A diacylglycerol kinase inhibitor, R59022, potentiates secretion by and aggregation of thrombin-stimulated human platelets

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The diacylglycerol kinase inhibitor R59022 (10 μ M) potentiates secretion and aggregation responses in human platelets challenged with sub-maximal concentrations of thrombin. Potentiation correlates closely with increased formation of diacylglycerol, increased phosphorylation of a 40 kDa protein, a known substrate for protein kinase C, and with decreased formation of phosphatidic acid, the product of diacylglycerol kinase. Phosphorylation of myosin light chains, formation of inositol phosphates and the mobilization of Ca^{2+} by thrombin are not affected by R59022 (10 μ M). These data support a role for protein kinase C in platelet aggregation and secretion, and provide further evidence that endogenous diacylglycerols bring about the activation of this enzyme. These data also add further argument against a role for phosphatidic acid in platelet activation.

INTRODUCTION

Activation of human platelets by thrombin is associated with hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2), leading to the generation of 1,2-diacylglycerol (DG) (Rittenhouse-Simmons, 1979; Bishop *et al.*, 1986) and inositol trisphosphate (IP_3) (Agranoff *et al.*, 1983; Watson *et al.*, 1984; Siess & Binder, 1985). Both of these products are thought to act as second messengers. DG activates protein kinase C (Nishizuka, 1983, 1984), and IP_3 mobilizes Ca^{2+} from non-mitochondrial stores within cells (Berridge & Irvine, 1984), including the platelet (O'Rourke *et al.*, 1985; Brass & Joseph, 1985). The predominant substrate for protein kinase C in the platelet is a 40 kDa protein of uncertain function (Sano *et al.*, 1983; Imaoka *et al.*, 1983), whereas the mobilization of Ca^{2+} brings about the phosphorylation of a 20 kDa protein, myosin light chain, mediated predominantly by myosin light-chain kinase (Daniel *et al.*, 1977; Castagna *et al.*, 1982; Naka *et al.*, 1983).

Evidence that protein kinase C is involved in thrombin-stimulated secretion and aggregation includes the following. (1) Relatively high concentrations of membrane-permeant DGs and phorbol esters, which have in common with DGs the ability to activate protein kinase C, stimulate secretion and aggregation (Kaibuchi *et al.*, 1983; Rink *et al.*, 1983). (2) In threshold concentrations, membrane-permeant DGs and phorbol esters synergize with Ca^{2+} to produce platelet activation (Kaibuchi *et al.*, 1983; Knight & Scrutton, 1984). (3) Activation of platelets by a wide variety of platelet agonists, including thrombin (Sano *et al.*, 1983), platelet-activating factor (Lapetina & Siegel, 1983), collagen (Watson *et al.*, 1985) and thromboxanes and endoperoxides (Siess *et al.*, 1983), correlates closely with the hydrolysis of PIP_2 and phosphorylation of the 40 kDa protein.

Direct evidence for a role for protein kinase C in platelet activation by cell-surface agonists, however, is not yet available, and alternative lines of evidence suggest a cautionary note before this hypothesis can be accepted. These include the following. (1) As yet there has been no identification of a substrate for protein kinase C with a known role in secretion or aggregation. (2) Pretreatment of platelets with phorbol ester or DG before challenge with thrombin inhibits subsequent platelet activation (Watson & Lapetina, 1985), suggesting that under certain circumstances protein kinase C has a negative-feedback role. The site of this feedback is believed to occur at the level of phospholipase C activation (Watson & Lapetina, 1985; MacIntyre *et al.*, 1985), and also by enhanced metabolism of IP_3 (Molina y Vedia & Lapetina, 1986). (3) The mass of DG undergoes a surprisingly small change after platelet activation by thrombin (Rittenhouse-Simmons, 1979; Bishop *et al.*, 1986), and even high thrombin concentrations cause only a doubling of intracellular DG. It is difficult to envisage how such a small change in relative mass can account for the marked activation of protein kinase C observed.

