Collagen stimulates [³H]inositol trisphosphate formation in indomethacin-treated human platelets

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The present study investigates the pathway of metabolism of inositol phospholipids in human platelets exposed to collagen. Platelet activation by collagen was preceded by a lag phase usually lasting 10–20 s. Formation of [³H]inositol trisphosphate (IP₃) was not observed during this period, but occurred in parallel with the onset of aggregation, release of ATP and phosphorylation of a 20000 Da and a 40000 Da protein. Indomethacin treatment partially inhibited all of these responses. Aggregation and ATP release, but not IP₃ formation, were further inhibited in indomethacin-treated platelets loaded with the fluorescent Ca²⁺ indicator, quin2. Under these conditions there was no detectable mobilization of Ca²⁺. These results demonstrate that activation of platelets by collagen is associated with rapid hydrolysis of polyphosphoinositides by phospholipase C, thereby producing IP₃. This observation is discussed in relation to IP₃ as a possible Ca²⁺-mobilizing agent.

The action of collagen on human platelets is associated with an initial lag phase followed by shape change, aggregation and release reactions which occur in parallel with formation of 1,2-diacylglycerol, phosphatidic acid, arachidonic acid metabolites and phosphorylation of a 40000 Da protein (Lloyd *et al.*, 1973; Broekman *et al.*, 1980; Rittenhouse & Allen, 1982; Sano *et al.*, 1983; Siess *et al.*, 1983). The mechanism of this activation is believed to involve the initial adhesion of a limited number of platelets to collagen resulting in stimulation of inositol phospholipid-specific phospholipase C. This is then followed by activation of phospholipase A₂, and subsequent production of arachidonic acid metabolites (endoperoxides and thromboxanes) which serve to amplify the response through activation of non-adherent platelets, again by phospholipase C stimulation (Sano *et al.*, 1983; Siess *et al.*, 1983).

It is presently unknown, however, which of the inositol phospholipids are hydrolysed by phospholipase C on exposure to collagen. Recent evidence has suggested that phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-bisphosphate, but not phosphatidylinositol, form the

substrates for phospholipase C attack in a variety of tissues following receptor activation (for review, see Berridge, 1984). A similar conclusion has been reached for human platelets challenged with thrombin (Agranoff *et al.*, 1983; Watson *et al.*, 1984b). Interestingly though, the action of collagen, unlike thrombin, can evoke a significant release of ATP without the apparent mobilization of intracellular Ca²⁺ as assessed by using the fluorescent Ca²⁺ probe quin2 (Rink *et al.*, 1983). In view of the known ability of IP₃ to mobilize Ca²⁺ in a variety of tissues (Streb *et al.*, 1983; Joseph *et al.*, 1984; Burgess *et al.*, 1984), this raises the intriguing possibility that collagen may stimulate a different pathway of inositol phospholipid metabolism in platelets from that stimulated by thrombin. This possibility has been investigated in the present study.

Materials and methods

Platelets were isolated from human volunteers on the day of the experiment as previously reported (Siess *et al.*, 1983). They were suspended in a modified Tyrode-Hepes buffer (composition in mM: NaCl, 134; NaHCO₃, 12; KCl, 2.9; Na₂HPO₄, 0.34; MgCl₂, 1; Hepes, 5; glucose, 5; EGTA, 1; buffered to pH 7.4) and labelled with

Abbreviations used: IP₁, inositol monophosphate; IP₂, inositol bisphosphate; IP₃, inositol trisphosphate.

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either [^{32}P]orthophosphate (0.5 mCi/ml for 60 min) or [^3H]inositol (66 $\mu\text{Ci}/\text{ml}$ for 3 h). Platelets were then washed and resuspended at a final concentration of $8 \times 10^8/\text{ml}$ and left at room temperature. Aliquots (0.5 ml) were transferred to aggregometer tubes and left at 37°C for 10 min in the presence of 2 mM- Ca^{2+} . They were subsequently placed in a Chronolog aggregometer at 37°C and stirred for 1 min; when necessary, indomethacin (20 μM) was added at the commencement of stirring. Additions were made and the reaction terminated at various times by transferring the contents of the tube to 1.88 ml of chloroform/methanol/conc. HCl (50:100:1) with a Pasteur pipette. Aggregation and ATP release were monitored continuously throughout the experiment by means of light transmission and luciferase fluorescence, respectively. Platelet shape change was reflected as a decrease in light transmission and aggregation as an increase. [^3H]Inositol phosphates were analysed as described by Watson *et al.* (1984b) and protein phosphorylation as described by Lapetina (1984).

