MOLECULAR PHARMACOLOGY, 20:302–309

Regulation of Cyclic AMP Metabolism in Human Platelets

Sequential Activation of Adenylate Cyclase and Cyclic AMP Phosphodiesterase by Prostaglandins

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Received December 18, 1980; Accepted March 27, 1981

SUMMARY

ALVAREZ, R., A. TAYLOR, J. J. FAZZARI, AND J. R. JACOBS. Regulation of cyclic AMP metabolism in human platelets: sequential activation of adenylate cyclase and cyclic AMP phosphodiesterase by prostaglandins. *Mol. Pharmacol.* **20**:302–309 (1981).

Prostacyclin (PGI_2) produces a transient elevation in cyclic AMP in washed human platelets. Intracellular cyclic AMP increases rapidly to a maximal level after 1–2 min of incubation and then gradually declines. This temporal pattern appears to involve a sequential activation of adenylate cyclase and cyclic AMP phosphodiesterase. The following three observations support this interpretation: (a) inhibition of endogenous phosphodiesterase activity with 1-methyl-3-isobutylxanthine eliminates the transient elevation pattern and permits accumulation of the cyclic nucleotide, (b) PGI_2 indirectly stimulates the activity of a cyclic AMP phosphodiesterase when preincubated with intact platelets, and (c) the gradual decrease in endogenous cyclic AMP coincides with an increase in phosphodiesterase activity. The potency and efficacy of prostaglandins (PGs) as stimulators of phosphodiesterase activity correlate with their capacity to increase intracellular cyclic AMP ($PGI_2 > PGD_2 \geq PGE_1$). PGD_2 increases cyclic AMP to a higher maximal level than does PGE_1 in intact cells, but stimulates adenylate cyclase to a lower maximal velocity in broken-cell preparations. This discrepancy was observed under a variety of assay conditions. When washed platelets were preincubated in a hypotonic buffer containing EDTA prior to the assay, the discrepancy was eliminated. Thus, under appropriate incubation conditions, the potency and efficacy of several naturally occurring PGs as stimulators of adenylate cyclase correspond to their ability to increase the intracellular concentration of cyclic AMP ($PGI_2 > PGD_2 \geq PGE_1 \gg PGE_2 \geq PGA_1 > PGF_{2\alpha}$). Similarly, their potency as inhibitors of ADP-induced platelet aggregation corresponds to their capacity to elevate cyclic AMP to a critical, submaximal level. These results lend further support to the concept that cyclic AMP mediates the effect of PGs on platelet function and that activation of adenylate cyclase is the primary biochemical event responsible for the accumulation of cyclic AMP.

INTRODUCTION

Endothelial cells of the vascular wall have the capacity to synthesize and release PGI_2[1] into the circulation (1–6). PGI_2 inhibits the aggregation of platelets induced by a variety of substances *in vitro* (1–3) and by adenosine diphosphate *in vivo* (7). This effect may be important in the regulation of early events in hemostasis and thrombosis (8).

At the biochemical level, prostaglandins stimulate platelet adenylate cyclase [ATP pyrophosphate-lyase (cyclizing) EC 4.6.1.1] activity (9–12) and elevate the intracellular concentration of cyclic AMP (10–13). An increase in cyclic AMP accompanies the inhibition of platelet aggregation by PGI_2 (13). The order of potency of PGE_1, PGA_1, and $PGF_{1\alpha}$ as inhibitors of aggregation corresponds to their order of potency in elevating cyclic AMP (10, 14). The addition of cyclic AMP (15), dibutyryl cyclic AMP (16), or an inhibitor of cyclic nucleotide phosphodiesterase (3′:5′-nucleotidohydrolase, EC 3.1.4.17) (10) to platelet-rich plasma prevents aggregation. These observations support the concept that cyclic AMP mediates the effect of prostaglandins on platelet function.

Several investigators have presented evidence which strengthens the original proposal (11) that human platelets have a distinct receptor for PGD_2. The supporting

[1] The abbreviations used are: PGI_2, prostacyclin; PG, prostaglandin; AMP-PNP, 5′-adenylylimido-diphosphate; MIX, 1-methyl-3-isobutylxanthine; PRP, platelet-rich plasma.

0026-895X/81/020302-08$02.00/0

data have been obtained primarily from platelet aggregation or prostaglandin binding studies.

Although it is generally assumed that adenylate cyclase activation by prostaglandins constitutes the primary biochemical event responsible for the accumulation of intracellular cyclic AMP, an important discrepancy has not been resolved. PGD_2 exhibits greater potency and efficacy than does PGE_1 in increasing cyclic AMP in intact cells but is less effective in stimulating adenylate cyclase activity in broken-cell preparations (17). These divergent results indicate either that the inhibition of platelet aggregation by PGD_2 cannot be entirely attributed to activation of adenylate cyclase (17) or that the poor response to PGD_2 in the enzyme assay is an artifact.

This study was initiated to examine the correlation between prostaglandin-induced activation of adenylate cyclase and the accumulation of cyclic AMP in human platelets. During the course of this study, prostaglandins were found to increase cyclic AMP phosphodiesterase activity when preincubated with intact cells. Preliminary reports of these findings have been presented elsewhere (18–20).

