

PROSTAGLANDINS, CYCLIC AMP AND HUMAN PLATELETS

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By

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LIST OF ABBREVIATIONS

AC.....adenylate cyclase
ADP.....adenosine diphosphate
ATP.....adenosine triphosphate
cyclic AMP.....adenosine 3',5'-cyclic monophosphate
5-HT.....serotonin, 5-hydroxytryptamine
PDE.....cyclic AMP phosphodiesterase
PGE₁, PGE₂, PGE_{2α}.....prostaglandins E₁, E₂ and F_{2α}
PPP.....platelet-poor plasma
PRP.....platelet-rich plasma
t.l.c.....thin layer chromatography

ABSTRACT

Preincubation of PRP with [14 C]adenine made it possible to monitor cyclic [14 C]AMP levels in intact platelets. Because the accumulation of cyclic [14 C]AMP in response to PGE₁ or PGE₂ was linear for at least 30 sec in the presence of a PDE inhibitor, the kinetics of the activation of AC by PGE₁ or PGE₂ could be investigated. Evidence was found for one PGE₁ receptor and two PGE₂ receptors as well as for a mutual competitive inhibition between the effects of PGE₁ and PGE₂. In the absence of a PDE inhibitor, PGE₂ reduced PGE₁-elevated levels of cyclic [14 C]AMP more than in the presence of a PDE inhibitor suggesting an activation of PDE as an additional mode of action. PCF was virtually inactive with platelet AC.

²⁰
In the absence of a PDE inhibitor, both PGE₁ and PGE₂ caused an increase in cyclic [14 C]AMP levels that peaked at 30 sec before declining to a steady state level of about half the maximum. In the presence of a PDE inhibitor, cyclic [14 C]AMP levels rapidly rose to plateau steady state levels by one minute. The results suggest that a decrease in AC activity, which would account for these effects occurs between 30 sec. and 1 min after addition of PGE₁.

The aggregating agents, ADP and epinephrine markedly reduced cyclic [14 C]AMP levels elevated by PGE₁ and caffeine. ADP was found to cause a non-competitive inhibition of the activation of AC by PGE₁. The effects of epinephrine were mediated by α -adrenergic receptors. A computer simulation of these effects suggested various mechanisms of actions which are discussed.

Platelet cyclic [14 C]AMP levels measured after the addition of ADP were found to correlate better with the inhibition of that aggregation by PGE₁ + papaverine than cyclic [14 C]AMP levels measured before the addition of the aggregating agent. Comparison of the relationship between inhibition of aggregation and increase in cyclic [14 C]AMP levels caused by PGE₁ and by PGE₁ + papaverine suggested a significant delay in the action of cyclic AMP on platelet aggregation. A given intracellular level of cyclic [14 C]AMP was more effective against aggregation caused by low rather than high ADP concentrations.

Di-homo- γ -linolenic acid, the fatty acid precursor of PGE₁, only increased the basal levels of cyclic [14 C]AMP very slightly, even in the presence of a PDE inhibitor.

Arachidonic acid, the fatty acid precursor of PGE₂ and PGE_{2G}, caused aggregation which was inhibited by aspirin. Arachidonic acid competitively inhibited the activation of AC by PGE₁.

The implications of these results in light of the effects of

PGE₂ and aspirin on ADP-induced aggregation were investigated and are discussed.

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Chapter I

GENERAL INTRODUCTION

1. BLOOD PLATELETS

As a research tool platelets have a number of advantages; they are easily obtained as a homogeneous preparation, they exhibit a number of characteristic reactions in response to physiological stimuli (e.g., adhesion, shape change, aggregation and a secretory process 'the release reaction') and these reactions can be greatly modified by drugs thus providing a good model system for pharmacological analysis.

Platelets are the smallest (2-3 μ in diameter) formed elements of the blood. They are non-nucleated, membrane bound bodies derived from megakaryocytes in the bone marrow. They contain mitochondria, amine storage granules ('dark bodies'), lysosomes (α -granules), microtubules and microfilaments (Behnke, 1967; Mustard & Packham, 1970). A contractile protein 'thrombosthenin', that probably functions in aggregation and the release reaction, is also present (Mustard & Packham, 1970). Platelets derive their energy chiefly from glucose by way of glycolysis and oxidative phosphorylation (Cherayak, 1965; Marcus & Zucker, 1965) and have the capacity to synthesize proteins (Booyss et al., 1968) and lipids (Lewis & Majerus, 1969). They have

a mean lifespan in the human circulation of approximately 10 days (Mustard & Packham, 1970).

The fundamental property of the blood platelet is its ability to respond to various stimuli by the formation of aggregates. Thus platelets are normally separate from one another in the circulation and will not adhere to normal endothelium but when the endothelium has been damaged they will adhere to the exposed subendothelial tissues (i.e. collagen fibres, basement membrane or microfibrils; Zucker & Borrelli, 1962; Tranzer et al., 1967; Spaet et al., 1971). Platelets then undergo a selective secretory process the 'release reaction', liberating into the plasma from cytoplasmic granules adenine nucleotides, serotonin and epinephrine as well as lysosomal enzymes. ADP, serotonin and epinephrine are themselves capable of inducing aggregation and the 'release reaction' (Mustard & Packham, 1970). Thrombin also induces release and provides one pathway for the interaction of platelets and plasma coagulation factors in hemostasis. Serotonin and epinephrine are biogenic amines with vasoconstrictor activity and may help to reduce the loss of blood from damaged vessels. Of the adenine nucleotides that are released ADP is the most important. It produces a change in platelet shape (i.e. a transformation from thin discs to

spiculated spheres), an increase in platelet stickiness and platelet aggregation. ADP has also been shown to activate factor XII on platelet membranes thus involving platelets in the intrinsic blood coagulation pathway (Walsh, 1972). The alteration of the platelet membrane is believed to expose a membrane-bound lipoprotein (Marcus, 1972), platelet factor 3, which accelerates thrombin generation. Thus injury to the blood vessel wall leads to platelet adhesion and then by way of the release reaction to platelet aggregation, and when stabilized by the formation of fibrin (Hovig et al., 1968) to the formation of a hemostatic plug or thrombus.

There is increasing evidence that the platelet plays a critical role in almost all responses of the blood to injury. Through its role in hemostasis the platelet is involved in the response to vascular injury, while through phagocytosis and the release of proteolytic enzymes and permeability factors (Mustard et al., 1965, 1968) it is involved in the response to the introduction of foreign materials and chemicals into the blood stream, as well as to many metabolic products that enter the blood particularly under stressful and abnormal conditions (Mustard & Packham, 1970).

Clinically platelet function has been considered mainly in relation to several hemostatic disorders (thrombo-

cytopenia, thrombasthenia and various thrombocytopathies), thrombosis and the development of atherosclerosis.

Platelets have also been studied in relation to vascular prostheses, uraemia, multiple sclerosis and aspirin ingestion.

Several hypotheses have been proposed to explain ADP-induced platelet aggregation. Gaarder and Laland (1964) suggested that platelet-bound ADP permits the formation of calcium bridges between platelets and hence aggregation. Spaet and Lejnieks (1966) observed that ADP was converted to AMP by platelets and proposed that the resultant free energy release was used in some way in the process of aggregation. Chambers (1967) suggested that ADP induced platelet aggregation by product inhibition of an ecto-ATPase which otherwise maintained the platelet membrane in a non-adhesive state. White (1968) proposed that ADP interfered with a membrane calcium pump permitting calcium ions to enter the platelet and activate the ATPase of thrombosthenin thus initiating a contractile process which led to aggregation. Booyse and Rafelson (1969) have suggested that platelet aggregation resulted from the combination of the actin and myosin of thrombosthenin on adjacent platelets. Guccione et al. (1971) postulated the presence of a membrane bound nucleotide diphosphokinase as the site of ADP action.

Despite the multiplicity of hypotheses the mechanism

of platelet aggregation is still not known. Agents causing aggregation range in size from small molecules such as ADP and epinephrine to large proteins such as thrombin and collagen. How such widely differing agents, which are not pharmacologically similar in any other tissue, induce a similar response in platelets is not obvious though it has been suggested that the effects of all these agents may be mediated by ADP (Haslam, 1967). A number of drugs inhibit aggregation caused by all aggregating agents. These drugs include adenosine, PGE_1 , and caffeine (Mustard & Packham, 1970), the pharmacological actions of which, on platelets are now believed to be mediated through increases in the intracellular level of cyclic AMP (Mills & Smith, 1971; Salzman, 1972; Haslam, 1973). Recently a great deal of work has been done investigating the effects of agents that either cause or inhibit platelet aggregation on platelet cyclic AMP levels. Certain agents that cause aggregation are capable of lowering previously raised cyclic AMP levels (Robison et al., 1969; Marquis et al., 1969; Haslam & Taylor, 1971b; Cole et al., 1971; Harwood et al., 1972; Mills & Smith, 1971, 1972). A few workers have reported that aggregating agents depress basal cyclic AMP levels (Salzman, 1972). However, although it has now been accepted as a general hypothesis that cyclic AMP mediates the inhibition of platelet aggregation by a number of compounds

including PGE_1 , the role of any decrease in basal cyclic AMP levels in regulating platelet function is still disputed (Salzman, 1972; Haslam, 1973).

2. CYCLIC AMP

A heat-stable factor mediating the action of epinephrine and glucagon on the activation of liver phosphorylase was found in 1956 (Rall et al., 1956). This heat-stable factor, cyclic AMP, appears throughout the animal kingdom and is also present in micro-organisms. The biological role of cyclic AMP has developed from a relatively simple one to the point where it appears to be a key regulatory agent in most mammalian tissues (Robison et al., 1971). Sutherland and co-workers have clarified the action of many hormone-target-organ combinations. The general system is one in which the hormones travel from their cells or origin to the cells of the target tissue(s) where they act to increase the intracellular level of cyclic AMP by stimulating AC, an enzyme in the cell membrane that catalyzes the formation of cyclic AMP from ATP (Sutherland, 1970).

It is not certain how the receptors for these hormones are related to AC. One model proposes an external

regulatory subunit which acts as the receptor for the hormone and an associated internal catalytic subunit that is basically similar in all tissues, whereas the structure of the regulatory subunit would vary from tissue to tissue thus accounting for the hormonal specificity. For example, glucagon does not stimulate AC in adrenal cortical preparations, just as ACTH does not stimulate hepatic AC (Haynes et al., 1960). In tissues in which two or more structurally different hormones are known to stimulate AC there would be a different receptor subunit for each hormone associated with the same type of catalytic subunit (Robison et al., 1969). Pohl and Rodbell (1971) have described the importance of phospholipids in the action of hormones on AC (e.g. glucagon and liver adenylate cyclase) suggesting a 'transducer' effect of some nature between the hormone receptor and the catalytic subunit of the cyclase.

Cyclic AMP is hydrolysed to 5'-AMP by another enzyme, PDE. This enzyme can be inhibited by a number of drugs (e.g. methyl xanthines, Butcher & Sutherland, 1962; papaverine, Kukovetz & Poch, 1970; pyrimidopyrimidines such as EA231, Mills & Smith, 1971). These two enzymes then, AC and PDE, determine the cyclic AMP level in cells.