The various fractions containing [^3H]inositol phosphates eluted from the Dowex columns were characterized by comparison with standards by using h.p.l.c. as previously described (Watson *et al.*, 1984b). The h.p.l.c. apparatus consisted of a Water Associates model 6000A solvent delivery system

and a UGK injector. Separations were performed on a Whatman analytical SAX column utilizing a 30 min gradient from 0.02 M to 0.80 M-ammonium phosphate, pH 3.35, at a flow rate of 1 ml/min.

Platelets labelled with [^3H]inositol and also loaded with the fluorescent Ca^{2+} indicator, quin2, were prepared as follows. After labelling with [^3H]inositol as described above, platelets were washed and resuspended at $2 \times 10^8/\text{ml}$ in Tyrodes buffer without EGTA. Quin2, tetra-acetoxymethyl ester, was added at a concentration of 15 μM and the platelets were left at 37°C for 10 min. EGTA (1 mM) and prostacyclin (100 ng/ml) were then added and the platelets washed and resuspended in Tyrodes buffer containing EGTA (1 mM). Typically, these conditions loaded platelets with 1–1.5 mM-quin2. Analyses of [^3H]inositol phosphates were then performed as above. For the fluorescent studies, platelets were resuspended at $1.4 \times 10^8/\text{ml}$ and fluorescence was monitored as described by Rink *et al.* (1983). Following the addition of collagen, Ca^{2+} or buffer, the cuvettes were gently shaken and replaced into the fluorimeter; no stirring was carried out.

Quin2, tetra-acetoxymethyl ester, was from Amersham International. Other materials were from previously described sources (Watson *et al.*, 1984b; Siess *et al.*, 1983).

Fig. 1. Platelet aggregation, ATP release and quin2 fluorescence induced by collagen (20 $\mu\text{g}/\text{ml}$)

Shown are representative traces from five to ten separate experiments. The increased light transmission apparent at the time of collagen addition reflects dilution of the platelet density. The trace shown in the box represents quin2 fluorescence; estimated [Ca^{2+}] are shown on the right. (a) Untreated platelets. Note the lag phase before the release of ATP and the onset of shape change which is rapidly followed by aggregation. (b) Indomethacin (20 μM)-treated platelets. Note the loss of shape change. The increased light transmission reflects both platelet adhesion to collagen and aggregation. (c) Indomethacin (20 μM)-treated platelets loaded with quin2. Note the much slower release of ATP and onset of adhesion and aggregation relative to non-quin2-loaded platelets.

Results

Action of collagen on control platelets

The onset of aggregation and release reactions in human platelets exposed to collagen (20 $\mu\text{g}/\text{ml}$) were preceded by a lag phase usually lasting 10–20 s (Fig. 1). There was no apparent formation of [^3H]inositol phosphates or [^{32}P]protein phosphorylation during this period (Figs. 2 and 3, respectively). Release of ATP and commencement of shape change occurred in parallel with increased formation of [^3H]IP₂, [^3H]IP₃ and [^{32}P]phosphorylation of a 20000 Da and a 40000 Da proteins (Figs. 1, 2 and 3, respectively). In contrast, formation of [^3H]IP₁ lagged behind these responses in two out of five experiments, as exemplified in Fig. 2.

Action of collagen on indomethacin-treated platelets

Pretreatment of platelets with a maximally effective dose of indomethacin (20 μM) decreased the release of ATP by collagen (20 $\mu\text{g}/\text{ml}$) to $24 \pm 2\%$

($n = 4$) of control values, and also aggregation (for example traces, see Fig. 1). This reduced responsiveness of platelets was associated with decreased formation of all three [^3H]inositol phosphates and decreased 20000 Da and 40000 Da protein phosphorylation (Figs. 2 and 3, respectively). Again, the onset of ATP release by collagen occurred in parallel with increased formation of [^3H]IP₂, [^3H]IP₃ and [^{32}P]phosphorylation of the 20000 Da and 40000 Da proteins (Figs. 1, 2 and 3, respectively). Formation of [^3H]IP₁ under these conditions often lagged behind formation of [^3H]inositol polyphosphates (Figs. 2 and 4).

Fig. 5 shows further confirmation that collagen is able to produce formation of [^3H]IP₃ in indomethacin-treated platelets. H.p.l.c. analyses of the 1 M-ammonium formate/0.1 M-formic acid fraction eluted from the Dowex columns were found to consist of one main peak of radioactivity in collagen-stimulated platelets which co-migrated with the standard [^{32}P]IP₃ prepared from red blood cell ghosts (Downes *et al.*, 1982).

