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Research Article

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ABSTRACT The present data disagree with earlier suggestions that thrombin's effect on platelets is to cause a decrease in intracellular cyclic 3′,5′-adenosine monophosphate. Washed human platelets or platelet-rich plasma were incubated at 37°C with human thrombin. After centrifugation, the supernates were assayed for nucleotides and calcium released. The platelet pellets, and in some experiments the supernates as well, were assayed by radioimmunoassay for intracellular cyclic AMP. In the washed platelet system, increasing doses of thrombin to 0.5 U/cc induced increasing release of nucleotides and calcium. This was accompanied by an average twofold increase in intracellular cyclic AMP levels. Prostaglandin E_1, which inhibited 30–50% of release, induced a four- to fivefold increase in cyclic AMP levels that was additive to the cyclic AMP-stimulatory effect of thrombin. Theophylline, which inhibited only 20–40% of nucleotide release, was synergistic with thrombin in the intracellular accumulation of cyclic AMP. The time-course of cyclic AMP accumulation in response to thrombin was slower than thrombin-induced nucleotide release. Similar findings were made in the platelet-rich plasma system where thrombin stimulation of nucleotide release also resulted in a marked accumulation of intracellular cyclic AMP. Thrombin did not appear to stimulate the release of intracellular cyclic AMP.

The mechanism underlying these observations was not apparent. The thrombin had no measurable inhibitory effect on platelet phosphodiesterase activity in either intact washed cells or the platelet homogenate supernates. Furthermore, thrombin inhibited, rather than stimulated, platelet adenyl cyclase activity in both intact washed cells and washed platelet particulate fractions. Of note, however, was the finding that thrombin did not completely inhibit the adenyl cyclase activity of prostaglandin-stimulated cells. Further work is needed to clarify the significance of this observation.

Nonetheless, the accumulation of intracellular cyclic AMP in response to thrombin observed in the present study suggests that the antagonistic actions of various agents on the platelet release reaction, thought to underlie platelet function, may depend upon a mechanism more intricate than a straightforward mediation through directly opposite effects on platelet cyclic AMP.

INTRODUCTION

In recent years, several investigators have implicated cyclic AMP as a factor in the control of normal platelet function (1, 2). Increments in cyclic AMP levels have been observed in response to various agents that inhibit platelet aggregation (3–5). Conversely, some agents which stimulate or augment platelet aggregation have been associated with reduced cyclic AMP levels (1, 2, 6, 7). This has led to the concept that the antagonistic actions of various substances on platelet function may be mediated by opposite effects on cyclic AMP concentrations.

The results of the present study do not support this concept. Washed human platelets were found to react to human thrombin by accumulating *increased* intracellular levels of cyclic AMP rather than the decreased levels others have reported. This increase was observed with amounts of thrombin previously shown to induce platelet aggregation (8). The increase occurred in conjunction with the release of platelet nucleotides and calcium into the surrounding medium, a process thought either to precede or accompany platelet aggregation (9). The present study thus stresses the need for further evaluation of the biochemical events that characterize and determine platelet function as well as the

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role of cyclic AMP as a possible physiological determinant of normal platelet activity.

METHODS

Freshly drawn blood from normal male subjects without drug exposure was anticoagulated with a 10% solution of EDTA (0.15 cc per 10 cc blood) and cooled to 4°C. Washed platelets were obtained as previously described (10) and resuspended in a 25 mM Tris-HCl buffer, pH 7.4, containing 140 mM NaCl and 0.3 mM EDTA.

Duplicate portions of the washed platelet suspensions, containing 700,000–1,500,000 platelets per mm^3, were kept either at 4°C or preincubated at 37°C for 5 min. For dose-responses studies, human thrombin (provided by Dr. D. Aronson, Division of Biologic Standards, National Institutes of Health), ranging between 0.0625 and 1.0 U/cc final incubation concentration, was added to the platelets and incubation continued for 5 min. The time-course of thrombin-induced nucleotide and calcium release was studied by incubating platelet suspensions with 0.25–0.50 U/cc of thrombin at 37°C for times varying between 5 sec and 5 min. Theophylline (from Matheson, Coleman and Bell, Cincinnati, Ohio), 3.0×10^{-3} M final concentration, or prostaglandin E_1 (from Dr. J. Pike. The Upjohn Company, Kalamazoo, Mich.), 3.0×10^{-6} M final concentration, was added to some tubes before initial incubation. Appropriate controls at 4 and 37°C with or without thrombin, prostaglandin, and theophylline were run concurrently in each experiment. After incubation, all samples were cooled to 4°C to stop the release reaction and spun at 2250 *g* for 15 min.

Similar studies were performed using citrate (0.1 cc of 10% Na citrate in normal saline per 10 cc blood) and normal saline rather than an EDTA-anticoagulated system. Experimental procedures and materials were otherwise identical to those already described.

Supernates were added to 2.5 M perchloric acid (0.5 M final concentration) and the total nucleotide content read at 260 mμ on a Beckman spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) (11). These portions were then diluted 1:2 with 0.15 M lanthanum chloride and assayed for calcium content on a Perkin-Elmer 303 atomic absorption spectrophotometer (Perkin Elmer Corp., Norwalk, Conn.) (12). In some experiments, a portion of the supernate was also assayed for cyclic AMP content as described below together with the platelets from the same samples.

Platelet pellets were kept at 4°C. Their cyclic AMP was extracted by a modification of procedures described by Steiner, Kipnis, Utiger, and Parker (13). After addition of 0.7 cc of 50 mM Tris-HCl buffer, pH 7.4, without EDTA, the platelets were sonicated at 4°C for 15 sec on a Sonifier W140D sonicator set at "25" (Heat Systems—Ultrasonics, Inc., Plainview, N. Y.). An equal volume of 10% trichloroacetic acid (TCA) was then added to each portion and, after thorough mixing, the suspensions were spun at 2250 *g* for 15 min. The supernates were extracted with three changes of anhydrous ether to remove the TCA, heated for 3 min at 65°C to evaporate any ether that remained, rapidly frozen in dry ice and acetone, and lyophilized.

Cyclic AMP concentrations were determined by a modification of the radioimmunoassay procedures developed by Steiner et al. (13). The 2′0-succinyl tyrosine methyl ester of cyclic AMP was synthesized and iodinated with ^{125}I to prepare the cyclic AMP tracer (ScAMP-^{125}I).[1] Antibody to cyclic AMP (ScAMP-Ab) was produced by injecting human serum albumin succinyl cyclic AMP in Freund's adjuvant intradermally into rabbits at 2 to 4-week intervals and harvesting the serum at 4 6 wk.

