INHIBITION OF PLATELET ENERGY PRODUCTION AND RELEASE REACTION BY PGE_1, THEOPHYLLINE AND cAMP

Sidney M. Wolfe and N. Raphael Shulman

Metabolic Diseases and Clinical Hematology Branches
National Institute of Arthritis and Metabolic Diseases
National Institutes of Health
Bethesda, Maryland

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Summary

Thrombin induces a rapid, simultaneous release from platelets of adenine nucleotides and calcium in a constant molar ratio.

PGE_1, theophylline, and dibutyryl cyclic AMP inhibit this release reaction, the attendant lactate production, and the subsequent aggregation of platelets.

Compounds which result in increased intracellular levels of cAMP appear to block aggregation by inhibiting the release reaction and energy production.

Introduction

The aggregation of human platelets induced by thrombin, ADP, and other agents (Emmons, et al., 1967; Kloeze, 1967) can be inhibited by prostaglandin E_1 (PGE_1). We recently found that PGE_1 causes an 18-fold increase in platelet adenyl cyclase activity and suggested that inhibition of aggregation was related to increased intracellular levels of cAMP (Wolfe and Shulman, 1969). Others have confirmed the stimulatory effect of PGE_1 on platelet adenyl cyclase activity, have shown that dibutyryl cAMP causes inhibition of platelet aggregation, and that theophylline augments inhibition of aggregation by PGE_1 (Cole, et al., 1970; Marquis, et al., 1969; Robison, et al., 1969; Salzman and Neri, 1969; Zieve and Greenough, 1969).

Platelet aggregation by thrombin is preceded by the release of ADP,

ATP, and other compounds (Holmsen, et al., 1969). Thrombin also causes release of calcium from platelets (Mürer, 1969), and increased lactate production (Bettex-Galland and Lüscher, 1960; Warshaw, et al., 1966), but the temporal relationship between these events and aggregation has not been determined. To define the mechanism by which PGE_1 inhibits aggregation it was of interest to see whether PGE_1 and other compounds which increase intracellular cAMP would inhibit the release of nucleotides and calcium as well as energy production.

Methods

Platelets used in experiments were from normal human adult males and were isolated as previously described (Wolfe and Shulman, 1969). Platelets were suspended at a count of 0.5 to 1.0 x $10^6/mm^3$ in buffer containing NaCl, 140 mM; Tris HCl pH 7.4, 25 mM; and EDTA 0.3 mM. To measure reaction rates aliquots of reaction mixtures that had been incubated at 37°C. were added to tubes in an ice bath at intervals and centrifuged in the cold at 1000 x g for 5 minutes to remove platelets. $HClO_4$ at a final concentration of 0.5 M was added to the supernatant and precipitated protein was removed by centrifugation at 3000 x g for 10 minutes. Absorption at 260 mμ was measured in the supernatant and the amount of adenine nucleotides calculated from the absorption of ATP standards in 0.5 M $HClO_4$. It has been shown that the 260 mμ-absorbing material consists mainly of ADP and ATP (Mürer, 1968). On the same samples used for measuring adenine nucleotides, calcium was determined with a Perkin-Elmer Model 303 atomic absorption spectrophotometer and lactate was determined enzymatically (Hohorst, 1963). In experiments involving theophylline or dibutyryl cAMP, both of which absorb at 260 mμ, an enzymatic determination of ADP was used as an index of nucleotide release (Holmsen and Stormorken, 1965).