Within the cell, cyclic AMP functions as a regulator

of biochemical processes specific to that particular cell and thus differs from many biochemical agents (e.g. ATP, Ca^{++}) which may at times play a regulatory role but which have other more essential functions. Hormones operating through cyclic AMP are in most tissues associated with an increase in its intracellular concentration secondary to the stimulation of AC. The effects of such hormones on such target tissues can be mimicked or potentiated by the inhibition of PDE which blocks the degradation of the nucleotide. However, some agents including the catecholamines, prostaglandins and insulin are capable of lowering the level of cyclic AMP in some cells either by an inhibition of AC or an activation of PDE. For example, PGE_1 (Butcher & Baird, 1968) and insulin (Butcher et al., 1966) lower epinephrine-elevated levels of cyclic AMP in isolated fat cells, and epinephrine in the presence of a β -adrenergic blocking agent will lower basal cyclic AMP levels in fat cells, pancreatic islets and in the toad bladder (Turtle & Kipnis, 1967).

The mechanism of action of cyclic AMP in tissues where it is recognized as a second messenger has not been established in all cases and hence an explanation of all the known effects of cyclic AMP in terms of a single common mode of action is not yet possible. However cyclic AMP-dependent enzymes are known to exist in most tissues in the form of protein kinases that mediate the phosphorylation of proteins.

substrates by ATP (Robison et al., 1971; Kuo & Greengard, 1969). It appears that cyclic AMP increases protein kinase activity by binding to a regulatory subunit of the enzyme with the result that the catalytic subunit dissociates in an active form (Gilman, 1970; Reimann et al., 1971). The physiological substrates for the protein kinase are in most tissues unknown, though in a few cases they have been identified as regulating enzymes e.g. muscle phosphorylase kinase (Walsh et al., 1968), glycogen synthetase (Larner, 1968) and hormone sensitive lipase (Corbin & Krebs, 1969; Tsai et al., 1970). Ribosomes in rabbit reticulocytes (Kabat, 1970), microtubules in brain (Goodman et al., 1970) and histones (Langan, 1968) have also been shown to act as substrates but the physiological significance of these effects is uncertain as the cyclic AMP-activated protein kinases appear to be of low specificity. However, specificity could be conferred by the intracellular localization of the protein kinase, and it is interesting in this connection that the membranes of the cardiac sarcoplasmic reticulum appear to contain a cyclic AMP-dependent protein kinase which is associated with the activation of calcium transport (Kirchberger et al., 1973).

3. PROSTAGLANDINS

Although the biological effects of prostaglandins were first seen by Kurzrok and Lieb in 1930 it was not until 1960 that Bergström and Sjövall isolated and determined the chemical structure of the first prostaglandins. All members of the prostaglandin family contain 20 carbon atoms and all have the same basic carbon skeleton of the hypothetical parent compound, prostanoic acid, which consists of a five member ring with two aliphatic side chains. Various arrangements of double bonds, hydroxyl and ketone groups divide the family into four classes A, B, E, and F. Six E and F compounds, the six primary prostaglandins, are derived from 8,11,14-eicosatrienoic acid; 5,8,11,14-eicosatetraenoic acid (arachidonic acid) and 5,8,11,14,17-eicosapentaenoic acid (Bergström et al., 1964) which can be found in most tissues. These can be converted to the eight secondary naturally occurring prostaglandins that have been identified to date (Figure 1). Each class A, B, E or F differs in the substitution and saturation of the cyclopentane ring while each member of a class differs from the others in the number of unsaturated double bonds in the aliphatic side chains, e.g. PGE_1 , PGE_2 and PGE_3 .

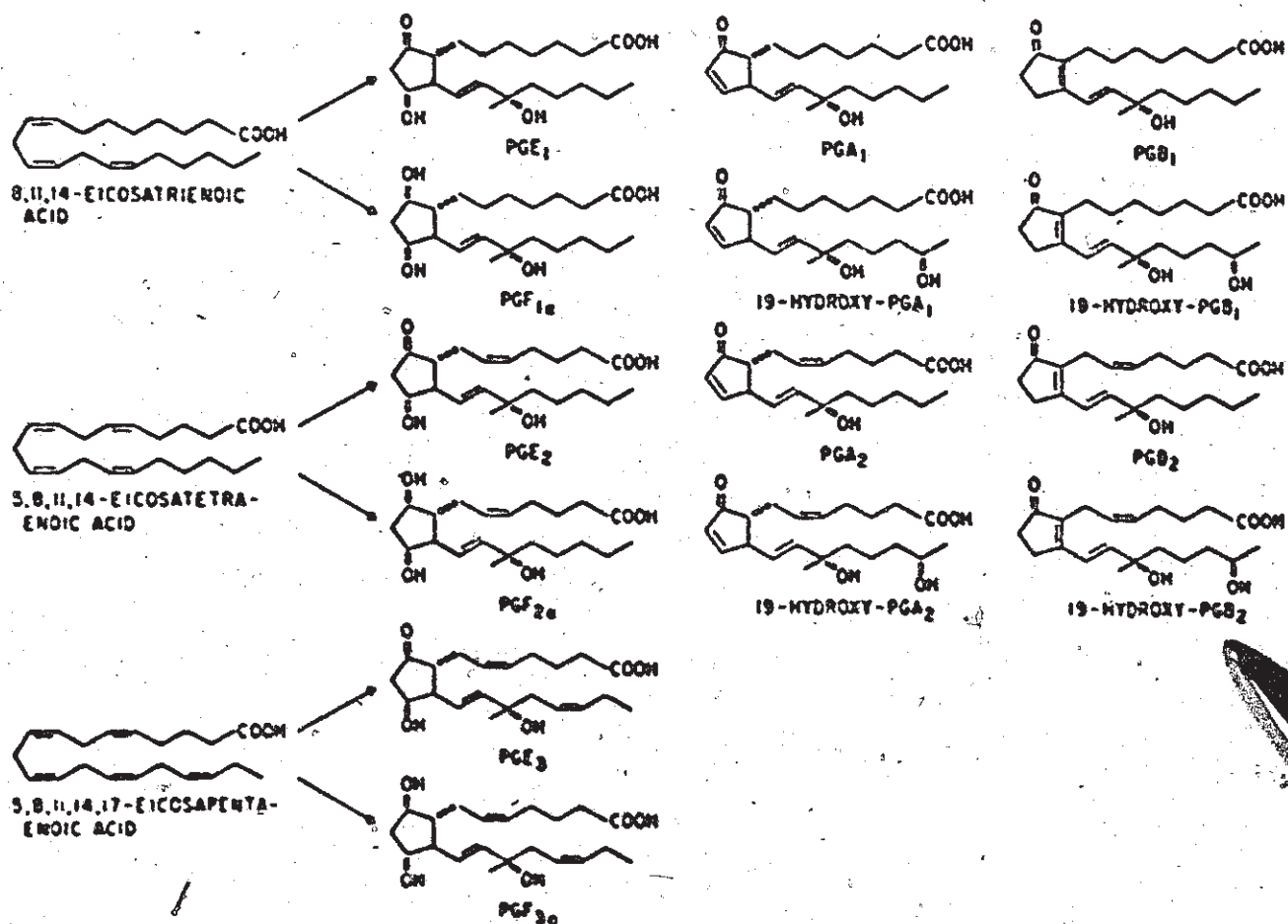


FIGURE 1. Structural formulas of the naturally occurring prostaglandins and their precursors.

Through the work of Samuelsson and Hamberg the basic mechanism for the synthesis of prostaglandins from their fatty acids precursors has been formulated (Figure 2). Phospholipase A liberates the polyunsaturated fatty acid precursors from membrane phospholipids prior to incorporation of molecular oxygen and cyclization (Bartels et al., 1970; Bergström et al., 1964; Samuelsson, 1965). Privett et al.,

(1972) working on hypophysectomized rats have implicated a pituitary function in the regulation of prostaglandin synthesis and hence the expression of the biological activity of the essential fatty acids.

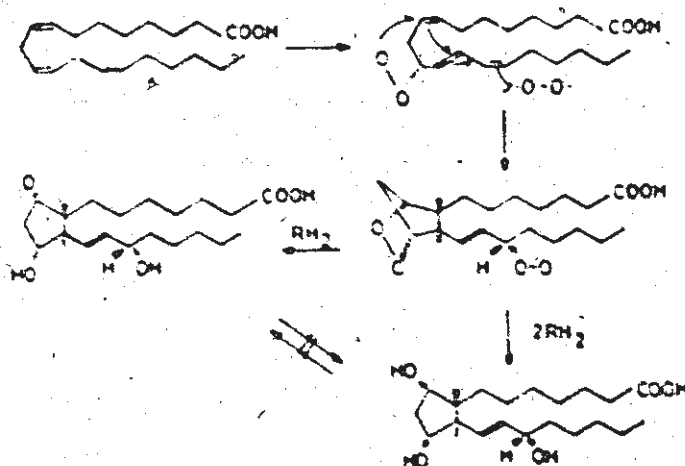


FIGURE 2. Mechanism proposed for the conversion of 8,11,14-cicosatrienoic acid to PGE_1 and PGF_{1a} .

Samuelsson (1971) has identified 7 α -hydroxy-5,11-diketotetranorprosta-1,16-dioic acid as the major human urinary metabolite of PGE_1 and PGE_2 . Figure 3 shows that PGE_2 is metabolized by way of a 15-hydroxydehydrogenase followed by reduction of the Δ^{13} double bond and β and ω oxidation. It is unlikely that the E and F prostaglandins function as circulating hormones in that they are 90% metabolized in one passage through the lungs and liver (Davson et al., 1968 and Ferreira et al., 1967).

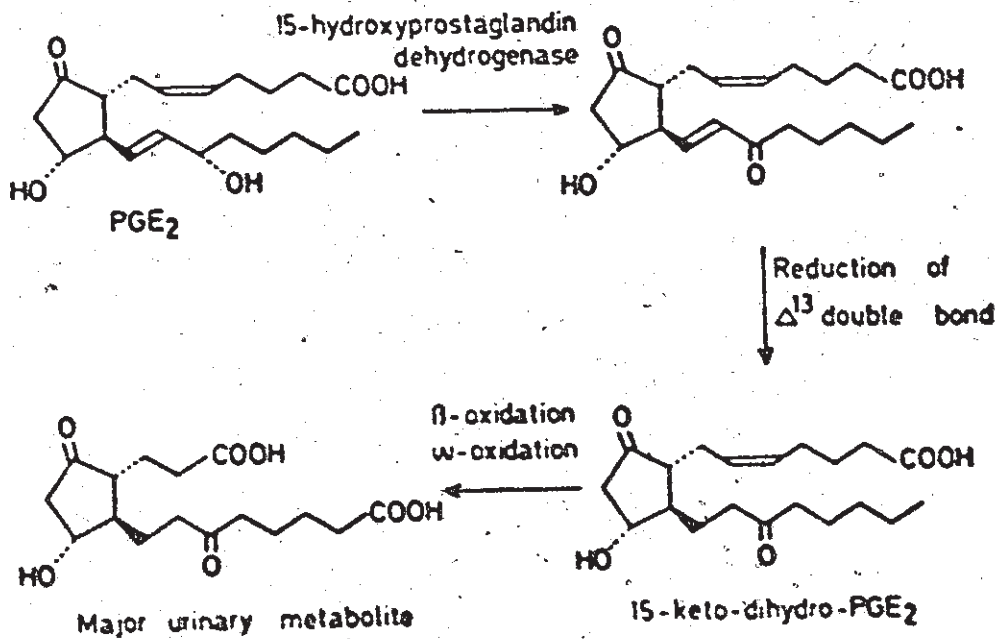


FIGURE 3. Reactions involved in the formation of the major urinary metabolite (7α-hydroxy-5,11-diketotetranor-prosta-1,16-dioic acid) from PGE₂.

