# PLATELET AGGREGATION

## I. REGULATION BY CYCLIC AMP AND PROSTAGLANDIN $E_1$

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Aggregation of human platelets is inhibited by cyclic AMP and by its dibutyryl derivative. Prostaglandin $E_1$ ($PGE_1$) inhibits platelet aggregation and stimulates cyclic AMP synthesis by human platelet membrane fractions apparently by stimulation of adenyl cyclase. Caffeine, in contrast, inhibits platelet phosphodiesterase resulting in increased cyclic AMP levels; $PGE_1$ and caffeine are synergistic. $PGA_1$ and $PGF_{1\alpha}$ are also active but at higher concentrations.

Platelet aggregation in human, rat, and pig plasma is induced by ADP and inhibited by $PGE_1$ (1). Aggregation induced by epinephrine or collagen, the increased adhesiveness of platelets to glass, and the reduced electrophoretic mobility of platelets effected by ADP are also reduced by $PGE_1$ (2,3). Among the other prostaglandins, $PGA_1$ and $PGF_{1\alpha}$ are less inhibitory (1,4). The activities of a number of hormones whose effects are presumably mediated by cyclic AMP are inhibited by prostaglandins (3,5,6).

We have observed that cyclic AMP or its dibutyryl derivative inhibit platelet aggregation induced by ADP. These observations were of particular interest since it had been stated that $PGE_1$ increased cyclic AMP levels in platelets though no data had been presented supporting the allegation (7). Accordingly we investigated the possibility that $PGE_1$ inhibition of platelet aggregation was mediated by increased levels of cyclic AMP.

## Materials and Methods

Adenosine-5'triphosphate-8-$^{14}$C was obtained from Calbiochem; cyclic AMP and ATP from P-L Biochemicals; and adenosine 3'-5'-cyclic monophosphate-H$^3$ from Schwarz Bioresearch.

Whole human blood collected in 4% citrate was obtained from a local blood bank. $PGE_1$ was kindly provided by Dr. Sune Bergström, Dr. David Kritchevsky, and Dr. John Pike of Upjohn Company. The latter also provided experimental samples of $PGA_1$ and $PGF_1\alpha$.[1]

Platelet rich plasma (PRP) was obtained by centrifugation of whole blood at 300 x g for 20 minutes, and platelets were obtained by centrifugation of PRP for 10 minutes at 2400 x g. The platelet pellet was resuspended in 0.32M sucrose and homogenized at 4$^o$ with a motor driven, tight fitting, teflon pestle. Various cell fractions were obtained by centrifugation at 1000 x g and 10,000 x g in a Sorvall RC2-B and at 100,000 x g in the Spinco L-1 ultra-centrifuge using rotor SW-39L. Particulate fractions were resuspended in 0.32M sucrose for assay. Protein concentrations were determined by the method of Lowry (8).

Adenyl cyclase activity was assayed essentially by the method of Krishna *et al.* (9). Aliquots of platelet fractions were added to a medium containing: tris-HCl buffered at pH 7.3, 4 x $10^{-2}$M; $MgSO_4$, 4 x $10^{-3}$M; theophylline, $10^{-2}$M or caffeine, 2 x $10^{-2}$M; ATP-8-$^{14}$C, 1-2 x $10^{-3}$M (S.A. 2 mc/mmole) in a final volume of 0.5 ml. Assays were carried out at 37$^o$ and the incubations terminated after 20 minutes with the addition of 1 µmole cyclic AMP and heating in a boiling water bath for five minutes.

Phosphodiesterase activity was assayed by measuring changes

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[1] $PGE_1$ from all sources showed identical activities.

in $H^3$-cyclic AMP when incubated with caffeine, $PGE_1$, or imidazole as compared with controls. The 100,000 x g supernatant fraction of platelet homogenates contains the bulk of the phosphodiesterase and was therefore utilized for assay. The incubation medium contained: tris-HCl buffered at pH 7.3, 4 x $10^{-2}$M; $MgSO_4$, 4 x $10^{-3}$M; $H^3$-cyclic AMP, 1 x $10^{-3}$M (S.A. 1 mc/mmole); and aliquots of 100,000 x g supernatant. Cyclic AMP remaining was isolated and analyzed as described above.

Platelet aggregation was measured in a Chrono-Log Aggregometer using a modification of the method of Born and Cross (10). Undiluted human platelet rich plasma was utilized for assay at 37°.

