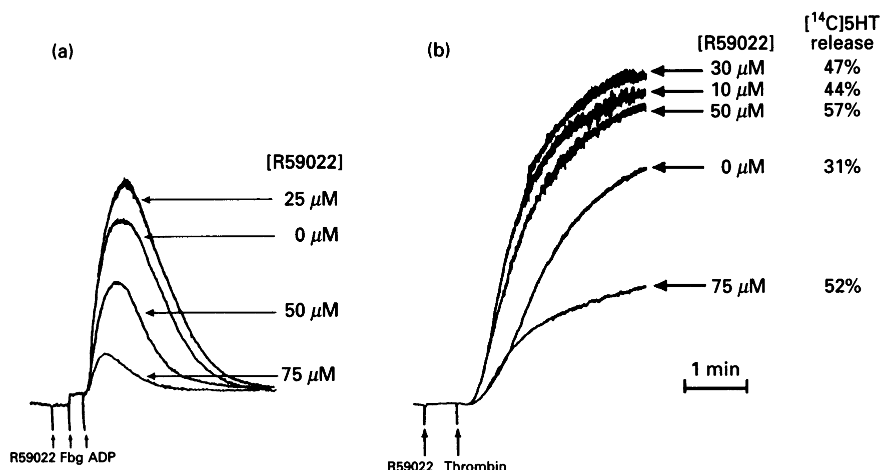

Figure 1 Effect of R59022 on aggregation induced by ADP and thrombin

R59022 (0–75 μM) was added 30 s before the aggregating agent and changes in light transmission through the stirred platelet suspensions were recorded. Aggregating agent: (**a**) 2 μM ADP in the presence of fibrinogen (Fbg, 400 μg/ml); (**b**) 0.05 units/ml thrombin. The percentage release of [^{14}C]5-hydroxytryptamine ([^{14}C]5HT) is shown beside the thrombin-induced aggregation curves. Release was negligible with ADP.

RESULTS

Effects of diacylglycerol kinase inhibitor R59022

At a concentration of 25 μM, R59022 did not cause platelets to change shape or aggregate. Release of [^{14}C]5-hydroxytryptamine was less than 1% ($n = 8$).

R59022 was used with the expectation that if ADP caused the formation of diacylglycerol, the effect of endogenous diacylglycerol would be prolonged or potentiated if its conversion to phosphatidic acid were blocked. In eight experiments with human platelets, R59022 (25 μM) either had no effect or only had a slight enhancing effect on the extent of aggregation induced by 2–4 μM ADP (Figure 1a). Mean values (±S.E.M., $n = 6$) for release of [^{14}C]5-hydroxytryptamine were 0.7 ± 0.4% for ADP and 2.1 ± 0.3% for R59022 and ADP. Higher concentrations of R59022 (50–75 μM) inhibited aggregation induced by ADP (Figure 1a). However, at concentrations of 10–50 μM, R59022 potentiated the aggregation and release induced by 0.05 unit/ml of thrombin (Figure 1b, Table 1); at a concentration of 75 μM, R59022 inhibited aggregation in response to 0.05 units/ml of thrombin, but the potentiation of the release of [^{14}C]5-hydroxytryptamine persisted (Figure 1b).

R59022 (25 μM) had no effect on the rise in cytosolic Ca^{2+} induced by ADP.

Table 1 Effect of diacylglycerol kinase inhibitor R59022, DHG and R59022 plus DHG on release of [^{14}C]5-hydroxytryptamine induced by thrombin

Results are means ± S.E.M. ($n = 4$) of the percentage of [^{14}C]5-hydroxytryptamine released in 3 min by 0.05 units/ml of thrombin, added to prelabelled platelets 30 s after the additions listed. The aggregation was maximal in all experiments. Paired difference analyses with the control are shown. The difference between DHG with thrombin and R59022 + DHG with thrombin is also statistically significant ($P < 0.025$).