MATERIALS AND METHODS

[α-^{32}P]-Adenosine 5′-triphosphate (10–30 Ci/mmole), [G-^{3}H]- or [^{32}P]-adenosine 3′:5′-monophosphate (10–50 Ci/mmole), and [8-^{3}H]-adenine (10–25 Ci/mmole) were purchased from New England Nuclear Corporation (Boston, Mass). AMP-PNP and [α-^{32}P]-AMP-PNP (10–25 Ci/mmole) were obtained from ICN Corporation, Chemical and Radioisotope Division. PGI_2 and PGD_2 (racemic) were provided by the Institute of Organic Chemistry, Syntex Research (Palo Alto, Calif.). PGI_2 (natural enantiomer) was a gift from Dr. Robert Rodvien, Institute of Health Research (San Francisco, Calif.). PGD_2 (natural enantiomer) was a gift from Dr. John Westwick, Royal College of Surgeons of England (London, England). All other prostaglandins (enantiomerically pure), nucleotides, phosphoenolpyruvate, and pyruvate kinase (lyophilized) were obtained from Sigma Chemical Company (St. Louis, Mo.). Unless stated otherwise, prostaglandin data were obtained with the natural enantiomers, except for PGI_2, which was the racemic mixture.

Prostaglandins were dissolved immediately before use in ethanol to 0.01 M. Dilutions were made into either ethanol, ethanol-water, or Buffer A (see below). PGI_2 was dissolved in 50 mM Tris-HCl buffer, pH 9.7, at 5° to 1 mM and was stored in 0.1-ml aliquots at −20°. Dilutions were made into the same ice-cold buffer solution immediately before the assay. PGI_2 solutions were used once and then discarded. MIX (obtained from Aldrich Chemical Company, Inc., Milwaukee, Wisc.) was dissolved in dimethyl sulfoxide. Solutions (1 M) were warmed to 37° prior to assay. In cyclic AMP studies, platelet suspensions were stirred during the pipetting step to keep MIX in solution. Control experiments were performed with dimethylsulfoxide.

Platelet Suspensions

Blood from donors who had not taken aspirin or similar medications for at least 2 weeks was collected by venipuncture into evacuated glass tubes [Vacutainer, Becton, Dickinson, Rutherford, N. J.] containing EDTA (7.7 mM final concentration). Platelet suspensions were then obtained by one of the following methods. Method I: PRP was obtained by centrifuging the blood in polycarbonate tubes at 200 × g for 15 min at 4°. All subsequent steps were performed at 4°. A platelet pellet was obtained by further centrifugation of the PRP at 1000 × g for 15 min. The pellet was resuspended in a volume of Buffer A (0.137 M NaCl, 12.3 mM Tris-HCl buffer, pH 7.4 at 37°, 1.54 mM EDTA, and 20 mM glucose) equal to the original PRP volume. At this stage, the platelet suspension was used in the adenylate cyclase, cyclic AMP accumulation, or cyclic AMP phosphodiesterase assays. Unless stated otherwise, platelet suspensions were obtained by Method I. For the phosphodiesterase experiments, blood was pretreated with 0.1 mM aspirin for at least 20 min at 22° (Method II) to inhibit platelet prostaglandin synthesis prior to obtaining PRP by centrifugation.

Platelet Membrane Suspensions

Hypotonic lysis. When hypotonically lysed platelet membranes were required for the adenylate cyclase assay, the platelet suspension in Buffer A (above) was centrifuged at 750 × g for 15 min in a polycarbonate test tube. The pellets were dispersed with a Vortex mixer and resuspended in either 10 mM Tris-HCl buffer, pH 7.4, containing cold 10 mM EDTA (Tris-EDTA buffer) or 10 mM Tris-HCl buffer (Tris buffer) equivalent to the original PRP volume. The suspensions were preincubated for 30 min in an ice-water bath and then centrifuged at 6000 × g for 15 min at 4°. The pellets were resuspended in cold 10 mM Tris-HCl buffer to one-tenth the original PRP volume and used in the adenylate cyclase assay. Electron micrographs revealed that platelets preincubated under hypotonic conditions with Tris-EDTA buffer resemble intact cells suspended in isotonic Buffer A. In contrast, platelets preincubated in Tris buffer contained granules and vesicles, but they appeared to be largely devoid of electron-dense cytoplasmic material. In accord with these observations, the protein content of the pellet fraction after preincubation in Tris buffer and centrifugation was less than one-half that of the pellet fraction after preincubation in Tris-EDTA buffer and centrifugation. The loss of intracellular protein in the absence of EDTA was attributed to increased permeability of the plasma membrane as a consequence of hypotonic lysis. In addition to its capacity to chelate divalent cations, the addition of EDTA increases the ionic and osmotic concentration of the preincubation medium.

Freeze-thaw procedure. Platelets were also ruptured with a freeze-thaw procedure. The platelet suspension in Buffer A was centrifuged at 750 × g for 15 min. The pellet was rapidly frozen by placing the centrifuge tube in a Dry Ice-acetone bath for 3 min and then in a water bath at 22° for 3 min. The pellet was resuspended in ice-cold Buffer A to the original (PRP) volume and centrifuged at 6000 × g for 15 min. The final pellet was resuspended in Buffer A to one-tenth the original (PRP) volume and used in the adenylate cyclase assay.