## Results and Discussion

The high concentrations of cyclic AMP necessary to inhibit

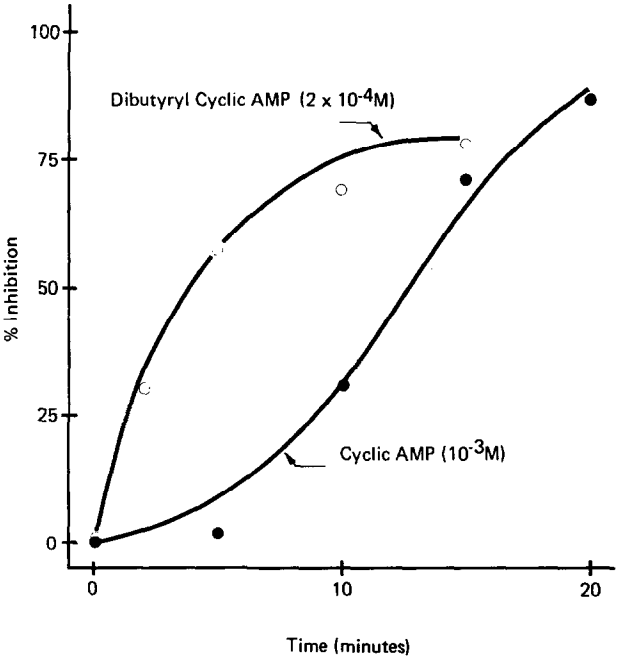


Figure 1. Cyclic AMP and Dibutyryl Cyclic AMP inhibition of ADP induced platelet aggregation. Nucleotides were tested against platelet aggregation induced by the addition of 1 µg ADP.

ADP-induced platelet aggregation (Fig. 1) is in keeping with the observations of others (5) that the cell membrane is relatively impermeable to the cyclic nucleotide. Interestingly, dibutyryl cyclic AMP exhibits a greater activity than cyclic AMP *per se* probably reflecting its more lipophilic character.

The accumulation of cyclic AMP, stimulated by NaF, increases linearly with increasing concentrations of 100,000 x g particulate fraction obtained from sonicated platelet homogenates (Fig. 2A). The level of labeled cyclic nucleotide synthesized by the platelet fraction also increases linearly with time (Fig. 2B). The bulk of adenyl cyclase activity in platelet homogenates is found in heavier particulate fractions (1000 x g and 10,000 x g). Sonication of these homogenates results in the redistribution of adenyl cyclase in lighter particulate

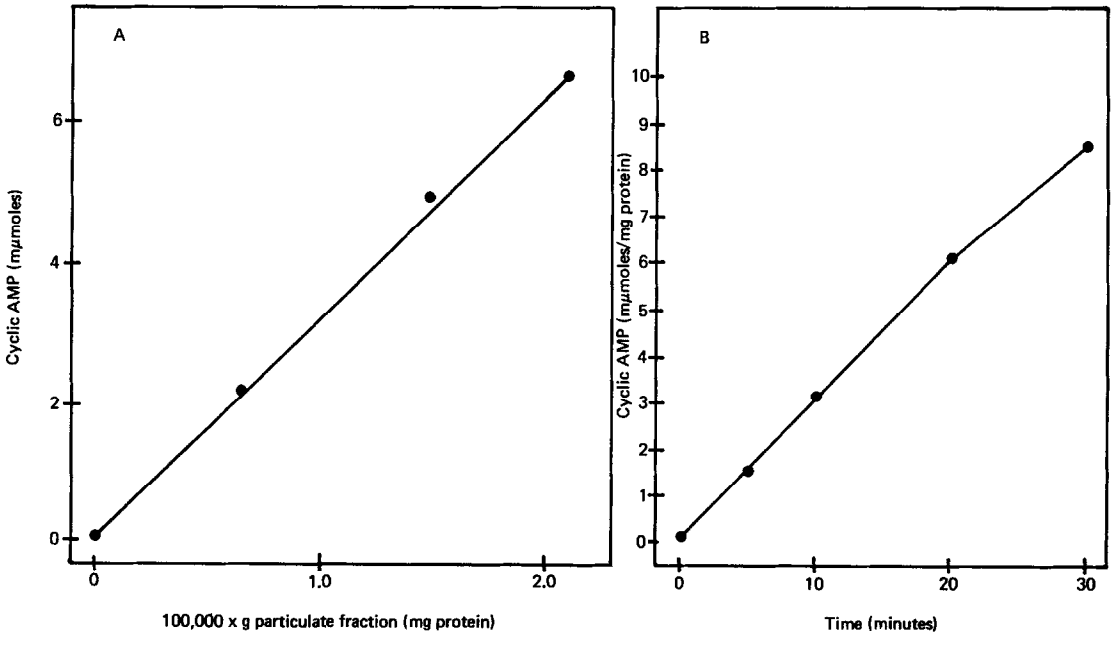


Figure 2. Effects of enzyme concentration and incubation time on cyclic AMP-$^{14}$C synthesis from ATP-$^{14}$C. (A and B) The 100,000 x g fraction was obtained after sonication of platelet homogenates. Sodium fluoride ($10^{-2}$M) was present in all vessels.