Action of collagen on indomethacin-treated platelets loaded with quin2

Loading indomethacin-treated platelets with the Ca^{2+} indicator, quin2, decreased the rate and

Fig. 2. Time course of collagen (20 $\mu\text{g}/\text{ml}$)-induced formation of [^3H]inositol phosphates in control and indomethacin (20 μM)-treated platelets

This experiment is representative of four other similar experiments that gave qualitatively similar results. Each point is the mean $\pm$ S.E.M. for triplicate determinations. The results shown have been corrected for the fraction of water-soluble phase analysed (80%) and for the fraction of eluant from the Dowex columns counted for radioactivity (see the Materials and methods section). ■, Control platelets; ●, indomethacin-treated platelets.

Fig. 3. Time course of collagen (20 $\mu\text{g}/\text{ml}$)-induced protein phosphorylation in control and indomethacin (20 μM)-treated platelets

This experiment is representative of two others that gave qualitatively similar results. Each point is the mean $\pm$ S.E.M. for triplicate determinations. Where S.E.M. values are not shown, they were less than 5% of the mean. 20kDa, 20000 Da protein; 40kDa, 40000 Da protein. ■, Control platelets; ●, indomethacin-treated platelets.

Fig. 4. Time course of collagen (20 µg/ml)-induced formation of [^3H]inositol phosphates in control and quin2-loaded platelets; both groups of platelets were treated with indomethacin (20 µM)

This experiment is representative of two others that gave qualitatively similar results. Each point is the mean $\pm$ S.E.M. for triplicate determinations. Platelets were loaded with 1–1.5 mM-quin2 as described in the Materials and methods section. ■, Controls; ●, quin2. Other conditions were as in Fig. 2.

amount of ATP released by collagen (20 µg/ml) to $64 \pm 13\%$ of that observed in indomethacin-treated controls (for example traces, see Fig. 1). In contrast, there was no apparent inhibition of formation of all three [^3H]inositol phosphates by collagen (20 µg/ml) in quin2-loaded platelets under these conditions (Fig. 4). In agreement with Rink *et al.* (1983), collagen (20 µg/ml) caused no significant increase in the concentration of Ca^{2+} in platelets treated with indomethacin (20 µM) (i.e. a cyclo-oxygenase inhibitor); in three experiments, the Ca^{2+} concentration in indomethacin-treated platelets exposed to collagen (20 µg/ml) was 146 ± 20 nM compared with 120 ± 13 nM in controls ($P > 0.05$); an example experiment is shown in Fig. 1.

Comparison of the actions of collagen, phorbol dibutyrate and 1,2-didecanoylglycerol on indomethacin-treated platelets

Fig. 6 again demonstrates that ATP release and the onset of aggregation in indomethacin-treated

Fig. 5. H.p.l.c. analyses of Dowex 1M-ammonium formate/0.1M-formic acid fractions obtained from indomethacin-treated platelets challenged with collagen (50 µg/ml) or buffer

Platelets were challenged with collagen (50 µg/ml) or buffer in the presence of indomethacin (20 µM) for 60 s and [^3H]inositol phosphates were extracted as described in the Materials and methods section. Five samples were pooled, applied to Dowex-1 anion-exchange columns and subsequently eluted as described. The eluants from the 1M-ammonium formate/0.1M-formic acid fraction were then analysed by h.p.l.c. This experiment is representative of two others. The arrows mark the elution positions of IP_2 and IP_3 standards prepared from red blood cell ghosts (Downes *et al.*, 1982) which were determined in adjacent runs. The small contamination of IP_2 was not always present; it is unclear if this reflects partial hydrolysis of IP_3 following application to the h.p.l.c. column or an inadequate elution of IP_2 from the Dowex column.

platelets exposed to collagen (20 µg/ml) are preceded by a lag phase of approx. 10–20 s (see also Fig. 1). The release of ATP by collagen peaked after approximately 90 s. In marked contrast, neither phorbol dibutyrate (200 nM) or 1,2-didecanoylglycerol (10 µM) induced any detectable release of ATP by 2 min, and both agents had slight, if any, effect on aggregation by this time (Fig. 6). Yet, these concentrations of 1,2-didecanoylglycerol and phorbol dibutyrate produce maximal phosphorylation of the 40000 Da protein in human platelets within 60 s (Watson *et al.*, 1984a; Lapetina, 1984, respectively).

Rink *et al.* (1983) have previously reported that 12-*O*-tetradecanoylphorbol 13-acetate and 1-oleoyl-2-acetyl-glycerol both produce ATP release and aggregation responses in platelets. These responses, however, were usually preceded by a lag phase of at least 2 min. In contrast, Sano *et al.* (1983) have reported that the time course of 5-hydroxy[^3H]tryptamine release in platelets by 1-oleoyl-2-acetyl-glycerol agrees well with the time course of phosphorylation of the 40000 Da protein.

