Role of Cyclic Nucleotides in Rapid Platelet Adhesion to Collagen

By Renata Polanowska-Grabowska and Adrian R.L. Gear

Adhesion of human platelets to type I collagen under arterial flow conditions is extremely fast, being mediated primarily by the αβ1 integrin (glycoprotein IIa/IIIa). We have investigated the involvement of cyclic nucleotides in platelet adhesion to soluble native collagen immobilized on Sepharose beads using a new microadhesion assay under arterial flow conditions. To prevent platelet stimulation by thromboxanes and adenosine diphosphate (ADP), experiments were performed with aspirin-treated platelets in the presence of ADP-removing enzyme systems such as creatine phosphate/creatine phosphokinase or apyrase. Rapid reciprocal changes in platelet adenosine 3′5′-cyclic monophosphate (cAMP) and guanosine 3′5′-cyclic monophosphate (cGMP) occurred during adhesion. cAMP levels in adherent platelets were 2.4-fold lower than in effluent platelets or in static controls, whereas cGMP levels were increased 2.4-fold. These results suggest that contact between platelets and collagen stimulates guanylate cyclase and inhibits adenylyl cyclase. This occurs in the absence of the platelet release reaction. We also studied short-term effects of agents that regulate cyclic nucleotide synthesis, prostaglandin E1 (PGE1) and sodium nitroprusside (SNP). After only 3.8 seconds at 10 to 30 dyne/cm², PGE1 (10 μmol/L) increased cAMP 16.4-fold, whereas SNP (50 μmol/L) increased cGMP ninefold and caused a 3.2-fold increase in cAMP. Both PGE1 and SNP rapidly (<5 seconds) inhibited platelet adhesion in a dose-dependent manner that was correlated with the increase in cyclic nucleotides. Our data suggest that cAMP and cGMP play a regulatory role in the initial phases of platelet adhesion to collagen mediated by the αβ1 integrin receptor.

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Addition of Mg²⁺, adhesion to collagen is mediated mainly by the αβ1 integrin complex and is extremely rapid, with exponential half-times as short as 0.2 seconds.

It is well known that platelet responses to various agonists can be regulated by the adenylate and guanylate cyclase pathways. In particular, it has been shown that collagen fibers can lower adenosine 3′5′-cyclic monophosphate (cAMP) levels in intact platelets and inhibit adenylyl cyclase in platelet membranes. It has also been shown that collagen-induced aggregation is associated with increases in platelet guanosine 3′5′-cyclic monophosphate (cGMP). Physiologically, these two pathways can be stimulated by factors produced by vascular endothelium, namely, prostacyclin (PGI2) and endothelium-derived relaxing factor, the most important component of which is nitric oxide (NO). These two agents can inhibit platelet function by increasing the concentrations of intracellular cAMP and cGMP, respectively.

Studies of the effect of PGIs on platelet adhesion to collagen have yielded conflicting results. In particular, it has been shown that collagen fibers can lower adenosine 3′5′-cyclic monophosphate (cAMP) levels in intact platelets and inhibit adenylyl cyclase in platelet membranes. It has also been shown that collagen-induced aggregation is associated with increases in platelet guanosine 3′5′-cyclic monophosphate (cGMP). Physiologically, these two pathways can be stimulated by factors produced by vascular endothelium, namely, prostacyclin (PGI2) and endothelium-derived relaxing factor, the most important component of which is nitric oxide (NO). These two agents can inhibit platelet function by increasing the concentrations of intracellular cAMP and cGMP, respectively.

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the primary response to collagen. Several investigators argue that PGs do not affect the initial (contact) phase of adhesion, but do inhibit subsequent steps related to platelet activation such as spreading, aggregation, and the release reaction. 