Platelet Aggregation

Blood was collected into evacuated tubes containing sodium citrate (30 mM final concentration). PRP was

collected after centrifugation for 15 min at 200 × *g* at room temperature. Platelet concentration was determined with a Coulter counter (Coulter Electronics Inc., Hialeah, Fla.). Siliconized glassware or plastic test tubes were used in all procedures. Aggregation was followed by the turbidimetric procedure of Born (21) using a Payton aggregation module. Prostaglandins were added to stirred PRP (450 rpm) at 37° and incubated for 5 min prior to induction of aggregation by ADP (5 μM). The total volume was 1 ml. The degree of inhibition was determined by measuring the change in percentage transmission of the primary phase of ADP-induced aggregation after 5 min of incubation. Concentration-response experiments for prostaglandins were repeated at least twice with platelets obtained from different donors. The results presented are representative data.

Adenylate Cyclase Assay

The composition of the incubation medium in a total volume of 0.2 ml was as follows: 40 mM Tris-HCl buffer, pH 7.4 at 37°, 2 mM cyclic AMP, 0.1 mM GTP, 0.5 mM (0.1 μCi) [α^{32}P]ATP, 4 mM $MgSO_4$, 20 mM phosphoenolpyruvate, and 6.2 units of pyruvate kinase to regenerate ATP. The reaction was initiated by the addition of 60 μl of the platelet membrane suspension. Platelet membranes were suspended in Buffer A if obtained by the freeze-thaw procedure or in 10 mM Tris-HCl buffer if obtained by hypotonic lysis. Incubations were performed at 37° for 10 min. Reactions were terminated by placing the tubes in an ice-water bath and immediately adding 0.8 ml of 1% (w/v) sodium dodecyl sulfate. The tube contents were mixed and 0.05 μCi [^{3}H]cyclic AMP was added to each tube to monitor recovery. Labeled cyclic AMP was isolated in accordance with the method described by Ramachandran (22). Assays were performed in duplicate or in triplicate. Enzyme activity was linear for at least 10 min and proportional to enzyme concentration in the range of 30–120 μg of protein. The protein content was estimated colorimetrically with human serum albumin as a standard (23).

Cyclic AMP Phosphodiesterase Assay

Platelet suspensions were adjusted to 10^9 cells/ml of Buffer A, and 90 μl (~0.2 mg of protein) were incubated in the presence or absence of the test prostaglandin (10 μl) diluted in Buffer A for 10 min at 30°. All pipetting steps or transferring of test tubes from one bath to another bath were performed sequentially at 15-sec intervals. After the preincubation, the tubes were placed in a Dry Ice-ethanol bath to freeze rapidly the suspensions and rupture the platelet membranes. After all of the tubes were processed in this way, the frozen suspensions were allowed to thaw by sequentially immersing the tubes in a 30° water bath for 1 min. At this time, 0.9 ml of phosphodiesterase incubation medium warmed to 30° and containing 12 mM Tris-HCl buffer, pH 7.7, 0.5 mM $MgCl_2$, 0.137 M NaCl, 20 mM glucose, and 1.1 μM [^{3}H]-cyclic AMP (0.2 μCi) was added to each tube. The contents were mixed and incubated for 5 min at 30°. The phosphodiesterase reaction was terminated by adding 10 μl of 0.1 M EDTA, pH 7.0, mixing, and immediately immersing the tubes in a boiling water bath for 90 sec. Labeled cyclic AMP was isolated from alumina columns in accordance with the method of Filburn and Karn (24). Assays were performed in triplicate. Hydrolysis of cyclic AMP was less than 10% with the nonactivated enzyme preparations after 5 min of incubation. No extracellular phosphodiesterase activity could be demonstrated if the freeze-thaw procedure was omitted.

Cyclic AMP Assay

Accumulation of [^{3}H]cyclic AMP from a prelabeled, intracellular pool of [^{3}H]ATP was measured by a modification of previously described procedures (25). Human platelets suspended in Buffer A were incubated with [^{3}H]adenine (5 nM, 1 μCi/ml) for 30 min at 37°. The percentage incorporation of [^{3}H]adenine into the platelets was determined after 30 min by centrifuging 1 ml of the suspension at 1000 × *g* for 15 min and measuring the radioactivity on 0.1-ml aliquots of the supernatant. The average percentage incorporation was 62 ± 5% for 20 experiments. The labeled platelet suspension (0.98 ml) was added to a test tube containing the test agent or solvent (20 μl) and stirred with a Vortex mixer. The incubations were initiated sequentially at 37° and terminated by placing the tubes in a Dry Ice-ethanol bath. The tubes were then placed in a boiling water bath for 5 min and allowed to cool before the addition of 20 μl of a stock solution of ^{32}P-labeled cyclic AMP, 10 mM (~2000 dpm), to monitor recovery. The contents were mixed and centrifuged for 10 min at top speed in a clinical centrifuge. Labeled cyclic AMP was isolated by the three-column technique (aluminum oxide, Dowex 1, and Dowex 50 ion exchange chromatography) described by Mao and Guidotti (26). Assays were performed in duplicate. Accumulation of ^{3}H-labeled cyclic AMP is expressed as a percentage of the [^{3}H]adenine incorporated into platelets, as previously described (25).

Presentation of Data

The results presented for the biochemical assays in Figs. 1–4 are representative data. Each experiment, performed in triplicate or in duplicate, was repeated at least twice with similar results. Relative efficacy and potency were confirmed by simultaneously testing each of the various prostaglandins at appropriate concentrations with platelets from a single donor. Standard deviations were generally less than 3–5% of the mean values.