Prostaglandins are widely distributed in biological systems. They exhibit a wide spectrum of actions being released in response to simple physiological stimuli (Ramwell & Shaw, 1970). Already they or one of their more stable or more potent derivatives have been used therapeutically for the induction of labour and/or abortions and their use has been suggested in the treatment of infertility, peptic ulcer, asthma, shock, hypertensive

disease and congestive heart failure (Higgins & Braunwald, 1972).

A single mechanism explaining the diverse action of prostaglandins on various tissues has not been formulated. A number of observations concerning the effects of prostaglandins on membrane and intracellular cations have suggested mechanisms which would involve the release of membrane-bound calcium (Coceani & Wolfe, 1966; Strong & Bohr, 1967; Ramwell & Shaw, 1970). A great deal of evidence has now accumulated however, which indicates that the pharmacological effects of prostaglandins are manifested in modulating the cyclic AMP levels in many cell types in response to neural and hormonal influences (Ramwell & Shaw, 1967; Bergström, 1967; Shio & Ramwell, 1971; Robison et al., 1971).

Although it is apparent that hormonal activation of tissues may be effected through an activation of AC (Robison, 1967; Sutherland & Robison, 1968), it is now well established that hormonal stimulation of many tissues and organs is also associated with changes in endogenous prostaglandins, qualitatively as well as quantitatively (Shio et al., 1971). Studies regarding the effect of tissue stimulation on endogenous prostaglandins have indicated that more prostaglandins are released than can be initially extracted (Ramwell & Shaw, 1970; Smith &

Willis, 1970), suggesting in fact a hormonal activation of the biosynthetic process rather than release from a preformed store. Prostaglandins have been found to mimic the action of hormones in certain tissues in increasing cyclic AMP formation through the activation of AC (Field et al., 1969; Kuehl et al., 1972), while in other tissues they have been found to inhibit the hormonal stimulation of cyclic AMP formation, for instance by vasopressin (Orloff et al., 1965). Kuehl and co-workers (1970) reported that in the mouse ovary 7-oxa-13 prostynoic acid competitively antagonized the stimulatory effect of luteinizing hormone (LH) and PGE_1 on cyclic AMP accumulation thus indicating that prostaglandins may play a key role in the LH-cyclic AMP sequence. These experiments have been reproduced by Burke et al. (1971) with thyroid-stimulation-hormone and by Vale et al. (1971) using TSH-releasing hormone. Results of such studies have led to the conclusion that in those tissues where prostaglandins are synthesized on humoral stimulation they may in turn serve to amplify or attenuate the duration of the concomitant cyclic AMP response and the resulting physiological response (Shaw et al., 1972; Kuehl et al., 1972).

4. THE PRESENT STUDY

It was the purpose of this study to examine several aspects of the relationship between prostaglandins and cyclic AMP in relation to human platelet function. With regard to the prostaglandins, at the start of this work it was known that PGE_1 was a potent inhibitor of platelet aggregation (Klöße, 1966), that PGE_2 inhibited the first wave but enhanced the second wave of ADP-induced aggregation (Shio & Ranwell, 1972) and that PGE_2 and $PGF_{2\alpha}$ were produced in platelets in response to thrombin stimulation (Smith & Willis, 1970). It was known that PGE_1 increased the intracellular concentration of platelet cyclic AMP but that these increases did not correlate well with the inhibition of aggregation in some instances (Ball et al., 1970; Mills & Smith, 1972). It was also known that PGE_2 decreased PGE_1 -elevated levels of cyclic AMP in rat platelets (Shio & Ranwell, 1972) and that ADP and epinephrine decreased PGE_1 -elevated levels of cyclic AMP (Robison et al., 1969; Marquis et al., 1970; Haslam & Taylor, 1971). Against this background the objectives of the present investigation were briefly (a) to confirm and extend the known relationships between PGE_1 , PGE_2 and $PGF_{2\alpha}$ stimulation of AC and platelet cyclic AMP levels, (b) to examine the mechanisms by which ADP and epinephrine are capable of

lowering PGE₁-elevated cyclic AMP levels, (c) to examine further the relationship between PGE₁-increased cyclic AMP levels and the inhibition of platelet aggregation and (d) to examine the effects of prostaglandin precursors on platelet cyclic AMP levels and platelet function. The technique of prelabelling the platelet metabolic pool of ATP with high specific activity [¹⁴C]adenine was devised as the most sensitive assay for cyclic AMP available (Kuo & DeRenzo, 1967; Ball et al., 1969). It was however, first necessary to verify the applicability of this approach in a series of preliminary experiments.

Chapter II

MATERIALS AND METHODS

1. MATERIALS

Adenine nucleotides were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. and were dissolved in isotonic saline (0.154 M NaCl), and adjusted to pH 7.0 with NaOH.

Radioactive compounds. [U-¹⁴C]Adenine (250 μ Ci, 287 μ Ci/mmol) was obtained from Amersham/Searle Corp., Arlington Heights, Illinois, U.S.A. and [8-³H]adenosine 3':5'-cyclic monophosphate (250 μ Ci, 28 Ci/mmol) was obtained from Schwarz/Mann, Orangeburg, N.Y., U.S.A. 5-Hydroxytryptamine-3'-C¹⁴ (55 μ Ci/mmol) was a kind gift from Dr. J. Mustard's laboratory.

Adenosine 3',5'-cyclic monophosphate phosphodiesterase.

0.2-0.5 units/mg protein was obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. and was dissolved immediately before experiments at 5 U/ml isotonic saline.

Adenosine 3',5'-cyclic monophosphate phosphodiesterase

Inhibitors. Theophylline and caffeine (anhydrous) were obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A. Papaverine was obtained from BDH Chemicals, Toronto, Ontario, Canada and RA133 was obtained from Dr. Karl Thomae GmbH, Biberach an der Riss.

Adrenergic antagonists. Phentolamine was obtained as a gift from Ciba Geigy in Montreal and (-)-propranolol as a gift from I.C.I. Ltd., Pharmaceuticals Division, Macclesfield, England.

(-)-Epinephrine bitartrate was obtained from Sigma Chemical Co., St. Louis, Missouri, U.S.A.

Prostaglandins and Prostaglandin Precursors. PGE₁, PGE₂ and PGF_{2α} were kindly provided by Dr. John Pike of the Upjohn Company, Kalamazoo, Michigan, U.S.A. and were dissolved in 95% ethanol and neutralized to pH 7.0. A prostaglandin inhibitor, 7-oxa-13 prostynoic acid was a kind gift of Dr. J. Fried, University of Chicago, Chicago, Ill., U.S.A. Two prostaglandin precursors were used: 5,8,11,14-eicosatetraenoic acid (arachidonic acid) grade I from Sigma Chemical Co., and 8,11,14-eicosatrienoic acid from Applied Science Laboratories Inc., State College, Penn., U.S.A. A control fatty acid (8,11-eicosadienoic acid) was also obtained from Sigma Chemical Co.

General Chemicals. Analytic reagents for general use were obtained from either Fisher Scientific Co., Fair Lawn, N.J., U.S.A. or Sigma Chemical Co., St. Louis, Missouri, U.S.A.

2. METHODS

Preparation of heparinized platelet-rich plasma. Human blood was collected by free-flow venipuncture using siliconized KC-17 gauge needles (Fenwal Laboratories, Morton Grove, Ill., U.S.A.), from healthy male or female volunteers who had not taken any aspirin or aspirin-like drugs during the previous week. Silicone rubber tubing and siliconized glassware were used throughout the procedure. The blood was collected into an isotonic saline solution of heparin (1000 U/ml, Sigma Chemical Co.), one hundred volumes of blood being mixed with one volume of anticoagulant. The blood was then centrifuged at room temperature in 50 ml glass tubes at $183 \times g$ for 15 min in a swing-out head Sorvall RC-3. The resultant PRP was transferred using siliconized Pasteur pipettes to a stoppered siliconized Erlenmeyer flask which was then kept in a water bath at 37°C .

In the preparation of washed platelets the acid-citrate-dextrose (ACD) solution of Aster and Jandi (1964) was used, six volumes of blood being mixed with one volume of anticoagulant. Suspensions of washed human platelets were prepared according to Mustard, Perry and Ardlie (1972).

Preparation of platelet-poor plasma. After the removal of PRP the remaining blood was recentrifuged at 1650 x g for 10 min and the resultant PPP was removed and stored at 37°C. PRP was diluted with PPP to give $3.6-4.0 \times 10^8$ platelets per ml when necessary.

Platelet counting. PRP was diluted 1 to 20 with a solution of 3.8% (w/v) citrate and 0.154 M NaCl mixed 1 part to 9 parts and counted in a Spencer hemocytometer (AO Instrument Co., Buffalo, N.Y., U.S.A.) under a phase-contrast microscope.

Aggregation Technique. Platelet aggregation was measured turbidometrically (Born 1962), using an adsorptionmeter (Payton Associates Ltd., Scarborough, Ont., Canada) and a potentiometer recorder (Photovolt Corp., N.Y.C., N.Y., U.S.A.) operated in the logarithmic mode. 0.85 ml samples of PRP with saline and any other additions to a final volume of 1 ml were maintained in cuvettes (1 cm diameter) at 37°C by a thermostatically controlled heating block surrounding the cuvette and stirred from below by a magnetic stirrer rotating at 900 rpm. A beam of light was passed through a yellow (606) filter and the platelet suspension onto a photoelectric cell and the decrease in optical density due to platelet aggregation was measured against a PPP blank. Each sample of platelet suspension was stirred for 1 min before

the addition of an aggregating agent to ensure that a stable turbidity level was achieved.

Purification of [U-¹⁴C]adenine. Five t.l.c. plates (20 x 20 cm, 250 μ thick) were prepared using MN cellulose 300 HR (Macherey Nagel and Co., Rexdale, Ont.), heat activated (30 min at 100°C) and cleaned by running the second dimension in Solvent II (Krebs & Hemo, 1953; isobutyric acid: 1 M ammonia: 0.1 M EDTA in v/v ratios of 125:75:2). The plates were allowed to dry and again heat activated after which 50 μ l (50 μ Ci) of the stock [¹⁴C]adenine solution (250 μ Ci) was applied to each plate. The plates were then run in the first dimension in Solvent I (Turtle & Kipnis, 1967; 1-butanol: acetone: glacial acetic acid: 14.8% NH₃: H₂O in v/v ratios of 90:30:20:1:60) for 2 1/2 hours, air dried and run in the second dimension in Solvent II for 4 hours. The [¹⁴C]adenine spots were identified under UV light, scraped from the plate and eluted 4 x 0.5 ml saline by centrifugation at 12,000 x g for 4 min in an Eppendorf 3200 centrifuge. The volumes of the eluates were measured and a 5 μ l sample of each as well as the stock solution was counted, this allowed for the determination of recoveries and, given the specific activity of the starting material, the preparation of a 200 μ M solution of [¹⁴C]adenine. Any traces of cellulose were removed from the bulked eluates by centrifugation at 12,000 x g for 15 min.