The role of cGMP in platelet adhesion is not completely understood. Because many agonists, including collagen, may increase cGMP, it was originally thought that an increase helps cause platelet activation. However, subsequent studies showed that cGMP may inhibit platelet function. Stimulation of soluble guanylate cyclase by NO or by sodium nitroprusside (SNP) blocks platelet aggregation and causes disaggregation of platelet aggregates. An increase in cGMP via the soluble guanylate cyclase is believed to increase cAMP levels by inhibition of the cGMP-dependent cAMP phosphodiesterase. The latter increase might then lead to the inhibition of platelet function by activation of the cAMP-dependent protein kinase. Radomski et al reported that platelet adhesion to collagen under stirred conditions is totally blocked by NO. Recently, de Graaff et al showed that NO reduced platelet adhesion to the matrix of cultured endothelial cells under flow conditions in whole blood.

Most previous investigations on the role of cyclic nucleotides in collagen-platelet interactions have been performed under stirred, static, or low shear conditions. The adhesion (contact) times being studied were usually of the order of minutes or even hours. However, in vivo platelet adhesion to the exposed subendothelium must be extremely fast. Also, because the life-times of regulators such as prostacyclin and nitric oxide in circulation are short, ie, 2 to 3 minutes and seconds, respectively, this suggests that regulation or changes in cyclic nucleotides during adhesion have to occur very rapidly. To our knowledge, there are no reports about the role of cyclic nucleotides for short (<1 minute) adhesion times and whether rapid changes in cyclic nucleotides may regulate adhesion.

In the present study, using our microadhesion column under conditions of arterial flow, we have found that attachment to collagen leads to reciprocal changes in cAMP and cGMP in adhering aspirin-treated platelets. We also found that rapid adhesion is inhibited up to 80% by high doses of PGE, and may be completely blocked by SNP within 5 seconds.

MATERIALS AND METHODS

**Materials.** PGE, PGI, SNP, aspirin, indomethacin, apyrase (grade VII; from potato), hirudin, bovine serum albumin (BSA; fatty acid free), creatine phosphokinase (CPK), creatine phosphate (CP), nordihydroguaiaretic acid (NDGA), and Sepharose 4B-200 were from Sigma Chemical Co (St Louis, MO). BritCN was from Aldrich (Milwaukee, WI). GRGDSP peptide was from Peninsula Labs (Belmont, CA). P2 (anti-GPIIb/IIIa complex) monoclonal antibody (MoAb) and SZ2 (anti-GPIb) MoAb were purchased from Amac (Westbrook, ME). 6F11 MoAb reactive with platelet membrane GPIIb/IIIa complex (αIIbβ3) was a kind gift from Dr Barry S. Coller (State University of New York, Stony Brook). Native salt-soluble type I collagen from rat skin was kindly donated by Dr Gary Balian (University of Virginia, Charlottesville).

**Preparation of human platelets.** Human venous blood was anti-coagulated with 0.10 v/v of citric acid-citrate-dextrose (ACD) buffer to a final citrate concentration of 11.5 mmol/L in whole blood, and centrifuged at 350g twice for 3 minutes and once for 5 minutes. The resultant platelet-rich plasma was gently mixed with 0.05 vol of ACD, apyrase (7.5 U/mL ADPase activity), indomethacin (1 μg/mL), and PGI (0.3 μg/mL) and centrifuged at 620g for 20 minutes. The platelet pellet was then washed in ACD buffer containing 0.3% BSA and apyrase (7.5 U/mL), and centrifuged again as previously described. Finally, the platelets were resuspended in a modified HEPES-Tyrode medium (137 mmol/L NaCl, 2.7 mmol/L KCl, 12 mmol/L NaHCO3, 0.42 mmol/L NaH2PO4, 5.5 mmol/L glucose, 10 mmol/L HEPES, pH 7.4) containing 2 mmol/L Mg2+, 0.15% BSA, and inhibitors of aggregation 7.5 U/mL apyrase (ADP-removing enzyme), 0.01 U/mL hirudin (thrombin inhibitor), and 500 μmol/L GRGDSP peptide (inhibitor of fibrinogen binding to activated platelets). The platelets were kept under a mixture of 5% CO2 and 95% air at room temperature and used within 4 to 5 hours after final resuspension. To help eliminate the effect of collagen-induced generation of thromboxane A2, experiments were performed with platelets treated with 100 μmol/L aspirin for 20 minutes at 37°C. In some experiments, adhesion studies were performed on inhibition of aspirin-treated platelets with ADP-removing enzyme system 40 U/mL creatine phosphokinase plus 10 mmol/L creatine phosphate or with 20 μmol/L NDGA (lipoxgenase inhibitor). Platelets were incubated with different MoAbs for 10 minutes at 37°C.