RESULTS

Platelet aggregation and cyclic AMP. Several naturally occurring prostaglandins were examined for their capacity to inhibit ADP-induced platelet aggregation (Fig. 1A) and compared with their ability to increase the accumulation of cyclic AMP from a prelabeled, intracellular pool of ATP in washed platelets (Fig. 1B). [The results obtained with this technique closely resemble those obtained by measuring the effect of PGI_2, PGD_2, and PGE_1 on the endogenous cyclic AMP content by a protein-binding assay (13).] The same order of potency was obtained in each assay ($PGI_2 > PGD_2 \geq PGE_1 \gg PGE_2 \geq PGA_1 > PGF_{2\alpha}$). The maximal accumulation of cyclic AMP in the presence of PGI_2 was substantially greater than that obtained with saturating concentrations of either PGD_2 or PGE_1. It is necessary, therefore,

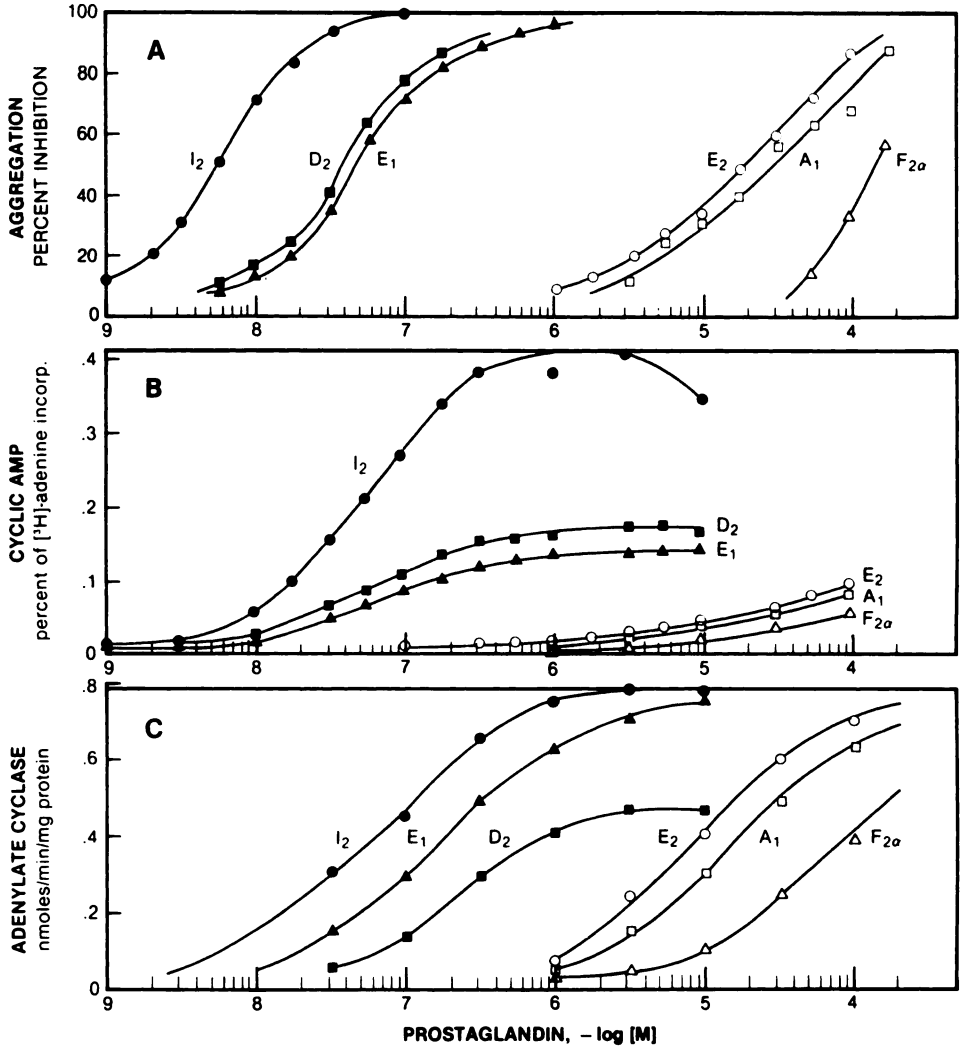

FIG. 1. *Effect of prostaglandins on platelet aggregation, intracellular cyclic AMP, and adenylate cyclase activity*

A. Percentage inhibition of platelet aggregation induced by 5 μM ADP in platelet-rich plasma.

B. Accumulation of ^{3}H-labeled cyclic AMP from a prelabeled, intracellular pool of [^{3}H]ATP after 1 min of incubation with washed platelets in Buffer A.

C. Activation of adenylate cyclase using 0.5 mM [^{32}P]ATP as the substrate.

The reaction was performed for 10 min at 37°. Platelet membranes were obtained by sequentially freezing and thawing platelets. Methods used to obtain platelet-rich plasma, washed platelets, membrane suspensions, and assay procedures are described under Materials and Methods. PGI_2 used in these experiments was the racemic mixture. The natural enantiomer was approximately twice (2.2 times) as potent as the racemic mixture in the aggregation assay. Basal levels of cyclic AMP or adenylate cyclase activity were subtracted from the data presented in B and C. Data for each of the more potent prostaglandins (I_2, D_2, E_1, and E_2) in each biochemical assay were obtained with platelets from a different donor.

to indicate both potency and efficacy in describing the effect of prostaglandins on cyclic AMP accumulation.

The increase in cyclic AMP in the presence of 50 nM PGI_2 is submaximal but evidently sufficient to prevent ADP-induced aggregation (Fig. 1A and B). A good correlation exists between the capacity of these prostaglandins to inhibit platelet aggregation and their ability to increase the intracellular accumulation of cyclic AMP to a critical, submaximal level.