Purification of [^3H]adenosine 3',5'-cyclic monophosphate.

One ml (250 μCi) of cyclic [^3H]AMP was spotted in two places on a t.l.c. plate and run in Solvent I. The spots were identified with the aid of a mixed nucleotide marker, scraped off and eluted with 3 x 1 ml of water. The eluates were bulked and diluted with water to give 25,000 dpm per 50 μl (i.e. 0.225 $\mu\text{Ci}/\text{ml}$).

Cyclic [^{14}C]AMP Assay. Cyclic [^{14}C]AMP was measured in platelets in which the metabolic/ATP pool had been prelabelled with [^{14}C]adenine (Kuo & DeRenzo, 1967; Ball et al., 1969). Platelet-rich plasma containing $3.6\text{-}4.0 \times 10^8$ platelets per ml was incubated for 90 min at 37°C with 2 μM [^{14}C]adenine by which time more than 90% of the ^{14}C was platelet bound (i.e. removed by centrifugation). Samples (0.85 ml) of this PRP with various additions to a final volume of 1 ml were incubated for specific times before mixing with 0.2 ml of ice cold 3 N perchloric acid. 50 μl of cyclic [^3H]AMP (0.01 μCi) was added to each sample as an internal standard permitting the determination of the recoveries of cyclic [^{14}C]AMP.

After standing 15 min in ice the precipitated proteins of each sample were removed by centrifugation at $12,000 \times g$ for 4 min. The supernatants were added to columns containing 1.5 ml of cation exchange resin (Biorad AG 50W-X8, H^+ form,

200-400 mesh, (Calbiochem Ltd., San Diego, California, U.S.A.) from which the cyclic AMP was eluted with 1 mM K-phosphate buffer, pH 7.3 in the 6-15 ml fraction.

To each eluate 0.3 ml 0.25 M $ZnSO_4$ and 0.3 ml 0.25 M $Ba(OH)_2$ was added. The mixture was centrifuged at 4°C for 10 min at 730 x g in order to remove the precipitate and absorbed nucleotides (Krishna, Weiss & Brodie, 1968). This procedure was repeated and the supernatants of each sample were lyophilized in 20 ml scintillation vials.

Residues were assayed for 3H and ^{14}C using a liquid scintillation counter. Cyclic [^{14}C]AMP values were corrected for the recoveries of cyclic [3H]AMP, which varied from 45-65%, and were expressed as a percentage of the platelet bound ^{14}C . Samples of PRP and PPP (50 μ l) were taken from the incubation PRP just prior to and after the experimental incubations, thus permitting the amount of ^{14}C taken up by the platelets in each sample to be determined.

Individual sample incubations lasted from 20 sec in kinetic studies up to 12 min in time course studies and were carried out between 90 and 110 minutes after the addition of [^{14}C]adenine to the PRP.

In some experiments the isolation of cyclic AMP involved passage down 2 ml columns of Biorad AC 50W-1B resin, the 4.5 to 10.5 ml fraction being eluted with water and lyophilized, after which the residues were subjected to t.l.c.

(Turtle & Kipnis, 1967) before being counted. Recoveries by this method were 30-50%.

Ba(OH)₂ and ZnSO₄ Solutions. ZnSO₄ was made into solution at 0.25 M and Ba(OH)₂ was made into solution at approximately 0.25 M being filtered to remove barium carbonate. The [Ba(OH)₂] was then adjusted to neutralize the ZnSO₄ to phenolphthalein when the two solutions were mixed in equal volumes, the pH of the supernatant being 7.5.

Liquid scintillation counting of ³H and ¹⁴C. To the lyophilized residue of each sample 1.5 ml water was added along with 10.5 ml of a dioxane based phosphor (Scales, 1967). The phosphor solution was prepared by dissolving 8 g of butyl PBD (New England Nuclear, Boston, Mass., U.S.A.) and 100 g of naphthalene (Amersham/Searle Corp., Arlington Heights, Ill., U.S.A.) in one liter of dioxane (Fisher Scientific, Toronto, Ontario, Canada). Particulate samples, such as the scrapings off t.i.c. plates were counted in the presence of 50 g silica/l dioxane (Thixotropic Gel Powder, Packard Instrument Co., Downers Grove, Ill., U.S.A.).

Vials were counted in a Beckman LS-230 liquid scintillation counter giving efficiencies for ¹⁴C and ³H of approximately 44% and 16% respectively under dual label conditions. The limiters on the counter were set for 100,000 cpm for ³H and 10,000 cpm for ¹⁴C, with a 50 minute

maximum for each sample. A series of both ^3H and ^{14}C quenched standards was run with each experiment to give a quench-correction curve. Corrections for background, channel cross-over and quenching were applied using a Hewlett Packard 9810A desk computer (Hewlett Packard (Canada) Ltd., Rexdale, Ontario, Canada).

Total cyclic AMP Assay. Measurements of total cyclic AMP in samples were performed according to the technique of Brown, Albano and Ekins (1971), using binding protein prepared from bovine adrenals stored in 1 ml aliquots at -50°C .

Each assay tube contained an amount of sample estimated to contain 1-5 pmoles of cyclic AMP, 100 μl of a 1 in 20 dilution of binding protein, and a saturating amount of cyclic [^3H]AMP (8.0 pmoles of 28 Ci/mole). Other tubes were similarly prepared containing known amounts of unlabelled cyclic AMP (0.2 - 10 pmoles) in addition to cyclic [^3H]AMP for preparation of a standard curve. After incubation for 1 1/2 hr at 0°C unbound cyclic AMP was removed by centrifugation with charcoal and a sample of the supernatant was counted. The percentage radioactivity bound against the known amount of cyclic AMP present in the standards was plotted. The cyclic AMP in the experimental samples was then calculated from the standard curve.

Computer simulation of changes in cyclic [¹⁴C]AMP levels.

Because the kinetics of the activation of AC by PGE₁ and the activity of PDE may be described by Michaelis-Menten equations, it was possible to write a Fortran program describing changes in the cyclic AMP levels with respect to PGE₁ concentration and to time. In its more complex form the equation developed allowed for variations in the ATP concentration, for the presence of two species of PDE (Aner & Mayol, 1973) and for the inhibitory effect of competitive PDE inhibitors on both of these enzymes. In the examples given in the present text a simpler equation, assuming a constant ATP concentration and considering only one species of PDE, was used:

$$\text{i.e.} \quad \frac{dc}{dt} = \frac{V_i}{\left(1 + \frac{K_{m_a}}{x}\right)} - \frac{V_o}{1 + \frac{K_m}{c} \left(1 + \frac{1}{K_I}\right)}$$

WHERE: c = concentration of cyclic AMP.

V_i = V_{max} for the activation of AC by PGE₁ at the ATP concentration prevailing in the platelets.

K_{m_a} = concentration of PGE₁ giving half maximal ($V_i/2$) activation of AC.

x = concentration of PGE₁.

V_o = V_{max} of PDE.

K_m = concentration of cyclic AMP giving half maximal activity of PDE.

i = concentration of PDE inhibitor.

K_i = dissociation constant of the PDE inhibitor -
PDE complex.

Employing the CDC 6400, a 4th order Runge-Kutta method was used to integrate the equation. The numerical values and their origins or the various constants involved are:

V_i = 9% (i.e. % cyclic
[^{14}C]AMP/30 sec)

experimentally determined

K_m = 0.33 μM

experimentally determined

x = 0.5 μM

an arbitrary value

V_o = 30%

an estimated value which gave steady state levels of cyclic [^{14}C]AMP similar to those encountered experimentally

K_m = 1.08%

Jard & Bernard (1970); μM value of $30 \times 10^{-6}\text{M}$ was converted to % cyclic [^{14}C]AMP on basis of Figure 1, Chapter III

i = 20,000 μM

an arbitrary value

K_i = 1,000 μM

value for caffeine given by Butcher et al., 1962

Chapter III

PRELIMINARY EXPERIMENTS

1. CONFIRMATION OF IDENTITY AND PURITY OF MATERIAL ISOLATED AS CYCLIC [^{14}C]AMP

The purity of the material isolated as cyclic [^{14}C]AMP as described under 'Methods' was determined by treating it with PDE. The reduction in ^{14}C radioactivity was compared with the reduction in ^3H radioactivity present in the pure cyclic [^3C]AMP added to monitor the recovery of cyclic [^{14}C]AMP.

After preincubation of PRP with [^{14}C]adenine, four PRP samples were treated with PGE₁ and four with saline. The incubations were stopped with perchloric acid as usual before cyclic [^3H]AMP was added to each sample. After centrifugation the supernatants from each group were pooled, neutralized with KOH to precipitate KClO_4 and then redivided into four identical samples. Two from each group were then incubated with PDE (0.11 units/sample) in the presence of 1.5 mM MgSO_4 and 56 mM tris-HCl, pH 7.4 (final concentrations) for different time intervals. The reactions were stopped with perchloric acid (0.5N, final) and 1 μmole of unlabelled cyclic AMP was added as an internal control to determine the subsequent recovery of cyclic AMP from each sample (by measurement of the extinction at 260 m μ), before

the procedure for the isolation of cyclic AMP was carried out as described under 'Methods.'

The results (Table 1) show that treatment with PDE for either 30 min or 90 min resulted in the destruction of over 99% of the added cyclic [^3H]AMP. In the samples not incubated with PGE_1 , treatment with PDE resulted in a 81% loss of ^{14}C , indicating that percentage of the basal cyclic [^{14}C]AMP was really cyclic AMP. In the samples that were incubated with PGE_1 , 98.5% of the [^{14}C]labelled material was destroyed on treatment with the enzyme, indicating that almost all of what was isolated as cyclic [^{14}C]AMP was in fact cyclic AMP.

Previous workers using similar methods have found basal cyclic [^{14}C]AMP levels of about 0.1% of the platelet ^{14}C (Ball et al., 1970; Mills & Smith, 1971). This is 3-4 times the values encountered in this study and it may therefore be assumed that the 80% purity of the basal cyclic [^{14}C]AMP in these experiments is appreciably better than that of earlier studies. Ball et al., (1970) did note that a purification of [^{14}C]adenine similar to that used in this work reduced the apparent basal cyclic [^{14}C]AMP levels.

Duration of Incubation (min)	Saline				PGE ₁ (1μM)			
	+PDE		-PDE		+PDE		-PDE	
	30'	90'	30'	90'	30'	90'	30'	90'
³ H dpm	149	18	13,926	13,683	44	24	14,384	12,847
¹⁴ C dpm	32	32	177	169	44	39	3,000	2,635
¹⁴ C dpm as a % of total platelet ¹⁴ C	.005	.005	.032	.031	.007	.007	0.522	0.513
% loss of cyclic [³ H]AMP	98.9	99.9	--	--	99.9	99.8	--	--
% loss of ¹⁴ C dpm	81.8	81.1	--	--	98.6	98.5	--	--

TABLE 1. The effect of added PDE on basal and PGE₁-elevated levels of cyclic [¹⁴C]AMP isolated by the technique used by the author.

