Inhibition of human platelet adenylate cyclase by collagen fibres

Effect of collagen is additive with that of adrenaline, but interactive with that of thrombin

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Collagen fibres in suspension have been shown to inhibit adenylate cyclase in human platelet preparations. Direct inhibition by collagen fibres was observed when intact platelets were used, although secondary events such as ADP secretion or prostanoid formation were important contributors to the inhibition of adenylate cyclase after treatment of platelets with collagen. The nature of the direct inhibition caused by collagen has been investigated in platelet membrane preparations, with the following results. (1) Collagen fibres inhibit platelet membrane adenylate cyclase in a dose-dependent manner. (2) Inhibition of adenylate cyclase by thrombin, adrenaline or collagen fibres could be abolished in the presence of guanosine 5'-[β -thio]diphosphate; half-maximal inhibition was obtained at about 100 μ M for the inhibitory action of thrombin, and at about 500 μ M for that of either adrenaline or collagen. (3) The action of each ligand was blocked to a similar extent by pertussis-toxin treatment of the platelet membranes. Taken together, these results indicate that the action of collagen, like that of thrombin and adrenaline, is G-protein-dependent. (4) Inhibition of adenylate cyclase by collagen fibres was additive with that caused by adrenaline, but co-operative with that caused by thrombin, suggesting that inhibitory pathways exist for collagen and adrenaline which are distinct from, but interactive with, that for thrombin. (5) Modification of the collagen fibres by pepsin treatment attenuated the effects of collagen, whereas heat-denaturation of the collagen fibres completely abolished their effects. These data suggest that the effects of collagen are specific, and depend on the detailed structure of the collagen fibres.

INTRODUCTION

Collagen is a potent activator of platelets, causing secretion from the platelet granules and aggregation of platelet suspensions, and collagens of the blood vessel walls are considered to be primary physiological stimuli for the haemostatic process (see Rink & Hallam, 1984). The collagen-platelet interaction is also considered important in the pathology of the blood vessel wall (Barnes, 1988). Collagen is known to interact with trans-membrane signalling pathways in platelets, although a precise understanding is lacking of the receptor populations on the platelet surface responsible for binding collagen and their subsequent activation of guanine-nucleotide-binding proteins (G-proteins) and effector enzymes (for a detailed review of this topic, see Siess, 1989). The platelet is controlled by the balance of membrane-bound signalling pathways. Phospholipases are considered crucial to the activatory process in platelets, with phospholipase A_2 -like activity in response to such agents as collagen and thrombin leading to the formation of thromboxane A_2 , which is itself a potent activatory stimulus. Phospholipase C activities may also be stimulated by collagen, with consequent increases in internal Ca^{2+} concentration and protein kinase C activity in platelets (see Kroll & Schafer, 1989). These pathways are considered important primary components of the platelet activation process; subsequent secretion of the platelet granule contents leads to amplification of these events, since the granules contain, for example, ADP and 5-hydroxytryptamine (5HT), both of which have activatory properties (see Zucker & Nachmias, 1985).

These signals are counterbalanced in the circulating platelet by the adenylate and guanylate cyclase pathways. These are stimulated principally by prostacyclin and nitric oxide respectively, which may be derived primarily from the vascular endothelium

(see Siess, 1989). It is considered that the activatory process in platelets is facilitated by inhibition of adenylate cyclase, although an absolute requirement for such inhibition before platelet activation can occur is not proven. The platelet products, ADP, 5HT and thromboxane A_2 , which are secreted during activation, together with thrombin, generated during the coagulation process, are each inhibitory towards adenylate cyclase (see Tremblay & Hamet, 1987). It has been proposed that collagen itself is also a direct inhibitor, although there is little detailed evidence for this. For example, Chiang *et al.* (1975) used collagen to promote aggregation and secretion of 5HT in platelets, and found a concomitant decrease in their cyclic AMP content. The explicit purpose of their experiments was to examine secretion, and the lower levels of cyclic AMP observed in the presence of collagen and collagen-derived materials may simply have reflected inhibition of adenylate cyclase secondary to this process. Platelet activation also leads to extreme changes in their morphology, and it is not certain that such physico-chemical alterations in the platelet membrane do not affect the adenylate cyclase system; the membrane environment is known to be an important determinant of adenylate cyclase activity (Houslay & Gordon, 1983).

In another study, Misselwitz *et al.* (1987) partially controlled for the effects of secretion from whole platelets by including indomethacin, a cyclo-oxygenase inhibitor, together with rather low levels of phosphocreatine and creatine kinase, an ADP-scavenger system, and, although they observed decreased cyclic AMP levels after adhesion to collagen-coated substrates, this might have resulted from inhibition of adenylate cyclase by 5HT released from endogenous sources, or from increased phosphodiesterase activity.

More compelling evidence for a direct effect of collagen was provided by the early study by Salzman & Levine (1971), who used freeze-permeabilized platelets without further separation of

Abbreviations used: GDP[S], guanosine 5'-[β -thio]diphosphate; G-proteins, guanine-nucleotide-binding proteins; 5HT, 5-hydroxytryptamine; IBMX, 3-isobutyl-1-methylxanthine; IC_{50} , concentration of GDP[S] giving half-maximal inhibition; PGE₁, prostaglandin E₁; PPP, platelet-poor plasma; PRP, platelet-rich plasma.

membranes to demonstrate inhibition of adenylate cyclase. For a direct effect of collagen to be inferred from that work it is necessary, and seems likely, that other secondary events such as thromboxane generation and granule secretion were completely uncoupled from the primary collagen binding by permeabilization of the platelets. However, that study pre-dates the present understanding of the inhibitory pathways of adenylate cyclase (see Northup, 1985), so that more detailed experiments are necessary to establish the precise effects of collagen on the inhibition of platelet adenylate cyclase.