Adenylate cyclase activation. Concentration-response curves for prostaglandins as stimulators of adenylate cyclase were obtained with a platelet membrane suspension. Broken-cell preparations were obtained by a sequential freeze-thaw procedure followed by resuspension of the pellet in Buffer A. The order of potency was $PGI_2 > PGE_1 \gg PGE_2 \geq PGA_1 > PGF_{2\alpha}$ (Fig. 1C). Additional experiments, performed with AMP-PNP as the substrate, revealed the same order of potency.

Maximal stimulation of enzyme activity by PGD_2 with either substrate was less than that obtained with PGE_1 and did not correspond to its efficacy with respect to cyclic AMP accumulation in intact cells. Removal of EDTA (1.54 mM) from the Buffer A resuspension medium further diminished the subsequent response to PGD_2. This observation suggested that changes in divalent cation concentration (which are likely to occur during the lysis procedure) may alter prostaglandin stimulation of adenylate cyclase.

To examine directly the effect of EDTA, washed platelets were preincubated in a hypotonic buffer (10 mM Tris-HCl, pH 7.4) for 30 min in the presence or absence of 10 mM EDTA. The suspensions were centrifuged at 6000 × *g* for 15 min. The pellets were resuspended in 10 mM Tris-HCl buffer and used in the adenylate cyclase assay. Incubation of washed platelets in a hypotonic buffer containing EDTA prior to cell lysis yields membrane-

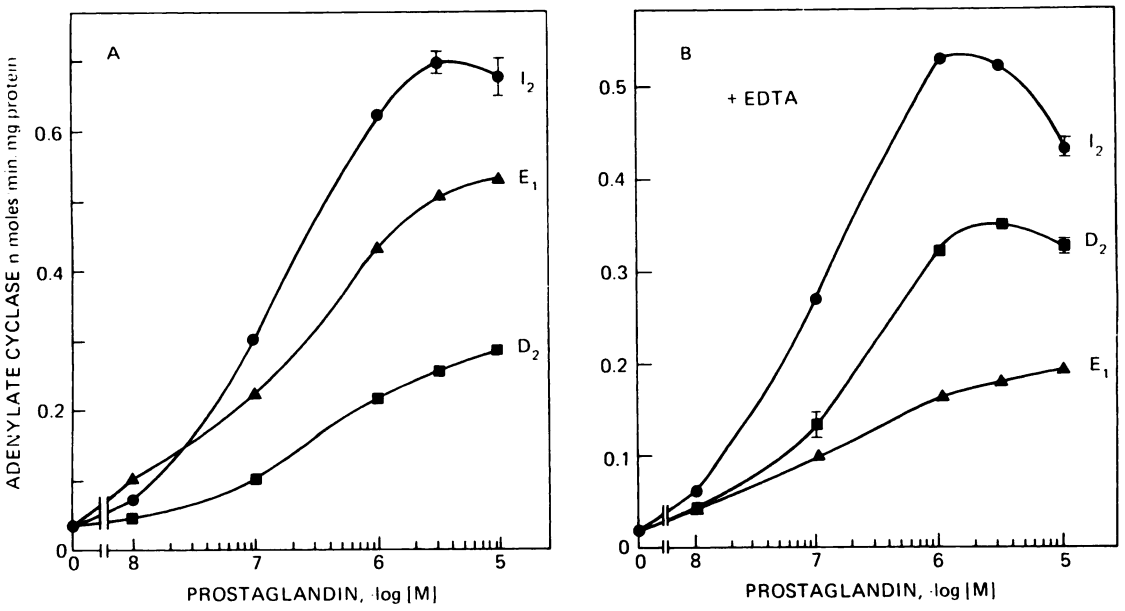

FIG. 2. *Effect of suspending platelets in EDTA on subsequent activation of adenylate cyclase by PGI_2, PGD_2, and PGE_1*

Platelet suspensions in Buffer A were centrifuged and the pellets were resuspended in either 10 mM Tris-HCl buffer or 10 mM Tris-HCl buffer containing 10 mM EDTA. The suspensions were incubated in an ice-water bath for 30 min. Pellet fractions were obtained by centrifugation, resuspended in Tris-HCl buffer, and used in the adenylate cyclase assay as described under Materials and Methods, "*Hypotonic lysis.*" Prostaglandin activation was examined with broken-cell preparations prepared from platelets in the absence (A) or presence (B) of 10 mM EDTA. ATP was used as the substrate. The *vertical bars* represent standard deviations of the mean. Basal activities were 35.1 and 18.2 pmoles/min/mg of protein in A and B, respectively. The protein content after incubation with EDTA was twice that of the Tris buffer control preparation. This appears to be due to the contribution of intracellular protein and it accounts for the lower specific activity shown in B (see Materials and Methods).

bound adenylate cyclase preparations which exhibit an enhanced response to PGD_2 (Fig. 2). Maximal stimulation by PGD_2 increased from 8.2- to 19.3-fold. This was accompanied by an increase in stimulation by PGI_2 from 19.9- to 29.1-fold. Stimulation by PGE_1 decreased from 15.3- to 10.6-fold. Similar results were obtained with lower concentrations of EDTA (1.54 mM).[2] With the EDTA pretreatment, the order of potency and efficacy of PGI_2, PGD_2, and PGE_1 correspond to their effects on cyclic AMP accumulation.