2. RELATIONSHIP BETWEEN CYCLIC [14 C]AMP LEVELS WITH TOTAL CYCLIC AMP LEVELS IN PLATELETS.

Variations in the cyclic [14 C]AMP level were induced by 30 sec incubations with various PGE₁ concentrations in the presence and absence of caffeine. The cyclic AMP was then isolated as described under 'Methods' and the cyclic [14 C]AMP content of one half of the Dowex 50 column eluate of each sample was calculated. The other half of each sample eluate was used for the determination of total cyclic AMP as described under 'Methods.' Each incubation was performed in triplicate and the mean values \pm S.E.M. obtained were plotted (Fig. 4). A linear regression was applied to the data with the resulting equation: $y=174.7 x + 32.4$. The correlation coefficient which describes the 'goodness of fit' of the equation to the data was 0.99, indicating an excellent correlation between the cyclic [14 C] AMP levels and the total cyclic AMP levels. The intersection with the ordinate at 32.4 pmoles represents the unlabelled cyclic AMP present in plasma (Maslan, unpublished).

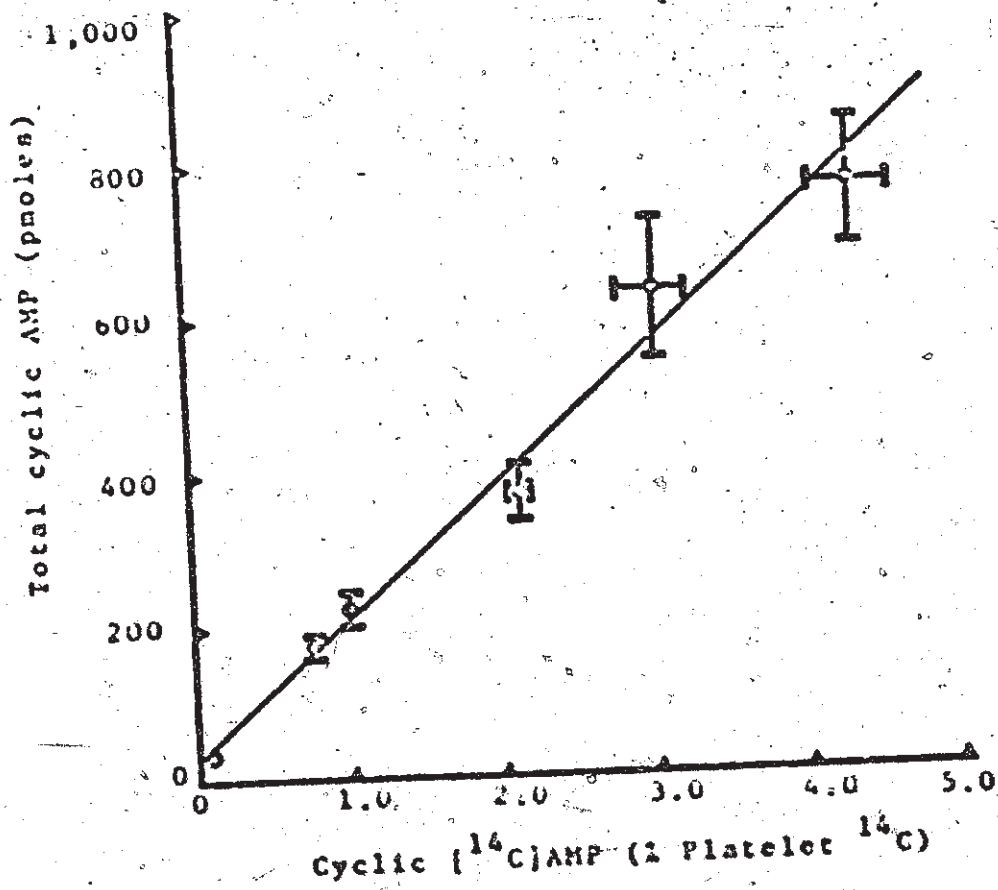


FIGURE 4. Correlation of cyclic [¹⁴C]AMP levels with total cyclic AMP in samples of PRP that were incubated with various PGE₁ concentrations + caffeine for 10 sec. There were 1.11×10^8 platelets/sample.

3. RELEASE OR LEAKAGE OF CYCLIC [14 C]AMP FROM PLATELETS IN PRP.

Estimation of the extracellular cyclic [14 C]AMP was carried out by two different techniques.

Firstly, PRP samples were incubated with and without PGE₁ in the presence of added PDE (0.1 unit/sample) and cyclic [3 H]AMP (to monitor the effectiveness of the PDE for varying lengths of time. After stopping the reaction in the usual manner, 1 μ mole of unlabelled cyclic AMP was added to determine the subsequent recovery of cyclic AMP from each sample (by measurement of the extinction at 260 m μ), before the procedure described under 'Methods' was carried out for the isolation of cyclic AMP. It was assumed that if there was a reduction in cyclic [14 C]AMP levels in the samples treated with PDE relative to the controls, then that amount of cyclic [14 C]AMP had been outside the platelets and thus available to the enzyme. The results (Table 2) show that between 8 and 21% of the cyclic [14 C]AMP was found extracellularly depending on the duration of the incubation and the strength of the PGE₁ stimulation.

Additions	Saline		0.5µM PGE ₁		2µM PGE ₁	
	6	12	6	12	6	12
Duration of Incubation(min)						
Cyclic [¹⁴ C]AMP (X platelet [¹⁴ C] of sample treated with PDE	.028	.028	0.164	0.151	0.379	0.361
Cyclic [¹⁴ C]AMP (X platelet [¹⁴ C] of sample not treated with PDE	.031	.031	0.172	0.177	0.414	0.444
X breakdown of cyclic [³ H] AMP	64	93	63	93	67	92
X cyclic [¹⁴ C]AMP lost due to PDE treatment (2's are corrected for cyclic [³ H] AMP breakdown)	16	11	8	16	12	21

TABLE 2. Percentage of sample cyclic [¹⁴C]AMP found extracellularly as determined by treatment with PDE.

The second method that was used involved incubating samples with PGE_1 \pm caffeine for varying lengths of time, centrifuging the samples (1 min in the Eppendorf), measuring the cyclic [^{14}C]AMP in the supernatant plasma and comparing the values obtained with control PRP samples. The results (Table 3) compare reasonably well with the preceding method although Salzman (1972) has claimed that centrifugation causes loss of platelet cyclic AMP.

These results are in contrast to those of Cole et al. (1971) who found over 50% cyclic AMP extracellularly. The results show that in this study the leakage of cyclic [^{14}C]AMP was not a major problem in the first few minutes of incubation with PGE_1 and was negligible in the presence of caffeine despite the very high levels of intracellular cyclic [^{14}C]AMP.

Additions	Saline		0.5µM PGE ₁		2µM PGE ₁		0.5µM PGE ₁ + Caff	
	A	B	A	B	A	B	A	B
Experiment								
Duration of Incubation(min)	2	3	2	3	2	3	2	3
Cyclic [¹⁴ C]AMP (2 platelets ¹⁴ C) in plasma	.002	.007	.013	.021	.042	.092	.027	.020
Cyclic [¹⁴ C]AMP (2 platelets ¹⁴ C) in PRP controls	.019	.029	.159	.180	.177	.414	3.079	4.057
2 cyclic [¹⁴ C] AMP of control found in the plasma	11	24	8	12	24	7	0.9	0.5
		23				21		4.0

TABLE 1. Percentage of sample cyclic [¹⁴C]AMP found extracellularly as determined by centrifugation. Data from two experiments (A and B) are presented.

Chapter IV

EFFECTS OF PROSTAGLANDINS E_1 , E_2 , AND $F_{2\alpha}$ ON PLATELET CYCLIC
[14 C]AMP LEVELS

1. INTRODUCTION

In 1966 Kloeze observed that PGE_1 inhibited ADP-induced aggregation in citrated pig, rat and human PRP while PGE_2 enhanced the aggregation in pig and rat but not in human PRP. Shio and Ramwell (1972) later observed however, that PGE_2 inhibited primary but potentiated the secondary aggregation induced by ADP in human citrated PRP. This was interesting because it was the first time that PGE_1 and PGE_2 , differing only in the saturation of the 5,6 bond, were shown to have opposing effects on the same cell type.

PGE_1 was shown to be potent stimulator of human platelet AC (Butcher et al., 1967; Wolfe & Shulman, 1969; Marquis et al., 1969; Scott, 1970; Moskowitz et al., 1971; Salzman & Levine, 1971), and was also shown to increase the intracellular level of cyclic AMP in intact platelets (Robison et al., 1969). Other investigators have used radioactive adenine (Ball et al., 1970; Haslam & Taylor, 1971b; Moskowitz et al., 1971; Mills & Smith, 1971) or adenosine (Vigdahl et al., 1969) to label endogenous ATP and have obtained essentially the same results: PGE_1 was found to stimulate the incorporation of radioactivity into

cyclic AMP reflecting an increased rate of synthesis of the cyclic nucleotide in the intact cells. PGE_1 stimulates AC activity in homogenates of human platelets at concentrations ($<10^{-8}\text{M}$) that are known to inhibit aggregation (Wolfe & Shulman, 1969; Zieve & Greenough, 1969). Methyl xanthines (e.g. theophylline, caffeine) were found to act synergistically with PGE_1 in increasing the accumulation of cyclic AMP in intact platelets (Ball et al., 1970; Robison et al., 1971) as would be expected from their actions as PDE inhibitors. In this regard two forms of PDE have been identified by several laboratories, in a number of tissues (Jard & Bernard, 1970; Thompson & Appleman, 1971) including human blood platelets (Amer & Marquis, 1972; Amer & Mayol, 1973). Platelet PDE-I has a K_m of 0.61 mM while platelet PDE-II has a K_m of 3.2 μM . On aggregation, platelet PDE-II which is partly membrane bound, is exposed to the plasma. PGE_1 and caffeine were both reported to decrease PDE-II activity (Amer & Marquis, 1972).

2. RESULTS

The effects of PGE_1 , PGE_2 and of $\text{PGF}_{2\alpha}$ on cyclic [^3H]AMP levels in human platelets were examined in the absence of a PDE inhibitor (Figure 5) and in the presence of

a PDE inhibitor, papaverine (Figure 6). Cyclic [14 C]AMP levels were measured 30 sec after the addition of prostaglandin and/or papaverine and were expressed as percentages of the total radioactivity in the platelets. PGE₁ markedly increased cyclic [14 C]AMP levels both in the presence and absence of a PDE inhibitor. PGE₂ had a much weaker effect on cyclic [14 C]AMP levels than PGE₁ while PGF_{2 α} was virtually inactive.

