Evidence that adhesion of electrically permeabilized platelets to collagen is mediated by guanine nucleotide regulatory proteins

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Adhesion of electrically permeabilized platelets to collagen was found to be essentially independent of free Ca^{2+} concentration in the medium. Addition of stable GTP analogues increased the proportion of adhering cells about 5-fold. This effect was inhibited by guanosine 5'-[β -thio]diphosphate, cytochalasin D or monoclonal antibodies to glycoprotein Ia. In contrast, the protein kinase C inhibitor staurosporine had only a small effect on the GTP-analogue-enhanced adhesion of the permeabilized cells to collagen. These results suggest that a guanine nucleotide regulatory (G)-protein is directly linked to the collagen receptor and is involved in the actin-dependent recruitment of additional collagen receptors.

INTRODUCTION

Adhesion of platelets to collagen is the primary event in the formation of a haemostatic plug. In the past few years, significant progress has been made in elucidating the mechanism of platelet adhesion to collagen (Santoro, 1988). Key to this progress was the development of assay systems in which adhesion of platelets to collagen could easily be studied. This development, along with the discovery of patients with platelets lacking the ability to adhere to collagen (Niewenhuis *et al.*, 1986; Kehrel *et al.*, 1988), led to the demonstration that glycoprotein Ia/IIa, an integrin also known as $\alpha 2\beta 1$, is a collagen receptor (Staatz *et al.*, 1989).

Adhesion of platelets to collagen appears to involve more than a simple interaction between glycoprotein Ia/IIa and collagen. Energy depletion or inhibition of actin polymerization with cytochalasins greatly slows the rate at which platelets adhere to collagen (Shadle & Barondes, 1982; Smith *et al.*, 1991). One possible explanation for these observations is that receptors need to be rearranged in the lateral plane of the platelet membrane in order to achieve full and rapid adhesion. This proposed rearrangement of receptors may be mediated by contractile proteins. Initial attachment of collagen to a few receptors would cause transduction of a signal into the platelets that would initiate the rearrangement process.

A common mechanism by which cells transduce extracellular signals into intracellular events is via the action of guanine nucleotide regulatory proteins (G-proteins). However, studies of G-proteins in intact cells are hampered by the inability to gain access to the cytoplasm of the cell. A model system which has been fruitful in demonstrating the involvement of G-proteins in platelets is the electrically permeabilized platelet (Knight & Scrutton, 1986). In this system, platelets are subjected to several pulses of a high-voltage electrical discharge, which results in the formation of small holes in the membrane of the cell. These holes allow the passage of small molecules, but do not allow the passage of most proteins. In this paper, we demonstrate that electrically permeabilized platelets retain the ability to adhere to collagen. Moreover, this adhesion is blocked by a monoclonal antibody to glycoprotein Ia as well as by cytochalasin D, indicating this adhesion occurs by the same receptors as in intact cells. We have used this system to demonstrate that activators of

G-proteins, i.e. guanosine 5'-[γ -thio]triphosphate (GTP[S]) and guanosine 5'-[$\beta\gamma$ -imido]triphosphate (p[NH]ppG), enhance the adhesion of platelets to collagen and that guanosine 5'-[β -thio]diphosphate (GDP[S]) inhibits GTP[S]-enhanced platelet adhesion to collagen. The GTP[S]-enhanced adhesion also is inhibited by both cytochalasin D and the monoclonal antibody to glycoprotein Ia. Evidence is presented that these effects are largely independent of Ca^{2+} or protein kinase C-dependent pathways, and we therefore suggest that there may be direct coupling between the receptor and the cytoskeleton via G-proteins.

METHODS

Materials

Radiolabelled oleic acid and adenine were from Amersham, Arlington Heights, IL, U.S.A., and collagen was from Horm-Chemie, Munich, Germany. Arg-Gly-Asp-Ser (RGDS) was from Bachem Bioscience, Philadelphia, PA, U.S.A. The thromboxane antagonist SQ 29,548 was a gift from Dr. D. Harris of Bristol, Myers, Squibb (Princeton, NJ, U.S.A.). The other chemicals were from Sigma Chemical Co., St. Louis, MO, U.S.A. Cytochalasin D was dissolved in dimethyl sulphoxide as a stock solution at 5 mg/ml and routinely added to platelet suspensions 10 min before exposure to collagen. The monoclonal antibody against GPIa was purchased from AMAC Inc., Westbrook, ME, U.S.A.