The adenylate cyclase assay is both convenient and reproducible. In the study described below, we sought to determine whether it could be used to reflect early and direct events following the interaction between platelet membranes and collagen. Firstly, we wished to establish the nature of the proposed inhibition of adenylate cyclase using human platelet membranes and to see whether it was susceptible to alterations in collagen structure which might ultimately enable us to test domains of the collagen molecule for interaction with the platelet surface.

The signalling pathways referred to above are activated by ligands binding to specific receptors on the platelet surface. It is well established that this may lead to the activation of specific classes of trimeric G-proteins, which in turn modulate the activity of specific effector enzymes. These events lead to changes in the cytosolic environment and to changes in platelet function, often the result of altered protein kinase activity. Adenylate cyclase is the best-characterized of these signalling pathways, and positive responses of adenylate cyclase are known to be mediated by G_s , its stimulatory G-protein (Gilman, 1987). In contrast, signals inhibitory to adenylate cyclase (see Northup, 1985) in platelets are considered, for adrenaline at least, to be mediated by G_{i2} (Simonds *et al.*, 1989) one of several genetically distinct inhibitory G-proteins (see Milligan, 1988) which share the property that their action is blocked by the covalent modification introduced by pertussis toxin. Such pertussis-toxin-sensitive G-proteins have also been implicated in the activation of other signalling pathways, such as phospholipase A_2 (see Axelrod *et al.*, 1988) and phosphoinositidase C, although for the latter this remains a contentious issue, since no G-protein has been identified to date which performs this function in all cell types. The G-protein population of the platelet is highly complex; as well as G_s , it is known to contain G_{i2} , G_{i3} (Simonds *et al.*, 1989), G_z (Carlson *et al.*, 1989), together with a plethora of 20–25 kDa guanine-nucleotide-binding proteins (Bhullar & Haslam, 1987; Ohmori *et al.*, 1989) as well, perhaps, as a G_o -like entity (Kajiyama *et al.*, 1990).

Many of the signalling pathways activated in platelets by collagen are considered to be G-protein-dependent, although in very few studies has activation of G-proteins by collagen been demonstrated. Where this has been described (e.g. Lapetina *et al.*, 1986; Walker & Bourguignon, 1990), direct linkage between collagen receptors and G-proteins has not necessarily been established, since, as indicated above, secondary events in the platelet activation process may lead to stimulation of G-protein-dependent signalling pathways. To avoid this uncertainty, it was our intention in the present study to investigate a primary event in the collagen-platelet interaction, and to provide insights into the G-protein(s) activated by collagen binding to the platelet membrane.

EXPERIMENTAL

Materials

Guanine nucleotides, cyclic AMP, phosphocreatine and creatine kinase were from Boehringer Mannheim U.K. (Lewes, East

Sussex, U.K.); prostaglandin E_1 (PGE_1), 3-isobutyl-1-methyl-xanthine (IBMX), ATP (grade I), thrombin and adrenaline were from Sigma (Poole, Dorset, U.K.); cyclic [3H]AMP was from Amersham International (Aylesbury, Bucks., U.K.); [α - ^{32}P]ATP was from ICN Flow (High Wycombe, Bucks., U.K.) or Amersham.

Collagen fibre suspensions

Collagen fibres, at about 10 mg/ml, extracted from bovine tendons, were generously supplied by Ethicon, Somerville, NJ, U.S.A. These were diluted to about 2 mg/ml in 0.01 M-acetic acid, and dialysed against the same solution exhaustively, before being diluted to 1 mg/ml and stored at 4 °C as a stable suspension of collagen fibres until required. Dilutions were made freshly before use. Pepsin digestion was performed as described previously (Zijenah *et al.*, 1990). Heat denaturation was performed by immersion of a polypropylene tube containing collagen fibres at 1 mg/ml in a boiling-water bath for 2 h. After this, the denatured collagen was kept at 60 °C until needed, and then dispensed into adenylate cyclase assay tubes at 30 °C immediately before use. This procedure was adopted to minimize renaturation of collagen which might have occurred on cooling.

Platelet membrane preparation

Platelet concentrates were obtained from the Regional Blood Transfusion Service, Hills Road, Cambridge, U.K., within 24 h of collection. Typically three packs of platelets were used for a single preparation by the method used previously in this laboratory (Stein & Martin, 1983; Farndale *et al.*, 1987), and batches were stored at –80 °C until required. Membrane protein was measured by the method of Lowry *et al.* (1951).