The radioimmunoassay incubation system, in a final volume of 500 μl, contained $5–10 \times 10^3$ cpm of the ScAMP-^{125}I tracer, antibody to ScAMP diluted with 0.1 M 2-(*N*-morpholino) ethanesulfonic acid (MES buffer), pH 6.1, to provide a concentration with > 20% binding, and a portion of lyophilized sample dissolved in 0.1 M MES buffer. Assay tubes were kept at 4°C and allowed to incubate for at least 18 hr.

After incubation, 1.5 cc of a 16% polyethylene glycol solution containing 0.8 mg/ml bovine gamma globulin was added to each incubation mixture to precipitate the cAMP-antibody complex (14). The portions were centrifuged at 2200 *g* for 1 hr, the supernates were discarded, and the tubes allowed to drain for at least 2 hr. The samples were counted for ^{125}I in a Packard 5022 gamma counter (Packard Instrument Co., Inc., Downers Grove, Ill.). Samples for total counts, background, total binding, and a semi-logarithmic standard curve with a range of 0.4–204.8 pmoles cyclic AMP were prepared for each assay.

Separate experiments were done to confirm the identity of the substance being assayed as cyclic AMP, according to the methods of Aurbach and Houston (15). Washed platelets, which had been incubated for 5 min at 37°C with thrombin (0.5 U/cc), were sonicated at 4°C as above and then heated at 105°C for 3 min to destroy endogenous phosphodiesterase activity (16). After cooling, samples were incubated at 37°C for 20 min with *active* beef heart phosphodiesterase (PDE) purified according to Butcher and Sutherland (17), *boiled* exogenous PDE, or buffer. Trichloroacetic acid was then added to each portion and the samples were centrifuged, extracted, lyophilized, and assayed for cyclic AMP together with the specimens above.

Because of the differences between the results obtained in the present washed platelet system and data reported in previous studies using platelet-rich plasma (2–4), further experiments were performed using platelet-rich plasma anticoagulated with EDTA, maintained at 37°C and exposed either to buffer or thrombin. Other portions of platelet-rich plasma were kept at 4°C. After a 5 min incubation, the platelet-rich plasma was centrifuged at 2200 *g* for 10 min. The resulting platelet pellets and the platelet-poor plasma were separately extracted and assayed for cyclic AMP as described above.

In an attempt to determine the mechanism of action of thrombin on the changes observed in platelet cyclic AMP, several experiments were performed to measure the effect of thrombin on platelet adenyl cyclase and phosphodiesterase activity. Intact washed platelets were incubated for 5 min with or without human thrombin (0.5 U/cc), separated from their supernate, and sonicated. The resulting homogenate was centrifuged for 15 min at 15,000 *g* to obtain a particulate fraction and a supernatant fraction (10). The particulate fraction was assayed for adenyl cyclase activity by the conversion of ATP-α-^{32}P to 3′,5′-cyclic AM^{32}P (10), applying methods described by Krishna, Weiss, and Brodie

[1] *Abbreviations used in this paper:* MES buffer, 2-(*N*-morpholino) ethanesulfonic acid; PDE, phosphodiesterase; PGE_1, prostaglandin E_1; ScAMP-Ab, antibody to cyclic AMP; ScAMP-^{125}I, cyclic AMP tracer; T_3, triiodothyronine; TCA, trichloroacetic acid.

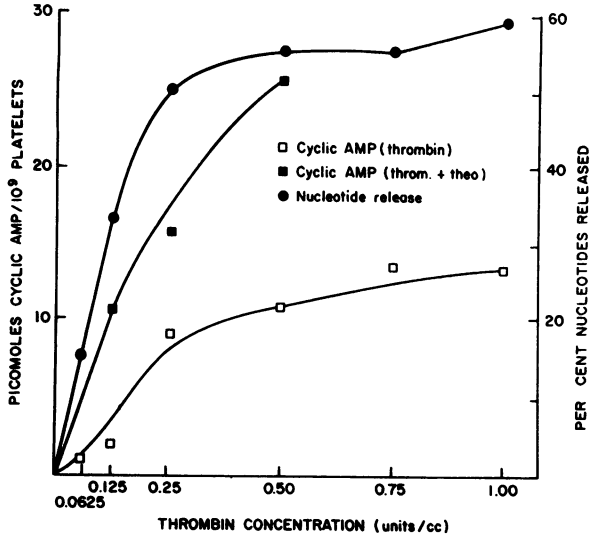

FIGURE 1 Dose-response relationship of thrombin-induced release reaction and intracellular cyclic AMP accumulation. Washed human platelets were incubated with human thrombin in doses ranging from 0.0625 to 1.00 U/cc. The right ordinate axis measures the per cent of total platelet nucleotides released (total platelet nucleotide content was 8–20 mμmoles/10^8 platelets). The left ordinate axis measures intracellular picomoles of cyclic AMP/10^9 platelets. All plotted values represent net release or net cyclic AMP accumulation (average base line at 37°C without thrombin was 10 pmoles cyclic AMP/10^9 platelets).

(18). The stimulatory effect of prostaglandin E_1 or NaF on the cyclase activity of these fractions was also assessed, using procedures described in previous reports (10, 18). Phosphodiesterase activity in the supernatant fraction was measured by the conversion of 3',5'-^{3}H-cyclic AMP to AMP-^{3}H, using an assay system described by Chase, Fedak, and Aurbach (19).

In other experiments, washed intact platelets that had *not* been preincubated with thrombin were similarly sonicated and centrifuged and the resulting particulate and supernatant fractions assayed respectively for adenyl cyclase and phosphodiesterase activity as above. In these studies, however, thrombin was added to some of the incubation tubes to ascertain the effect of thrombin in the assay itself.

RESULTS

Washed platelet suspensions incubated for 5 min at 37°C with increasing doses of thrombin release increasing amounts of nucleotides into the surrounding medium (Fig. 1). This reaction is maximal when 50–60% of the platelet's total nucleotide content has been released, and occurs at a thrombin concentration of 0.25–0.50 U/cc.

In such studies, nucleotides and calcium are released in parallel, with a constant molar ratio of 0.6–0.7. At 4°C, release in response to thrombin is negligible. Incubation of platelets at 37°C in the *absence* of thrombin also results in but minimal release.