Results

Figure 1 shows the rapid simultaneous release of adenine

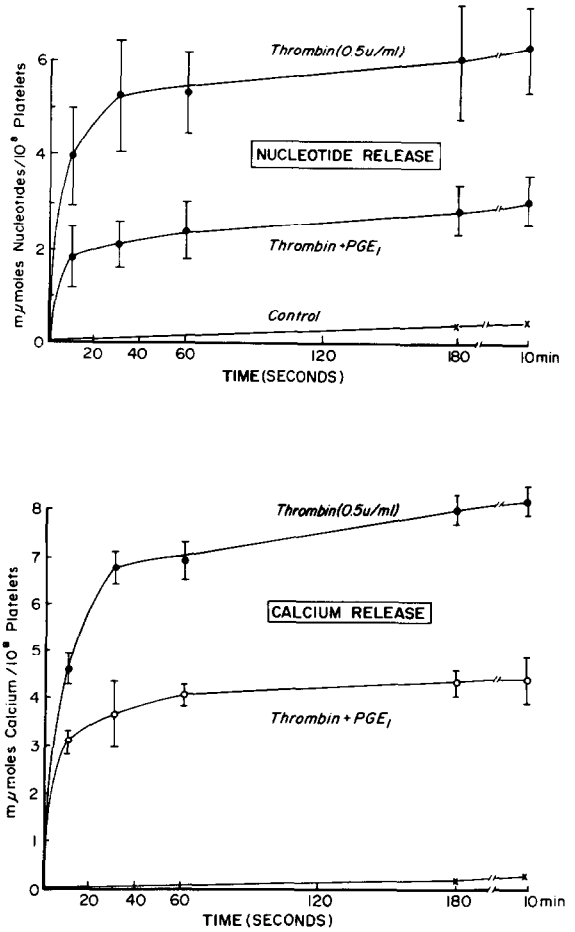

Fig. 1.--Effect of PGE_1 on release of nucleotides (upper graph) and calcium (lower graph) from washed human platelets. Each point is the mean of four experiments and the SEM is indicated by vertical brackets. PGE_1 was used at a final concentration of 3×10^{-6}M and added three minutes prior to the addition of thrombin.

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nucleotides and calcium from platelets following the addition of thrombin. The molar ratio of released calcium to released nucleotides remained constant from 10 seconds through 10 minutes after the addition of thrombin, averaging 1.27 ± .08 (mean ± SEM). Preincubation of platelets with PGE_1 resulted in 40-50% inhibition of the release of both calcium and nucleotides.

The ability of a fixed concentration of PGE_1 (3×10^{-6}M) to

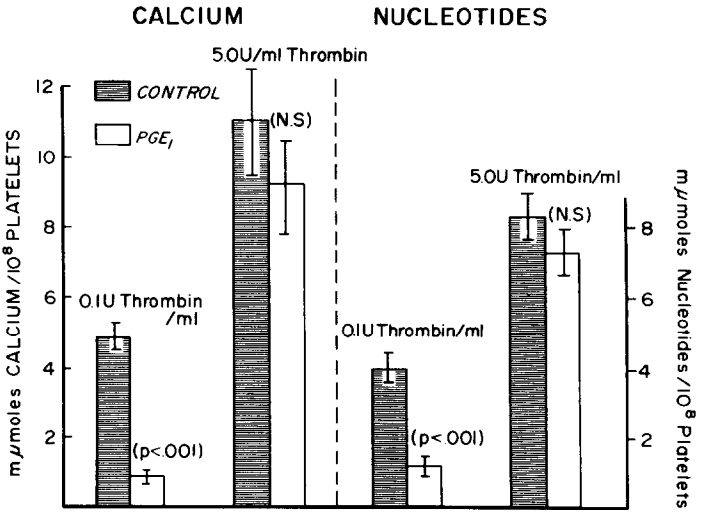

Fig. 2.--Effect of thrombin concentration on PGE_1 inhibition of calcium and nucleotide release. Platelets were incubated with PGE_1, 3×10^{-6} M, for 3 minutes before adding thrombin. Data represents mean ± SEM of 4 experiments for each set of conditions. Nucleotides and calcium were measured on the same samples.

inhibit the release reaction was related to the amount of thrombin used. When the concentration of thrombin was 1/5 that used in Figure 1 (0.1 U/ml), it resulted in 70-80% inhibition by PGE_1 of both nucleotide and calcium release, as seen in Figure 2, but a thrombin concentration 10 times that used in Figure 1 (5 U/ml) resulted in no significant inhibition by PGE_1 (Fig. 2).

Thrombin is known to stimulate lactate production in platelets (Bettex-Galland and Lüscher, 1960; Warshaw, et al., 1966), but the kinetics of this stimulation shortly after addition of thrombin and its relation to the release reaction have not been determined. It can be seen in Figure 3 that there is a burst of lactate production apparent as early as 10 seconds after the addition of thrombin and that PGE_1 partially inhibits this burst. The maximum rate of lactate production is attained by 30 seconds, and, as shown in Figure 1, nucleotide release is essentially complete within this interval.