Cyclic AMP assay

Platelets from a single pack of concentrate were pelleted by centrifugation at 1000 g for 15 min and resuspended in 145 mM-NaCl/5 mM-KCl/10 mM-glucose/1 mM-MgSO₄/0.5 mM-EGTA/10 mM-Hepes/NaOH, pH 7.4, containing BSA at 3.5 g/l, then counted and diluted to 5×10^8 platelets/ml. IBMX was added from an ethanolic stock solution (0.1 M) to 0.1 mM final concentration, and the platelet suspension was held at 37 °C for 5 min. Incubations were started by adding about 150 μ l of platelets to PGE_1 (with other additions in the same buffer) and 20 μ l of 0.01 M-acetic acid with or without collagen fibres at 1000 μ g/ml. After suitable times, the reaction was stopped by transferring the tubes to a boiling-water bath for 5 min, centrifuging and taking 50 μ l portions of the supernatants for assay by a modification (Farndale *et al.*, 1992) of the competitive binding assay of Brown *et al.* (1971).

Adenylate cyclase assay

This was conducted as we have described previously (Farndale *et al.*, 1987; Wadman *et al.*, 1991). Briefly, 35 ± 3 μ g (mean $\pm$ S.E.M. from 39 experiments) of platelet membrane protein per assay was incubated at 30 °C with 1 mM-Mg²⁺, 100 μ M-ATP, 500 μ M-cyclic AMP, 0.1 μ M- PGE_1 , 10 μ M-GTP, 2×10^5 d.p.m. of [α - ^{32}P]ATP, creatine kinase (0.6 mg/ml) and 8 mM-phosphocreatine in 50 mM-Tris/HCl, pH 8.0, in a total volume of 100 μ l. The reaction was stopped with 100 μ l of 2% (w/v) SDS containing 40 mM-ATP and 1.4 mM-cyclic AMP. The products were separated by the method of Salomon *et al.* (1974) and quantified by liquid-scintillation counting.

Adenylate cyclase activity in four membrane preparations

Table 1 shows the properties of four preparations of human platelet membranes. These preparations behaved similarly; each showed about a 10-fold increase in adenylate cyclase activity

Table 1. Adenylate cyclase activity in four membrane preparations

The adenylate cyclase activity of about 50 μg of membrane protein per assay tube from each of four platelet membrane preparations was determined during 20 min as described in the Experimental section. All assays were performed in the presence of 10 μM -GTP, 0.1 μM -PGE₁, 10 μM -adrenaline (Adr) or thrombin (1 unit/ml; Thr) were included as indicated in the Table. Each value represents the mean adenylate cyclase activity ($\pm$ S.E.M. from four determinations), and is expressed in pmol of cyclic AMP/min per mg of protein

Prep. no. ...	Adenylate cyclase (pmol/min per mg)			
	1	2	3	4
Basal	15.67 $\pm$ 0.81	18.03 $\pm$ 0.13	14.67 $\pm$ 0.43	14.96 $\pm$ 0.33
PGE ₁	125.27 $\pm$ 0.92	140.03 $\pm$ 1.86	155.33 $\pm$ 1.10	154.76 $\pm$ 4.21
PGE ₁ + Adr	53.47 $\pm$ 0.72	63.91 $\pm$ 1.12	85.89 $\pm$ 1.68	90.43 $\pm$ 1.92
PGE ₁ + Thr	62.40 $\pm$ 1.65	71.35 $\pm$ 2.52	61.98 $\pm$ 0.37	76.65 $\pm$ 2.94

when 0.1 μM -PGE₁ was included in the assay, and each was substantially inhibited by 10 μM -adrenaline or 1 unit of thrombin/ml. This was taken to indicate that the receptor populations of the preparations were actively coupled to both stimulation and inhibition of adenylate cyclase. In the work described below, at least four preparations were used individually in each experimental procedure, unless indicated otherwise in the legends. Where a single experiment is used to illustrate the results, it was representative of the performance of the four individual preparations.

Platelet aggregation

Blood (18 ml), drawn from the antecubital vein of a volunteer who had not taken aspirin recently, was anticoagulated with 2 ml of 3.8% (w/v) trisodium citrate. Platelet-rich plasma (PRP) was prepared by centrifugation at 1000 g for 1 min, and platelet-poor plasma (PPP) was prepared by centrifuging for a further 10 min. The reactions were conducted as we have described previously (Fitzsimmons *et al.*, 1986), by adding 3 μl of collagen fibre suspensions at known concentration to 150 μl of PRP in a stirred aggregometer (Bryston) cuvette. The chart recorder output was calibrated by using PPP and PRP, and aggregation was measured as an increase in light transmittance.

RESULTS

Collagen-stimulated platelet aggregation

Fig. 1 shows the capacity of the bovine tendon type I collagen fibres used throughout this study to cause aggregation in freshly prepared human PRP. A near-maximal response was obtained with collagen at 4 $\mu\text{g}/\text{ml}$. These data show that the suspension of collagen fibres used for all the experiments described here was an active aggregatory preparation.

Inhibition of cyclic AMP production in whole platelets

Fig. 2 shows the increase in cyclic AMP content of washed human platelets stimulated by the addition of 0.1 μM -PGE₁ in the presence of 0.1 mM-IBMX, an inhibitor of cyclic AMP phosphodiesterase. The control curve shows a rapid and significant rise to about 10 times the resting cyclic AMP level; the latter parameter was re-measured at the end of the time course, and was not significantly altered from the initial level. The inclusion of collagen at 100 $\mu\text{g}/\text{ml}$ with 0.1 μM -PGE₁ exerted a marked inhibitory effect on the generation of cyclic AMP in these platelets, so that a plateau level about 6 times basal was reached. The inhibition by collagen occurred rapidly, being significant at the earliest time point tested. The control cyclic AMP levels, with PGE₁ alone, are of the same order as those obtained by Karniguian *et al.* (1983), using fresh washed platelets activated

with 0.1 μM -prostacyclin. This experiment suggested that receptors for both PGE₁ and collagen were functional in the platelets used in this study after storage for 24 h.