Prostaglandin E_1 (PGE_1, 3×10^{-6} M) inhibits 30–50% of maximal thrombin-induced nucleotide and calcium release at 37°C (Fig. 2). Theophylline at 3×10^{-3} M in the same experiments inhibits maximal platelet nucleotide release by 20–40%. Neither agent measurably affects the release reaction of platelets incubated with thrombin at 4°C or without thrombin at 37°C.

In the same platelet samples, a comparable set of relationships characterizes the effects of thrombin on the accumulation of intracellular cyclic AMP during the release reaction. At 4°C, levels of cyclic AMP range between 8 and 12 pmoles per 10^9 platelets (Fig. 3). The addition of thrombin (or prostaglandin E_1, or theophylline) at this temperature does not change these levels. Increments of only 1–2 pmoles per 10^9 platelets are seen when the samples are incubated *without* any of these agents at 37°C.

Small amounts of thrombin at 37°C induce the accumulation in platelets of gradually *increasing* cyclic AMP concentrations (Fig. 1). At 0.25 U of thrombin per cc of platelet suspension, intracellular cyclic AMP levels are increased nearly twofold above base line. Higher concentrations of thrombin up to 1.0 U/cc appear to stimulate only slightly greater increases above this level. No cyclic AMP appears in the supernate during thrombin stimulation.

In the presence of prostaglandin E_1, which alone causes a four- to fivefold increase in cyclic AMP, the stimulatory effect of thrombin appears to be slightly additive (Fig. 3). In contrast, thrombin together with

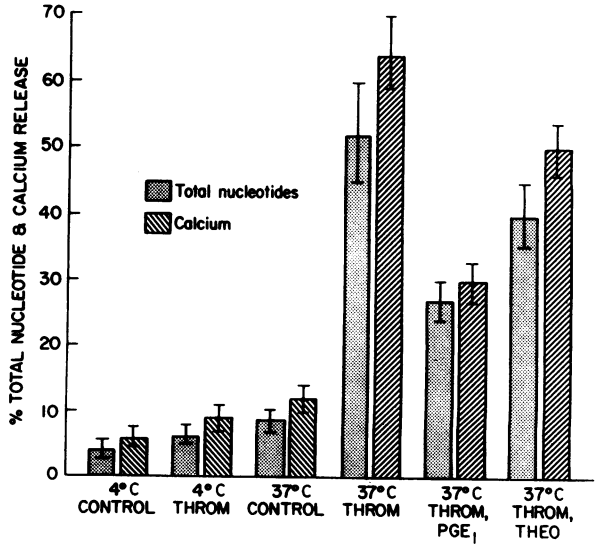

FIGURE 2 Effect of thrombin, prostaglandin E_1, and theophylline on the platelet release reaction. The per cent of total nucleotide and calcium release in response to thrombin (0.5 U/cc), prostaglandin E_1 (3×10^{-6} M), and/or theophylline (3×10^{-3} M) is given as the mean of 10 different determinations ±1 SD.

theophylline induce a synergistic increase in intracellular platelet cyclic AMP levels (Fig. 3). Similarly, in the dose-response studies the presence of theophylline *amplifies* the amount of cyclic AMP accumulated in response to each dose of thrombin tested (Fig. 1). The linearity of the dose-response relationship with concentrations of thrombin up to 0.25 U/cc is maintained.

The time-course of the release reaction of washed platelets in response to thrombin is extremely rapid. At least 50–60% of the release of nucleotides and calcium is complete within 5 sec of incubation with thrombin (0.5 U/cc) (Fig. 4). At least 80–90% of release has occurred by 15 sec. By 30 sec, the total amount of releasable nucleotides and calcium, amounting to approximately 60% of the platelet's total content of these substances (20), appears in the extracellular medium. Incubation to 5 min does not change these levels.

In these time-course studies, cyclic AMP accumulation in response to thrombin seems to be slower than the release of nucleotides and calcium (Fig. 4). At a time when the release reaction is 50–60% complete (5 sec after the addition of thrombin), the amount of intracellular cyclic AMP observed approaches only 10–20% of levels reached with longer incubation times. At 15 sec, when the release reaction is 80–90% complete, the amount of cyclic AMP increase is only 60–70% of ultimate concentrations. Not until 30 sec of incubation do cyclic AMP levels approach the expected twice-base line or greater concentrations observed as a maximal response to thrombin.

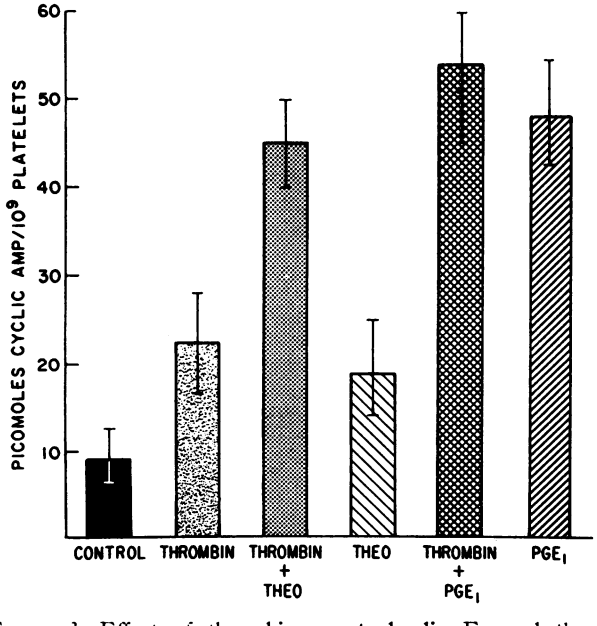

FIGURE 3 Effect of thrombin, prostaglandin E_1, and theophylline on platelet cyclic AMP. Platelet pellets from six of the experiments represented in Fig. 2 were assayed for cyclic AMP content. Values in picomoles cyclic AMP per 10^9 platelets represent the mean ±1 SD.

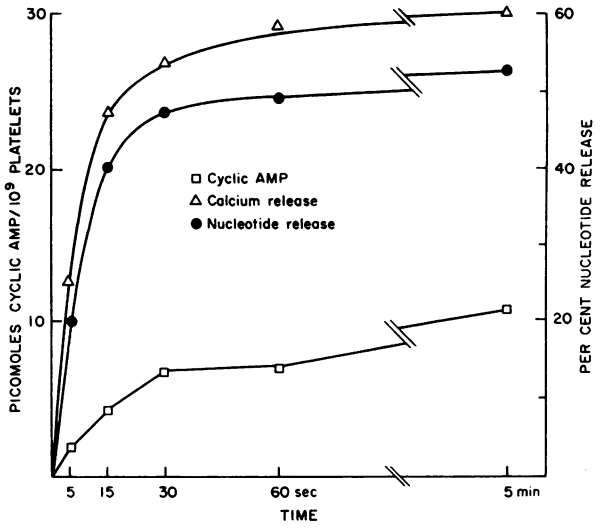

FIGURE 4 Time-course of thrombin-induced release and intracellular cyclic AMP accumulation. Washed human platelets were incubated at 37°C with thrombin (0.5 U/cc) for times varying between 5 sec and 5 min. The right ordinate axis measures per cent nucleotides (and calcium) released. The left ordinate axis represents picomoles of intracellular cyclic AMP/10^9 platelets. All plotted values represent net release or net cyclic AMP accumulation (average control base line values of cyclic AMP accumulation at 37°C was 12 picomoles/10^9 platelets).

