PLATELET AGGREGATION

II. Adenyl Cyclase, Prostaglandin E_1, and Calcium

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Intracellular cyclic AMP synthesis from ATP generated by incubation of *intact human platelets* with adenosine-^{14}C is increased by Prostaglandin E_1 (PGE_1). The level of cyclic AMP synthesized is temporally related to the degree of inhibition of platelet aggregation effected by PGE_1. Aggregation is dependent upon the presence of calcium ions, which inhibit both the stimulation of adenyl cyclase and the inhibition of aggregation by PGE_1.

We have recently demonstrated (1) that cyclic AMP and its dibutyryl derivative inhibit ADP-induced platelet aggregation and that PGE_1, a potent inhibitor of ADP- and epinephrine-induced aggregation, stimulates the synthesis of cyclic AMP by *platelet membrane fractions*. In addition, we have shown that whereas caffeine increases the level of cyclic AMP by inhibiting platelet phosphodiesterase, PGE_1 exerts no inhibitory action on phosphodiesterase, and when used together, PGE_1 and caffeine are synergistic in increasing cyclic AMP accumulation.

In the further exploration of the proposal that PGE_1 inhibits platelet aggregation *via* stimulation of adenyl cyclase, we have studied the temporal relationship of cyclic AMP synthesis and inhibition of ADP-induced aggregation in response to PGE_1. In addition, the requirement for calcium in aggregation led us to investigate the effects of calcium ions on platelet adenyl cyclase activity.

Materials and Methods

Adenosine-8-^{14}C and adenosine-5'-triphosphate-8-^{14}C were obtained from Calbiochem and International Chemical and Nuclear Corp., and cyclic AMP from P-L Biochemicals. Whole human blood collected in 4% citrate was obtained from a local blood bank. Whole blood was processed as previously reported (1). The platelet rich plasma obtained was centrifuged at 800 g for five minutes and the platelet pellet carefully resuspended in a Tris-HCl (37.5 mM, pH 7.4) buffered-balanced salt solution containing 6.7 mM EDTA as described by Rossi (2).

The resuspended platelets were pre-incubated for two hours at 37° with adenosine-8-^{14}C (S.A. 20 mc/mmole). Aliquots of this preparation were then added to prepared tubes for the assay of intracellular adenyl cyclase activity. The final incubation medium contained 5 x 10^{-2}M caffeine and 1 x 10^{-2}M $MgSO_4$ and other components as stated in tables and figures. Platelets were incubated for ten minutes and the incubation terminated after the addition of 1 µmole cyclic AMP by heating in a boiling water bath for five minutes. The tubes were centrifuged at 2500 g for five minutes and the supernatant fractions analyzed for ^{14}C-labeled cyclic AMP using the method of Krishna, *et al* (3).

Undiluted human platelet rich plasma was utilized for the assay of platelet aggregation as measured in a Chrono-Log Aggregometer at 37° by a modification of the method of Born and Cross (4) to be described elsewhere.

Results and Discussion

PGE_1 stimulates the synthesis of cyclic AMP from ATP-^{14}C by *platelet membrane fractions* (1,5) and also increases

cyclic AMP synthesis in *intact platelets* previously incubated for two hours with adenosine-^{14}C (Fig. 1). The accumulation of cyclic AMP increases significantly at low concentrations of PGE_1 and reaches a maximum at approximately 1 µg (3 x 10^{-6}M) PGE_1. NaF, similarly to PGE_1, stimulates adenyl cyclase activity of platelet membrane fractions (1); unlike PGE_1, however, NaF does not stimulate cyclic AMP synthesis in intact platelets suggesting different mechanisms of action for PGE_1 and NaF.

Holmsen, *et al* (6) observed that ATP is not incorporated into nucleotides of isolated platelets resuspended in saline and therefore it is not surprising that ATP-^{14}C, in contrast to adenosine-^{14}C, is not incorporated into cyclic AMP in isolated platelets resuspended in buffered salt solution even with the addition of PGE_1. This observation suggests an intracellular localization for adenyl cyclase.

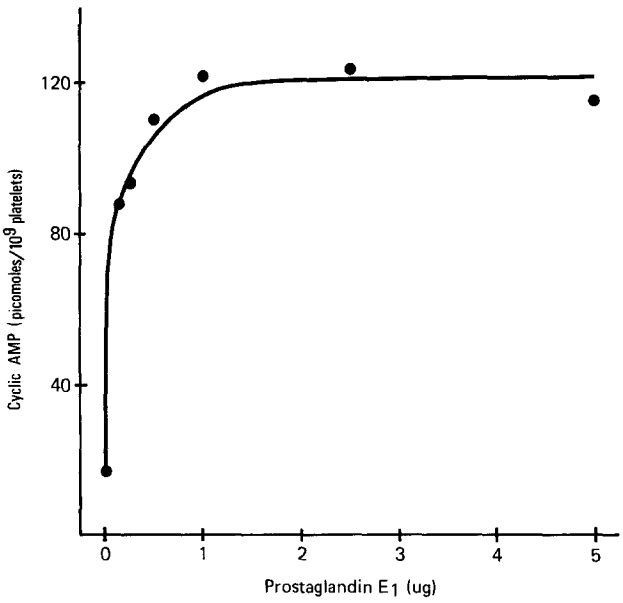

Figure 1. Prostaglandin E_1-stimulation of cyclic AMP synthesis in intact platelets.