Cyclic AMP accumulation and phosphodiesterase activity. In addition to being more powerful, the stimulation of cyclic AMP accumulation in washed platelets by PGI_2 is longer-lasting than the increase obtained with PGE_1 (Fig. 3A). The transient elevation pattern in the presence of PGI_2 is characterized by a rapid increase followed by a gradual decline.

To examine the role of cyclic AMP phosphodiesterase activity, cyclic AMP formation was measured in the presence of a competitive inhibitor of this enzyme, MIX. Cyclic AMP accumulates steadily in the presence of 10 mM MIX when incubated with PGI_2, PGD_2, or PGE_1 (Fig. 3B). These results suggest that the decline observed in intracellular cyclic AMP (Fig. 3A) is due to its enzymatic hydrolysis.

Comparison of the magnitude of cyclic AMP accumulation in the presence and absence of MIX revealed that more than 90% of the cyclic nucleotide formed in platelets via stimulation of adenylate cyclase does not accumulate but is, instead, rapidly hydrolyzed by cyclic AMP phosphodiesterase.

[2] R. Alvarez, A. Taylor, J. J. Fazzari, and J. R. Jacobs, unpublished observations.

Although MIX strongly enhances the accumulation of cyclic AMP in the presence of PGD_2, the phosphodiesterase inhibitor also selectively reduces its molar potency. The mechanism of this effect of MIX is not known. It is concentration-dependent and most pronounced at the highest concentration (10 mM) tested.[2]

MIX is a relatively potent inhibitor of cyclic AMP phosphodiesterase in human platelets. With a substrate concentration of 1 μM, the EC_{50} for MIX was 8 μM. However, relatively high concentrations of MIX are required to obtain a maximal effect on cyclic AMP accumulation in intact platelets. This may reflect poor intracellular uptake of the inhibitor. MIX is maximally effective at 10 mM when combined with 1 μM PGE_1 for incubation times up to 2 min. Longer incubation times yield higher cyclic AMP concentrations, but the EC_{50} values increase concomitantly, and 10 mM is no longer a saturating concentration. This time-dependent apparent decrease in the potency of MIX as an inhibitor may be the consequence of the gradual accumulation of cyclic AMP and competition for the catalytic site. It is also consistent with a gradual increase in phosphodiesterase activity following exposure to PGE_1.

Activation of cyclic AMP phosphodiesterase. Initial attempts to demonstrate activation of platelet cyclic AMP phosphodiesterase by prostaglandins were unsuccessful. Instead, we observed inhibition (3–10%) of phosphodiesterase activity in the presence of 1 μM PGE_1 or no significant effect at 0.1 μM. Amer and Marquis (27) have previously reported that PGE_1 decreases the activity of a low K_m phosphodiesterase in broken cell preparations. PGE_1 failed to activate either membrane-bound or soluble phosphodiesterase when the enzyme was iso-

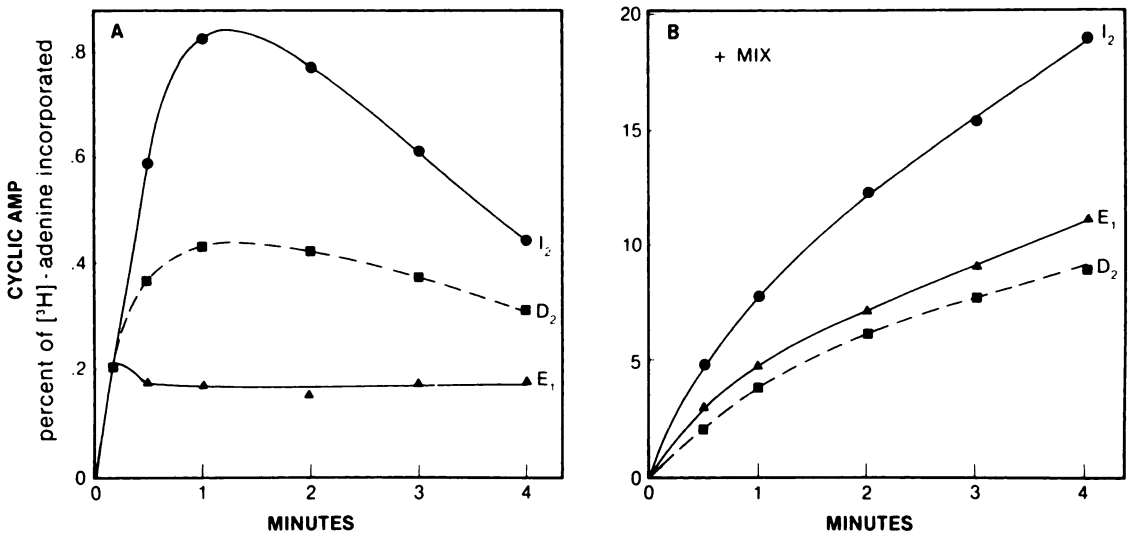

FIG. 3. *Time course of the effect of 1 μM PGI_2, PGD_2, and PGE_1 on ^{3}H-labeled cyclic AMP accumulation from a prelabeled pool of [^{3}H]ATP in washed platelets in the absence (A) and presence (B) of 10 mM MIX at 37°*

The basal level of ^{3}H-labeled cyclic AMP prior to the addition of the prostaglandins was 0.025% of [^{3}H]adenine incorporated.

lated by hypotonic lysis, glass homogenization, or freeze-thawing of platelets.[2]

To examine the possibility that intact cells were required to observe enzyme activation, platelets were preincubated with 1 μM PGI_2, PGD_2, or PGE_1 for 10 min. The cells were then rapidly frozen to rupture the membrane and the suspension was thawed at 30°. Phosphodiesterase activity measured in these broken-cell preparations was 52, 44, and 33% higher than control values after exposure to PGI_2, PGD_2, and PGE_1, respectively.