Platelet preparation

Human blood was obtained from healthy volunteers who denied taking any drug during the previous week. The blood was anticoagulated with citric acid/citrate/dextrose (Aster & Jandel, 1964) and centrifuged at 180 *g* for 15 min at room temperature to obtain platelet-rich plasma. The platelet-rich plasma was incubated with 1 mM-aspirin for 30 min at 37 °C to inhibit cyclooxygenase and centrifuged at 800 *g* for 20 min at room temperature to obtain a platelet pellet.

The pellet was resuspended in 3 ml of wash buffer (5.5 mM-dextrose, 128 mM-NaCl, 4.26 mM- Na_2HPO_4 , 7.46 mM- NaH_2PO_4 , 4.77 mM-trisodium citrate, 2.35 mM-citric acid, pH 6.5) containing 0.35% BSA and incubated for 1 h with 5 μCi of [^3H]-

Abbreviations used: GTP[S], guanosine 5'-[γ -thio]triphosphate; p[NH]ppG, guanosine 5'-[$\beta\gamma$ -imido]triphosphate; GDP[S], guanosine 5'-[β -thio]diphosphate; BAPTA, bis-(*o*-aminophenoxy)ethane-*NNN'*-tetra-acetic acid.

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oleic acid/ml at 37 °C. This suspension was centrifuged at 800 *g* for 20 min at room temperature.

Preparation of electrically permeabilized platelets

The platelet pellet was resuspended in 3 ml of permeabilization buffer (140 mM-NaCl, 14 mM-MgSO₄, 5 mM-glucose, 1 mM-EGTA, 0.37% BSA, 25 mM-Pipes, 5 mM-Hepes, 10 μM-leupeptin and 28.5 mM-NaHCO₃, pH 6.4). The cell suspension was subjected to 12 electrical discharges of a 4 μF capacitor at a field strength of about 20000 V/cm in a home-made apparatus similar to that described by Knight & Scrutton (1980). The cells were separated from the suspension buffer by centrifugation (800 *g*, 20 min, 4 °C) and resuspended in a buffer containing either 140 mM-potassium glutamate, 10 mM-bis-(*o*-aminophenoxy)-ethane-*NNN'*-*N'*-tetra-acetic acid (BAPTA), 7 mM-MgCl₂, 10 μM-leupeptin, 5 mM-ATP, 20 mM-creatine phosphate, 50 units of creatine kinase/ml (to convert any secreted ADP into ATP), 5 μM SQ 29,548 (a thromboxane A₂ antagonist), 120 μM-RGDS (a fibrinogen-receptor antagonist) and 10 mM-Hepes (pH 7.2), or 140 mM-potassium glutamate, 17 mM-MgCl₂, 10 mM-EDTA, 10 μM-leupeptin, 5 mM-ATP, 50 units of creatine kinase/ml, 20 mM-creatine phosphate, 5 μM SQ 29,548, 120 μM-RGDS and 10 mM-Hepes (pH 7.2). Cells were equilibrated for 8 min on ice with the appropriate CaCl₂ solution before use. Free Ca²⁺ concentrations were calculated by using a computer program (Perrin & Sayce, 1967).

Collagen-adhesion assays

The microtitre-plate assay was based on a modification of the method of Tandon *et al.* (1989) and was performed in triplicate. Microtitre plates were coated with 2 μg of collagen, unless noted, in a volume of 50 μl for 2 h at room temperature. After aspiration of the collagen, the wells were treated with 250 μl of Ca²⁺-free Tyrode's solution containing 0.5% BSA for 1 h at room temperature. After aspiration of this solution, 50 μl of the suspension of [³H]oleic acid-labelled platelets was added and incubation was continued for 1 h at room temperature without agitation. Subsequently, the platelets remaining in suspension were removed by aspiration and the wells were washed with 3 × 250 μl of Ca²⁺-free Tyrode's buffer. The platelets adhering to the wells were solubilized by two 30 min treatments at room temperature with 100 μl of 2% SDS, and radioactivity was determined by liquid-scintillation counting. Blanks, in which the adhesion of platelets to wells not coated with collagen was measured, were typically less than 0.1% of the radioactivity. In experiments in which secretion was measured, platelets were prelabelled with 5-hydroxy-[¹⁴C]tryptamine and, after the incubation period, a sample of the platelet suspension (50 μl) was removed from the microtitre plate and added to 4 vol. of 0.25% formaldehyde/10 mM-EDTA. After centrifugation at 16000 *g* for 2 min at 20 °C, the amount of secretion was determined as the percentage of the total 5-hydroxy[¹⁴C]tryptamine present in the gel-filtered platelets.