We have proposed (1) that PGE_1 inhibits aggregation *via* stimulation of adenyl cyclase yielding higher cyclic AMP levels, and that the cyclic nucleotide then blocks aggregation by an unknown mechanism. Such a sequence, if correct, would require that the increase in cyclic AMP occur prior to or simultaneously with the observed inhibition of aggregation. The results presented in Fig. 2 indicate an immediate rise in cyclic AMP in intact platelets upon the addition of PGE_1, a threefold increase in cyclic AMP being observed within 15 seconds. Peak cyclic AMP levels are attained within five minutes and it is important to note that this correlates very well with the peak inhibition of aggregation observed with PGE_1 (Fig. 2). These observations coupled with those previously reported (1) fulfill all of the criteria suggested by Sutherland, *et al* (7) for implicating cyclic AMP mediation

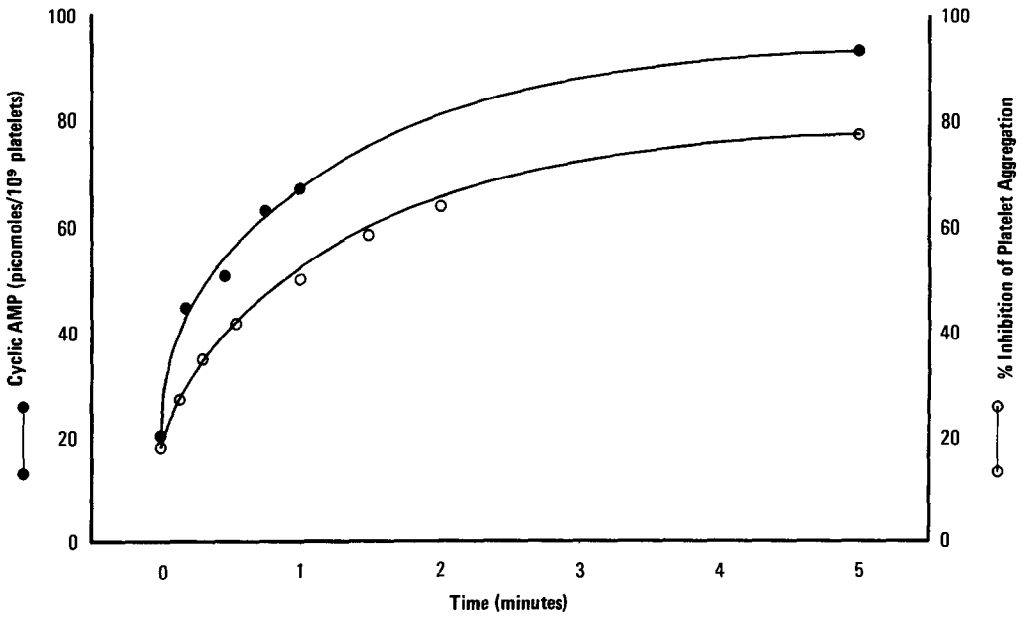

Figure 2. The simultaneous increase in intracellular cyclic AMP and inhibition of ADP-induced aggregation by Prostaglandin E_1. PGE_1 was present in a final concentration of 1×10^{-8}M.

of an hormonal effect.

Calcium ions are an absolute requirement for the aggregation of platelets regardless of the inducing agent (8,9). The mechanisms proposed for the aggregation phenomenon have, therefore, involved calcium ion participation. Born and Cross (4) have proposed that ADP binds to platelets and participates in intercellular bridges involving calcium ions and a protein; Spaet (10) suggests that ADP conversion to AMP releases energy which in turn drives a binding reaction involving calcium ions, and Salzman (11) implicates a calcium requiring actomyosin-like "ecto ATPase" in aggregation, suggesting that inhibition of this enzyme results in membrane unfolding and