Basal or stimulated enzyme activity was proportional to incubation time up to 10 min and with protein concentration in the range of 25–250 μg/ml. Enhanced phosphodiesterase activity was observed at low substrate concentrations (0.1–10 μM cyclic AMP) and was accordingly attributed, at least in part, to a low K_m form of the

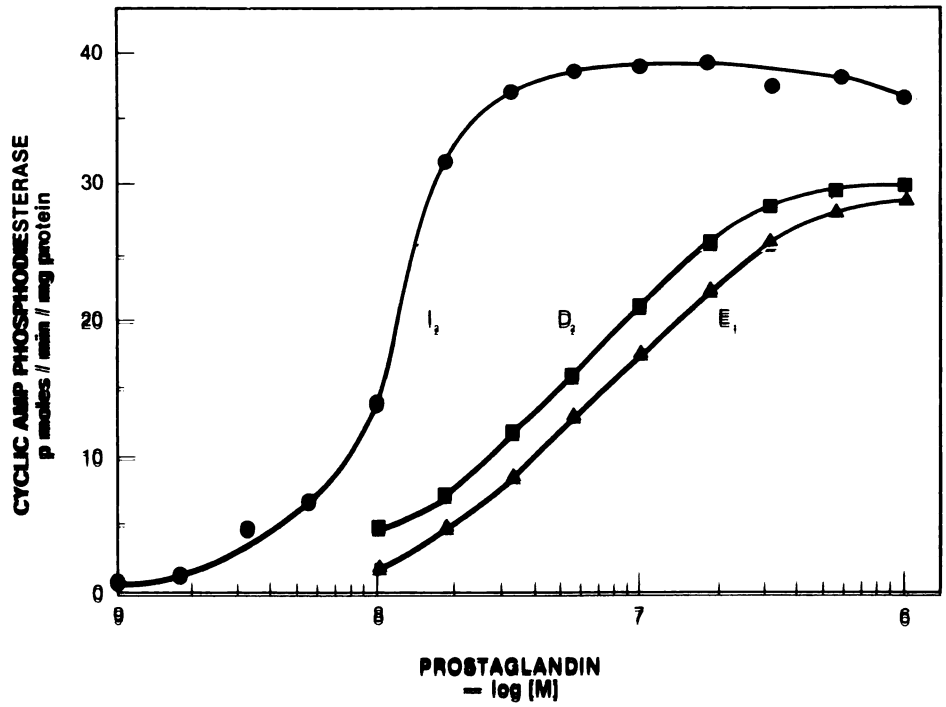

FIG. 4. *Cyclic AMP phosphodiesterase activity in lysed platelets after preincubation with different prostaglandins*

The prostaglandins (all natural enantiomers) were preincubated for 10 min at 30° with intact platelets in Buffer A at the concentrations indicated. Platelet suspensions were obtained by Method II. The assay was performed for 5 min at 30° with 1.0 μM cyclic AMP after lysing the cells with the freeze-thaw procedure described under Materials and Methods. Basal activity (67 pmoles/min/mg of protein) was subtracted from the data presented. Data for each prostaglandin were obtained with platelets from a different donor. Their relative potency and efficacy were confirmed with appropriate concentrations of each prostaglandin with platelets from a single donor.

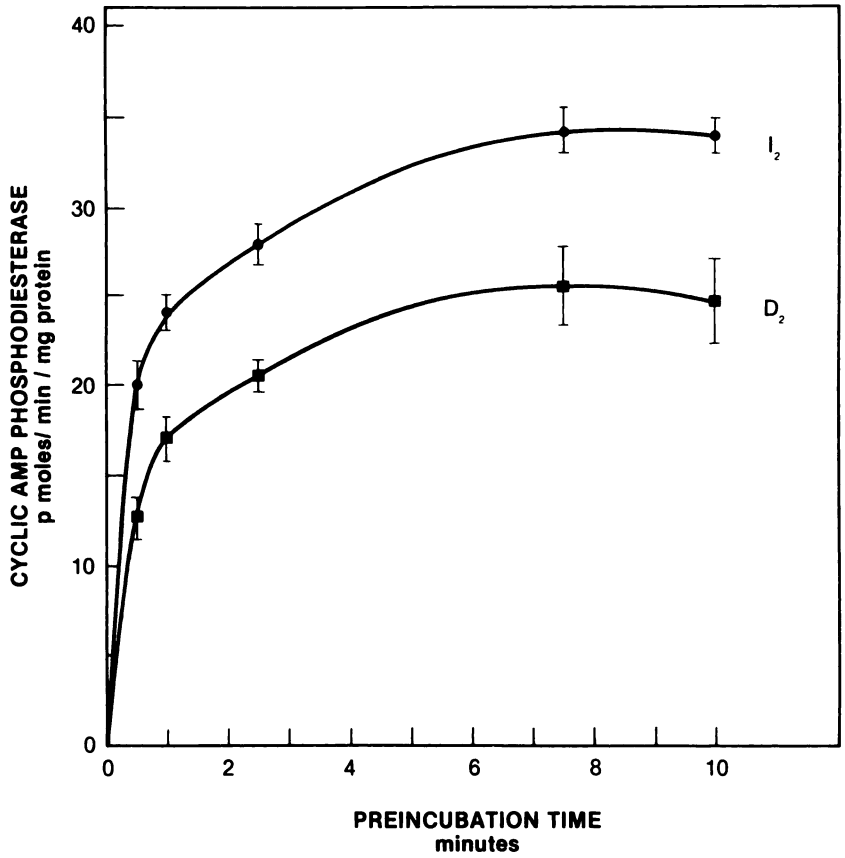

FIG. 5. *Effect of preincubation time in the presence of 1 μM PGI_2 (●) or PGD_2 (■) on cyclic AMP phosphodiesterase activity in lysed platelets*