RESULTS

The use of electrically permeabilized platelets in adhesion studies requires that these cells adhere to collagen in a manner similar to that of intact cells. Fig. 1 shows that adhesion of permeabilized platelets to collagen was relatively independent of the free Ca²⁺ concentration in the medium. A buffer containing the Ca²⁺ chelator BAPTA was used in these experiments. BAPTA has unique advantages over other Ca²⁺-buffer systems in that, when the added Ca²⁺ is varied in an effort to manipulate the free Ca²⁺ concentration, the BAPTA system does not give large changes in either pH or free Mg²⁺ concentration (Daniel

et al., 1987). We also measured the Ca²⁺-dependence of 5-hydroxytryptamine secretion from platelets in the microtitre wells and found that, as with other permeabilized-platelet assay systems (Knight & Scrutton, 1986), this secretion was strongly dependent on the free Ca²⁺ concentration (results not shown). Fig. 1 also shows that adhesion of the permeabilized platelets was inhibited by a monoclonal antibody to glycoprotein Ia, one of the subunits of the putative collagen-adhesion receptor, glycoprotein Ia/IIa, as well as by cytochalasin D, an inhibitor of adhesion of intact platelets to collagen. Although the adhesion data are qualitatively similar to data obtained with intact

Fig. 1. Adhesion of electrically permeabilized platelets to collagen

Platelets were suspended in the buffer containing BAPTA as chelator. Microtitre wells were coated with 5 μg of collagen/well. Platelets were preincubated with 0.9% NaCl (●), 10 μg of anti-GPIa monoclonal antibody/ml (○), or 20 μM-cytochalasin D (□) for 10 min at 20 °C before addition to microtitre wells for measurement of adhesion to collagen as described in the Methods section; 100% relative adhesion represented 0.7% of added platelets. The results are means ± s.d. of triplicate determinations and are representative of three experiments.

Fig. 2. Effect of stable GTP analogues on platelet adhesion to collagen

(a) Response to GTP[S]; (b) response to p[NH]ppG. The abscissa indicates the amount of collagen applied to each well. All samples were preincubated for 10 min at 20 °C before addition to the microtitre wells. Concentration of the analogues were 0 μM (○), 10 μM (□), 50 μM (●) or 100 μM (■). All measurements were performed at a fixed Ca²⁺ concentration, calculated to be 6 μM, with EDTA as a Ca²⁺ chelator; 100% relative adhesion represented 6.3% of added platelets. The results are means ± s.d. of triplicate determinations and are representative of three experiments.

Fig. 3. Inhibition by GDP[S] of GTP[S]-enhanced adhesion of electrically permeabilized platelets to collagen

Platelets were incubated at 20 °C for 5 min with either (●) 10 μM- or (□) 100 μM-GTP[S], followed by an additional 10 min with the indicated concentrations of GDP[S] before addition to the microtitre wells. All measurements were performed at a fixed Ca^{2+} concentration, calculated to be 6 μM; 100 % relative adhesion represented 6.7 % of added platelets. The results are means $\pm$ S.D. of triplicate determinations and are representative of three experiments.

Fig. 5. Inhibition of adhesion of electrically permeabilized platelets to collagen by cytochalasin D

Platelets were incubated at 20 °C for 10 min with the indicated concentrations of cytochalasin D, followed by an additional 5 min with either (□) 0.9 % NaCl or (●) 10 μM-GTP[S] before addition to the microtitre wells. All measurements were performed at a fixed Ca^{2+} concentration, calculated to be 0.67 μM; 100 % relative adhesion represented 5.6 % of added platelets. The results are means $\pm$ S.D. of triplicate determinations and are representative of three experiments.

Fig. 4. Inhibition of adhesion of electrically permeabilized platelets to collagen by an anti-glycoprotein-Ia monoclonal antibody

Platelets were incubated at 20 °C for 10 min with indicated concentrations of the anti-glycoprotein-Ia antibody (Anti-GPIa mAb), followed by an additional 5 min with (●) 0.9 % NaCl, (□) 10 μM- or (○) 100 μM-GTP[S] before addition to the microtitre wells. All measurements were performed at a fixed Ca^{2+} concentration calculated to be 0.67 μM; 100 % relative adhesion represented 5.0 % of added platelets. The results are means $\pm$ S.D. of triplicate determinations and are representative of three experiments.

platelets, a major difference was that only 0.5–1 % of the permeabilized cells adhered, whereas normally approx. 10 % of intact cells adhere in this system.