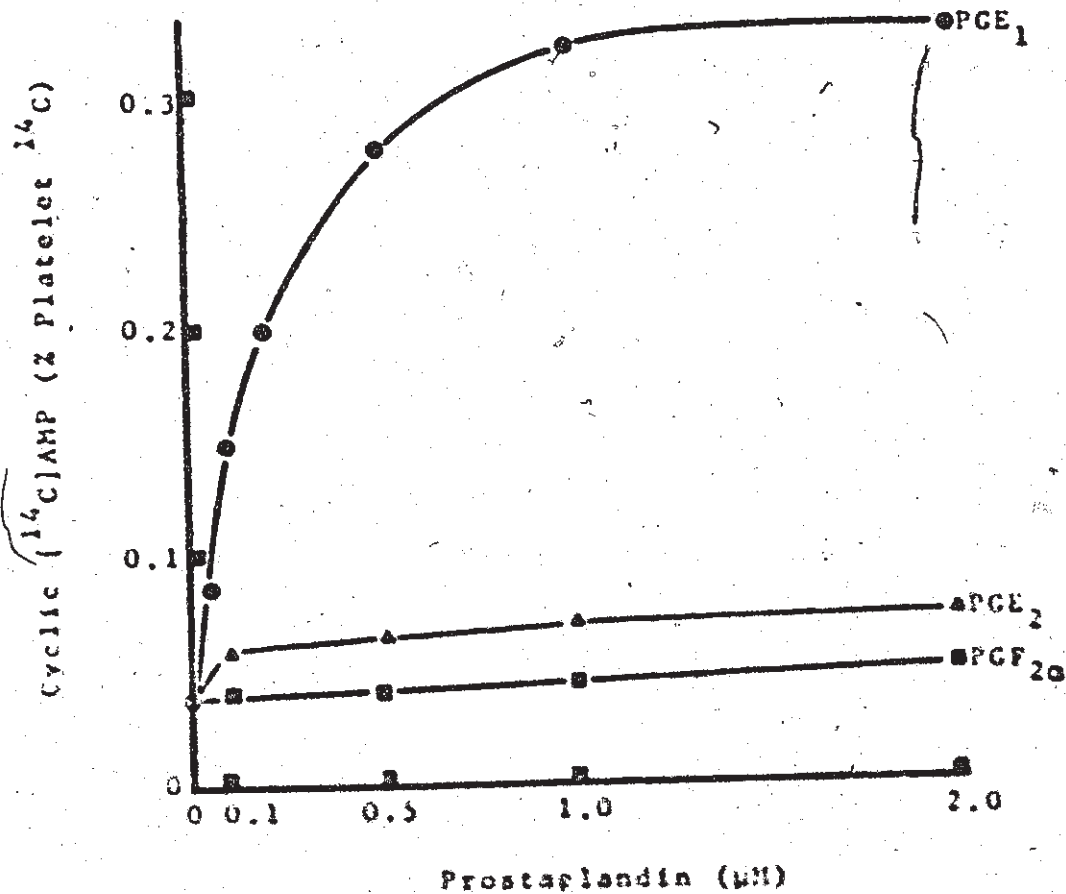


FIGURE 5. Effects of increasing concentrations of PGE₁, PGE₂ and of PGF_{2 α} on cyclic [14 C]AMP levels in human platelets.

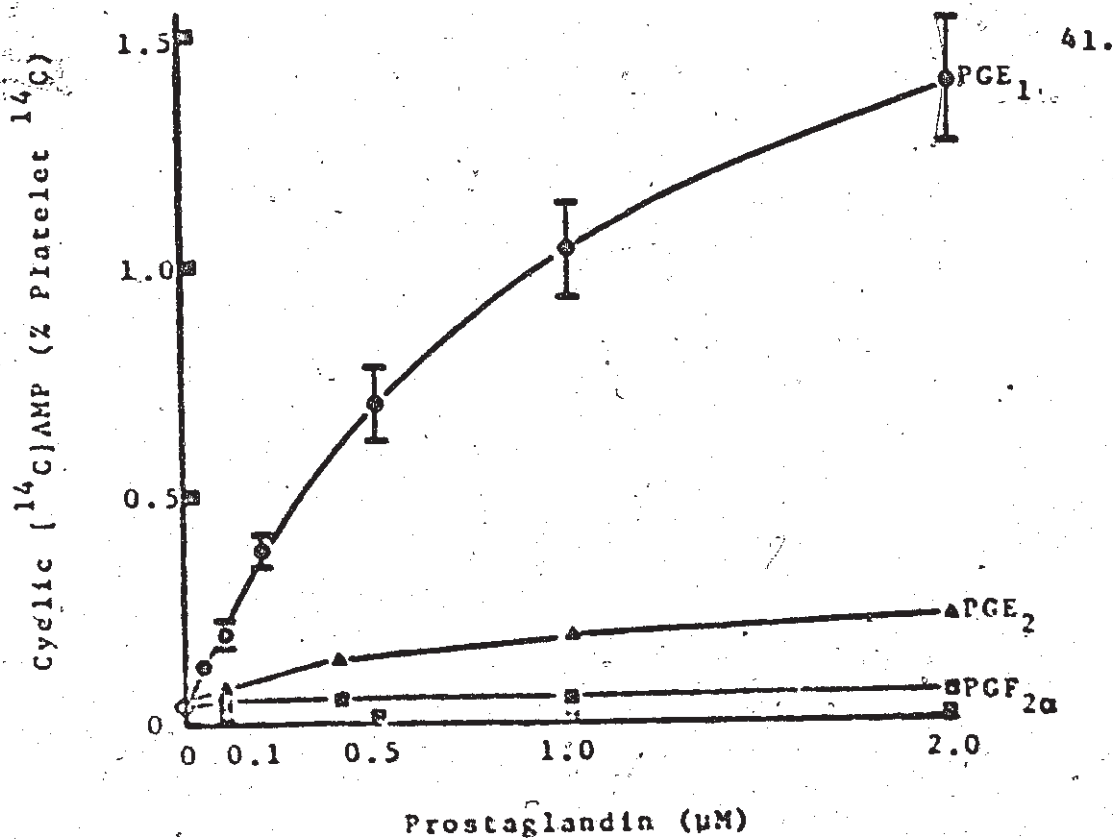


FIGURE 6. Effects of increasing concentrations of PGE₁, PGE₂ and of PGF_{2α} on cyclic [¹⁴C]AMP levels in the presence of papaverine (2 mM) in human platelets. Results with PGE₁ are from three experiments.

The effect of PGE₁ was examined in the presence of two other PDE inhibitors, caffeine and RA233 (Figure 7). PGE₁ with caffeine (20 mM) produced the largest synergic increase in cyclic [¹⁴C]AMP levels. A synergic effect was also seen with PGE₂ in the presence of caffeine or papaverine.

but not with $\text{PGF}_{2\alpha}$ in the presence of papaverine (Figure 8).

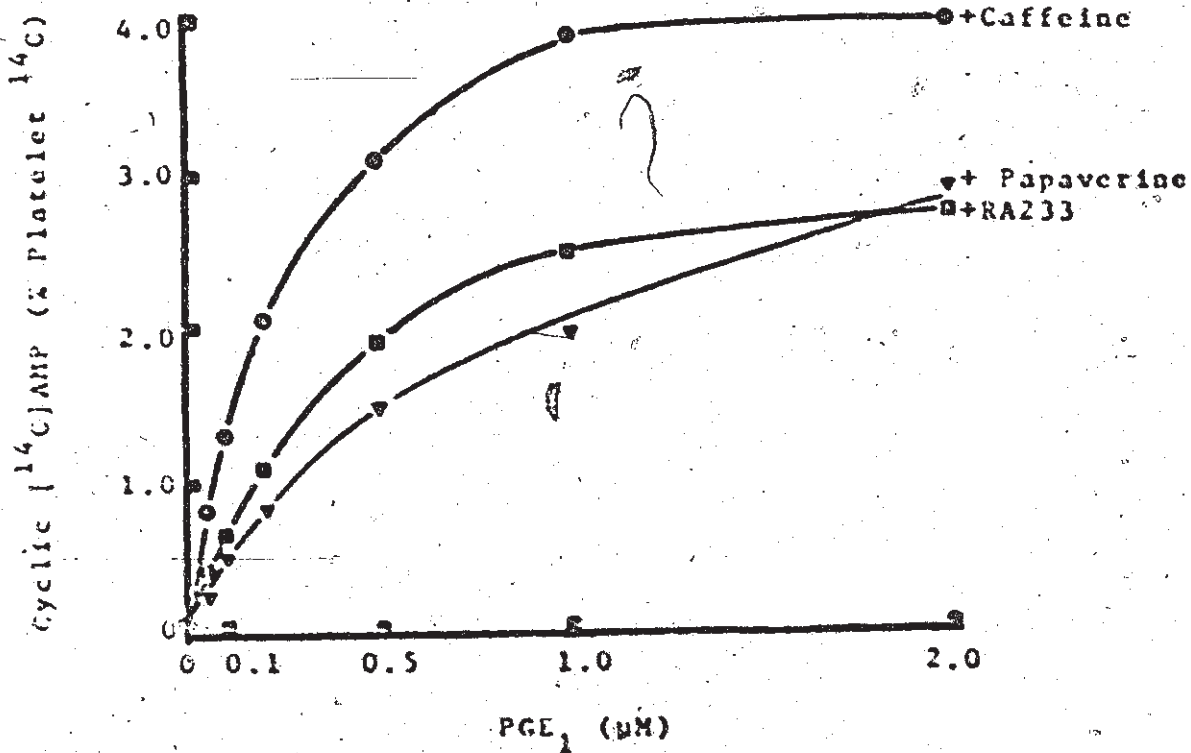


FIGURE 7. Effects of increasing concentrations of PGE_1 in the presence of caffeine (20 mM), RA233 (0.5 mM) or papaverine (2 mM) on cyclic $[^{14}\text{C}]\text{AMP}$ levels in human platelets.

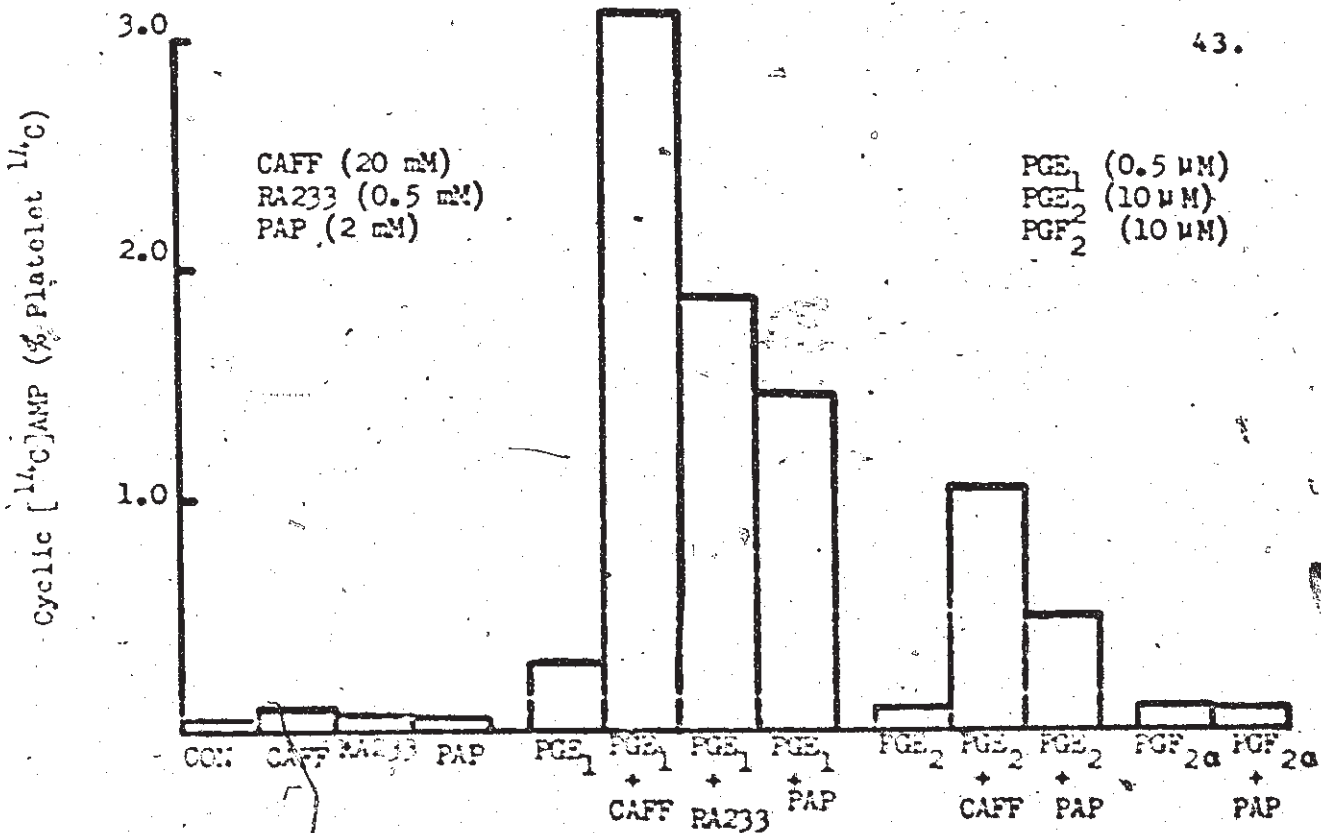


FIGURE 8. Effects of PGE₁, PGE₂ and PGF_{2α} in the presence and absence of various PDE inhibitors on cyclic [¹⁴C]AMP levels in human platelets after 30 sec incubations. Data from three different experiments was utilized in preparing this figure.