As seen in the dose-response studies, the addition of theophylline to samples in the time-course studies also amplifies the thrombin-induced increments of cyclic AMP that occur (Fig. 5). Even at 5 sec, when there appears only a small increment in cyclic AMP levels in response to thrombin alone, theophylline greatly magnifies the levels of cyclic AMP observed. At the same time, the theophylline inhibits the release reaction. Through this dual effect, theophylline completely obscures the lag, seen in the presence of thrombin alone, between cyclic AMP accumulation and the occurrence of platelet nucleotide release.

The cyclic AMP concentration in the supernate of control platelets either at 4 or 37°C is less than 0.4 pmoles/10^9 platelets. Incubation of these samples with thrombin or theophylline does not induce an increase in extracellular cyclic AMP levels, despite the increase in intracellular levels described above. The effect of prostaglandin E_1 on extracellular concentrations was not measured.

When citrate and a normal saline buffer are used instead of the EDTA and Tris buffer in the washed platelet system, no increase in intracellular cyclic AMP is seen in response to thrombin. Base line levels of

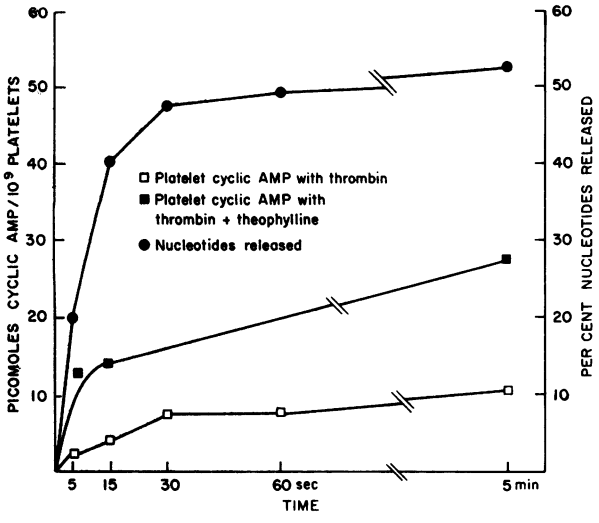

FIGURE 5 Effect of theophylline on the time-course of cyclic AMP accumulation in response to thrombin. In some of the samples described in Fig. 4, theophylline (3×10^{-3} M) was added to the incubation system and its effect on the time-course of cyclic AMP accumulation measured.

cyclic AMP, however, are maintained in all of the platelet samples. Furthermore, the release of calcium and nucleotides in response to thrombin occurs to the same extent as in the EDTA-anticoagulated system.

In the plasma system the effect of thrombin is even more striking than in the washed platelet preparations (Table I). At 4°C, base line levels are similar to the cyclic AMP concentrations observed in the washed platelet systems described above. These levels are also comparable to cyclic AMP levels seen in plasma platelets maintained at 37°C. The addition of thrombin to these platelets at 37°C induces a twofold and as great as a fourfold increase in cyclic AMP levels. The use of sonication before the addition of TCA in the cyclic AMP extraction procedures does not seem to alter the basic phenomenon observed in the present investigation. Extracellular plasma concentrations of cyclic AMP in the same experiments are high enough (150–200 pmoles/cc) to prevent an interpretation of small changes in extracellular cyclic AMP levels in response to thrombin. The effect of citrate in the plasma medium was not evaluated.

If the increase in radioimmunoassayable material which occurs with thrombin in either the washed or the plasma system is indeed cyclic AMP, it would be predicted that exposure to endogenous or exogenous phosphodiesterase (PDE) would eliminate this increment. Washed platelets heated to 95°C to destroy endogenous PDE (16) when incubated with either buffer or boiled exogenous PDE, maintain the cyclic AMP levels accumulated in response to thrombin (Fig. 6). Incubation with *active* exogenous PDE destroys the increments that thrombin induces. Prolonged incubation at 37°C of platelets *not* preheated, allows *endogenous* platelet PDE to destroy the increased cyclic AMP levels that occur with thrombin. In fact, the 20 min of incubation reduces these levels to half base-line. As previously reported in other tissues (15), this establishes the identity of the substance being assayed as cyclic AMP.

Particulate fractions obtained from platelets exposed to thrombin have less than half the basal activity of adenyl cyclase compared to platelets incubated without thrombin (Table II A). Similarly, the stimulation of platelet cyclase activity by prostaglandin E_1 added to these preparations is less than half as great as in prostaglandin-stimulated control platelet particulate fractions. It is important to note, however, that the stimulation of cyclase activity by prostaglandin in these experiments is *not completely* inhibited by the previous exposure to thrombin. Rather, there is still an 8- to 10-fold increase in cyclase activity in response to the exogenous PGE_1, despite the presence of the active thrombin (0.25–0.50 U/cc). Moreover, the stimulation of enzyme activity by NaF is only mildly depressed by prior incubation of the intact platelets with thrombin. Similar changes have also been seen by other investigators (8).

The addition of thrombin (0.5 U/cc) to the cyclase assay incubation mixture itself also has a major effect on adenyl cyclase activity (Table II B). Basal activity is depressed by 60%. Prostaglandin-stimulated activity, however, is inhibited by only 40%. As in the intact platelets, all of the PGE_1-stimulated activity is not abolished by simultaneous exposure to thrombin. Cyclase activity is still increased to 8 times basal levels. Furthermore, NaF-stimulated enzyme activity is again only mildly depressed.

TABLE I

Thrombin Effect on Intracellular Cyclic AMP in Platelet-Rich Plasma

Exp. No.	4°C control	37°C control	37°C thrombin
	pmoles cyclic AMP/10⁹ platelets		
1	12.3	11.1	52.0
2	5.4	5.5	12.2
3	10.8	10.5	22.0
4	15.7	22.5	37.7

In the above experiments, platelet-rich plasma was incubated for 5 min at 4 or 37°C with or without thrombin. Intracellular concentrations of cyclic AMP were measured by radioimmunoassay as was done in the washed platelet system. Extraction procedures were also the same; in experiments 3 and 4, however, the sonication step was omitted.