Dependence of inhibition of platelet adenylate cyclase on secreted products

To determine whether prostanoid formation was an intermediate in the inhibition of adenylate cyclase by collagen, platelet suspensions were incubated at 37 $^{\circ}\text{C}$ with or without 10 μM -indomethacin together with IBMX as above for 5 min before mixing with PGE₁ and other additions. In each of two such experiments, the inhibition of cyclic AMP production caused by the addition of collagen was about 10% less when indomethacin was included.

To test whether the platelet granule contents also had a role in these events, experiments were conducted on three separate preparations of platelets incubated with IBMX and 10 μM -indomethacin as above. Levels of ADP (2 μM) which were maximally inhibitory (Cooper & Rodbell, 1979) and of ATP (100 μM) which were supramaximally antagonistic towards the ATP receptor (Cusack & Hourani, 1982) were included in some incubations, and the effect of collagen (100 $\mu\text{g}/\text{ml}$) was de-

Fig. 1. Platelet aggregation caused by bovine tendon collagen (type I) fibres

Collagen fibres (3 μl) were added to a stirred suspension of platelets in PRP (150 μl) in an aggregometer as described in the Experimental section, to give the indicated final collagen concentration. The resultant changes in transmittance are shown.

Fig. 2. Collagen fibres decrease [cyclic AMP] in PGE₁-stimulated washed platelets

Washed human platelets (final concn. 4×10^8 /ml, in 145 mM-NaCl/5 mM-KCl/10 mM-glucose/1 mM-MgSO₄/0.5 mM-EGTA/10 mM-Hepes/NaOH, pH 7.4, containing BSA at 3.5 g/l and 100 μ M-IBMX) were added to 0.1 μ M-PGE₁ with or without collagen (100 μ g/ml), mixed and incubated at 37 °C for the indicated times. Samples were then boiled for 5 min, centrifuged, and cyclic AMP was assayed as described in the Experimental section. Data represent means $\pm$ S.E.M. from triplicate determinations for control (■) and collagen-treated (○) platelets.

Table 2. Inhibition of adenylate cyclase in intact platelets with or without ADP and ATP

Platelets were washed and resuspended in buffer to a concentration of 1.5×10^8 /ml as described in the Experimental section, and incubated for 5 min at 37 °C with 100 μ M-IBMX and 10 μ M-indomethacin. Platelets (150 μ l) were pipetted into 50 μ l of the same buffer containing 0.1 μ M-PGE₁, 2 μ M-ADP, 100 μ M-ATP and collagen at 100 μ g/ml as indicated, incubated for a further 8 min, and the reaction was stopped by boiling. Cyclic AMP was measured as described in the Experimental section. Each value represents the mean level of cyclic AMP ($\pm$ S.E.M. from at least three determinations), and is expressed in pmol of cyclic AMP/10⁸ platelets.

	Cyclic AMP		<i>P</i> <
	Control	+ collagen	
Basal	0.46 $\pm$ 0.11	—	
PGE ₁	16.0 $\pm$ 0.39	7.5 $\pm$ 0.29	0.001
+ ADP	7.2 $\pm$ 0.27	5.9 $\pm$ 0.21	0.02
+ ATP	9.9 $\pm$ 0.16	8.8 $\pm$ 0.21	0.02
+ ADP + ATP	9.9 $\pm$ 0.25	8.4 $\pm$ 0.16	0.005

terminated. The results are shown in Table 2. It was found that collagen alone caused a 53 % decrease in the cyclic AMP levels found after incubation with 0.1 μ M-PGE₁; ADP alone caused a rather greater effect, but in the presence of ADP, collagen caused significant further decrease in adenylate cyclase activity. ATP completely abolished the action of ADP, but allowed further inhibition by collagen in both the presence and the absence of ADP. These data indicate an important role for secreted granule products as intermediates of the inhibition of platelet adenylate cyclase by collagen, but also show that significant inhibition by collagen persisted under conditions where the action of ADP was effectively antagonized. This degree of inhibition was comparable with that found in the membrane preparations (see below).

Dose-dependent inhibition of adenylate cyclase in platelet membranes

To examine the inhibition of adenylate cyclase more directly, and in more detail than is possible in intact platelets, experiments

Fig. 3. Inhibition of platelet membrane adenylate cyclase by collagen fibres

Platelet membranes (typically 25 μ g of protein/assay), prepared as described in the Experimental section, were incubated at 30 °C for 15 min with 0.1 μ M-PGE₁, 10 μ M-GTP, 1 mM-Mg²⁺, 500 μ M-cyclic AMP, 100 μ M-ATP and 2×10^5 d.p.m. of [α -³²P]ATP, creatine kinase (0.6 mg/ml), 8 mM-phosphocreatine and 50 mM-Tris/HCl, pH 8.0, together with a final concentration of 1 mM-acetic acid (control) or the indicated level of collagen fibres suspended in 1 mM-acetic acid. Adenylate cyclase activity was determined as described in the Experimental section. Each data point, expressed as pmol of cyclic AMP/min per mg of membrane protein, represents the mean difference $\pm$ S.E.M. between control and collagen-treated membranes obtained from four experiments, each with four replicates.