The magnitude of the difference in adhesion between intact and electrically permeabilized platelets suggested that other factors play a role in amplifying the adhesion response. G-proteins mediate many intracellular signalling pathways. In order to determine if G-proteins regulate the adhesion of platelets to collagen, we measured the effects of stable GTP analogues GTP[S] and p[NH]ppG on the adhesion of electrically permeabilized platelets to collagen. Fig. 2 shows that adhesion of

Fig. 6. Effect of staurosporine on responses of electrically permeabilized platelets

Platelets were incubated at 20 °C for 10 min with the indicated concentrations of staurosporine, followed by an additional 5 min with 100 μM-GTP[S] before addition to the microtitre wells. Both adhesion (□) and secretion of 5-hydroxy[^{14}C]tryptamine (●) were measured in the same samples. All measurements were performed at a fixed Ca^{2+} concentration, calculated to be 0.67 μM; 100 % relative adhesion represented 4.0 % of added platelets, and 100 % relative secretion represented 76 % of total 5-hydroxy[^{14}C]tryptamine. The results are means $\pm$ S.D. of triplicate determinations and are representative of three experiments.

permeabilized platelets is enhanced by both GTP analogues, which produced about a 5–6-fold increase in the number of cells that adhered to the microtitre wells. GTP[S] was slightly more potent than p[NH]ppG. Fig. 3 demonstrates that the enhancement of platelet adhesion to collagen by GTP[S] was inhibited by the stable GDP analogue, GDP[S]. Fig. 4 shows that the monoclonal antibody to glycoprotein Ia inhibits the GTP[S]-stimulated adhesion to collagen as effectively as control cells. Similarly, we found that cytochalasin D inhibited adhesion of GTP[S]-stimulated cells as effectively as control cells (Fig. 5).

In the previous experiments that have used electrically permeabilized platelets, most investigators have attributed the observed effects of guanine nucleotides and agonists to an activation of

phospholipase C followed by a diacylglycerol-dependent activation of protein kinase C (Haslam *et al.*, 1990). To test whether activation of protein kinase C mediated the enhancement of adhesion of the permeabilized platelets to collagen, we tested the effect of the protein kinase C inhibitor staurosporine on GTP[S]-stimulated adhesion (Fig. 6). We found that, whereas staurosporine was able to inhibit completely GTP[S]-stimulated secretion of 5-hydroxytryptamine, it inhibited GTP[S]-stimulated adhesion of the permeabilized cells by less than 30%.

DISCUSSION

Adhesion of platelets to collagen is a complex phenomenon. The initial step is certain to be interaction of a specific peptide sequence on collagen with a specific receptor present on the platelet surface. Staatz *et al.* (1991) have demonstrated that the sequence Asp-Gly-Glu-Ala may be the sequence of collagen recognized by a platelet receptor. We have proposed (Smith *et al.*, 1991) that, after initial attachment of a single platelet receptor to collagen, a signal is transduced into the cell that causes the rearrangement of additional receptors so that they are brought into alignment with additional attachment sequences on the collagen fibres. This hypothesis was based on the fact that the rate of platelet adhesion to collagen is inhibited either by agents that interfere with actin polymerization or by depletion of the cell of its energy reserves, and is consistent with a model proposed by Rubin *et al.* (1981). The mechanism by which the cell responds to the initial signal, i.e. binding of collagen to its receptor, is unknown. However, we have recently shown that collagen-induced stimulus-response coupling is not inhibited by elevated cyclic AMP (Smith *et al.*, 1992).