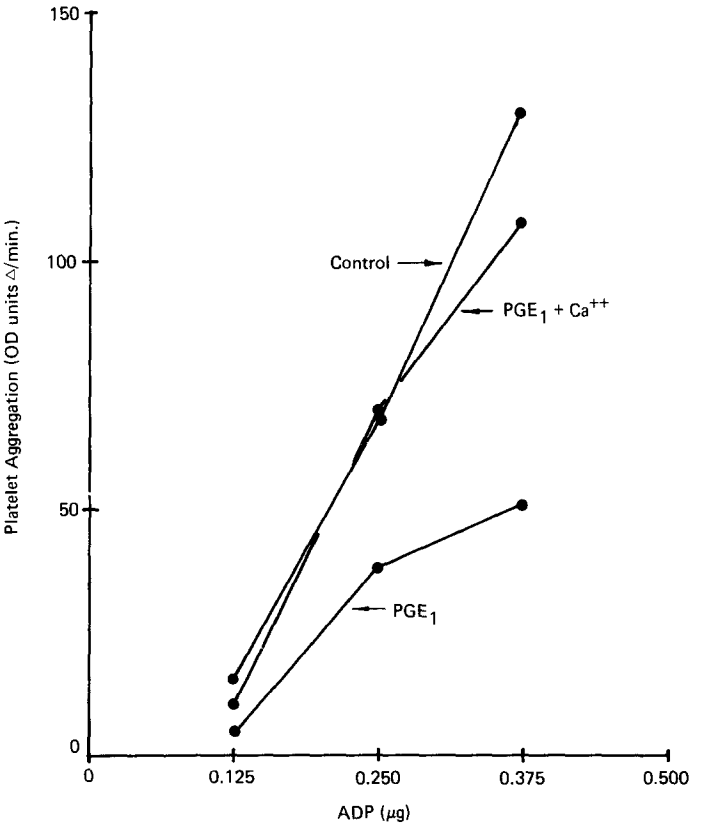

Figure 3. Decrease in PGE_1-inhibition of ADP-induced platelet aggregation by Ca^{++}. Concentrations utilized: PGE_1, 2 x 10^{-8}M; $CaCl_2$, 5 x 10^{-4}M.

exposure of binding sites with affinities for calcium and fibrinogen. Interestingly, Rabinowitz, *et al* (12) have recently shown that most of the adenyl cyclase activity in skeletal muscle is associated with the calcium-accumulating granules derived from the sarcoplasmic reticulum, which are involved in excitation-contraction coupling and muscular relaxation. In view of the importance of calcium ions to any proposed model of aggregation, we have investigated the effects of calcium ions upon PGE_1-inhibition of aggregation and stimulation of cyclic AMP synthesis.

We have observed that calcium ions decrease the inhibition of ADP-induced aggregation mediated by PGE_1 (Fig. 3). This was most apparent at lower concentrations of ADP but

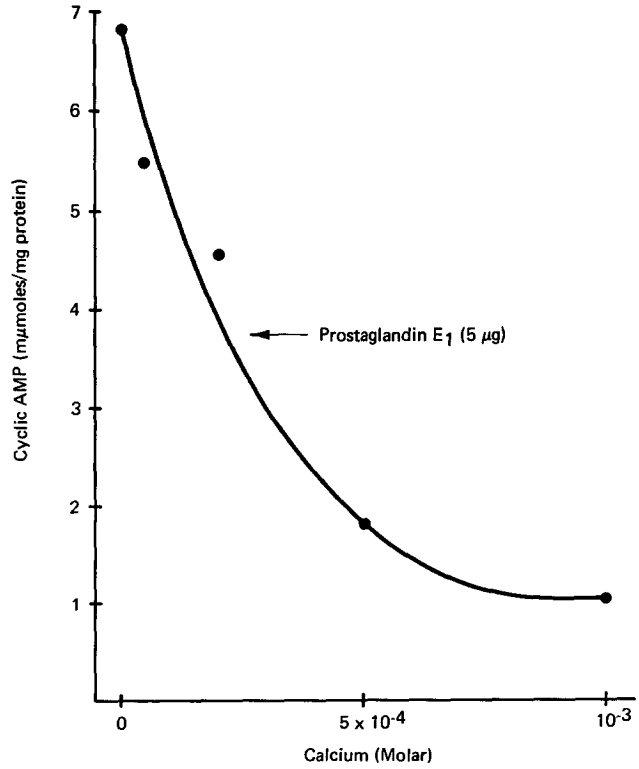

Figure 4. The inhibition of PGE_1-stimulated platelet membrane (10,000 g pellet) adenyl cyclase activity by Ca^{++}.

even at higher concentrations, where PGE_1 inhibited to a great degree, calcium ions almost completely prevented the inhibition of PGE_1. Of even greater interest is the observation that calcium ions inhibit the stimulation of cyclic AMP synthesis by PGE_1 in platelet membrane fractions (Fig. 4) in a dose related fashion. Consequently the known calcium involvement in the aggregation of platelets may include its action on adenyl cyclase as well as on other enzyme systems (*e.g.*, Ca^{++} - Mg^{++} dependent "ecto-ATPase"). Alternatively, it is conceivable that calcium transport may be regulated by the prostaglandins and therefore examination of the effects of various agents on calcium transport by platelet endoplasmic reticulum may be enlightening in revealing more precisely the locus of calcium action.

Acknowledgement

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