Addition	[^{14}C]5-Hydroxytryptamine released (%)	P
DMSO (control)	43.8 ± 6.4	
R59022 (25 μM)	53.2 ± 5.5	< 0.01
DHG (25 μM)	53.5 ± 5.2	0.02
R59022 (25 μM) + DHG (25 μM)	60.7 ± 5.8	< 0.001

Effects of DHG and OAG

In four out of nine experiments, DHG and/or OAG (25 μM) caused a gradual, slight increase in light transmission in the presence of fibrinogen (400 μg/ml) (Figure 2a). The initial decrease in light transmission (characteristic of the shape change induced by agonists such as ADP or thrombin) did not occur in any of the experiments and the oscillations characteristic of disc-shaped platelets were not decreased. Incubation with these diacylglycerols for 2 min or longer before the addition of fibrinogen abolished their aggregating effects (Figure 2a). In the other five experiments, the platelets did not change shape or aggregate in response to DHG or OAG. The percentage release of [^{14}C]5-hydroxytryptamine (mean ± S.E.M., 1.8 ± 0.5%, $n = 9$) in 3 min was very low in all the nine experiments.

Addition of DHG or OAG with ADP or within 2 min before ADP (2–4 μM) (in the presence of fibrinogen) greatly potentiated the extent of ADP-induced aggregation (Figures 2b and 2c). This potentiation was accompanied by a small percentage release of [^{14}C]5-hydroxytryptamine during the first 3 min after the addition of ADP (mean ± S.E.M. for ADP alone, 1.23 ± 0.7%, $n = 8$; for DHG added 30 s before ADP, 5.9 ± 5.5%, $n = 8$). For platelets that aggregated in response to DHG alone, the percentage release in response to DHG and ADP was higher (10.0 ± 2.6%, $n = 4$) than for platelets that did not aggregate in response to DHG alone (1.9 ± 0.4%, $n = 4$). When DHG or OAG was incubated with the platelets before the addition of ADP, the potentiation decreased with increasing incubation time, until by 3–5 min both the rate and extent of aggregation were less than the control samples, to which only fibrinogen and ADP had been added (Figures 2b and 2c) and the release of granule contents was similar to the control values. However, deaggregation was much slower than in the control samples (Figures 2b and 2c). The addition of R59022 with DHG enhanced the potentiating effect