No effect on normal phosphodiesterase activity is apparent in platelets incubated while intact with thrombin. Similarly, platelet homogenates assayed for phosphodiesterase activity in the presence of thrombin appear to have no alteration in enzyme action compared with control platelets.

DISCUSSION

Results of the present study raise questions about the commonly-held view that stimulation of platelet function is mediated by a lowering of intracellular cyclic AMP. At concentrations of thrombin that cause maximal release of nucleotides and calcium from platelets, and are known to cause complete platelet aggregation, there occurs at least a twofold increase in platelet cyclic AMP levels both in washed platelet preparations and platelets from platelet-rich plasma. This increase lags by several seconds the occurrence of nucleotide and calcium release.

Several questions are posed by these data. First, what explanations are there to account for the discordance between results in the present study, describing a thrombin-induced *increase* in intracellular cyclic AMP, and the generally-accepted hypothesis that aggregating agents work by *lowering* platelet cyclic AMP content?

TABLE II

Effect of Thrombin on Platelet Adenyl Cyclase Activity

Substance tested with thrombin	Thrombin concn. used	Adenyl cyclase activity assay		
		Buffer	PGE_1	NaF
	U/cc	*pmoles cyclic AMP/10 min per mg protein*		
A. Intact washed platelets	None	130	2300	880
	0.25	56	1160	730
	0.50	38	1032	632
B. Particulate fraction from washed platelets	None	155	1580	695
	0.50	56	940	545
	5.0	56	675	450

In A, intact washed platelets were incubated with or without thrombin, chilled to 4°C, centrifuged, separated from the supernate, resuspended in fresh Tris buffer, and sonicated. The particulate fraction obtained was assayed for adenyl cyclase activity in the absence of thrombin. Prostaglandin E_1 or NaF was added to some portions in the assay to stimulate cyclase activity.

In B, platelets were washed and resuspended in Tris-HCl buffer pH 7.4. They were not preincubated with thrombin. A sonicated pellet was obtained as in A and assayed for adenyl cyclase activity, but this time in the presence or absence of thrombin. Prostaglandin E_1 or NaF was added to some portions in the assay as above to stimulate cyclase activity.

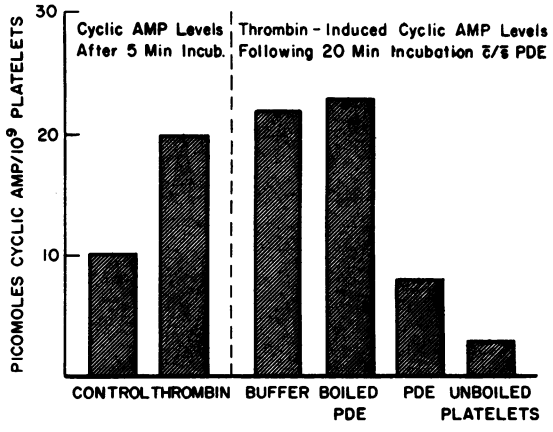

FIGURE 6 Effect of exogenous phosphodiesterase on radioimmunoassayable unknown (presumably cyclic AMP). In two separate experiments, exogenous beef heart phosphodiesterase (PDE) was shown to decrease thrombin-stimulated intracellular cyclic AMP to base line (control) levels. Incubation of the thrombin-stimulated cells (heated to destroy endogenous phosphodiesterase) with buffer or boiled PDE did not decrease the thrombin-induced cyclic AMP increments. Prolonged incubation of unheated platelets with either buffer, exogenous PDE, or boiled exogenous PDE not only destroyed the thrombin-induced increments but also reduced cyclic AMP concentration to one-half control levels.

Second, by what mechanism does thrombin induce the observed accumulation of intracellular cyclic AMP? And third, but most important, does cyclic AMP play a determining or mediating role in platelet function and, if so, what is this role?

Aggregating agents: increased or decreased cyclic AMP? Previous investigations have reported that agents with antagonistic actions on platelet function work through opposite effects on cyclic AMP levels: substances which inhibit platelet function increase cyclic AMP whereas those that stimulate platelet function cause decreased cyclic AMP (2–4). The contradictory results in the present investigation, in which thrombin as an aggregating agent was found to *increase* intracellular platelet cyclic AMP, prompted a reexamination of the methods used in the present work to see if the data merely represent artifact.

The preparation of washed platelets entailed several centrifugation steps. Others have suggested that centrifugation induces the loss of intracellular cyclic AMP, presumably via an activation of platelet phosphodiesterase (1). However, the base line levels of cyclic AMP in the washed platelets were similar to those of platelets kept in plasma and not subjected to repeated centrifugation, both in the present study (Table I) and in work previously reported by Cole, Robison, and Hartmann (21). Furthermore, in each experiment duplicate portions at 4 and 37°C with and without thrombin and/or theophylline were always run to validate the

increase in cyclic AMP observed in response to thrombin. The presence of these controls in each experiment appeared to nullify the possible decremental effect of centrifugation that otherwise might perhaps have obscured the phenomena being reported.

In order to study the activity of various platelet enzymes in our washed preparations, as well as the effects of time and thrombin dose on the release reaction and the accumulation of cyclic AMP, the platelets in the present investigation were kept chilled at 4°C when not being incubated. Such chilling has also been reported to effect platelet cyclic AMP levels (1). As was true in the case of centrifugation, however, the internal controls in each experiment in the present work permitted reliable observations of the stimulatory effect of thrombin on cyclic AMP levels since *all* of the samples had been chilled. Moreover, chilling seemed to have no effect on the base line cyclic AMP levels; nor did it decrease the prostaglandin-stimulated cyclic AMP increments also reported by others (*2*, 4).

Procedures used to extract platelet cyclic AMP were basically similar to methods described for use with platelets (1, *22*) as well as other tissues (13). The only major change was the use of sonication in order to study the enzyme activities in the various samples. However, this seemed to have no effect on cyclic AMP levels (Table I), presumably because the procedures were conducted at 4°C throughout, preventing thereby any sonication-stimulated activity of inhibitory or stimulatory enzymes. Similarly, the radioimmunoassay techniques used in the present work had already been well-described and reliably used in other cell types (13, 23). These procedures provided reproducible results in the present study as well, both in the washed platelet system and in platelet-rich plasma. The validity of these results as being truly representative of the changes in platelet cyclic AMP described above was shown by destroying the thrombin-induced cyclic AMP increments with exogenous beef heart phosphodiesterase (Fig. 6), using methods previously employed in other tissues (15).