**Rapid cyclic nucleotide formation by PGE or SNP under flow conditions.** Stimulation of synthesis of platelet cAMP and cGMP by different concentrations of PGE and SNP for short reaction times (1 to 4 seconds) was tested using a quenched-flow approach. It involves pumping washed platelets and an inducing agent at 37°C through a narrow diameter tube of precise length, the distance for a given flow rate corresponds to an exact reaction time. The internal diameter of the reaction tube used in these experiments was about 0.3 mm and gave wall shear rates of 2,520 s-1 for a pumping speed of 6.7 μL s-1. The reaction was stopped with 10% (w/vol) trichloroacetic acid (TCA) in isotonic saline solution.

**Adhesion assay.** The continuous-flow adhesion approach was essentially as described previously. BritCN-activated Sepharose 4B beads were coated either with native salt-soluble collagen type I from rat skin or with BSA and were used as adhesive substrates in the microadhesion column. A two-syringe system using 1-mL syringes was used. One syringe containing washed platelets and the second syringe containing SNP or PGE, in isotonic NaCl or buffer, were connected via teflon tubing to the microadhesion column. Their contents were immediately mixed for a maximum pre-exposure time of 3.5 seconds and pumped through the collagen beads. Flow rates were regulated by a variable-speed syringe pump from 6.7 to 2.0 μL s-1 and gave shear rates at the surface of the beads from 3,400 to 1,020 s-1. The different contact (adhesion) times were obtained by changing the amount of beads or the flow rates in the microcolumn and were calculated from the formula 

\[
V = \frac{Q}{t_c} = \frac{V_t}{t_c}
\]

where \( V_t \) (μL) represents the free volume (volume accessible to the flow) and \( Q \) (μL s-1) represents the flow rate through the column. Adhesion to collagen was determined by counting single platelets in the suspension before and after exposure to the collagen-coated beads using a resistive particle counter (Particle Data, Elmhurst, IL), and was expressed as the percentage of platelets bound to collagen. Because nonspecific adhesion to the BSA-coated beads in an Mg2+-containing medium was 3% to 5%, the extent of platelet adhesion to collagen was corrected for this contribution at each experimental point.

To analyze the changes in concentrations of cyclic nucleotides during adhesion, we usually pumped 2 to 3 × 108 platelets through a microcolumn of 50 μL of collagen-coated beads at 3.35 μL s-1 for about 2.5 minutes. This corresponded to an average adhesion time of 1.25 minutes. The shear rate generated by this condition was
Platelets in the column effluents were treated with ether. The recovery of cAMP and cGMP for platelets that had adhered to the beads resulting from both the ether extraction and from cAMP or cGMP trapping by the beads was 85%. The cAMP and cGMP recovery for platelets in suspension was 90%. cGMP concentrations were determined in the resultant aqueous extracts by commercially available radioimmunoassay kits (Advanced Magnetics, Cambridge, MA) after acetylation of samples. For cAMP determination, the aqueous extracts were mixed with equal volumes of 50 mmol/L acetate buffer, pH 6.5, and the cAMP content was then measured by radioimmunoassay as described. Values for platelet cyclic nucleotides were corrected for the recovery of cAMP and cGMP.

Statistics. Values are given as means ± SD with the number of observations given in parentheses. Means were compared by the Student's t-test for unpaired data.

RESULTS

Basic adhesion kinetics and effects of antibodies. The adhesion kinetics of aspirin-treated platelets in plasma-free medium containing Mg²⁺, GRGDSP peptide, hirudin, and apyrase in the presence of different MoAbs are shown in Fig 1. Both the initial rate and extent of adhesion were unchanged when CP/CPK was used as ADP-removing enzyme system with or without lipoygenase inhibitor NDGA. Platelet adhesion to collagen was inhibited by the anti-GPIIb/IIa antibody 6F1 (5 to 50 μg/mL) in a dose-dependent manner. Major inhibition (about 80%) occurred at 25 μg/mL of the 6F1 antibody. We did not observe any inhibition of adhesion by either anti-GPIb (SZ2) or anti-GPIb/IIa (P2) antibody at concentrations up to 25 μg/mL. This finding indicates that adhesion in our system was mediated primarily by the GPIb/IIa (α₃β₁) receptor.