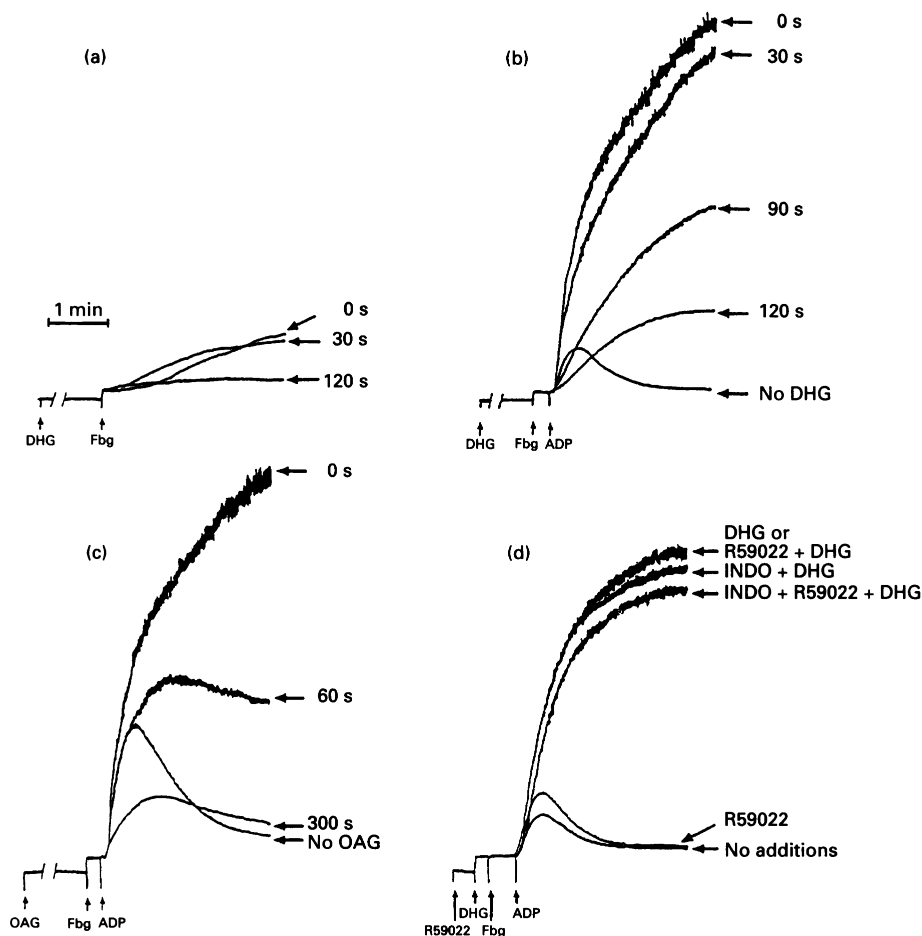

Figure 2 Effect of diacylglycerols and inhibitors on ADP-induced platelet aggregation

Changes in light transmission through the stirred platelet suspensions were recorded. (**a**) DHG (25 μM) was added at various times before fibrinogen (Fbg); similar results were obtained with OAG; (**b**) DHG (25 μM) was added at various times before ADP (4 μM); (**c**) OAG (25 μM) was added at various times before ADP (3 μM); (**d**) R59022 (25 μM) was added 40 s before ADP and DHG (25 μM) was added 30 s before ADP (3 μM). The effect of incubating platelets with indomethacin (INDO, 10 μM) for 5 min before the other additions is also shown. Fibrinogen (400 μg/ml) was added to all samples as indicated.

of DHG in some experiments, had little additional effect in others (Figure 2d) and somewhat inhibited the potentiation in others. None of R59022, DHG and the combination of R59022 and DHG resulted in the formation of thromboxane B_2 in either the presence or the absence of ADP. Indomethacin (10 μM) did not inhibit the potentiation of ADP-induced aggregation by DHG or OAG either in the absence or in the presence of R59022 (Figure 2d). In five experiments, the low percentage (3.5 ± 1.2 %) of [^{14}C]5-hydroxytryptamine released by 25 μM DHG or OAG followed at 30 s by ADP (2–4 μM) was reduced to 2.0 ± 1.0 % by indomethacin. With maximum aggregation responses (results not shown), thrombin-induced release of [^{14}C]5-hydroxytryptamine was enhanced by DHG and further enhanced by the combination of R59022 and DHG ($P < 0.025$, Table 1).

Either DHG or OAG, added 30 s before ADP, inhibited the rise in cytosolic [Ca^{2+}] caused by low concentrations of ADP (Figure 3). In two experiments with 0.5 μM ADP, the rise in cytosolic [Ca^{2+}] appeared to be completely blocked.

At a concentration of 3 μM, ADP caused no significant increase in the phosphorylation of pleckstrin, whereas DHG (25 μM) caused a large increase (Figure 4). The addition of DHG, followed 30 s later by ADP, caused an even greater phosphorylation of pleckstrin at the time (30–60 s after ADP) corresponding to maximal aggregation of samples to which only ADP was added. The phosphorylation then decreased to the level of that caused by DHG alone.

Effects of staurosporine

Staurosporine, added at a concentration of 25 nM at 30 s before ADP (2–4 μM), had little or no effect on ADP-induced aggregation or on the rise in cytosolic [Ca^{2+}], whereas this concentration strongly inhibited aggregation and release of dense granule contents induced by a low concentration of thrombin (Figures 5a and 5b). Higher concentrations of staurosporine were progressively inhibitory, but there was little difference in the extent of inhibition of ADP-induced aggregation at 200 nM and 400 nM staurosporine and ADP-induced aggregation was not abolished by these high concentrations (Figure 5a). Staurosporine at 25 nM also reduced the DHG- or OAG-induced potentiation of ADP-induced aggregation (Figure 5c) and partially blocked the DHG-induced inhibition of the rise in cytosolic [Ca^{2+}] caused by ADP (Figure 3).