As a final check on the reality of the phenomenon observed in the washed platelet system, however, the same experimental and radioimmunoassay procedures were applied to the platelet-rich plasma system used in previous investigations. The results obtained (Table I), were again in conflict with the hypothesis suggested by earlier workers. The thrombin was repeatedly found to induce at least a twofold increase in intracellular cyclic AMP. Moreover, base line concentrations of cyclic AMP were comparable to those observed in the washed platelet system.

Such data suggest that the phenomenon described in the present study was not necessarily due to methodological artifact. This conclusion, however, prompted a search of earlier investigations for possible reasons to explain the discrepancies reported. Several possible explanations were uncovered by such a review.

The conceptual role of cyclic AMP as a possible mediator of platelet function was first suggested by studies that documented the stimulation of cyclic AMP levels by prostaglandin E_1, an inhibitor of platelet aggregation (*2*–4, *24*). Similar platelets, with intracellular cyclic AMP levels already elevated by prior exposure to prostaglandin E_1, were then incubated with one of the aggregating agents (*2*, 6). This resulted in a decrease in the prostaglandin-elevated cyclic AMP concentrations. The interpretation of these data led to the hypothesis that aggregating and inhibiting substances exert their opposing effects on platelet function through opposite effects on platelet cyclic AMP.

In these studies the effect of prostaglandin on stimulating greater cyclic AMP production was fairly straightforward. However, the effect of the releasing agents on decreasing cyclic AMP levels was based on evidence somewhat less direct, since the effects of these agents on *base line* levels of cyclic AMP were not assessed. The more direct evidence in the present investigation, testing thrombin alone on base line cyclic AMP concentrations, as well as recent reports by Haslam and Taylor (25) describing an epinephrine-induced increase above base line in platelet cyclic AMP levels after incubation of platelet-rich plasma with epinephrine alone, strongly challenge this hypothesis.

In addition, several differences of methodology may have led to the discordance of results being discussed. For example, in other investigations incubation times usually were long, at least 15 to 20 min. During this time, endogenous platelet phosphodiesterase could have destroyed any increase in cyclic AMP that had occurred in response to the aggregating agent. This, in fact, was seen in the present study when platelet homogenates incubated with buffer for 20 min at 37°C after the initial 5 min experimental incubation with or without thrombin displayed half-base line or lower levels of cyclic AMP (Fig. 6). A somewhat similar finding was reported by Haslam and Taylor who described an accumulation of cyclic AMP in platelets in response to epinephrine during the 1st min of incubation and an inhibition of this accumulation during the 2nd min (25).

Second and perhaps most important, the anticoagulant that was used could have effected the results observed in the various studies. This was suggested by several findings. First, when citrate rather than EDTA was used in the present washed platelet system, no increment in platelet cyclic AMP in response to thrombin was observed, even though all other conditions were identical and the release reaction proceeded normally.

Second, thrombin *was* found to have a pronounced stimulatory effect on cyclic AMP levels even in the plasma system when EDTA was the anticoagulant (Table I) in contrast to findings in previous studies where citrate was used (2, 3, 6). Thus, citrate may have prevented the stimulatory effects of the aggregating agents on platelet cyclic AMP levels.

Just how this may have occurred is suggested by earlier demonstrations of significant differences between citrate and EDTA in sequestering various cations (26). On this basis, subsequent investigators found that normal platelet function is effected by a delicate balance between Mg^{++} and Ca^{++} and that in vitro this balance is governed by the type of anticoagulant used in the system (27–29). Further insight was provided by the finding that the balance between Mg^{++} and Ca^{++} is critical in determining the activity of adenyl cyclase in other tissues (19, 30). In these studies, as well as in platelets in the present work, 10^{-4} M Ca^{++} partially inhibited and 10^{-2} M Ca^{++} completely inhibited PGE_1- and NaF-stimulated adenyl cyclase activity. Moreover, in previous studies using washed platelet preparations (10), just the use of citrate in preventing platelet clumping was found to be enough to prevent the striking stimulatory effect of PGE_1 on adenyl cyclase activity that was seen when EDTA was used instead. Such inhibition of cyclase activity, just by varying the Ca^{++} concentration, or more simply by changing the cation chelator, may have been adequate in the previous studies (2, 3, 6) to prevent cyclic AMP formation. Use of the more powerful chelating agent EDTA (26, 27) as the anticoagulant in both the washed platelet system as well as in the platelet-rich plasma in the present work may have circumvented this inhibition and allowed the stimulation of cyclic AMP that was observed.

How does thrombin stimulate cyclic AMP accumulation? Conceivably, the thrombin-induced accumulation of intracellular cyclic AMP might have been the result of decreased cyclic AMP release from cells because of exposure to thrombin. Data in the present study did not support this concept. Base line levels of extracellular cyclic AMP were less than 0.4 pmoles/10^9 platelets. The presence of theophylline in the bathing medium to inhibit any exogenous phosphodiesterase activity over the 5 min incubation time did not alter the amount of cyclic AMP found extracellularly. This would argue against the possibility that cyclic AMP is released constantly from platelets so as to inhibit aggregation and that thrombin, by interfering with this release, produces an increased intracellular cyclic AMP level.

For the most part, the increased levels of cyclic AMP observed in response to various substances in other cell types have been found to be due to either a stimulation of adenyl cyclase activity and the production of cyclic AMP or an inhibition of phosphodiesterase activity and the decreased destruction of cyclic AMP. In the present study, both of these possibilities as well were examined.

First, human thrombin was shown to have no measurable effect on platelet phosphodiesterase activity in supernatant fractions from platelet homogenates. Similarly, Brodie, Baenziger, Chase, and Majerus could not demonstrate any inhibitory effect (8). Such studies suffer from their inability to determine phosphodiesterase activity within the actual cell and its compartments. However, the data at hand suggest that the elevations in cyclic AMP did not result from an inhibition of cyclic AMP destruction.

The synergistic effect of thrombin on platelet cyclic AMP with theophylline, a phosphodiesterase inhibitor (24), suggested that the action of thrombin on the accumulation of cyclic AMP might be mediated by a stimulation of adenyl cyclase. It was found, however, that thrombin added either to intact washed platelets *before* assaying them for cyclase activity or to washed platelet homogenates *during* the cyclase assay inhibited rather than stimulated adenyl cyclase activity (Table II).