Electrically permeabilized platelets have provided a good model system with which to study the role of intracellular mediators on platelet function (Knight & Scrutton, 1986). This method has proved useful in many different cells, including platelets, where it helped to define the importance of both Ca^{2+} - and diacylglycerol-dependent pathways in platelet secretion. In addition, the first evidence implicating a G-protein in activation of phospholipase C was obtained in electrically permeabilized platelets by using stable GTP analogues. We have shown here that electrically permeabilized platelets retain the ability to adhere to collagen coated on microtitre wells. Adhesion of these cells is qualitatively similar to adhesion of intact cells, since it is inhibited by monoclonal antibodies to glycoprotein Ia (Fig. 4) and cytochalasin D (Fig. 5). This adhesion was relatively independent of the free Ca^{2+} concentration over the range normally found intracellularly. This result is in contrast with secretion of 5-hydroxytryptamine from permeabilized platelets, which is extremely dependent on the intracellular Ca^{2+} concentration. Previously, we had shown in intact cells that suppression of increases in intracellular Ca^{2+} above the basal level with BAPTA, a chelator of Ca^{2+} that can be introduced into platelets, had very little effect on the extent or rate of adhesion of platelets to collagen (Smith *et al.*, 1991). The results presented here are consistent with our earlier findings and indicate that changes in intracellular Ca^{2+} are not of major importance in regulating the adhesion of platelets to collagen. In addition, we found that the protein kinase C inhibitor staurosporine had only a small effect on adhesion of permeabilized platelets to collagen. In contrast, 5-hydroxytryptamine secretion, a response known to be dependent on the protein kinase C pathway (Ashby *et al.*, 1990), is fully inhibited by the concentrations of staurosporine that we have used. Although staurosporine may not be totally specific for protein kinase C, the fact that we obtain very little inhibition of adhesion by this agent indicates a relatively small role for protein kinase C-dependent events in platelet adhesion to collagen.

G-proteins are known to be important mediators in the transduction of signals in many different cell types. They have been shown to regulate specific effectors, including phospholipase C, adenylate cyclase and ion channels (Taylor, 1990). Recent evidence suggests that G-proteins may also regulate the level of actin polymerization either directly or indirectly. A *Drosophila* mutation linked to a defect in gastrulation manifests itself as a lack of co-ordination of cell shape and cell motility, and thus implies a dysfunction of the cytoskeleton (Parks & Wieshaus, 1991). The mutation has been shown to reside in a gene that encodes a protein similar in amino acid sequence to the α subunit of G-proteins, leading those authors to suggest that a G-protein-dependent regulation of the cytoskeleton is important for normal gastrulation. Chemotaxis in *Dictyostelium*, thought to involve actin polymerization via increases in nucleation activity, has been proposed as a G-protein-dependent event, based on the *fgd A* mutants (Hall *et al.*, 1989). Carlson *et al.* (1986) found that the β subunit of G-proteins co-fractionated with the cytoskeleton of S49 mouse lymphoma cells. Sheth *et al.* (1991) found that actin polymerization in U937 cells was associated with expression of $\text{G}_{i2\alpha}$ subunit upon cellular differentiation. Perhaps the most direct evidence for an involvement of a G-protein comes from the work of Bengtsson *et al.* (1990) on human neutrophils. They demonstrated that GTP analogues could increase the F-actin content of the neutrophil and, although this effect was not inhibited by pertussis toxin, it was inhibited by GDP[S]. Depression of the free Ca^{2+} level had essentially no effect on this response.

The results presented in this paper are consistent with our previous findings implicating an actin-dependent process in enhancement of the rate of platelet adhesion to collagen. The present paper extends these findings by demonstrating that adhesion of electrically permeabilized platelets to collagen is enhanced about 5-fold by stable GTP analogues. The stable GDP analogue GDP[S] inhibits enhancement of adhesion by the GTP analogue GTP[S]. The enhanced adhesion cannot be explained by GTP[S]-dependent activation of the phospholipase C pathway for two reasons. (1) Phospholipase C activation results in inositol 1,4,5-trisphosphate production and Ca^{2+} mobilization, but adhesion of permeabilized platelets to collagen was almost independent of the free Ca^{2+} concentration over the normal intracellular range, consistent with our studies with intact cells using BAPTA (Smith *et al.*, 1991). If changes in intracellular Ca^{2+} were important, we would expect to see dramatic increases in adhesion as Ca^{2+} was increased, as is the case for secretion of 5-hydroxytryptamine. (2) Phospholipase C activation also results in diacylglycerol production and activation of protein kinase C. The protein kinase C inhibitor staurosporine had only a small effect on adhesion at concentrations that completely inhibited 5-hydroxytryptamine secretion. Therefore these experiments are inconsistent with a major role for either Ca^{2+} or protein kinase C in GTP-dependent enhancement of platelet adhesion and suggest that a G-protein-dependent process directly regulates mobilization of collagen receptor, allowing for enhanced adhesion. We propose that G-proteins regulate availability of collagen-adhesion receptors through regulation of the cytoskeleton.

This work was supported in part by N.I.H. (grant HL-36579). We thank Dr. Mary Selak for helpful discussions during the course of these experiments.

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Received 9 December 1991/6 March 1992; accepted 23 March 1992