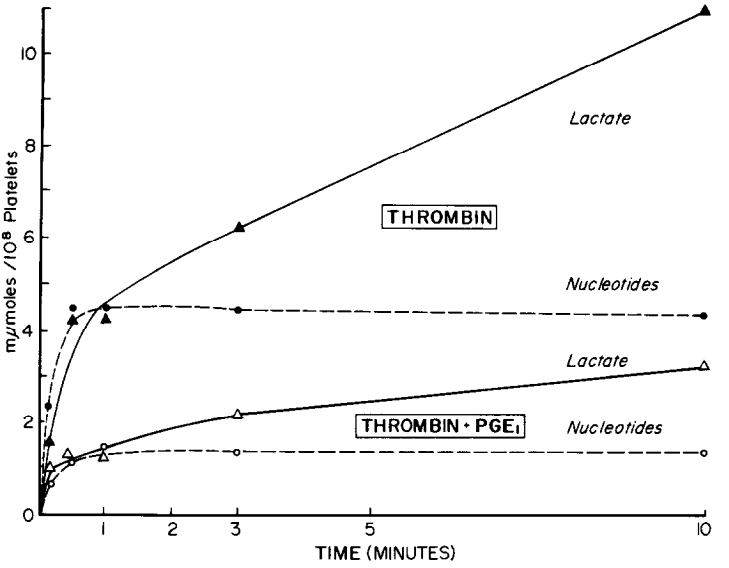

Fig. 3.--Effect of PGE_1 on nucleotide release and lactate production. Control values (no thrombin) have been subtracted to give the results shown. Final concentration of thrombin was 0.5 U/ml and PGE_1, 3×10^{-6}M.

Inhibition by PGE_1 of nucleotide release and lactate production is seen at all intervals tested from 10 seconds to 10 minutes.

TABLE 1

Effect of theophylline and cyclic AMP on release of ADP and on lactate production.

Preparation	mμmoles/10^8 Platelets	
	ADP Release	Lactate Production
Control	0.59	1.00
Thrombin (.7 U/ml)	3.94	3.20
Thrombin + Theo (.005M)	0.89	0
Thrombin + cAMP (.003M)	0.63	1.33

Theophylline (Theo) or dibutyryl cAMP were added 3 minutes prior to the addition of thrombin and the mixture incubated for one minute after addition of thrombin. Values are the means of two separate experiments, each analyzed in duplicate.

In order to further test the hypothesis that the inhibition of the release reaction and energy production is mediated by the stimulatory effect of PGE_1 on adenyl cyclase, the effects of theophylline and dibutyryl cAMP were studied. Table 1 shows that both of these compounds, which raise intracellular levels of cAMP, also inhibit the release of ADP and lactate production. All experiments were performed in 0.3 mM EDTA to prevent aggregation. Thus, it is apparent that both energy production and the release reaction are primary events rather than being secondary to aggregation.

Discussion

Within 10 seconds following the addition of thrombin to human platelets, both adenine nucleotides and calcium are released into the incubation medium in a constant molar ratio. The release of both nucleotides and calcium is essentially complete in less than 60 seconds and the percent inhibition by PGE_1 is the same for both. This data and the demonstration that only a small fraction of total platelet calcium is extractable by EDTA (Wallach, et al., 1958) are consistent with the suggestion (Mürer, 1969) that both calcium and nucleotides may originate in the intracellular alpha granules and be released together following the addition of thrombin.

Since platelet aggregation is thought to depend on calcium and ADP (Gaarder, et al., 1961), the inhibition by PGE_1 of their release may be the basis for the inhibitory effect of PGE_1 on aggregation. The present experiments show that PGE_1, theophylline, and dibutyryl cyclic AMP, all of which raise intracellular levels of cAMP, can each inhibit the release reaction and the attendant lactate production. Because the release reaction and the stimulation of lactate production occur so rapidly following addition of thrombin, the sequence of these reactions and the one which may be primarily inhibited by increased intracellular levels of cAMP is not yet clear.

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