These results were similar to those of earlier studies that also described an *inhibition* of cyclase activity by bovine thrombin (31–33). More recent work by Brodie et al., using human thrombin, also demonstrated an inhibition of cyclase activity in intact platelets by as little as 0.1 U/cc, a level comparable to that used in the present investigation. Moreover, the thrombin in these studies had its greatest effect on prostaglandin-stimulated cyclase activity, a phenomenon that was also seen to occur in the present work. The two sets of data differed in the effect of thrombin on sonicated platelet homogenates: the present data demonstrated marked inhibition of both base line and prostaglandin-stimulated cyclase activity in washed platelet particulate fractions; data from the work of Brodie et al. did not detect any inhibition. At present, we cannot reconcile or explain the differences. In any event, a direct effect of thrombin in stimulating cyclase activity to explain the increments of cyclic AMP observed could not be demonstrated. Indeed, the opposite seemed to be true.

However, further analysis of the data disclosed that perhaps an *indirect* effect by thrombin could have stimulated cyclase activity. In the assays for adenyl cyclase, prostaglandin E_1 was found to stimulate enzyme activity 15- to 18-fold. The addition of thrombin to the assay incubation medium, or the prior addition of thrombin to intact platelets, seemed to inhibit the prostaglandin-stimulated activity by 40–50%. Thus, prostaglandin-stimulated activity was *not* completely abolished; an 8- to 10-fold increase in activity above base line was still apparent. Evidently, the amount of human

thrombin used, presumably a physiological level (8), was not enough to override completely the effect of PGE_1 in the present study. This is significant, for recent investigations have reported that thrombin induced human platelets to produce and release prostaglandins E_2 and $F_{2\alpha}$ (34). In human platelets, both of these prostaglandins in high enough concentrations may be capable of stimulating adenyl cyclase (10) and the formation of cyclic AMP. This stimulatory effect in vivo might override the direct inhibitory effect of thrombin on cyclase activity seen in vitro and explain the phenomenon of a thrombin-induced increase in intracellular cyclic AMP observed in the present investigation.

In any case, the mechanism by which thrombin induced the accumulation of cyclic AMP in platelets in the present investigation remained conjectural. This is not a situation without precedent. Recent studies of the effect of triiodothyronine (T_3) in increasing cyclic AMP levels in fat cells could not document the means by which this occurred (35). The investigators did not consider the possibility that the T_3 might stimulate prostaglandin production. In a somewhat different investigation, an inhibitor of glucagon-stimulated cyclase activity was found in the cytoplasm of hepatocytes (36). The possible presence of a similar inhibitor in platelets could account for the absence of thrombin effect observed in the in vitro systems used in the present study.

What is the role of cyclic AMP in platelet function? Results in the present study challenge the hypothesis that a decrease in intracellular cyclic AMP is the factor that mediates platelet aggregation. The data indicate, rather, that we may be dealing with a spectrum of cyclic AMP accumulation in response to various agents with *opposite* effects on platelet activity. At the one end, aggregating agents such as thrombin in the present study and epinephrine in the report by Haslam and Taylor (25), induce the accumulation of small amounts of cyclic AMP, perhaps by stimulating the production and release of prostaglandins from the cells. At the other end, exogenous prostaglandin E_1, a potent inhibitor of platelet aggregation in vitro, stimulates the production of greater amounts of cyclic AMP.

If we assume that we are dealing with a homogeneous platelet population, as seems to be the case from morphological studies,[9] the cyclic AMP accumulated in response to thrombin in the present investigation may be a manifestation of several possible occurrences. For one, the cyclic AMP may in some way be involved with the secretory processes associated with the platelet release reaction. In other cell types, substances that stimulate cell secretion also have been found to increase the cellular content of cyclic AMP (37). A second possibility is that the elevations in cyclic AMP may be a nonspecific response to various agents and have nothing to do with controlling or mediating the process of aggregation at all. This possibility has been suggested independently by Boullin, Green, and Price (38) in their recent studies on ADP-induced platelet aggregation.

The third, and most intriguing possibility, involves postulation of a feedback mechanism of platelet function. Here, the accumulation of cyclic AMP in response to low-dose thrombin may represent an attempt by the platelet to make the aggregating process reversible.

At present, such postulations must remain conjectural. Any additional hypothesis as to the mechanism of action of thrombin, prostaglandins, and cyclic AMP as physiological determinants of normal platelet function must await further investigation.

[9] Droller, M. J. Ultrastructural study of the effects of aggregating agents and their inhibitors on the platelet release reaction. In preparation.

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REFERENCES

1. Salzman, E. W., E. B. Rubino, and R. V. Sims. 1970. Cyclic 3',5'-adenosine monophosphate in human blood platelet. III. The role of cyclic AMP in platelet aggregation. *Ser. Haematol.* **3**: 100.
2. Robison, G. A., A. Arnold, and R. C. Hartmann. 1969. Divergent effects of epinephrine and prostaglandin E_1 on the level of cyclic AMP in human blood platelets. *Pharmacol. Res. Commun.* **1**: 325.
3. Salzman, E. W., and L. L. Neri. 1969. Cyclic 3',5'-adenosine monophosphate in human blood platelets. *Nature (Lond.)*. **224**: 609.
4. Vigdahl, R. L., N. R. Marquis, and P. A. Tavormina. 1969. Platelet aggregation. II. Adenyl cyclase, prostaglandin E_1, and calcium. *Biochem. Biophys. Res. Commun.* **37**: 409.
5. Ball, G., G. G. Brereton, M. Fulwood, D. M. Ireland, and P. Yates. 1970. Effect of prostaglandin E_1 alone and in combination with theophylline or aspirin on collagen-induced platelet aggregation and on platelet nucleotides including adenosine 3',5'-cyclic monophosphate. *Biochem. J.* **120**: 709.
6. Marquis, N. R., J. A. Becker, and R. L. Vigdahl. 1970. Platelet aggregation. III. An epinephrine induced decrease in cyclic AMP synthesis. *Biochem. Biophys. Res. Commun.* **39**: 783.
7. Moskowitz, J., J. P. Harwood, W. D. Reid, and G. Krishna. 1971. The interaction of norepinephrine and prostaglandin E_1 on the adenyl cyclase system of human and rabbit blood platelets. *Biochim. Biophys. Acta.* **230**: 279.
8. Brodie, G. N., N. L. Baenziger, L. R. Chase, and P. W. Majerus. 1972. The effects of thrombin on adenyl