Fig. 6. Action of collagen, phorbol dibutyrate and 1,2-didecanoylglycerol on platelet aggregation and ATP release. Shown are representative traces from one experiment which is representative of five to ten others. Platelets were treated with indomethacin ($20\text{ }\mu\text{M}$) 1 min prior to the addition of agonist. (a) Collagen ($20\text{ }\mu\text{g/ml}$); (b) phorbol dibutyrate (200 nM); (c) 1,2-didecanoylglycerol ($10\text{ }\mu\text{M}$).

Fig. 7. Time course of U44069 ($10\text{ }\mu\text{M}$)-induced formation of [^3H]inositol phosphates in indomethacin ($20\text{ }\mu\text{M}$)-treated platelets

This experiment is representative of two others that gave the same qualitative results. Each point is the mean $\pm$ S.E.M. for triplicate determinations.

Action of U44069 on indomethacin-treated platelets

In contrast with collagen, the stable endoperoxide analogue U44069 produces an immediate release of ATP and onset of aggregation in indomethacin-treated platelets (results not shown).

Similarly, U44069 produced significant formation of [^3H]IP₂ and [^3H]IP₃ at the first time point investigated (i.e. 5 s) (Fig. 7). As with collagen, however, formation of [^3H]IP₁ by U44069 lagged behind the formation of the [^3H]inositol polyphosphates (Fig. 7).

Discussion

The present study has shown that, for indomethacin-treated platelets, the onset of ATP release and [^{32}P]phosphorylation of a 20000 Da and a 40000 Da protein induced by collagen occur at the same time as formation of [^3H]IP₂ and [^3H]IP₃. These data are therefore consistent with the initiating mechanism of collagen-induced platelet activation being mediated through the phosphodiesteratic cleavage of both polyphosphoinositides, or simply phosphatidylinositol 4,5-bisphosphate; the rapid formation of [^3H]IP₂ would then reflect rapid hydrolysis of [^3H]IP₃. The delayed formation of [^3H]IP₁ observed in some, but not all, experiments indicates that collagen may not induce the phosphodiesteratic cleavage of phosphatidylinositol, at least, at very early times; instead, the [^3H]IP₁ formed could reflect hydrolysis of [^3H]IP₂. This pathway of metabolism of inositol phospholipids induced by collagen resembles that suggested for thrombin on platelets (Watson *et al.*, 1984b) and also a number of other stimuli on other tissues (for review, see Berridge, 1984).

Previously, it had been shown that formation of [^3H]arachidonoyldiacylglycerol or [^{32}P]phosphatidic acid by collagen in human platelets was markedly blocked by cyclo-oxygenase inhibitors

(Sano *et al.*, 1983; Siess *et al.*, 1983), suggesting that liberated thromboxanes and endoperoxides also cause platelet activation through the phosphodiesteratic cleavage of inositol phospholipids. This conclusion has since been verified by the demonstration that synthetic analogues of endoperoxides increase the levels of [^{32}P]phosphatidic acid in platelets (Pollock *et al.*, 1984; Siess *et al.*, 1983, 1985). It was not surprising, therefore, that in the present study a maximally effective dose of indomethacin markedly inhibited, but did not fully block, formation of all three [^3H]inositol phosphates by collagen, thereby implying that thromboxanes and endoperoxides stimulate the phosphodiesteratic cleavage of polyphosphoinositides. This observation was confirmed by the demonstration that the stable endoperoxide analogue, U44069, increases the levels of [^3H]IP₂ and [^3H]IP₃ in indomethacin-treated platelets.

The observation that collagen stimulates IP₃ formation in indomethacin-treated platelets is of particular significance. IP₃ has recently received attention as a possible second messenger for inducing Ca²⁺ mobilization, because it has been demonstrated to evoke Ca²⁺ release from the endoplasmic reticulum of semi-permeabilized pancreatic acinar cells (Streb *et al.*, 1983) and semi-permeabilized hepatocytes (Joseph *et al.*, 1984; Burgess *et al.*, 1984). Yet, in aspirin-treated platelets, Rink *et al.* (1983) had reported that collagen is able to release ATP without apparent mobilization of Ca²⁺. This observation has been reproduced in the present study for indomethacin-treated platelets. There are a number of explanations for this apparent paradox.