In the presence of these PDE inhibitors PGE₁ causes a linear increase in cyclic [¹⁴C]AMP levels over at least the first 30 sec (Figure 9). Therefore, Michaelis-Menten kinetics were applied to the activation of AC by PGE₁ and Lineweaver-Burk plots of the data gave straight lines (Figure 10) indicating a single PGE₁ receptor for which the K_m and V_{max} values were calculated (Table 4). A time course to check for linearity was performed in each case in this

study that was subjected to kinetic analysis and increases in cyclic [^{14}C]AMP due to PDE inhibitors alone were subtracted from the data prior to any kinetic treatment.

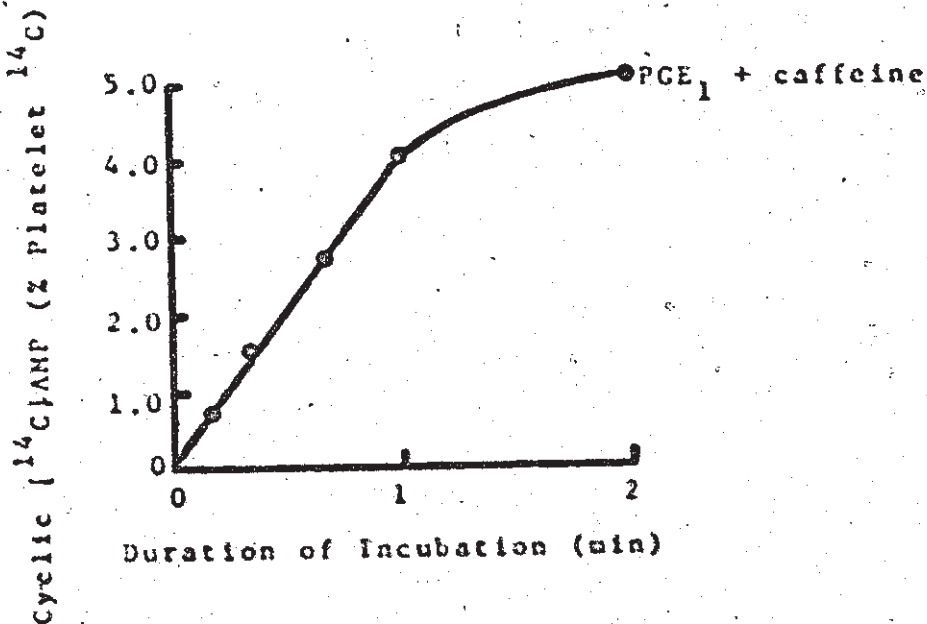


FIGURE 9. Time course over 2 min of the effect of PGE₁ (0.5 μM) plus caffeine (20 mM) on cyclic [^{14}C]AMP levels in human platelets.

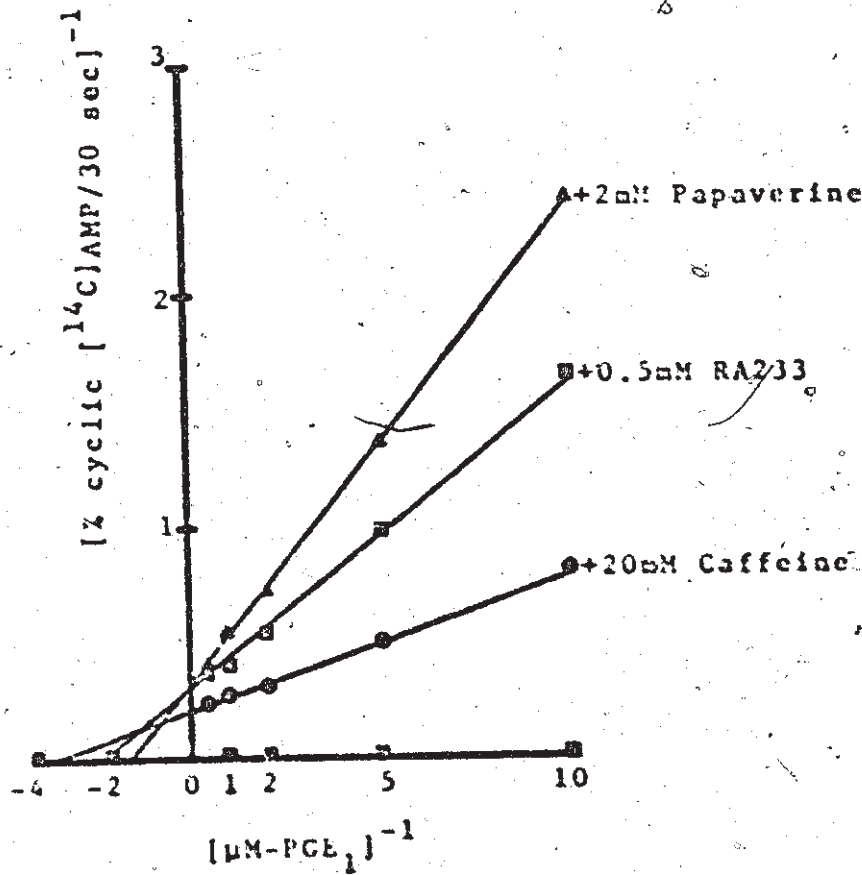


FIGURE 10. Lineweaver-Burk plots of the effects of PGE_1 plus caffeine (20 mM), papaverine (2 mM) or RA233 (0.5 mM) on AC activity in intact human platelets.

	Vmax (% cyclic [¹⁴ C]AMP/30 sec)	Km (μM)
PGE ₁ + caffeine	5.4	0.33
PGE ₁ + papaverine	3.1	0.71
PGE ₁ + RA233	3.57	0.51

TABLE 4. Km and Vmax values obtained from Figure 10 for the effects of PGE₁ plus various PDE inhibitors on AC activity.

The effects of various combinations of prostaglandins on cyclic [¹⁴C]AMP levels in platelets were then examined.

A range of concentrations of PGE₂ with a single concentration of PGE₁ in the presence or absence of papaverine (Figure 11) had minimal effects on the increase in cyclic [¹⁴C]AMP seen with PGE₁ alone. No inhibition and only a very slight increase in cyclic [¹⁴C]AMP was observed.

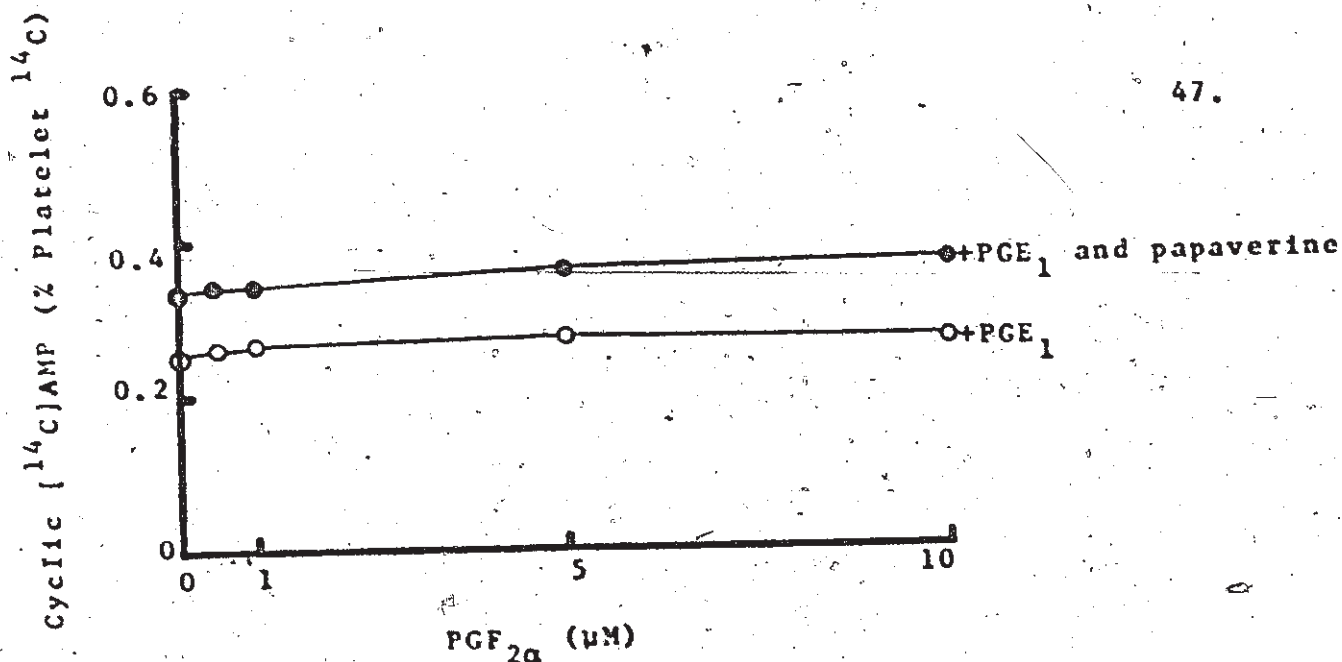


FIGURE 11. Effects of increasing concentrations of PGF_{2α} plus 0.2 μM PGE₁ with and without papaverine (2 μM) on cyclic [¹⁴C]AMP levels in human platelets.

Addition of increasing concentrations of PGE₂ with a single concentration of PGE₁ in the presence of papaverine resulted in a somewhat less than additive effect on cyclic [¹⁴C] levels (Figure 12). However, in the absence of papaverine (Figure 13), this combination resulted in a decrease in cyclic [¹⁴C]AMP from the level seen with PGE₁ alone. This effect was increased with increasing concentrations of PGE₂. The results suggest that PGE₂, in addition to stimulating AC, was also stimulating PDE.