Efficient control of cellular responses necessitates rapid turnover of regulating mediators, such as DG. Two enzymes operate to metabolize DG, DG lipase (Bell *et al.*, 1979) and DG kinase (Lapetina & Cuatrecasas, 1979), the latter producing phosphatidic acid (PA), which itself has been hypothesized to be a second messenger (Putney, 1981). If DG is a second messenger with a role in platelet activation, then the inhibition of its metabolism should potentiate platelet activation by surface agonists such as thrombin, analogous to methylxanthine potentiation of cyclic AMP-mediated responses. de Chaffoy de Courcelles *et al.* (1985) have shown that R59022 inhibits DG kinase in human platelets. The present study was therefore undertaken to investigate the action of R59022 on platelet activation by

thrombin in an attempt to obtain more direct evidence for an involvement of protein kinase C in secretion and aggregation.

METHODS

Blood (50 ml) was obtained from drug-free volunteers on the day of the experiment, by using 0.15 vol. of sterile citrate as anti-coagulant. Platelet-rich plasma was obtained by centrifugation at 200 *g* for 20 min, and platelets were separated by centrifugation at 800 *g* for 10 min in the presence of prostacyclin (Vargas *et al.*, 1982). Platelets were resuspended in a modified Tyrode buffer (135 mM-NaCl, 12 mM-NaHCO₃, 2.8 mM-KCl, 0.35 mM-NaH₂PO₄, 1 mM-MgCl₂, 5 mM-Hepes, 5 mM-glucose, pH 7.3), and labelled with [³²P] P_i (1 mCi/3 ml) or [³H]inositol (50 µCi/ml) for 1 h and 3 h respectively, in the presence of prostacyclin (Watson *et al.*, 1985). They were then centrifuged at 800 *g* for 10 min, and resuspended at a concentration of 2×10^8 – 4×10^8 /ml in the above buffer in the presence of indomethacin (10 µM). For experiments in which [³H]5-hydroxytryptamine secretion, [³H]DG formation or intracellular Ca²⁺ was measured, the platelets were incubated with [³H]5-hydroxytryptamine (10 µCi), [³H]arachidonic acid (50 µCi) or fura2 AM (6 µM) (Sage & Rink, 1986) respectively for 45–60 min in platelet-rich plasma before centrifugation.

All experiments, except for the fura2 studies, were carried out in a Chronolog Lumi-aggregometer in a final volume of 0.5 ml with stirring and at a temperature of 37 °C. The following protocol was used: R59022 or its solvent was added 60 s before thrombin or the Ca²⁺ ionophore A23187, and the platelets were left for a further 60 s. In some experiments, luciferin–luciferase reagent (0.2 mg) was included in order to measure the secretion of ATP. Light transmission was recorded throughout the experiment and displayed on a chart recorder. For platelets labelled with [³²P]P_i, the reaction was terminated by taking a 50 µl sample for analysis of protein phosphorylation, and the remaining portion was transferred to 1.8 ml of chloroform/methanol (1:2, v/v) for measurement of PA (Lapetina & Siegel, 1983). Protein phosphorylation was estimated by one-dimensional SDS/polyacrylamide-gel electrophoresis (11% gel), and PA formation by t.l.c. (Lapetina & Siegel, 1983). For analyses of [³H]inositol phosphates or [³H]DG, the reaction was terminated by transferring the platelets to 1.8 ml of chloroform/methanol/HCl (50:100:1, by vol.); inositol phosphates were separated by Dowex anion-exchange chromatography (Watson *et al.*, 1984), and [³H]DG was measured by t.l.c. (de Chaffoy de Courcelles *et al.*, 1985). DG was identified by co-chromatography with DG (detected with iodine) prepared from phosphatidylinositol by incubation with platelet phospholipase C (Siess & Lapetina, 1983). [³H]5-Hydroxytryptamine secretion was measured as described previously (Packham *et al.*, 1977). Fura2 studies were carried out in a Perkin–Elmer single-beam spectrofluorimeter (model LS-3) at a temperature of 37 °C and with constant stirring. Excitation was at 350 or 380 nm, and emission was at 510 nm; the manual shift between the two excitation wavelengths took less than 5 s and was only performed when a constant reading had been obtained. Intracellular Ca²⁺ concentrations were estimated either by the ratio of the readings at the two