Effects of platelet adhesion on cAMP levels. The cAMP content of unstimulated static platelets was 10.78 ± 1.40 pmol/10⁶ platelets. Platelets that adhered to the collagen-coated beads had a 2.4-fold lower cAMP level (P < .001) compared with resting platelets (static control, Table 1). Platelets in the column effluent, which were in short contact with the collagen beads, had only slightly (18%) but not significantly (P > .05) reduced cAMP levels. Another flowing (dynamic) control was provided by pumping platelets through a column filled with BSA-coated beads. The cAMP concentration in the effluent from this column (11.8 ± 2.8 pmol/10⁶ platelets) was not significantly different from the static controls (P > .05). The cyclic AMP content of platelets adhering to the collagen-coated beads in the presence of another ADP-removing enzyme system CP (10 mmol/L)/CPK (40 U/mL) and 20 μmol/L NDGA (lipoygenase inhibitor) was 4.75 ± 0.95 pmol/10⁶ platelets (3 independent experiments), which is not significantly different from values

| Table 1. Changes in Platelet Cyclic Nucleotides Caused by Adhesion to Collagen |
|-----------------|-----------------|-----------------|-----------------|-----------------|
| Cyclic Nucleotide | Static Control  | Flowing Control | Effluent Platelets | Adherent Platelets |
| cAMP            | 10.78 ± 1.40    | 11.80 ± 2.80    | 8.79 ± 2.80       | 4.55 ± 1.08      |
| cGMP            | 2.52 ± 0.34     | 2.72 ± 0.41     | 3.11 ± 0.42       | 6.16 ± 1.87      |

Washed aspirin-treated platelets in the presence of Mg²⁺, apyrase, hirudin, and GRGDSP peptide were pumped through the collagen-coated beads for 2.5 minutes at a contact time (t₀) of 1.35 seconds (37°C). Adhesion was terminated by the addition of 10% (wt/vol) ice-cold TCA and concentrations of cAMP and cGMP were determined by radioimmunoassay. The "flowing control" represents platelets pumped through BSA-coated beads. Data shown represent means ± SD of five separate platelet preparations.

Fig 1. Effect of MoAbs on rapid platelet adhesion to collagen. Washed aspirin-treated platelets in the presence of 2 mmol/L Mg²⁺, apyrase (7.5 U/mL), 500 μmol/L GRGDSP peptide, and 0.01 U/mL hirudin in isotonic saline solution. The quenched platelet lysates were separated from the beads and cell debris by centrifugation for 10 minutes at 6,000g. The supernatants were extracted with ether. The recovery of cAMP and cGMP for platelets in suspension was 90%. cGMP concentrations were determined in the resultant aqueous extracts by commercially available radioimmunoassay kits (Advanced Magnetics, Cambridge, MA) after acetylation of samples. For cAMP determination, the aqueous extracts were mixed with equal volumes of 50 mmol/L acetate buffer, pH 6.5, and the cAMP content was then measured by radioimmunoassay as described. Values for platelet cyclic nucleotides were corrected for the recovery of cAMP and cGMP.

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Effects of platelet adhesion on cGMP levels. The level of cGMP in unstimulated platelets was 2.52 ± 0.34 pmol/10⁹ cells. Platelets attached to collagen showed a 2.4-fold increase in cGMP compared with static controls (P < .001, Table 1). Platelets emerging in the column effluent had slightly (23%) but not significantly (P > .05) increased concentrations of cGMP. The increase in cGMP content was not observed in the effluent from the control BSA column.