(i) Collagen stimulates insufficient amounts of IP₃ to evoke Ca²⁺ mobilization. By making the assumption that the specific radioactivity of [^3H]IP₃ in the present studies is the same as that of [^3H]phosphatidylinositol (this appears valid as from experiments on ^{32}P -labelled platelets it is known that the phosphates in positions 4 and 5 of phosphatidylinositol 4-monophosphate and phosphatidylinositol 4,5-bisphosphate rapidly reach the specific radioactivity of cellular [^{32}P]ATP, thereby implying that all three lipids are in equilibrium), it is possible to calculate the concentration of IP₃ formed. Using the estimate of the content of phosphatidylinositol in human platelets of 17 nmol/10⁹ platelets, as determined by Bell & Majerus (1980), it is estimated that collagen induces the peak formation of between 5 and 20 μM -IP₃ in indomethacin-treated platelets. This concentration of IP₃ was maximally effective in evoking Ca²⁺ mobilization in the studies of Streb *et al.* (1983), Joseph *et al.* (1984) and Burgess *et al.* (1984).

(ii) IP₃ is unable to cause Ca²⁺ mobilization in

platelets. This possibility has not yet been tested in platelets, although Lapetina *et al.* (1984) have shown that IP₃ stimulates [^{32}P]phosphorylation of both the 20000 Da and 40000 Da proteins in semi-permeabilized platelets in a manner consistent with the mobilization of Ca²⁺. Lapetina *et al.* (1984) also suggested, however, the possibility that IP₃ may directly activate protein kinases independently of Ca²⁺.

(iii) The isomer of IP₃ produced in platelets by collagen is not the 1,4,5 isomer, and is inactive in mobilizing Ca²⁺. Irvine *et al.* (1984) have recently reported the presence of inositol 1,3,4-trisphosphate in rat parotid gland challenged with muscarinic agonists, and it is therefore plausible that this or another isomer may be produced in platelets stimulated with collagen. At present, it is unknown if any other isomers of IP₃ are able to mobilize Ca²⁺. Clearly, there is a need for direct chemical characterization of the IP₃ produced by collagen.

(iv) IP₃ stimulates Ca²⁺ release in platelets, but this was not detectable by using quin2 fluorescence. A possible source of error in the use of quin2 in the present studies is related to the observation by Rink *et al.* (1983) that aggregation of platelets interferes with the fluorescence of quin2. In an analogous manner, it is possible that adhesion and aggregation to collagen may also cause interference because of the close clustering of platelets.

At present, it is not possible to distinguish between these latter three possibilities, but the inability of maximally effective doses of phorbol dibutyrate and 1,2-didecanoylglycerol to mimic the time course of collagen-induced ATP release and aggregatory responses in indomethacin-treated platelets demonstrates that stimulation by collagen involves mechanisms additional to protein kinase C activation. One of these mechanisms is likely to be related to activation of the Ca²⁺/calmodulin-dependent myosin light chain kinase which phosphorylates the 20000 Da protein whose labelling is stimulated by collagen in the present study. Naka *et al.* (1983) have previously shown that, although protein kinase C can also phosphorylate this protein, it does so at a different site to that induced by thrombin and to a much lesser extent. One possible explanation, therefore, for the difference between collagen and stimulants of protein kinase C (i.e. diacylglycerols and phorbol esters) is that collagen causes mobilization of a very small amount of Ca²⁺ which is sufficient to activate myosin light chain kinase, and thereby phosphorylate the 20000 Da protein; alternatively, however, IP₃ may directly activate this kinase as suggested by Lapetina *et al.* (1984).

However, even if there is mobilization of Ca²⁺ by collagen, it is likely to be only a relatively small amount. Rink *et al.* (1983) reported that collagen

(10 µg/ml) caused no detectable mobilization of Ca^{2+} in platelets, but that in contrast thrombin (0.5 unit/ml) always produced a very slight increase in cytosolic Ca^{2+} , normally from 60 nM to just below 100 nM. This concentration of thrombin produces an approx. 5-fold greater increase in the levels of $[\text{^3H}]\text{IP}_3$ in platelets than does 10 µg/ml collagen (S. P. Watson & E. G. Lapetina, unpublished work), and additionally, a 4-fold greater increase in ATP release (Rink *et al.*, 1983).

In conclusion, the present study has shown that collagen is able to induce the phosphodiesteratic cleavage of polyphosphoinositides in indomethacin-treated human platelets, thereby liberating IP_3 . The liberation of IP_3 , however, does not result in a detectable mobilization of Ca^{2+} in quin2-loaded platelets, although the suggestion is made that this observation may be misleading because of the limitations in the use of quin2 to detect small increases in Ca^{2+} fluorescence in platelets that have adhered and aggregated to collagen.

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