TABLE 1

$PGE_1$ Stimulation of Cyclic AMP Synthesis

| $PGE_1$ (µg) | Cyclic AMP (mµmoles/mg protein)* |
|---|---|
| 0.0 | 0.4 |
| 0.2 | 1.5 |
| 0.5 | 1.9 |
| 2.0 | 3.4 |
| 5.0 | 4.7 |
| 20.0 | 6.5 |
| 50.0 | 6.9 |

*The values reported are averages and pooled results of 3 separate experiments. A 10,000 x g particulate fraction (non-sonicated) was used.

TABLE 2

The Effects of Caffeine, $PGE_1$, and Imidazole on Platelet Phosphodiesterase Activity

| Conditions | Cyclic AMP (mµmoles) Total | Δ from control |
|---|---|---|
| Control | 323 | -0- |
| Caffeine ($2 \times 10^{-2}$M) | 665 | + 342 |
| $PGE_1$ (25 µg) | 334 | + 11 |
| Imidazole ($2 \times 10^{-3}$M) | 239 | - 84 |

fractions (10,000 x g and 100,000 x g). No adenyl cyclase activity was found in the soluble 100,000 x g supernatant frac-

tion. Adenyl cyclase appears therefore to be associated with cellular membrane fractions as found in other mammalian tissues.

$PGE_1$ is an extremely potent stimulator of cyclic AMP synthesis by platelet membrane fractions (Table 1). This observation is in confirmation of recent results obtained by Wolfe and Schulman (11). Increased levels of cyclic AMP may result alternatively by blockade of phosphodiesterase, but the possibility is excluded since $PGE_1$ at concentrations up to $3 \times 10^{-4}$M exerts no effect on this enzyme (Table 2).

The increase in cyclic AMP observed with $PGE_1$ is the result then of direct or indirect stimulation of adenyl cyclase. When added together $PGE_1$ and caffeine are synergistic in increasing cyclic AMP levels as one might expect if both agents were affecting different loci (Table 3).

The relative activities of $PGE_1$, $PGA_1$, and $PGF_1\alpha$ on the synthesis of cyclic AMP by platelet membrane fractions (Table 4) correlates directly with their relative abilities to inhibit ADP-induced aggregation as first reported by Kloeze (1) and extended in this laboratory (4). These results together with our observations that cyclic AMP *per se* inhibits platelet aggregation lends support to our hypothesis that $PGE_1$ inhibits aggregation by providing higher levels of cyclic AMP and specifically by enhanced generation of the cyclic nucleotide rather than by blocking its further utilization.

In summary we have observed that 1) adenyl cyclase in broken cell preparations responds to stimulation by $PGE_1$. Further the order of potency of $PGE_1$, $PGA_1$, and $PGF_1\alpha$ are the same in inhibiting platelet aggregation as in stimulating platelet adenyl cyclase; 2) the activity of $PGE_1$ is potentiated by a phosphodiesterase inhibitor, caffeine; 3) the addition of

TABLE 3

The Synergism of Caffeine and $PGE_1$ in Effecting Cyclic AMP Synthesis*

| Conditions | Cyclic AMP (mμmoles/mg protein) |
|---|---|
| Control | 0.40 |
| Caffeine ($2 \times 10^{-2}$M) | 0.68 |
| $PGE_1$ (5 μg) | 5.19 |
| Caffeine ($2 \times 10^{-2}$M) + $PGE_1$ (5 μg) | 8.31 |

*Conditions were the same as described in legend of Table 1.

TABLE 4

The Relative Potency of Various Prostaglandins in Stimulating Cyclic AMP Synthesis

| Prostaglandin ($7.5 \times 10^{-6}$M) | Cyclic AMP (mμmoles/mg protein)* Total | Δ from control |
|---|---|---|
| None | 0.48 | --- |
| $PGE_1$ | 4.35 | 3.87 |
| $PGA_1$ | 2.10 | 1.62 |
| $PGF_1\alpha$ | 0.51 | 0.03 |

*The actual values are misleading in that extremely high concentrations of $PGA_1$ and $PGF_1\alpha$ are necessary to approach the stimulation observed with $PGE_1$. Conditions were the same as described in legend of Table 1.

exogenous cyclic AMP mimics the effects of $PGE_1$ in inhibiting platelet aggregation and 4) the level of cyclic AMP in whole

platelets changes in response to $PGE_1$ simultaneously with the physiological response (inhibition of platelet aggregation) (12). Thus the regulation of intracellular cyclic AMP levels may play a major role in the susceptibility of platelets to aggregation, and ultimately, in thrombogenesis.

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