Fig. 1. Concentration–response curve for R59022 inhibition of thrombin-induced formation of [³²P]PA

Platelets were pre-labelled with [³²P]P_i and challenged with thrombin (1 unit/ml) for 60 s in the presence of various concentrations of R59022. Each point represents the mean ± S.E.M. from three experiments performed in duplicate or triplicate. Statistical indications were made by a non-parametric Mann–Whitney U-test relative to thrombin alone (control): **P* < 0.05; ***P* < 0.01.

excitation wavelengths, or by the reading obtained at a single wavelength as described by Grynkiewicz *et al.* (1985); both methods gave essentially the same result. All experiments were performed in duplicate, triplicate or occasionally quadruplicate.

Statistical comparisons were made by a non-parametric Mann–Whitney U-test.

Materials

All radioactive materials were purchased from Amersham International. Thrombin, A23187 and phosphatidylinositol were from Sigma. R59022 was from Janssen and was dissolved as described by de Chaffoy de Courcelles *et al.* (1985). Fura2 AM was from Molecular Probes, CA, U.S.A. Luciferin–luciferase reagent was from Coulter, Luton, Beds., U.K. Sterile citrate was from Evans Medical, Greenford, Middx., U.K. Prostacyclin was kindly donated by The Wellcome Foundation, Beckenham, Kent, U.K. All other reagents were of analytical grade.

RESULTS

Effect of R59022 on thrombin-induced formation of PA

Initial experiments were carried out in order to find the optimum concentration of R59022 for further study. The ability of R59022 to inhibit the formation of [³²P]PA by a maximally effective concentration of thrombin is shown in Fig. 1. In reasonable agreement with the results of de Chaffoy de Courcelles *et al.* (1985), approx. 80% inhibition was observed at 100 µM-R59022, and 40% inhibition occurred with a concentration of approx. 10 µM. On its own, R59022 (100 µM) had no significant effect on the resting content of [³²P]PA (results not shown). At concentrations above 10 µM, R59022 caused a decrease in light transmission, the time course and magnitude of which are very reminiscent of thrombin-stimulated shape change of platelets from a disc to a spiny sphere with pseudopodia (results not shown). Morphological studies are required to verify whether this

Fig. 2. Effect of R59022 on thrombin-induced shape change and aggregation

Shown are example traces of R59022 (10 μ M) potentiation of platelet aggregation, but not shape-change responses, induced by sub-maximal concentrations of thrombin. Potentiation was observed in nine out of ten experiments. S, solvent for R59022; T, thrombin; R, R59022 (10 μ M). Thrombin concentration in (a) was 0.03 units/ml and in (b) 0.1 unit/ml.

is a classical shape-change response, since many compounds interfere with membrane structure and therefore light transmission. Since shape change is thought to be mediated by phosphorylation of myosin light chains and to be unrelated to protein kinase C activation (Daniel *et al.*, 1984), the above result casts doubt on the specificity of action of relatively high concentrations of R59022. For this reason, therefore, a concentration of R59022 (10 μ M) just below threshold for this effect was used for all subsequent experimentation. This concentration had no effect on resting PA contents or phosphorylation of the 20 kDa and 40 kDa proteins (Fig. 3).