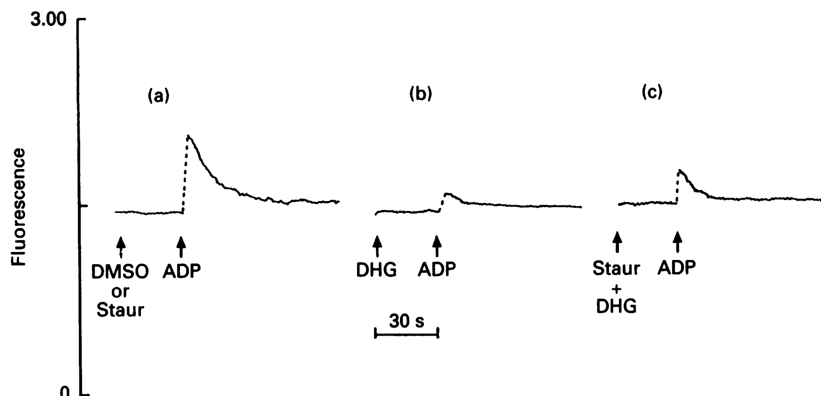

Figure 3 Effect of DHG and staurosporine on the increase in fluorescence of fura-2-AM-loaded platelets induced by ADP

(**a**) Staurosporine (Staur, 25 nM) or control solution was added 30 s before 3 μM ADP (the two recordings were superimposable); (**b**) DHG (25 μM) was added 30 s before ADP; (**c**) staurosporine (25 nM) and DHG (25 μM) were added 30 s before ADP. Fibrinogen (400 μg/ml) was present in all samples of platelet suspension. Similar results were obtained with OAG.

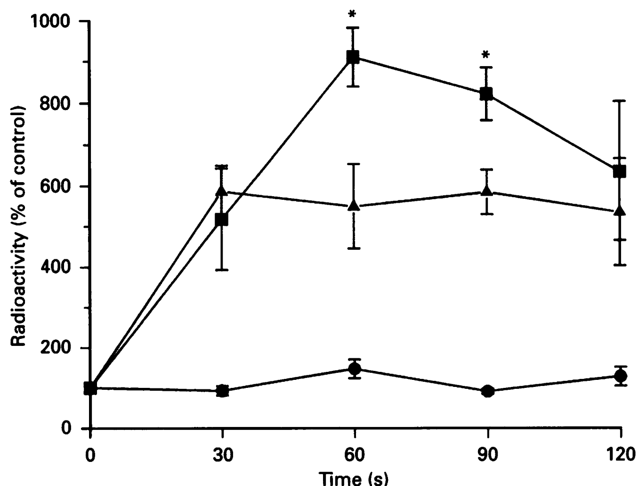

Figure 4 Phosphorylation of pleckstrin (47 kDa) by ADP, DHG and DHG plus ADP

●, ADP (3 μM); ▲, DHG (25 μM); ■, DHG + ADP. Fibrinogen (400 μg/ml) was present in all samples. DHG or DMSO (control) was added at zero time, 30 s before ADP. The c.p.m. in pleckstrin of control, unstimulated platelets (mean $\pm$ S.E.M., 329 $\pm$ 31, $n = 19$) was taken as 100%. Values shown are means $\pm$ S.E.M. for three experiments (ADP, DHG) and four experiments (DHG + ADP). The differences between the values for DHG and DHG + ADP at 60 and at 90 s are significant ($^*P < 0.05$); values for ADP alone are not significantly different from the unstimulated or zero time values.

Effects of okadaic acid

Incubation of human platelets with okadaic acid (1 μM), an inhibitor of protein phosphatases 1 and 2a, partially inhibited aggregation induced by 2–4 μM-ADP (in the presence of fibrinogen). Neither okadaic acid nor the combination of okadaic acid and ADP induced appreciable release of [^{14}C]5-hydroxytryptamine. Inhibition of ADP-induced aggregation was evident at 2 min of incubation and was stronger at 5 min (Figure 6a). Similar results were obtained with a low concentration of thrombin (0.05 unit/ml) and the percentage of [^{14}C]5-hydroxytryptamine released was reduced from 66 % without okadaic acid to 3 % by a 5 min incubation with okadaic acid before the addition of thrombin (Figure 6b).

DISCUSSION

These experiments were done under conditions in which only primary ADP-induced aggregation takes place; aggregation in response to ADP alone was followed by deaggregation and release of dense granule contents was approximately 1 %.

