Platelet Release Reaction and Intracellular cGMP

By Avery Weiss, Nancy Lewis Baenziger, and John P. Atkinson

Enhanced cAMP concentrations inhibit the aggregation and release reaction of isolated human platelets and platelet-rich plasma to all known inducing agents. An opposing role for cGMP in this phenomenon has been proposed by some but not by others, and the function of cGMP in this secretory process is unclear. To further elucidate the role of cGMP in the release reaction, the effect of increased concentrations of this cyclic nucleotide on 14C-serotonin release was evaluated utilizing isolated human platelets and highly purified human thrombin or commercially available bovine thrombin. Several recently described stimulators of guanylate cyclase, including sodium nitroprusside, sodium azide, nitrosothioguanidines, and ascorbic acid, were found to markedly augment platelet cGMP levels. Enhanced platelet cGMP concentrations produced by these drugs or by the exogenous addition of cGMP and its analogues neither caused these cells to secrete nor modulated the thrombin-induced serotonin release reaction. The inhibition of serotonin release by increased cAMP concentrations was not counteracted by increased cGMP levels. Platelet cGMP concentrations were unaltered by thrombin. These data indicate that cGMP is not an obligatory signal or a modulator of the thrombin-induced platelet release reaction.

AUGMENTATION of cAMP concentrations by pharmacologic agonists or by the addition of exogenous cAMP and its analogues inhibits the aggregation and release reaction of isolated platelets or platelet-rich plasma (PRP) to all known inducing agents. A positive regulatory role for cGMP in this secretory phenomenon has been proposed, since investigators have reported that increased cGMP concentrations potentiate the collagen- and epinephrine-induced aggregation and release reaction of PRP. While some have shown an apparent relationship between the release reaction in PRP and elevated cGMP levels, others have pointed out that kinetic data indicate that this response may be a consequence and not the mediator of the aggregation and release reaction. Moreover, not all investigators have found cGMP to potentiate or correlate with this reaction.

To determine if cGMP was a necessary signal or a modulator of this secretory process, we first found several drugs that elevated cGMP levels in isolated human platelets and then evaluated their effects and those of cGMP and its analogues on the thrombin-induced release reaction.

MATERIALS AND METHODS

Unless noted otherwise, reagents were obtained from Sigma Chemical, or Fisher Scientific, Co., St. Louis, Mo.

Platelets were purified from the peripheral blood of normal human donors by the method of Baenziger and Majerus. In the whole-cell experiments 0.45 ml of the platelet suspension (1.2 x 10^9 platelets/ml in Hanks' balanced salt solution (HBSS) or platelet resuspension buffer; see below and...
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ref. 9) was incubated for varying time intervals (usually 5 min) at 37°C with the test agents. Each condition was assayed in duplicate or triplicate, and three or four sets of controls (cells plus buffer) were employed in each experiment. The test agents were also dissolved and diluted in HBSS or platelet resuspension buffer, except for N-methyl-N-nitro-N-nitrosoguanidine (MNNG) (Aldrich Chemical, Milwaukee, Wisc.), which was dissolved in 0.1 ml acetone and then diluted in buffer. The highest final acetone concentration utilized was 0.1% (v/v), and this concentration had no effect on the release reaction, platelet cGMP concentrations, or cyclase activity. The reaction was stopped by rapid centrifugation (2200 g for 2 min) followed by snap-freezing of the cell pellet in an ethanol–dry ice bath. After boiling (3 min at 103°C) and sonicating (10 sec employing a Biosonic Model 1V, Bronwill, Rochester, N.Y.), the sample was centrifuged (2200 g for 10 min) and suitable aliquots from the supernatant analyzed for cGMP content by radioimmunoassay (RIA).10,11 Early time points were obtained by snap-freezing the entire cell suspension in liquid nitrogen; the suspension was then processed as described above. In selected experiments, cyclic nucleotides were extracted by perchloric acid precipitation, neutralization, and sequential purification by neutral alumina and Dowex-2 (Bio Rad Ag-X8, 100-200 mesh in chloride form) anion-exchange chromatography employing 0.5 N HCl to elute cGMP.12 Further details of these standard methods have been published.10-12 The recovery of tracer quantities (2000-5000 cpm) of [3H]-cGMP (21 Ci/mM) (Amersham-Searle, Arlington Heights, Ill.) and of known quantities of cGMP (usually 0.5 and 5 pmol) was 30%-55%.