Effect of R59022 on platelet activation by sub-maximal concentrations of thrombin

In nine out of ten experiments, R59022 (10 μ M) was observed to potentiate aggregation, but not shape change induced by sub-maximal concentrations of thrombin, as exemplified in Fig. 2. In six of these studies, the platelets had been pre-labelled with [32 P]P_i, and potentiation was accompanied by a significant ($P < 0.05$) decrease in formation of PA, to 36% of control values (Fig. 3a), a 338% increase in [32 P]phosphorylation of a 40 kDa protein ($P < 0.01$), and no apparent change in [32 P]phosphorylation of a 20 kDa protein (Fig. 3b). In one of these studies luciferin-luciferase reagent was present, and R59022 was observed to potentiate the secretion of ATP. In the other four studies mentioned above, the platelets had been prelabelled with [3 H]5-hydroxytryptamine, and the potentiation of aggregation by R59022 (10 μ M) was accompanied by a significant shift to the left in the thrombin dose-response curve for [3 H]5-hydroxytryptamine secretion (Fig. 4). In a separate set of experiments, R59022 (10 μ M) potentiated the formation of [3 H]DG by a concentration of thrombin (0.1 unit/ml) that was just maximal for aggregation (Table 1), thereby confirming the previous work of de Chaffoy de Courcelles *et al.* (1985).

In contrast, R59022 (10 μ M) had no significant effect on the dose-response curve for the mobilization of intracellular Ca²⁺ by thrombin in either the presence or

Fig. 3. Effect of R59022 on thrombin-induced formation of [32 P]PA and phosphorylation of 20 kDa and 40 kDa proteins

Platelets were pre-labelled with [32 P]P_i and challenged with concentrations of thrombin (0.04–0.1 unit/ml) threshold for aggregation. Data were only taken from the five (out of six) experiments in which potentiation of aggregation was observed, and are shown as means $\pm$ S.E.M. from duplicate or triplicate determinations. The effect of R59022 (10 μ M) on the formation of PA or phosphorylation of 20 kDa and 40 kDa proteins by threshold concentrations of thrombin was compared with effects of thrombin alone by using a non-parametric Mann-Whitney U-test. * $P < 0.05$; ** $P < 0.01$. C, control; R, R59022 (10 μ M); T, threshold thrombin concentrations; T_{max}, thrombin (1 unit/ml).

the absence of extracellular Ca²⁺ (Fig. 5). Similarly, R59022 (10 μ M) had no apparent effect on the formation of [3 H]IP₃ by thrombin (1 unit/ml) (Table 1).

Action of R59022 on platelet activation by the Ca²⁺ ionophore A23187

In the presence of cyclo-oxygenase inhibitors, the Ca²⁺ ionophore A23187 activates platelets independently of

Fig. 4. Effect of R59022 on the secretion of $[^3\text{H}]5$ -hydroxytryptamine by thrombin

Human platelets were pre-labelled with $[^3\text{H}]5$ -hydroxytryptamine as described in the Methods section. Results are shown as means $\pm$ S.E.M. from four experiments performed in duplicate: * $P < 0.05$. $\square$, Control; $\blacksquare$, plus R59022 ($10 \mu\text{M}$).

Fig. 5. Effect of R59022 on the mobilization of intracellular Ca^{2+} by thrombin

Platelets were loaded with fura2 and challenged with thrombin in either the presence ($\circ$, $\bullet$) or the absence ($\square$, $\blacksquare$) of extracellular Ca^{2+} . The peak intracellular Ca^{2+} was calculated as described in the Methods section. $\circ$, $\square$, Controls; $\bullet$, $\blacksquare$, plus R59022 ($10 \mu\text{M}$).

phospholipase C and without apparent formation of DG (Rittenhouse, 1984; Lapetina *et al.*, 1986). It was therefore decided to investigate the action of R59022 on platelet activation by A23187 in the presence of the cyclo-oxygenase inhibitor, indomethacin. In three out of four experiments, R59022 had no effect on secretion or aggregation induced by threshold concentrations of A23187 (results not shown). In the fourth experiment there was a slight potentiation of A23187-induced aggregation by R59022, but the magnitude of this effect was much less than that observed with thrombin.