The very slight potentiating effect of the diacylglycerol kinase inhibitor, R59022 (25 μM), on ADP-induced aggregation of human platelets (and inhibition by higher concentrations of R59022) indicates that diacylglycerol formation probably does not play a major role in this aggregation response. If diacylglycerol formation were involved, the kinase inhibitor would have potentiated aggregation by preventing the conversion of diacylglycerol to phosphatidic acid, a process that is known to occur rapidly (de Chaffoy de Courcelles et al., 1985). Potentiation by 10 μM R59022 of pleckstrin phosphorylation by 10 μM dioctanoylglycerol has been observed previously (Livne et al., 1991a). Our experiments with thrombin, in which R59022 potentiated aggregation and the thrombin-induced release of dense granule contents, indicate that the kinase inhibitor was active at the concentration used (25 μM) when a stimulus that does cause diacylglycerol formation was tested. Nunn and Watson (1987) have also observed potentiation by R59022 of aggregation induced by sub-maximal concentrations of thrombin and this potentiation was associated with increased formation of diacylglycerol and decreased formation of phosphatidic acid. In addition, since DHG and OAG potentiated ADP-induced aggregation of human platelets when added 30 s–2 min before ADP, if an appreciable amount of diacylglycerol had been formed by ADP stimulation, strong potentiation of ADP-induced aggregation of human platelets would have occurred when conversion of diacylglycerol to phosphatidic acid was inhibited by R59022. Thus several observations with R59022 provide evidence that, during primary aggregation, ADP has only a slight stimulatory effect on the formation of diacylglycerol and differs markedly from thrombin in this respect. In experiments with platelets prelabelled with arachidonic acid, Kawahara et al. (1983) detected hardly any diacylglycerol formation when platelets were stimulated with 5 μM ADP.

The inhibition of ADP-induced aggregation caused by a high concentration of R59022 may be a non-specific effect, since aggregation in response to thrombin was also inhibited, although the thrombin-induced release of dense granule contents remained potentiated.

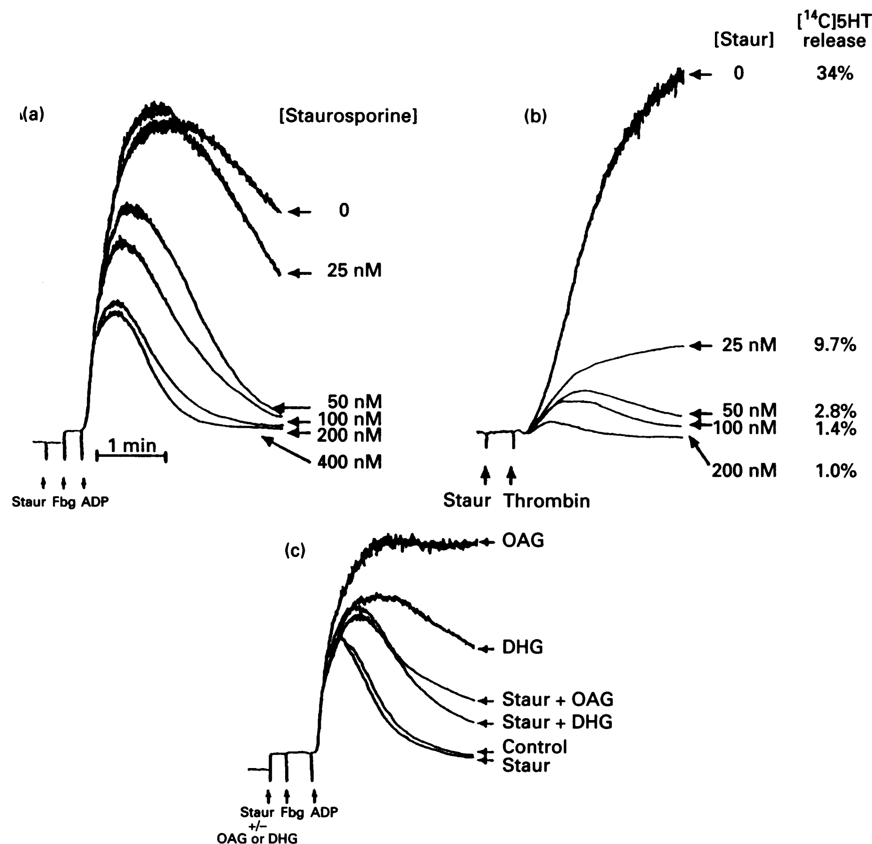

Figure 5 Effects of staurosporine on ADP and thrombin-induced aggregation and on potentiation of ADP-induced aggregation by diacylglycerols