were performed on platelet membrane preparations. Fig. 3 shows the inhibition of PGE₁-stimulated adenylate cyclase caused in platelet membranes by increasing doses of collagen. Individual preparations of platelet membranes each showed significant inhibition above a collagen level of about 30 μ g/ml. In the experiment shown, the concentration of collagen fibres was not tested above 100 μ g/ml, but separate experiments indicated that inhibition continued to increase at least as far as 200 μ g/ml, and appeared to be proportional to the collagen concentration used. Varying the platelet membrane concentration between 10 and 100 μ g of protein per assay did not affect the inhibition of adenylate cyclase caused by collagen at 100 μ g/ml (results not shown).

Time-dependence of inhibition

Fig. 4 shows that the inhibition of adenylate cyclase by collagen at 100 μ g/ml occurred without delay, and that the level of inhibition did not change significantly during 20 min incubation, although, since adenylate cyclase activity appeared to decline somewhat towards the end of the experiment, optimum separation of the curves was obtained after 15 min. For all subsequent experiments, therefore, the adenylate cyclase assay was conducted for 15 min.

Role of prostanooids and ADP in the action of collagen

Since our experiments with whole platelets demonstrated a partial role for both prostanooids and ADP in the action of collagen on adenylate cyclase, it is important to determine whether these factors are also active in the platelet membrane experiments, although at the outset we considered it improbable that secondary events such as prostanooid formation and secretion would remain actively coupled to collagen receptors after the membrane preparation process. To test this possibility, we conducted adenylate cyclase assays both in the presence of 10 μ M-adenosine 5'-[β -thio]diphosphate to saturate ADP receptors (Cooper & Rodbell, 1979), and with 500 μ M-ATP further to antagonize ADP, which potentially might be released by the addition of collagen in the event that the granule system were

Fig. 4. Time-dependence of inhibition of adenylate cyclase

Adenylate cyclase assays were performed as described in the legend to Fig. 3, except that the assays were terminated after the indicated times, and data (means $\pm$ S.E.M. for three determinations) are expressed as pmol of cyclic AMP/mg of membrane protein. Results from control ($\blacktriangle$) and collagen-treated ($\circ$) membranes are shown from one of two similar experiments.

intact. These experiments revealed an identical degree of inhibition of adenylate cyclase activity by collagen under all conditions tested (results not shown). Similarly, inhibition of adenylate cyclase in membrane preparations was not perturbed by the inclusion of $10\ \mu\text{M}$ -indomethacin or $1\ \text{mM}$ -EGTA in the assays, which would block the stimulation of prostanoid formation and phospholipase activity respectively. We conclude that the effects of collagen under these circumstances were direct.

Inhibition by adrenaline with and without collagen

Fig. 5 shows the inhibition of platelet adenylate cyclase observed in the presence of increasing doses of adrenaline, both with and without collagen at $100\ \mu\text{g}/\text{ml}$. This experiment showed the expected sigmoid curve for adrenaline alone, with near-maximal inhibition at $10\ \mu\text{M}$ -adrenaline and no further inhibition occurring as the concentration was increased to $1\ \text{mM}$. The addition of collagen caused further inhibition at all doses of adrenaline, so that the adrenaline inhibition curve was displaced downwards by the inclusion of collagen. Hence, with even maximal levels of adrenaline, further inhibition could be obtained by adding collagen.

Inhibition by thrombin with and without collagen

Fig. 6 shows the inhibitory response of platelet membranes to increasing doses of thrombin with and without collagen at $100\ \mu\text{g}/\text{ml}$. The control curve has a complex shape of closely similar form to that described by Aktories & Jakobs (1984). The curve obtained with thrombin concentrations below about $1\ \text{unit}/\text{ml}$ was sigmoid, similar to that obtained with adrenaline, but higher doses caused an additional, linear, decline in adenylate cyclase activity. This phase of the thrombin effect was found by Aktories & Jakobs (1984) to be independent of GTP, and was hence considered non-specific. In the presence of collagen, low doses of thrombin (which alone were without effect) caused a significant additional inhibition of adenylate cyclase activity over that caused by collagen alone; for example, thrombin at $0.03\ \text{unit}/\text{ml}$ alone was not significantly inhibitory, but the inclusion of thrombin at $0.03\ \text{unit}/\text{ml}$ with collagen at $100\ \mu\text{g}/\text{ml}$ caused a significant further decrease in adenylate cyclase activity. This

Fig. 5. Inhibition of platelet adenylate cyclase by adrenaline with or without collagen fibres

Adenylate cyclase assays were performed on $31\ \mu\text{g}$ of platelet membrane protein per assay as described in the legend to Fig. 3. [Adrenaline] was varied as indicated, and assays were performed with ($\circ$) or without ($\square$) collagen fibres (final concn. $100\ \mu\text{g}/\text{ml}$). Data are means $\pm$ S.E.M. from four replicates from one experiment, and similar results were obtained with three other membrane preparations.