The ability of exogenously added cGMP or cGMP analogues to increase intracellular levels was evaluated by assaying the quantity of these cyclic nucleotides remaining in thoroughly washed platelets. After a 10 min incubation at 37°C in platelet resuspension buffer, the platelets were washed three to five times in 5 ml wash buffer at 4°C. Approximately 100,000 cpm of [3H]-cGMP were added to selected tubes in order to monitor the adequacy of the washing procedure. When less than 100 cpm remained in the supernatant, the cGMP concentration was determined in the cell pellets as described above.

The cross-reactivity of the three cGMP analogues with the antibody used in the RIA for cGMP was assessed as described previously for other compounds.16 These results indicated that 8-bromo-cGMP, monobutryl cGMP, and dibutyryl cGMP were equal to or only slightly less than cGMP itself in their reactivity. Therefore as determined by our cGMP RIA the increased intracellular concentrations of the cGMP analogues following their exogenous addition to platelets represent definitive augmentation, and the levels obtained are minimum values.

Guanylate cyclase activity was assayed by the method of Kimura and Murad,17 in which the quantity of cGMP formed from GTP in the presence of lymphocyte extract is measured by RIA. In brief, a soluble platelet extract was prepared by sonicating 1-2 x 109 platelets/ml at 4°C in pH 7.6 TMD (Tris-HCl, 50 mM; MnCl₂, 4H₂O, 5 mM; dithiothreitol, 2 mM) buffer. Following centrifugation (16,000 g for 30 min at 4°C) the supernatant was removed and 0.01 ml distributed to test tubes containing GTP (1 mM), theophylline (5 mM), test agents, and buffer to a final volume of 0.1 ml. A GTP regenerating system was not found to be necessary, but a 10%-15% reduction in the quantity of cGMP measured was noted if theophylline was omitted from the reaction mixture. Following a 10-min incubation at 37°C, the reaction was stopped by the addition of threefold excess of cold sodium acetate buffer (0.05 M, pH 4.0), to prevent the nonenzymatic conversion of GTP to cGMP18 and immediate heating at 85°C for 3 min. Suitable aliquots were removed, diluted in 0.05 M acetate buffer pH 6.2, and assayed for cGMP by RIA.

The release reaction was examined employing platelets prelabeled with 14C-serotonin as described by Tollefsen et al.14 Isolated human platelets prelabeled with 5-[2-14C]-serotonin binoxalate (0.2 mCi/mg), New England Nuclear, Boston, Mass., were washed and resuspended to a final concentration of 1.1 x 10⁷ cells/ml in isotonic Tris-buffered saline (pH 7.4) containing 0.14 M NaCl, 0.015 M Tris-HCl, and 0.0055 M glucose (platelet resuspension buffer).15 In most experiments the platelets (0.40 ml) were incubated for 5 min at 37°C with 0.05 ml of the test agent. Serotonin release was determined 2 min after the subsequent addition of 0.05 ml thrombin (final concentrations between 4 and 0.01 units/ml) were used to determine the standard release curves. Two different sources of thrombin were employed in these experiments. One was a commercial bovine preparation (500 U/mg) obtained from Sigma and the other a highly purified human thrombin (2500-3000 U/mg) obtained according to methods identical to those recently published by Fenton et al.16 The human thrombin was a gift from J. Mileitch, and this material was kindly analyzed by Dr. J. S. Finlayson and found to be identical to his preparation. The magnitude of the serotonin release was determined by decanting the suspension into a 10-ml syringe, adding 4.5 ml of the Tris-buffered saline solution, and filtering the suspension through a
Millipore filter (RAWP 025-00; 1.2 μm pore diameter; Millipore, Bedford, Mass.) to collect the platelets. The filter was dissolved in 10 ml Bray’s solution and the counts determined by liquid scintillation spectrometry.

Phosphodiesterase activity, thin-layer chromatography, and protein determinations were performed by standard methods previously reported from this laboratory.

The data are expressed as the mean ± SEM. The means were compared using Student’s t test.

RESULTS

Human platelets were incubated with drugs reported to enhance intracellular cGMP concentrations in platelets and other tissues. Ascorbic acid, sodium nitroprusside, sodium nitrite, and N-methyl-N′-nitro-N-nitroso-guanidine (MNNG) increased cGMP concentrations in intact cells.