Effects of PGE₁ and SNP on rapid changes in platelet cyclic nucleotides. Before platelets reached the collagen-coated beads, they were mixed in a connecting tube with different concentrations of PGE₁ or SNP solutions for about 3.5 seconds. The kinetics of changes in cyclic nucleotides induced by these agents within 1.5 to 3.8 seconds are shown in Fig 2A and B. For a mixing time of 3.8 seconds, 10 μmol/L PGE₁ induced a 16.4 ± 1.4-fold increase in cAMP. PGE₁ was specific for increasing cAMP and did not influence cGMP levels (data not shown). However, SNP increased not only platelet cGMP but also caused a dose-dependent increase in cAMP. SNP at 50 μmol/L caused a ninefold increase in cGMP and a 3.2-fold increase in cAMP. The cyclic nucleotides levels were increased in a concentration-dependent manner (data not shown).

Inhibition of adhesion by PGE₁ and SNP. Platelets were pumped through the collagen-coated beads together with increasing concentrations of these agents after a brief pre-exposure for about 3.5 seconds. Both PGE₁ and SNP strongly and rapidly reduced adhesion in a dose-dependent manner, with about 80% inhibition caused by 20 μmol/L PGE₁ at a contact time (tₜ) of 1.35 seconds (Fig 3A). The extent of inhibition was correlated with the increase in cAMP of the flowing platelets (Fig 3B). Almost complete inhibition (90%) was observed for 100 μmol/L SNP at tₜ = 1.35 seconds (Fig 4A). At lower concentrations, the extent of inhibition was dose-dependent and correlated with the increases in cAMP and cGMP levels (Fig 4B).

Platelets attached to the collagen beads in the presence of PGE₁ had lower levels of cAMP compared with static controls treated with this drug or with platelets emerging in the column effluent (Fig 5). The cAMP content of PGE₁-treated platelets attached to collagen was significantly higher than in adherent platelets that had not been exposed to PGE₁. Platelets bound to collagen in the presence of SNP also had lower levels of cAMP than did SNP-treated static controls and effluent platelets. However, we observed that adhesion to collagen caused no significant changes in cGMP levels when SNP-treated platelets were pumped through the collagen-coated beads (data not shown).

DISCUSSION

We have found that adhesion of aspirin-treated platelets to collagen causes levels of cAMP to decrease compared with static controls (Table 1). Nonadherent platelets that may have had only brief contact with the collagen surface and platelets pumped through control, BSA-coated beads (flowing control) had unchanged cAMP levels. The decrease in cAMP concentration during adhesion is not associated with secretion from platelet dense granules as monitored by serotonin release⁹ or with secretion from α-granules as monitored by expression of platelet α-granule membrane protein GMP-140.⁴⁴ In addition, changes in cAMP could not be attributed to the released ADP, because the decrease in cAMP occurred in the presence of the ADP-removing enzyme systems CP/CPK and apyrase. The use of PGI₂ for an intermediate step during the preparation of the washed platelets might have suggested increased cAMP levels greater than the basal levels seen at the beginning of the experiment. The subsequent decrease in cAMP concentration could then be caused by lowering the previously elevated cAMP. However, we consider this possibility to be unlikely based on recent studies. In particular, it has been shown that
Fig 3. Inhibition of rapid platelet adhesion to collagen by PGE₁. (A) Time course of PGE₁-induced inhibition. Platelets were pumped through collagen-coated beads together with buffer (c) or with different concentrations of PGE₁: 1 μmol/L (v), 10 μmol/L (V), and 20 μmol/L (c) at 37°C. Preexposure time (time during which platelets were mixed with inhibitor) was about 3.6 seconds. (B) Percentage of PGE₁-induced inhibition at a contact time (t₁) of 1.35 seconds as a function of concentration. Data shown represent means ± SD of six independent platelet preparations.