Fig. 6. Inhibition of platelet adenylate cyclase by thrombin with or without collagen fibres

Adenylate cyclase assays were performed on $29\ \mu\text{g}$ of platelet membrane protein per assay as described in the legend to Fig. 3. [Thrombin] was varied as indicated, and assays were performed with ($\circ$) or without ($\blacktriangle$) collagen fibres (final concn. $100\ \mu\text{g}/\text{ml}$). Data are means $\pm$ S.E.M. from four replicates from one experiment, and similar results were obtained with three other membrane preparations.

suggested that some synergism between collagen and thrombin existed in the inhibition of platelet adenylate cyclase, and is a qualitatively different result from that obtained with adrenaline.

At thrombin doses close to the point of inflection of the control curve, i.e. about $1\ \text{unit}/\text{ml}$, corresponding to the limit of

Fig. 7. Inhibition of platelet adenylate cyclase by collagen fibres in the presence of either adrenaline or thrombin

Platelet membranes (typically 25 μ g of protein per assay) were incubated with collagen fibres (100 μ g/ml) in the absence or presence of the indicated level of either thrombin or adrenaline. Adenylate cyclase activity was determined as described in the legend to Fig. 3. Each data point represents the mean inhibition of adenylate cyclase activity in collagen-treated membranes (as a percentage of adenylate cyclase activity in the absence of collagen) obtained from four experiments (each with four replicates) performed with adrenaline ($\square$) or thrombin ($\blacktriangle$). The lines drawn are least-squares best fit.

Fig. 8. Effect of thrombin on the concentration-dependence of collagen-mediated inhibition of adenylate cyclase

Platelet membranes (typically 25 μ g of protein per assay) were incubated with the indicated level of collagen fibres ($\square$, control) or collagen fibres + thrombin at 0.01 unit/ml ($\circ$). Adenylate cyclase activity was determined as described in the legend to Fig. 3. Each data point, the inhibition of adenylate cyclase activity calculated as a percentage of activity obtained in the absence of collagen, represents the mean inhibition $\pm$ S.E.M. obtained from the mean values from four experiments, each with four replicates.

GTP-dependent inhibition, collagen caused further inhibition of adenylate cyclase, but at higher doses of thrombin no significant inhibition was obtained by the addition of collagen.

Relative inhibition by adrenaline and thrombin + collagen

Fig. 7 replots the mean data from four experiments, each similar to those depicted in Figs. 5 and 6, to emphasize the difference in behaviour of adrenaline and thrombin in the presence of collagen; the graph shows the percentage inhibition of platelet membrane adenylate cyclase caused by collagen fibres in the presence of increasing concentrations of either adrenaline or thrombin. The difference in the properties of the two ligands is clearly discernible: at low adrenaline concentration the effect of collagen was to cause about a 20% decrease in adenylate cyclase activity, and with increasing adrenaline concentration this proportion did not change significantly (assessed by linear regression, $r = 0.8$, $P < 0.1$), although it appeared to increase somewhat. In marked contrast, the effect of collagen in the

Table 3. Effect of pertussis toxin on inhibition of platelet adenylate cyclase

Platelet membranes were incubated for 30 min at 30 $^{\circ}$ C with 1 mM-NAD $^{+}$ under control conditions or with dithiothreitol-activated pertussis toxin (3 μ g/ml) as indicated, together with 1 mM-ATP, 1 mM-Mg $^{2+}$, 100 μ M-GTP, 10 mM-nicotinamide and 50 mM-Tris/HCl, pH 7.4. Membranes were centrifuged (10000 g for 10 min) and resuspended in 25 mM-Tris/HCl, pH 8.0. The adenylate cyclase activity of 36 μ g of membrane protein per assay tube was then determined during 15 min as described in the Experimental section. All assays were performed in the presence of 10 μ M-GTP and 0.1 μ M-PGE $_1$. Collagen fibres (100 μ g/ml), 10 μ M-adrenaline or thrombin (0.3 unit/ml) were included as indicated in the Table. Each value represents the mean adenylate cyclase activity ($\pm$ S.E.M. from four determinations), and is expressed in pmol of cyclic AMP/min per mg of protein. Δ represents the relative blockade by pertussis toxin of the inhibition caused by each of the ligands tested.

	Adenylate cyclase		Δ (%)
	Control	Pertussis toxin	
Basal	102.7 $\pm$ 0.9	106.7 $\pm$ 2.5	—
Collagen	71.9 $\pm$ 3.2	89.0 $\pm$ 1.3	43
Adrenaline	39.3 $\pm$ 0.3	67.5 $\pm$ 0.9	40
Thrombin	50.8 $\pm$ 1.8	78.1 $\pm$ 4.3	47

presence of the two lowest doses of thrombin tested, 0.003 and 0.01 unit/ml, was to cause greater inhibition of adenylate cyclase (about 30%). As thrombin dose increased, this proportion decreased rapidly, so that at 30 units/ml the additional effect of collagen was negligible. These data were negatively correlated ($r = 0.93$, $P < 0.01$), and again suggest co-operative and saturable inhibition of adenylate cyclase by thrombin and collagen, whereas no such interaction was observed with adrenaline.