### Table 1. Effect of Selected Drugs and Thrombin on Whole-Cell Platelet cGMP Concentrations and on Guanylate Cyclase Activity

<table>
<thead>
<tr>
<th>Agent (mM)</th>
<th>Whole-Cell cGMP Concentration</th>
<th>Guanylate Cyclase Activity</th>
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<tbody>
<tr>
<td></td>
<td>Stimulation Ratio</td>
<td>Stimulation Ratio</td>
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<tr>
<td>Sodium azide</td>
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<tr>
<td>10</td>
<td>1.9 ± 0.6</td>
<td>3.4 ± 0.3</td>
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<tr>
<td>1</td>
<td>2.0 ± 0.5</td>
<td>3.5 ± 0.9</td>
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<tr>
<td>0.1</td>
<td>1.7 ± 0.1</td>
<td>4.3 ± 1.0</td>
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<tr>
<td>0.01</td>
<td>1.4 ± 0.3</td>
<td>1.7 ± 0.4</td>
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<tr>
<td>Sodium nitroprusside</td>
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<tr>
<td>10</td>
<td>7.3 ± 1.2</td>
<td>3.6 ± 0.9</td>
</tr>
<tr>
<td>1</td>
<td>6.7 ± 1.1</td>
<td>4.4 ± 1.4</td>
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<tr>
<td>0.1</td>
<td>5.4 ± 1.0</td>
<td>4.3 ± 0.8</td>
</tr>
<tr>
<td>0.01</td>
<td>3.3 ± 0.6</td>
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<tr>
<td>0.001</td>
<td>1.7 ± 0.4</td>
<td>1.5 ± 0.2</td>
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<tr>
<td>Sodium nitrite</td>
<td>4.1 ± 1.3</td>
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<tr>
<td>1</td>
<td>1.9 ± 0.7</td>
<td>1.6 ± 0.4</td>
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<tr>
<td>0.1</td>
<td>1.3 ± 0.1</td>
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<tr>
<td>N-methyl-N′-nitro-N-nitroso-guanidine (MNNG)</td>
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<tr>
<td>10</td>
<td>8.3 ± 1.7</td>
<td>3.2 ± 0.4</td>
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<tr>
<td>1</td>
<td>7.5 ± 1.4</td>
<td>5.9 ± 2.9</td>
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<tr>
<td>0.1</td>
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<td>5.7 ± 1.4</td>
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<td>0.01</td>
<td>1.9 ± 0.4</td>
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<tr>
<td>0.001</td>
<td>1.3 ± 0.1</td>
<td>2.0 ± 0.9</td>
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<tr>
<td>Ascorbic acid</td>
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<tr>
<td>10</td>
<td>5.4 ± 1.5</td>
<td>1.5 ± 0.6</td>
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<tr>
<td>5</td>
<td>3.9 ± 1.0</td>
<td>1.9 ± 0.3</td>
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<tr>
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<td>1.1 ± 0.2</td>
<td>1.1 ± 0.1</td>
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<tr>
<td>Bovine thrombin (U/ml)</td>
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<tr>
<td>10</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.2</td>
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<tr>
<td>1</td>
<td>1.2 ± 0.1</td>
<td>0.8 ± 0.3</td>
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<td>0.1</td>
<td>0.9 ± 0.2</td>
<td>0.9 ± 0.1</td>
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<tr>
<td>0.01</td>
<td>0.8 ± 0.1</td>
<td>0.9 ± 0.2</td>
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*Mean ± SEM for four experiments. Platelets were incubated for 5 min with the drugs or bovine thrombin.