Changes in light transmission through the stirred platelet suspensions were recorded. (**a**) Staurosporine (Staur), 0–400 nM, was added 30 s before 3 μM ADP in the presence of fibrinogen (Fbg, 400 μg/ml); (**b**) staurosporine (0–200 nM) was added 30 s before 0.05 unit/ml thrombin; the percentage release of [^{14}C]5-hydroxytryptamine ([^{14}C]5HT) is shown beside the aggregation curves; (**c**) effect of staurosporine (25 nM) on the aggregation induced by ADP (3 μM) and on the potentiation of ADP-induced aggregation by DHG (25 μM) or OAG (25 μM) added 30 s before ADP. Fibrinogen (Fbg, 400 μg/ml) was added as indicated. Control: only DMSO and Fbg were added before ADP.

Slow and weak aggregation of human platelets induced by 4-phorbol 12-myristate 13-acetate (PMA) or various diacylglycerols has been reported by other investigators (Zucker et al., 1974; Lapetina et al., 1985; Valone and Johnson, 1987). However, Schächtele et al. (1988) reported strong aggregation in response to 30 μM OAG. We observed that fibrinogen was necessary for the weak aggregation response induced by DHG or OAG, that little release of dense granule contents occurred and that platelets from only some of our donors aggregated in response to the diacylglycerols. In keeping with the observations of other investigators with phorbol esters or OAG (MacIntyre et al., 1985; Valone and Johnson, 1987; Rink and Sage, 1990), DHG and OAG did not cause a detectable change in the concentration of cytosolic Ca^{2+} in platelets.

The addition of DHG or OAG 30 s before low concentrations of ADP (0.5 μM) abolished the ADP-induced rise in cytosolic [Ca^{2+}]. With slightly higher concentrations of ADP, the extent of the ADP-induced rise in cytosolic [Ca^{2+}] was diminished by the presence of DHG or OAG and this effect of diacylglycerols was counteracted by staurosporine, indicating that activation of protein kinase C was probably involved. Inhibition of the agonist-induced rise in cytosolic [Ca^{2+}] upon activation of protein kinase C has been noted by other investigators (MacIntyre et al., 1985; Krishnamurthi et al., 1986a; Valone and Johnson, 1987; Yoshida and Nachmais, 1987), but the reactions that are responsible for this effect have not been identified. Our results indicate that, surprisingly, this inhibition occurs concurrently with potentiation of aggregation by diacylglycerols.

The strong potentiation of ADP-induced aggregation of human platelets by DHG or OAG, added with ADP or within 2 min before ADP addition, is not surprising and similar effects of phorbol esters and OAG have been observed with other agonists (Krishnamurthi et al., 1986a,b; Siess and Lapetina, 1987). In experiments with PMA added to aspirin-treated, citrated, platelet-rich plasma, Siess and Lapetina (1987) observed that PMA, added 5–10 s before ADP, had a sensitizing effect on ADP-induced aggregation, which they described as synergism. Potentiation is probably due to the activation of protein kinase C. In our experiments, the potentiation by DHG or OAG of ADP-induced aggregation did not involve formation of thromboxane A_2, because the potentiation was practically unaffected by indomethacin. Potentiation by PMA of thrombin-induced secretion of [^{14}C]5-hydroxytryptamine has also been observed to be insensitive to indomethacin (Krishnamurthi et al., 1986a).