Effect of low [thrombin] on collagen dose-response curve

To test this interactive inhibition further, the converse experiment was conducted on each of four platelet membrane preparations. Collagen fibre concentration was varied in the presence and absence of a low dose (0.01 unit/ml) of thrombin, which was inactive on its own (see Fig. 6). Fig. 8 gives the pooled results of the four experiments, and shows the expected linear inhibition of adenylate cyclase by collagen alone. In the presence of thrombin, the curve shows a more rapid onset of inhibition with increasing collagen dose, but above 30 μ g/ml the two curves appeared convergent, since the data were not significantly different. The apparent effect of thrombin was to increase the sensitivity of platelet adenylate cyclase to collagen: collagen at 10 μ g/ml in the presence of thrombin caused the same inhibition of adenylate cyclase as collagen at 30 μ g/ml without thrombin.

Effect of pertussis toxin on inhibition of adenylate cyclase

To examine the role of G-proteins involved in these events, platelet membranes were incubated with 1 mM-NAD $^{+}$ and activated pertussis toxin (3 μ g/ml) for 30 min, and then their susceptibility to inhibitors of adenylate cyclase was tested. The data from one of four such experiments is shown in Table 3, showing that blockade by pertussis-toxin treatment of the inhibitory effects of collagen, adrenaline or thrombin was not complete. This presumably reflected incomplete ADP-ribosylation of the G $_i$ -like complement of the platelet membranes under the conditions tested. However, the relative activity of each of the inhibitors was attenuated to a rather similar extent, i.e. about 45%. This experiment indicated that the action of collagen is likely to be G-protein-mediated and, as might be expected,

Table 4. IC₅₀ for blockade of inhibitory effects by GDP[S]

Adenylate cyclase activity was determined for each of the four membrane preparations in the presence of 10 μ M-GTP and 0.1 μ M-PGE₁ as described in the Experimental section. Assays were carried out in the absence or presence of collagen fibre suspensions (100 μ g/ml) or 10 M-adrenaline or thrombin (0.3 unit/ml) as indicated. IC₅₀ was defined as that concentration of GDP[S] at which the inhibitory effects of the ligands tested were half-maximal, compared with activity in the absence of GDP[S]. A similar procedure yielded IC₅₀ for inhibition of PGE₁-activated adenylate cyclase by GDP[S]. The values given are the overall means $\pm$ S.E.M. calculated from quadruplicate determinations for each of the four membrane preparations. For adrenaline and collagen, each membrane preparation was tested twice, and for PGE₁-activated adenylate cyclase, four times. Statistical analysis using one-way ANOVA showed that differences between all pairs of means were significant ($P < 0.05$), except between the values obtained for collagen and adrenaline.

Ligand	IC ₅₀ (μ M)
Thrombin	107 $\pm$ 13
PGE ₁	187 $\pm$ 29
Collagen	496 $\pm$ 27
Adrenaline	515 $\pm$ 19

suggested a role for one of the pertussis-toxin-sensitive G_i-like G-proteins in these events.

Effect of guanosine 5'-[β -thio]diphosphate (GDP[S]) on inhibition of adenylate cyclase

To examine further the G-proteins activated by these ligands, platelet membranes were incubated with 0.1 μ M-PGE₁ and collagen, adrenaline or thrombin with increasing concentrations of the stable GDP analogue GDP[S], which inhibits G-protein activation (Eckstein *et al.*, 1979). Table 4 shows the concentration (IC₅₀) of GDP[S] which causes 50% blockade of the effects of each of the inhibitory ligands, obtained from each of four membrane preparations. These experiments showed that, as expected, the inclusion of GDP[S] substantially decreased the activation of adenylate cyclase by PGE₁. They also suggested that the inhibition of adenylate cyclase caused by collagen was equally susceptible to blockade by GDP[S] as that caused by adrenaline, although the precise value of IC₅₀ obtained for these ligands must be treated with some caution, since the activity of adenylate cyclase was rather low at high GDP[S] concentration. In marked contrast, the action of thrombin was much more sensitive to GDP[S].

Structural requirements for the effects of collagen

In a preliminary investigation of the structural determinants for the inhibition of platelet adenylate cyclase by collagen, membranes were treated in two sets of experiments with collagen fibres in suspension (100 μ g/ml) alongside either collagen fibres derived from the same preparation which had been treated with pepsin (to remove the non-helical N- and C-terminal telopeptides from the collagen fibres) or collagen fibres which had been denatured by heating to 100 °C for 2 h. Both treatments impaired the ability of collagen to inhibit adenylate cyclase; pepsin treatment halved the effect of collagen at 100 μ g/ml, but lower doses of the pepsin-treated material, tested down to 10 μ g/ml, were without significant effect. In contrast, heat-denatured collagen was without significant inhibitory activity (results not shown).

DISCUSSION

The data presented above demonstrate direct inhibition of adenylate cyclase, measured in intact platelets as well as in platelet membranes, by a suspension of collagen fibres. The degree of inhibition obtained in platelet membranes (26 $\pm$ 2%; $n = 65$) with collagen at 100 μ g/ml was less than that caused by optimal doses of other inhibitors, 10 μ M-adrenaline (42 $\pm$ 3%; $n = 19$) or thrombin at 0.03 unit/ml (41 $\pm$ 4%; $n = 18$), although the dose of collagen generally applied throughout this study was not maximal. The inhibitory effect of collagen was additive with that of adrenaline, but co-operative with that of thrombin; the activity of either collagen or a low level of thrombin was enhanced by the presence of the other, but, when platelet membranes were treated with a high dose of thrombin, collagen caused no further inhibition of adenylate cyclase. The difference between adrenaline and thrombin in this respect was striking.