The platelet suspension was obtained by Method II. The reaction was allowed to continue for 5 min at 30° with 1.0 μM cyclic AMP. The data from three experiments were combined and the mean values are shown. Each experiment (in triplicate) was performed with platelets from a different donor. The *vertical bars* represent standard errors of the mean. Basal activity (59 pmoles/min/mg of protein) was subtracted from the data presented. PGE_1 was also tested at 1 μM and induced a time-dependent increase in enzyme activity which was similar to that obtained with PGD_2 (data not shown).

enzyme. The activated state was stable at 4° for at least 30 min after the freeze-thaw procedure.

The effect of prostaglandins on cyclic AMP phosphodiesterase activity was measured after 10 min of preincubation with intact cells at 30° (Fig. 4). The order of efficacy and potency was $PGI_2 > PGD_2 \geq PGE_1$. PGE_2, PGA_1, and $PGF_{2\alpha}$ had no significant effect on enzyme activity at 1 μM.

Phosphodiesterase activation following exposure to prostaglandins develops rapidly during the 1st min of preincubation and then gradually increases to a maximal level after approximately 7 min (Fig. 5). A comparison of the time course of the effect of PGI_2 and PGD_2 on phosphodiesterase activity (Fig. 5) with cyclic AMP accumulation (Fig. 3A) reveals that the decline in cyclic AMP content coincides with an increase in phosphodiesterase activity.

DISCUSSION

The results of this study indicate that the potency of prostaglandins as inhibitors of ADP-induced aggregation correlates with their capacity to increase intracellular cyclic AMP to a critical, submaximal level. Similarly, under appropriate assay conditions, the efficacy and potency of these prostaglandins as stimulators of adenylate cyclase correspond to their ability to increase the intracellular concentration of cyclic AMP. These results lend further support to the concept that cyclic AMP mediates the effect of prostaglandins on platelet function and that activation of adenylate cyclase is the primary biochemical event responsible for the accumulation of cyclic AMP. A discrepancy between stimulation of cyclic AMP formation and adenylate cyclase activity by PGD_2 was observed under standard assay conditions. Preincubation of platelets in a hypotonic buffer containing EDTA yields an enzyme preparation which exhibits the expected response to PGI_2, PGD_2, and PGE_1 and eliminates the discrepancy.

The transient increase in platelet cyclic AMP which occurs in response to PGI_2 is characterized by a rapid increase followed by a gradual decline. The decrease in cyclic AMP is due to cyclic AMP phosphodiesterase activity and may involve an indirect activation of this enzyme by PGI_2. This interpretation is supported by three observations: (a) inhibition of phosphodiesterase activity with MIX eliminates the transient pattern and permits accumulation of the cyclic nucleotide, (b) prostaglandins indirectly stimulate the activity of a cyclic AMP phosphodiesterase when preincubated with intact platelets, and (c) the time course of phosphodiesterase activation coincides with the decrease in cyclic AMP.

The mechanism of phosphodiesterase activation is not clear. It may be mediated by cyclic AMP or may involve a change in the ratio of interconvertible forms of the platelet enzyme (28), enzyme phosphorylation via cyclic AMP-dependent protein kinase (29), or calmodulin (30). The order of potency of prostaglandins in increasing phosphodiesterase activity ($PGI_2 > PGD_2 \geq PGE_1 \gg PGE_2$) corresponds to that obtained for the receptor-mediated elevation in intracellular cyclic AMP (Fig. 1B) and suggests that the activation process is mediated by prostaglandin receptors which are similar to or identical with those which activate adenylate cyclase.

Phosphodiesterase activation by hormones which increase cyclic AMP formation has been observed in other isolated cell preparations. Incubation of rat adipocytes with catecholamines or ACTH increases phosphodiesterase activity (31, 32). A similar stimulation was obtained with rat hepatocytes incubated with glucagon (33). Nemecek *et al.* (34) have previously reported that PGE_1 decreases phosphodiesterase activity in human diploid fibroblasts.

Prostaglandin-stimulated adenylate cyclase activity in human platelet membrane preparations is linear as a function of time up to 10 min.[2] This pattern indicates that the activation process is virtually instantaneous. In contrast, the indirect activation of platelet phosphodiesterase develops over a period of several minutes. Sequential activation of these enzymes would permit the observed burst in intracellular cyclic AMP followed by a gradual decline to a new steady-state level. Thus, the increase in phosphodiesterase activity may represent a homeostatic mechanism in the regulation of cyclic AMP metabolism.

ACKNOWLEDGMENTS

We gratefully acknowledge the many useful discussions with Dr. John J. Bruno and his support of this project. This study was facilitated by the technical assistance of Ms. Gail Winter. Platelet electron micrographs were provided by Mrs. Friederike M. Freymark.

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