Fig 4. Inhibition of rapid platelet adhesion by SNP. (A) Time course of SNP-induced inhibition. Platelets were pumped through collagen-coated beads together with buffer (c) or with SNP at 1 μmol/L (v), 12.5 μmol/L (V), 50 μmol/L (c), and 100 μmol/L (v). Preexposure time was about 3.5 seconds. (B) Percentage of inhibition as a function of SNP concentration at a contact time (t₁) of 1.35 seconds. Data shown represent means ± SD of six independent experiments.
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The increase in cGMP content preceded aggregation measured by light scattering and reached a maximum when aggregation began. This suggested that the change in cGMP was not an effect of release reaction but rather a result of intracellular synthesis of cGMP induced by collagen. Recently, Radomski et al. showed that platelets stimulated with collagen synthesize NO from L-arginine, which activates the soluble guanylate cyclase. Our results support and extend these observations, indicating that direct binding of platelets to collagen via the α5β1 integrin complex stimulates cGMP synthesis in the absence of platelet aggregation and secretion. The changes in cGMP may, in turn, influence the platelet cytoskeleton organization, similar to findings in neuroblastoma cells. This may represent part of the major events in the subsequent phases of the adhesion process.

In our studies, PGE1 induced a very fast, dose-dependent inhibition of platelet adhesion to collagen that was correlated with the increase of cAMP (Fig 3A). The concentrations of PG required to inhibit adhesion were much higher than those necessary to block completely platelet aggregation, and no more than 80% reduction was seen with 20 μmol/L PGE1. The residual adhesion (about 10% to 15% of total platelet number pumped through the collagen-coated beads) was mediated by a mechanism that was insensitive to an increase in platelet cAMP. Our results agree with the data of Weiss and Turitto, who found similar inhibition of adhesion to collagen with 1 μmol/L PGI2 at a shear rate of 800 s⁻¹, and the data of Karniguian et al., who noted a strong inhibition of adhesion both for normal and thrombasthenic platelets to collagen type III. This issue requires further study with highly purified different collagen substrates.

Blocking the α5β1 collagen receptor with MoAb 6F1 also failed to cause complete inhibition of adhesion, the extent of which (about 80%) was very similar to that caused by high doses of PGE1. This finding suggests that the adhesion inhibited by the increase of cAMP may depend on collagen binding to the α5β1 integrin. We found rapid and almost complete inhibition of platelet adhesion by 100 μmol/L SNP (Fig 4A). This result agrees with the studies of Radomski et al., who found that NO totally blocks adhesion to collagen fibers in suspension, and the observations of de Graff et al., who found that NO strongly blocked adhesion to the extracellular matrix produced by endothelial cells in whole blood under flow conditions. SNP (50 μmol/L) increased both cGMP (up to 9-fold) and cAMP levels (up to 3-fold) under rapid flow conditions (Fig 2B). It is unlikely that the threefold increase in cAMP was the only cause of the observed inhibition, because an approximate 20-fold increase in cAMP was required to cause a similar inhibition when PGE1 was used. A threefold increase in cAMP induced by PGE1 resulted in less than 20%
inhibition of adhesion. Therefore, it appears that the cGMP increase is primarily responsible for the inhibition of the adhesion to collagen caused by SNP. Similar conclusions were reached by Maurice and Haslam in their study of inhibition of platelet-activating factor-induced aggregation by SNP. A threefold increase in cAMP induced by SNP was also observed by Maurice and Haslam in rabbit platelets, although some other studies in human platelets failed to detect any changes in cAMP by SNP. The increase in cAMP may result from inhibition by cGMP of the low-Km, cAMP-cGMP inhibitable phosphodiesterase. The mechanism for the inhibition of platelet adhesion to collagen by SNP is not clear. It is possible that it is caused by synergistic effect of cAMP and cGMP or by a cGMP-independent mechanism, such as stimulation of ADP-ribosylation. Intriguingly, the inhibition of platelet adhesion by increased levels of cGMP appears to contradict our observations that an increase in cGMP is induced by attachment to collagen (Table 1). It is possible that relatively small increases in cGMP potentiate adhesion to collagen or result from adhesion, whereas higher levels inhibit both adhesion and aggregation. This issue requires further study.

The results of our study indicate that platelet cAMP and cGMP undergo significant and reciprocal changes during the αβ1-mediated platelet-collagen interaction. The initial phase of platelet attachment to collagen under flow conditions may be blocked within seconds by PGE1 and SNP. Therefore, intravascular release of PGI2 and NO under arterial flow conditions may have powerful regulatory effects on the initial interaction of platelets with the exposed vessel wall.

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