† Mean ± SEM for four experiments at each point. Stimulation ratios of 1.7 or greater were at least significant at p < 0.05. Basal guanylate cyclase activity was 160 ± 24 pmol cGMP/min/mg protein.
azide, sodium nitroprusside (NP), sodium nitrite, and MNNG were found to increase cGMP many times over (Table 1). The most potent stimulatory agents were NP and MNNG, producing elevations of intracellular cGMP at concentrations as low as 0.01 mM. The response was usually maximal by 20 sec and persisted for at least 20 min, and the magnitude and kinetics of the response were similar in the absence of extracellular Ca²⁺. These compounds also stimulated guanylate cyclase activity in broken-cell preparations (Table 1). Platelet guanylate cyclase activity was maximal between pH 7.6 and 8.0 and linear with time (up to 20 min at 10 μg protein/assay) and enzyme concentration (2–50 μg protein/assay). In most cases, the magnitude of the increase in cyclase activity was proportional to the changes produced in intact cells, although sodium azide was a better stimulator of guanylate cyclase in broken-cell preparations and ascorbic acid in intact cells. As reported for other tissues, these agonists did not alter cGMP or cAMP phosphodiesterase activity in platelets (data not shown). The basal and agonist-stimulated cGMP produced in intact platelets and by the guanylate cyclase preparations comigrated in thin-layer chromatographic analysis identically to ³H-cGMP and to known quantities of cGMP comparable to those found in the platelet preparations. The cGMP immunoinhibitory activity from the whole- or broken-cell experiments was reduced 80–100% through treatment at 37°C for 60 min with beef heart ³',S' cyclic phosphodiesterase (0.05 mg) and comigrated with ³H-cGMP in ion-exchange column chromatography (Dowex 2; see Materials and Methods). Identical results were obtained if the cell
suspensions were acid precipitated and absorbed with neutral alumina and the

cGMP in the supernatant extracted by column chromatography (as outlined in

Materials and Methods) prior to RIA for cGMP.

An extensive series of experiments were undertaken in which cGMP was

analyzed in human platelets after the addition of human or bovine thrombin

(100 0.1 U/ml). In these studies NP (1 mM) was usually employed as the positive

time point as early as 10 sec and as late as 60 min were evaluated, a stan-
dard thrombin-induced serotonin release curve analysis was simultaneously

performed on the platelet preparation in question, and several buffers (platelet

wash buffer, platelet resuspension buffer, HBSS, Gey’s balanced salt solution,

and phosphate-buffered saline) with and without calcium were evaluated. Under

none of these circumstances were significant increases in platelet cGMP

concentrations observed in response to the addition of thrombin to the platelet

suspension (Table 1). Likewise, thrombin did not activate platelet guanylate cy-

clase.

The effect of drugs that raise cGMP and exogenously added cGMP and its

analogues on the platelet release reaction were studied (Figs. 1 and 2). Incubation

of platelets with stimulators of guanylate cyclase (10^{-2}–10^{-6} M) or cGMP, N^{6}-

\text{\textit{Fig. 2. Effect of drugs that raise cAMP or cGMP levels on thrombin-induced platelet release reaction. Control curve, mean ± SEM for three experiments in which bovine thrombin was added after a 5-min preincubation of drugs with platelets; percentage serotonin release was determined 2 min thereafter. Drugs increasing cGMP levels were employed at 1 mM except for ascorbic acid (utilized at final concentration of 10 mM). Curves generated in presence of drugs stimulating guanlyte cyclase fell within 95% confidence limits for simultaneously determined control curves. One identical experiment with human thrombin also showed no enhancement of serotonin release. Similar experiments were performed employing these drugs (10^{-2}–10^{-6} M) that increase cGMP levels; significant augmentation of release compared to control was not observed. Curves shown for drugs increasing cAMP levels (PGE_{1}, 100 \mu g/ml; theophylline, 5 \times 10^{-4} M) represent means from same three experiments used to generate control curve and are significantly different from it (p < 0.01). SEM bars not shown on these curves for clarity, but they were of magnitude similar to those presented for control. PGE_{1} (100 \mu g/ml), theophylline (5 \times 10^{-4} M), and PGE_{1} plus theophylline at same concentration as used alone produced 2.8 ± 0.4, 2.9 ± 0.5, and 14.4 ± 3.9 (mean ± SEM)-fold augmentation of basal cAMP levels. In three experiments drugs (10^{-4} M) that enhanced cGMP levels did not prevent inhibitory effect of compounds (PGE_{1} and theophylline) that raised cAMP concentrations.}}
O"-dibutyryl cGMP, O"-monobutyryl cGMP, and 8-bromo-cGMP (10^{-3} - 10^{-6} M) for 2, 5, or 15 min did not induce serotonin release in excess of control. Under the same incubation conditions platelet cGMP concentrations were increased many times by 10^{-3} M (as much as 20-fold) and 10^{-5} M cGMP and its analogues, with 8-bromo-cGMP producing the highest intracellular levels. Thus elevated intracellular concentrations of cGMP do not in themselves constitute an effective signal to induce the platelet release reaction.