Low concentrations of ADP (2–4 μM) had no significant effect on the phosphorylation of pleckstrin. Daniel et al. (1984) showed previously that ADP-induced shape change in the presence of EDTA was associated with only a very slight increase in the phosphorylation of this 47 kDa protein. We observed, however, that ADP significantly increased the phosphorylation of pleck-

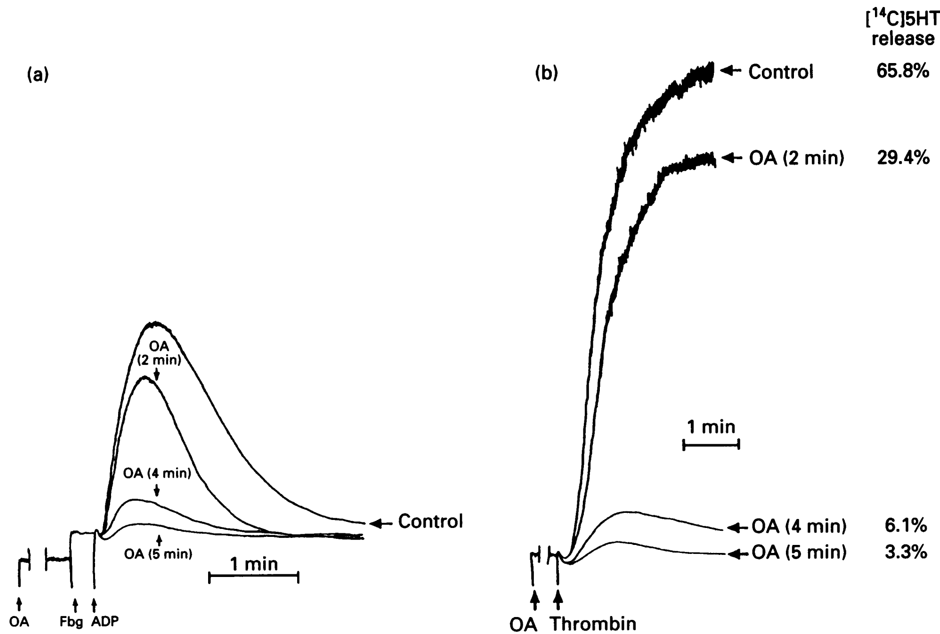

Figure 6 Effect of incubation with okadaic acid on aggregation induced by ADP and thrombin

Changes in light transmission through the stirred platelet suspensions were recorded. Okadaic acid (OA) was used at a concentration of 1 μM. (**a**) Aggregation with ADP (2 μM) in the presence of fibrinogen (Fbg, 400 μg/ml); (**b**) aggregation with thrombin (0.05 unit/ml). The incubation times with OA are shown beside the aggregation curves. The percentage release of [^{14}C]5-hydroxytryptamine ([^{14}C]5HT) is shown with the thrombin-induced aggregation curves. Control: OA replaced by its solvent, DMSO.

strin that was caused by DHG. This effect parallels the potentiation of aggregation by DHG.

The progressive decrease in potentiating effects and the eventual inhibition of aggregation by the diacylglycerols when they were incubated with platelets for several minutes before the addition of ADP is in keeping with the known negative feedback regulation exerted by PMA and other agents that stimulate protein kinase C (Zavoico et al., 1985; Watson and Lapetina, 1985; Powling and Hardisty, 1986; Krishnamurthi et al., 1986a; Bishop et al., 1990). Caramelo and Schrier (1988) have suggested that the inhibitory effects of PMA are due to its ability to decrease the rise in cytosolic Ca^{2+} caused by various agonists; this would explain the inhibition of aggregation that is observed when diacylglycerol or PMA is incubated with platelets for several min before the addition of an agonist. However it does not explain the observation that strong potentiation of aggregation of human platelets occurs at a time when inhibition of the rise in cytosolic Ca^{2+} is observed, that is when a diacylglycerol is added with ADP or shortly before ADP. It is apparent that extensive primary aggregation in response to low concentrations of ADP can take place despite strong inhibition of the rise in cytosolic [Ca^{2+}]. Rink and Sage (1990) do not list aggregation among the functional responses of platelets ('secretory exocytosis, shape change and myosin phosphorylation') that can occur with little or no measured rise in cytosolic [Ca^{2+}]. Our observations indicate that ADP-induced aggregation can be added to this list. Rink and Sage (1990) attribute the Ca^{2+}-independent secretion to activation of protein kinase C, but this explanation may not apply to ADP-induced aggregation, since the phosphorylation results indicate little or no phosphorylation of pleckstrin during primary aggregation induced by a low concentration of ADP.