Table 1. Effect of R59022 ($10 \mu\text{M}$) on the formation of $[^3\text{H}]\text{IP}_3$ and $[^3\text{H}]\text{DG}$ by thrombin

Platelets were prelabelled with $[^3\text{H}]\text{inositol}$ or $[^3\text{H}]\text{arachidonic acid}$, and subsequently challenged with either a high concentration of thrombin (1 unit/ml) or a concentration just above threshold for aggregation (0.1 unit/ml), respectively, for 60 s. $[^3\text{H}]\text{IP}_3$ and $[^3\text{H}]\text{arachidonic acid-labelled DG}$ were measured as described in the Methods section. Results (d.p.m.) are shown as means $\pm$ S.E.M. of three experiments performed in quadruplicate: * $P < 0.05$ relative to thrombin alone.

	Basal	Thrombin	Thrombin + R59022
IP_3	155 ± 34	380 ± 81	355 ± 67
DG	943 ± 81	1035 ± 53	$1356 \pm 116^*$

DISCUSSION

There are several criteria which must be satisfied before a physiological role for protein kinase C and DG in platelet aggregation and secretion by thrombin can be considered as proven. The present study has gone a long way to satisfying one of these criteria. The inhibition of the metabolism of DG through the use of a DG kinase inhibitor, R59022 (de Chaffoy de Courcelles *et al.*, 1985), potentiated platelet secretion and aggregation responses to sub-maximal concentrations of thrombin, but did not affect the shape-change response. These effects correlated closely with an increased formation of DG, increased phosphorylation of the 40 kDa protein, a known substrate for protein kinase C, and decreased formation of PA, the reaction product of DG kinase. There was no change in phosphorylation of the 20 kDa protein (which is not a major substrate for protein kinase C), the formation of inositol phosphates or mobilisation of intracellular Ca^{2+} . Taken together, these results suggest that R59022 ($10 \mu\text{M}$) can be used as a relatively selective inhibitor of DG kinase, and that increasing membrane DG produces increased activation of protein kinase C. The fact that these effects occur in conjunction with increased secretion and aggregation strongly indicate a causal relationship between these two responses, and adds further support to the hypothesis that protein kinase C is involved in platelet secretion and aggregation.

The possible involvement of protein kinase C in platelet shape change is controversial. Lapetina & Siegel (1983) observed a close correlation between the onset of shape change by platelet-activating factor and activation of protein kinase C. On the other hand, Daniel *et al.* (1984) reported that ADP-induced shape change is associated with strong phosphorylation of myosin light chain and with only minimal activation of protein kinase C; furthermore, platelet aggregation by phorbol ester is not preceded by shape change (Rink *et al.*, 1983), and this latter observation provides direct evidence against an immediate involvement of protein kinase C in this response. The possibility must still be considered, however, that protein kinase C may play a modulatory role in shape change in conditions in which Ca^{2+} mobilization and myosin light-chain phosphorylation occur. The present study provides evidence against this

possibility, however, by demonstrating that thrombin-induced shape change remains unchanged under conditions of increased protein kinase C activation. This conclusion needs to be confirmed by morphological studies, since shape change is not accurately represented by the simple monitoring of light transmission.

The decreased formation of PA by thrombin in the presence of R59022 provides further evidence against a second-messenger role for PA in platelet secretion and aggregation. Originally, it had been suggested that PA may be involved in the mobilization of Ca^{2+} , in view of its ability to move Ca^{2+} ions across an artificial membrane (Tyson *et al.*, 1976) and to release Ca^{2+} from a platelet membrane fraction (Gerrard *et al.*, 1978). The ability of PA to act as an ionophore, however, has been challenged (Holmes & Yoss, 1983), and evidence against PA producing Ca^{2+} mobilization in platelets has been reported (Watson *et al.*, 1985). The present study adds further argument against a second-messenger role for PA in platelets, by demonstrating that R59022 inhibits formation of PA by thrombin, without a change in the mobilization of intracellular Ca^{2+} . The release of intracellular Ca^{2+} is more likely to be related to the formation of IP_3 , which was not affected by R59022.

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