The effects of adrenaline and collagen were both inhibited by GDP[S], with IC₅₀ about 500 μ M, whereas the action of thrombin was completely abolished by this level of GDP[S], showing IC₅₀ about 100 μ M. These data therefore indicate a degree of co-incidence between the actions of adrenaline and collagen, with the pathway activated by thrombin being kinetically distinct. Preincubation of platelet membranes with pertussis toxin and NAD⁺ caused partial blockade of the inhibition of adenylate cyclase caused by each ligand. Both this and our observations of the effects of GDP[S] indicate that the inhibition of adenylate cyclase by collagen fibres, as well as by the other ligands, is likely to be mediated by G-proteins.

We do not understand the mechanisms underlying these events. The simplest interpretation might be that thrombin activates a different pertussis-toxin-sensitive G-protein in platelet membranes from that activated by either adrenaline or collagen. Synergism between thrombin and collagen might then occur as these two activated G-proteins converge upon adenylate cyclase. Evidence for more complex schemes for the action of thrombin on platelets involving the activation of multiple G-proteins has been produced by others (e.g. Houslay *et al.*, 1986). Lapetina (1990) proposed a scheme for the activation by thrombin of phosphoinositide C in platelets in which the activation of G_{i2} also involved a 20 kDa guanine-nucleotide-binding protein, Rap1b. Our data might be consistent with these previous proposals, and indicate that thrombin activates a G-protein having a lower affinity for GTP than either G_s or G_{i2}.

Although a substantial literature describes the activation of platelet signalling pathways by collagen, little is known about the identity of G-protein activated directly by the primary event of collagen binding to its receptor. This in part results from the lack of cell-free systems in which signalling pathways other than adenylate cyclase may be examined. The present work provides direct evidence for a pertussis-toxin-sensitive G-protein in the signal-transduction pathway activated by collagen. The identity of the G-protein involved must await further study, although it appears to be kinetically indistinguishable from the G_{i2} thought to mediate the effect of adrenaline in platelet adenylate cyclase (Simonds *et al.*, 1989).

Many authors have used high levels of collagen to demonstrate effects on other platelet signalling pathways; it is not unusual for concentrations in excess of 10 μ g/ml to be applied to these systems (e.g. Salzman & Levine, 1971; Chiang *et al.*, 1975; Lapetina *et al.*, 1986; Pollock *et al.*, 1986; Walker & Bourguignon, 1990). It should be noted that the concept of concentration cannot easily be applied to collagen, since in its physiological state and as applied here it exists as an extensively aggregated fibre. The majority of the mass of the collagen is likely to lie within the fibre, but it is only the surface of the fibre which can

interact with the platelet. It is difficult to compare doses of collagen between different experimental studies without a detailed knowledge of the state of aggregation of the collagens used.

However, it is clear within this study that the inhibitory response of platelet adenylate cyclase extends to at least a collagen level (100 $\mu\text{g/ml}$) well beyond that necessary to cause maximal platelet aggregation in PRP ($\sim 4 \mu\text{g/ml}$), although the minimum collagen level causing detectable inhibition of adenylate cyclase in the presence of thrombin may be of the same order as the latter. Several reasons might be advanced to explain this apparent discrepancy. Firstly, platelet aggregation is a highly co-operative event in which secretion of the platelet granule constituents, such as ADP, together with thromboxane production, plays an important amplifying role, which we have confirmed in the present study. The effects of these products have deliberately been avoided by using membrane preparations, so that the effects of exogenous materials may be observed directly. Secondly, it is possible that the platelet-collagen interaction involves other constituents of either plasma or platelet granules (such as fibrinogen, fibronectin, thrombospondin and von Willebrand factor), which have been substantially washed from the platelets during the preparation procedure. It is our experience (M. J. Barnes, unpublished work) that a fully aggregatory response of washed platelets can only be obtained with much higher doses of collagen than is needed for aggregation of platelets in PRP. This concept is supported by the work of Zijenah *et al.* (1990), who demonstrated a role for plasma constituents in promoting Mg^{2+} -independent platelet adhesion to bovine tendon collagen type I fibres.

Our preliminary data suggest that the triple-helical or fibrous state of the native collagen is essential for the inhibition of adenylate cyclase by collagen, since heat-denatured collagen fibres were inactive. The minor modification of collagen structure (removal of the non-helical N- and C-terminal telopeptides) caused by pepsin treatment may also be important in determining the extent or consequences of collagen binding to the platelet membrane. These findings are consistent with the effects of collagen on platelet aggregation, where previous studies have shown that heat-denatured collagen was inactive (see Santoro & Cunningham, 1980) and pepsin treatment of collagen fibres impaired their aggregatory activity (M. J. Barnes, unpublished work). This interpretation of our data should be treated with some caution, since it is plausible that other constituents of the collagen preparation may be important and are also removed or denatured by the treatments applied here. However, it can be concluded from the present study that the inhibitory action of collagen on platelet adenylate cyclase provides a system in which the structural requirements of collagen for interaction with the platelet surface might readily be investigated.

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