ADP-induced aggregation was not inhibited by 25 nM staurosporine, which strongly inhibited thrombin-induced aggregation and this concentration of staurosporine also inhibited the DHG-induced potentiation of ADP-induced aggregation of human platelets. With staurosporine a K_I value of 24 nM has been observed for inhibition of the Na^+/H^+ transporter induced by PMA or DHG (Livne et al., 1991b) and a K_I value of 70 nM has been reported for inhibition of phosphorylation of pleckstrin induced by dioctanoyl glycerol (Watson et al., 1988). Our observations and these K_I values indicate that the formation of the diacylglycerol and the activation of protein kinase C have little involvement in primary ADP-induced aggregation. In addition, ADP-induced aggregation was only partially inhibited by 50–400 nM staurosporine. Partial inhibition of ADP-induced aggregation by 1 μM staurosporine has been observed by Watson and Hambleton (1989) who have postulated that there may be phosphorylation-dependent and phosphorylation-independent pathways of platelet aggregation. Non-specific inhibitory effects on serine/threonine kinases by high concentrations of staurosporine have been recognized by other investigators (Watson et al., 1988; Kocher and Clemetson, 1991) and such effects may be responsible for the partial inhibitory effects we observed with 50–400 nM staurosporine. High concentrations of staurosporine (in the micromolar range) have been reported to inhibit ADP-induced aggregation of porcine platelets (IC_{50} of 11.6 μM), but to have no effect on thrombin-induced aggregation (Oka et al., 1986). In contrast, Watson et al. (1988) and Watson and Hambleton (1989) reported some inhibition of human platelet aggregation induced by 0.1–1 unit/ml thrombin by 1 μM staurosporine and complete inhibition of the secretion of dense granule contents. Thrombin is generally considered to be a stronger stimulus for platelets than is ADP, but we observed that the responses to thrombin that are associated with activation of protein kinase C were more readily inhibited by staurosporine than was ADP-induced aggregation. It appears, therefore, that ADP can aggregate platelets without much, if any, contribution

from protein kinase C. This conclusion is also borne out by the lack of significant phosphorylation of pleckstrin during the primary phase of aggregation induced by low concentrations of ADP (2–4 μM) and indirectly by earlier studies showing that little conversion of phosphatidylinositol 4,5-bisphosphate to inositol trisphosphate occurs (Fisher et al., 1985; Sweatt et al., 1986; Vickers et al., 1990).

In agreement with the observations of other investigators who have studied the effects of okadaic acid on thrombin-induced aggregation (Murata et al., 1992) and release of dense granule contents (Lerea, 1991; Murata et al., 1992), we found that incubation of platelets with okadaic acid inhibited aggregation induced by low concentrations of ADP or thrombin and inhibited thrombin-induced release of dense granule contents. Lerea (1991) has speculated that okadaic acid-sensitive protein phosphatases might be instrumental in the regulation of platelet activation. If so, aggregation in response to both ADP and thrombin are affected, although these agonists act on platelets in different and independent ways. Walker and Watson (1992) have suggested that okadaic acid inhibits the activation of phospholipase C in human platelets, but such an effect would not account for the inhibitory effect of okadaic acid on the primary phase of ADP-induced aggregation.

In conclusion, primary ADP-induced aggregation has little dependence on the formation of diacylglycerol or activation of protein kinase C. Activation of protein kinase C by DHG or OAG greatly potentiates ADP-induced aggregation, and formation of thromboxane A_2 is not responsible for this potentiation. Staurosporine inhibits the potentiating effects of the diacylglycerols. However, incubation of platelets with diacylglycerol for several minutes results in a negative feedback effect on ADP-induced platelet reactions. Surprisingly, at a time when diacylglycerols greatly potentiate ADP-induced aggregation, they strongly inhibit the ADP-induced rise in cytosolic [Ca^{2+}], indicating that the extent of primary aggregation is independent of the level to which cytosolic [Ca^{2+}] rises.

The skilful technical assistance of Ms Sheila DaCosta is gratefully acknowledged. This study was supported by grants from the Medical Research Council of Canada (MT2629) and the Heart and Stroke Foundation of Ontario (B1732).

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Received 17 June 1992/12 October 1992; accepted 13 October 1992