cyclase activity and a membrane protein from human platelets. *J. Clin. Invest.* **51**: 81.
9. Holmsen, H., H. J. Day, and H. Stormorken. 1969. The blood platelet release reaction. *Scand. J. Haematol. Suppl.* **8**: 3.
10. Wolfe, S. M., and N. R. Shulman. 1969. Adenyl cyclase activity in human platelets. *Biochem. Biophys. Res. Commun.* **35**: 265.
11. Murer, E. H. 1968. Release reaction and energy metabolism in blood platelets with special reference to the burst in oxygen uptake. *Biochim. Biophys. Acta.* **162**: 320.
12. Murer, E. H. 1969. Thrombin-induced release of calcium from blood platelets. *Science (Wash. D. C.).* **166**: 623.
13. Steiner, A. L., D. M. Kipnis, R. Utiger, and C. Parker. 1969. Radioimmunoassay for the measurement of adenosine 3',5'-cyclic phosphate. *Proc. Natl. Acad. Sci., U.S.A.* **64**: 367.
14. Desbuquois, B., and G. D. Aurbach. 1971. Use of polyethylene glycol to separate free and antibody-bound peptide hormones in radioimmunoassays. *J. Clin. Endocrinol. Metab.* **33**: 732.
15. Aurbach, G. D., and B. A. Houston. 1968. Determination of 3',5'-adenosine monophosphate with a method based on a radioactive exchange reaction. *J. Biol. Chem.* **243**: 5935.
16. Vigdahl, R. L., J. Mongin, and N. R. Marquis. 1971. Platelet aggregation. IV. Platelet phosphodiesterase and its inhibition by vasodilators. *Biochem. Biophys. Res. Commun.* **42**: 1088.
17. Butcher, R. W., and E. W. Sutherland. 1962. Adenosine 3',5'-phosphate in biological materials. I. Purification and properties of cyclic 3',5'-nucleotide phosphodiesterase and use of this enzyme to characterize adenosine 3',5'-phosphate in human urine. *J. Biol. Chem.* **237**: 1244.
18. Krishna, G., B. Weiss, and B. B. Brodie. 1968. A simple, sensitive method for the assay of adenyl cyclase. *J. Pharmacol. Exp. Ther.* **163**: 379.
19. Chase, L. R., S. A. Fedak, and G. D. Aurbach. 1969. Activation of skeletal adenyl cyclase by parathyroid hormone *in vitro*. *Endocrinology.* **84**: 761.
20. Holmsen, H., H. J. Day, and E. Storm. 1969. Adenine nucleotide metabolism of blood platelets. VI. Subcellular localization of nucleotide pool with different functions in the platelet release reaction. *Biochim. Biophys. Acta.* **186**: 254.
21. Cole, B., A. Robison, and R. C. Hartmann. 1971. Studies on the role of cyclic AMP in platelet function. *Ann. N. Y. Acad. Sci.* **185**: 477.
22. Gilman, A. G. 1970. A protein binding assay for adenosine 3',5'-monophosphate. *Proc. Natl. Acad. Sci. U. S. A.* **67**: 305.
23. Steiner, A. L., C. W. Parker, and D. M. Kipnis. 1970. The measurement of cyclic nucleotides by radioimmunoassay. *In* Role of Cyclic AMP in Cell Function. P. Greengard and E. Costa, editors. Advances in Biochemical Psychopharmacology, Vol. 3. Raven Press, New York. 89.
24. Mills, D. C. B., and J. B. Smith. 1971. The influence on platelet aggregation of drugs that affect the accumulation of adenosine 3',5'-cyclic monophosphate in platelets. *Biochem. J.* **121**: 185.
25. Haslam, R. J., and A. Taylor. 1971. Effects of catecholamines on the formation of adenosine 3',5'-cyclic monophosphate in human blood platelets. *Biochem. J.* **125**: 377.
26. Chaberek, S., and A. E. Martell. 1959. Organic Sequestering Agents. John Wiley & Sons, Inc., New York. 514-572.
27. Des Prez, R. M., R. E. Bryant, J. A. Katz, and T. E. Brittingham. 1967. Platelet aggregation by magnesium ion. *Thromb. Diath. Haemorrh.* **17**: 516.
28. Hovig, T. 1964. The effect of calcium and magnesium on rabbit blood platelets aggregation *in vitro*. *Thromb. Diath. Haemorrh.* **12**: 179.
29. Herrman, R. G., W. B. Lacefield, and V. G. Crowe. 1970. Effect of ionic calcium and magnesium on human platelet aggregation. *Proc. Soc. Exp. Biol. Med.* **135**: 100.
30. Marcus, R., and G. D. Aurbach. 1971. Adenyl cyclase from renal cortex. *Biochim. Biophys. Acta.* **242**: 410.
31. Zieve, P. D., and W. B. Greenough III. 1969. Adenyl cyclase in human platelets: activity and responsiveness. *Biochem. Biophys. Res. Commun.* **35**: 462.
32. Salzman, E. W., and L. Levine. 1971. Cycle 3',5'-adenosine monophosphate in human blood platelets. II. Effect of N^6-2'-O-dibutyryl cyclic 3',5'-adenosine monophosphate on platelet function. *J. Clin. Invest.* **50**: 131.
33. Salzman, E. W., P. Kensler, and L. Levine. 1971. Role of cyclic AMP in platelet function. *Proc. N. Y. Acad. Sci.* In press.
34. Smith, J. B., and A. L. Willis. 1971. Aspirin selectively inhibits prostaglandin production in human platelets. *Nat. New Biol.* **231**: 235.
35. Caldwell, A., and J. N. Fain. 1971. Triiodothyronine stimulation of cyclic adenosine 3',5'-monophosphate accumulation in fat cells. *Endocrinology.* **89**: 1195.
36. Bitensky, M. W., R. E. Gorman, A. H. Neufeld, and R. King. 1971. A specific, reversible, macromolecular inhibitor of hepatic glucagon responsive adenyl cyclase. *Endocrinology.* **89**: 1242.
37. Sutherland, E. W., G. A. Robison, and R. W. Butcher. 1968. Some aspects of the biological role of adenosine 3',5'-monophosphate (cyclic AMP). *Circulation.* **37**: 279.
38. Boullin, D. J., A. R. Green, and K. S. Price. 1972. The mechanism of adenosine diphosphate induced platelet aggregation: binding to platelet receptors and inhibition of binding and aggregation by prostaglandin E_1. *J. Physiol. (Lond.).* **221**: 415.