We next examined the possibility that cGMP agonists and analogues could modulate the release reaction. The addition of either bovine or purified human thrombin to the platelets treated with cGMP and its analogues (10^{-3} - 10^{-6} M) produced a serotonin release curve virtually superimposable on the control curve (Fig. 1). In contrast, 10^{-3} - 10^{-6} M N^6-O"-dibutyryl cAMP and cAMP shifted the curve to the right, indicating a reduction in serotonin release. The addition of 10^{-4} or 10^{-5} M dibutyryl cGMP, 8-bromo-cGMP, or cGMP simultaneously with dibutyryl cAMP (10^{-5} M), prostaglandin E, (100 μg/ml), or theophylline (5 × 10^{-3} M) also did not alter the inhibition of release caused by the compounds that increased cAMP levels. Moreover, none of the stimulators of guanylate cyclase utilized over the concentration range shown in Table 1 enhanced thrombin-induced serotonin release, while drugs that elevated cAMP levels produced consistent inhibition of this secretory process (Fig. 2). In a series of experiments employing 0-30-min preincubations, the drugs (10^{-2} - 10^{-6} M) that enhanced cGMP levels also did not potentiate the thrombin-induced release reaction (0.02, 0.2, and 2.0 U/ml were utilized in these experiments) or prevent the inhibition observed with the cAMP stimulatory drugs including theophylline. In fact, in some experiments 10^{-2} and 10^{-3} M sodium nitroprusside and 10^{-2} M MNNG produced inhibition of serotonin release.

DISCUSSION

This study shows that elevated intracellular concentrations of cGMP produced by the exogenous addition of cGMP and its analogues or by drugs that stimulate guanylate cyclase do not induce the platelet release reaction. Therefore increased intracellular cGMP levels do not constitute a sufficient signal to the platelet to initiate this release reaction. These results are supported by the unpublished studies of another group that noted that the marked increase in platelet cGMP caused by ascorbic acid failed to induce platelet aggregation.5

In these experiments we also tested the hypothesis that raised intracellular cGMP levels might modulate the thrombin-induced serotonin release. Others have reported that the platelet aggregation and release reaction induced by epinephrine or collagen was enhanced by cGMP and dibutyryl cGMP.5 4 In contrast, in our studies neither cGMP or its analogues nor potent stimulators of guanylate cyclase augmented serotonin release over a wide range of thrombin concentrations. The inability of raised cGMP concentrations to counteract the inhibitory effects of enhanced cAMP concentrations is further evidence against a critical involvement of cGMP in this reaction or evidence that it acts in a fashion opposite or antagonistic to cAMP. These data obviously do not rule out a role for cGMP in platelet release reaction induced by other effectors. However, the ability of cGMP to partially reverse the inhibitory effects of increased cAMP concentrations in other systems may involve an unrelated phenomenon, such as increasing
binding of the inducing stimulus, rather than acting as a primary modulator of the release reaction per se."\(^5\)

Most agents that induce aggregation and release in platelet-rich plasma have been found to produce transient increases (two- to fourfold) in intracellular cGMP levels.\(^{20,21}\) However, analysis of this phenomenon by several groups suggested that the elevation in cGMP followed platelet aggregation rather than mediating this reaction.\(^6\) Furthermore, Haslam has shown in dog platelets that an increase in cGMP is not required for either aggregation or release.\(^2\) Taken together, these data and our own would strongly support the conclusion that cGMP is not fundamentally involved in the release reaction.

One difference between our results and those reported by others\(^{20}\) concerns the effects of thrombin on platelet cGMP levels. We found no increase in platelet cGMP levels over a 10,000-fold concentration range of thrombin, even though serotonin release was verified and two thrombin preparations were used. The reason for this discrepancy is unclear.

The release reaction measured here in isolated platelets reflects the interaction of proteolytically active thrombin with its platelet receptor.\(^{14}\) Release and aggregation as measured in PRP induced by other effectors possibly involves additional metabolic pathways. Although cGMP may affect the release reaction in these pathways by mechanisms yet to be defined, in platelets this cyclic nucleotide is clearly not the obligatory mediator (second messenger) or modulator of the thrombin-induced secretory phenomenon. Most of the drugs shown here to activate guanylate cyclase and elevate cGMP levels in platelets similarly increase cGMP concentrations in human lymphocytes and granulocytes and rabbit alveolar macrophages (Atkinson JP: unpublished data). In previous studies these pharmacologic probes were not available. As exemplified by this report, these agents should provide powerful tools to aid in clarifying the role of cGMP in immunologic and/or inflammatory processes.

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