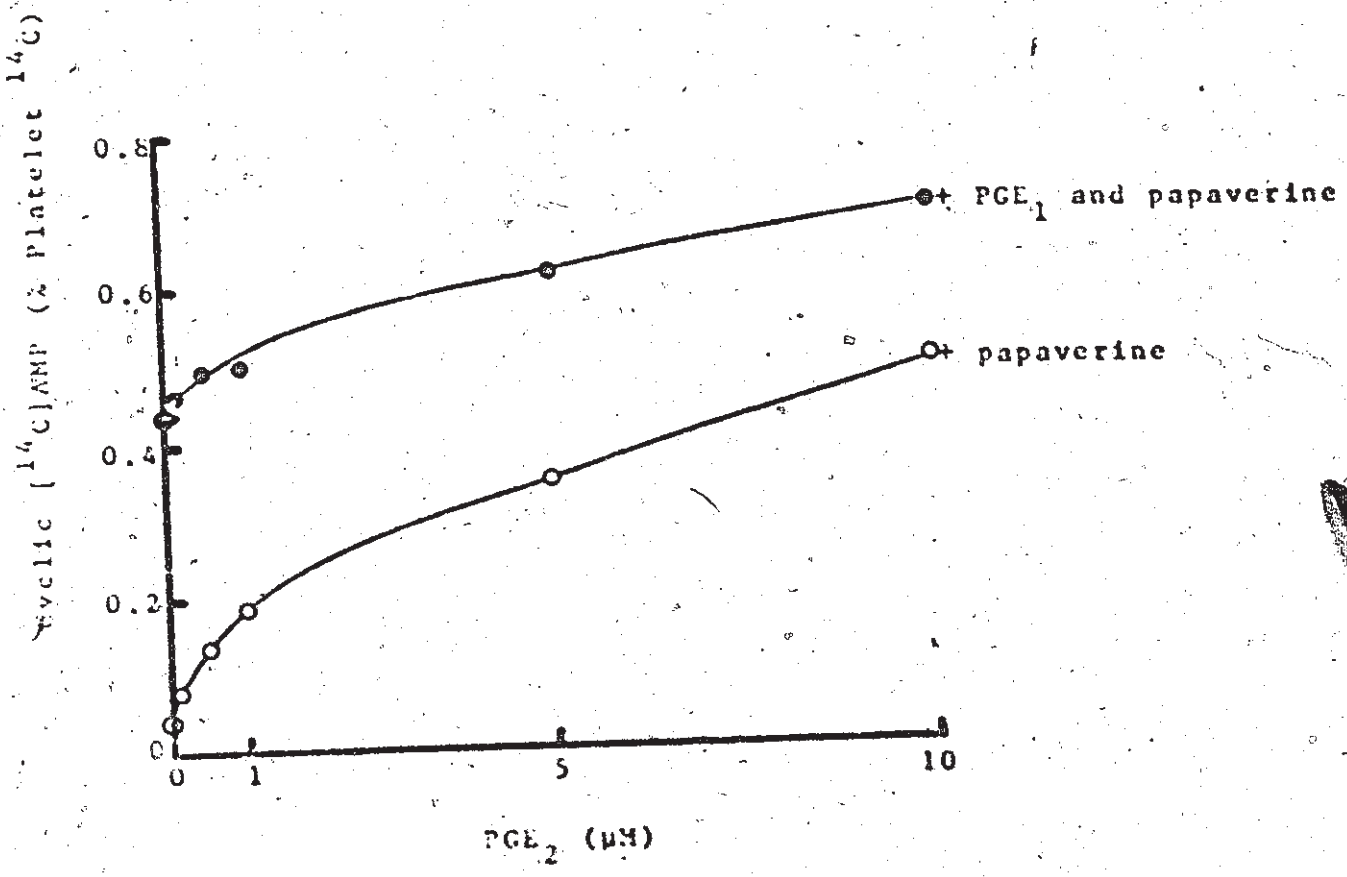


FIGURE 12. Effect of increasing concentrations of PGE₂ alone and of PGE₂ with 0.2 μM PGE₁, in the presence of papaverine (2 mM) on cyclic [¹⁴C]AMP levels in human platelets.

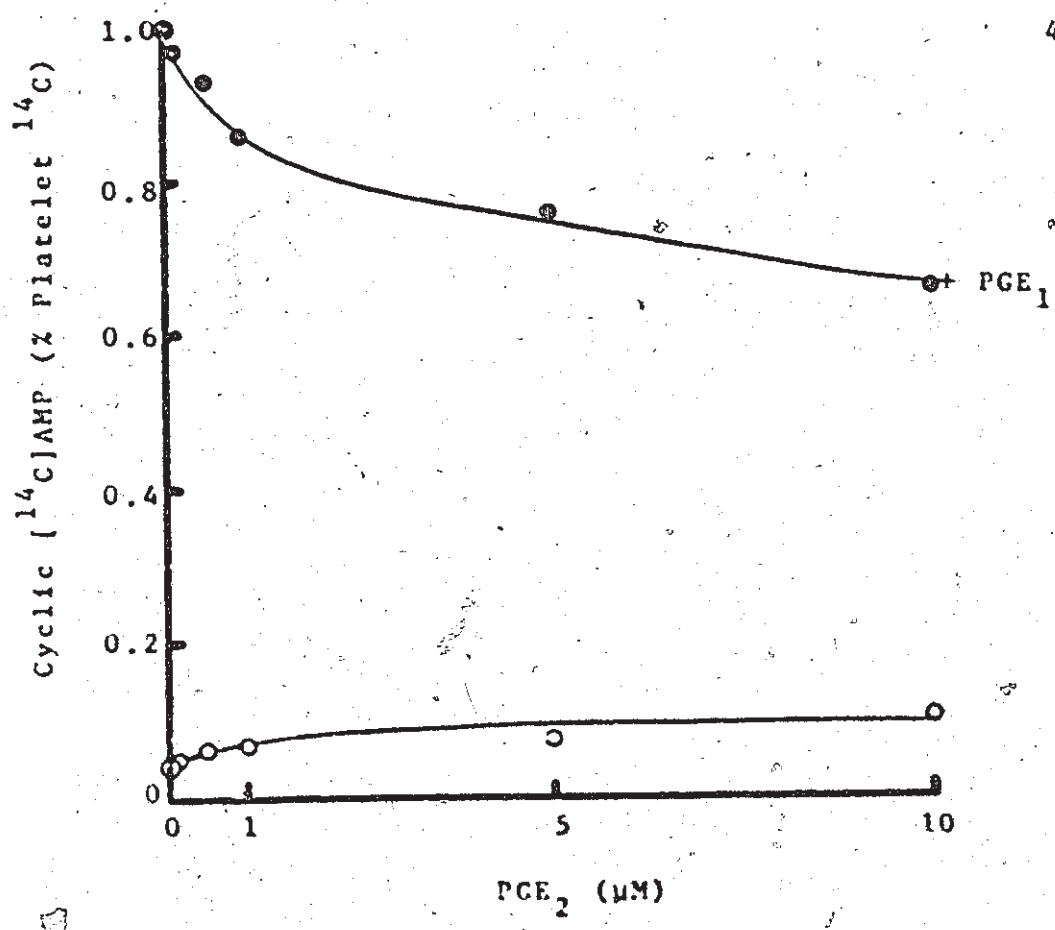


FIGURE 13. Effect of increasing concentrations of PGE₂ and of PGE₂ with 0.5 μM PGE₁ in the absence of papaverine on cyclic [¹⁴C]AMP levels in human platelets.

PGF_{2α} (0.5 μM) had no significant effect on the cyclic [¹⁴C]AMP formed with a range of concentrations of PGE₁ with or without papaverine (Figure 14).

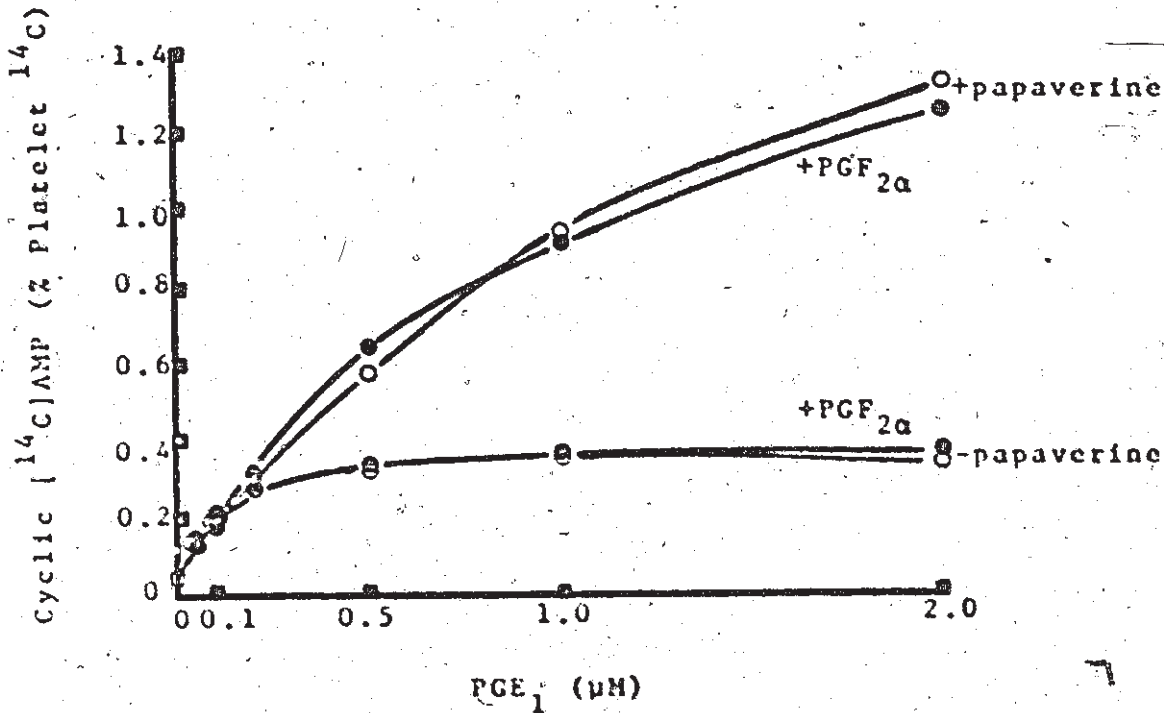


FIGURE 14. Effects of increasing concentrations of PGE₁ alone (○), and of PGE₁ plus 0.5 µM PGF_{2α} (●) with and without 2 mM papaverine on cyclic [¹⁴C]AMP levels in human platelets.

The effect of 0.5 µM PGE₂ or 10 µM PGE₂ with a range of concentrations of PGE₁ in the absence of a PDE inhibitor (Figure 15) was to cause a decrease in the control PGE₁-elevated levels of cyclic [¹⁴C]AMP above about 0.1 µM PGE₁. Below this PGE₁ concentration partially

additive effects were observed. The effect of 0.5 μM PGE_2 or 10 μM PGE_2 with a range of concentrations of PGE_1 in the presence of caffeine shows that the decrease in cyclic [^{14}C]AMP accumulation was abolished in the case of 0.5 μM PGE_2 and reduced in the case of 10 μM PGE_2 (Figure 16). In this case partially additive effects of PGE_1 and PGE_2 were observed below 0.4 μM PGE_1 .

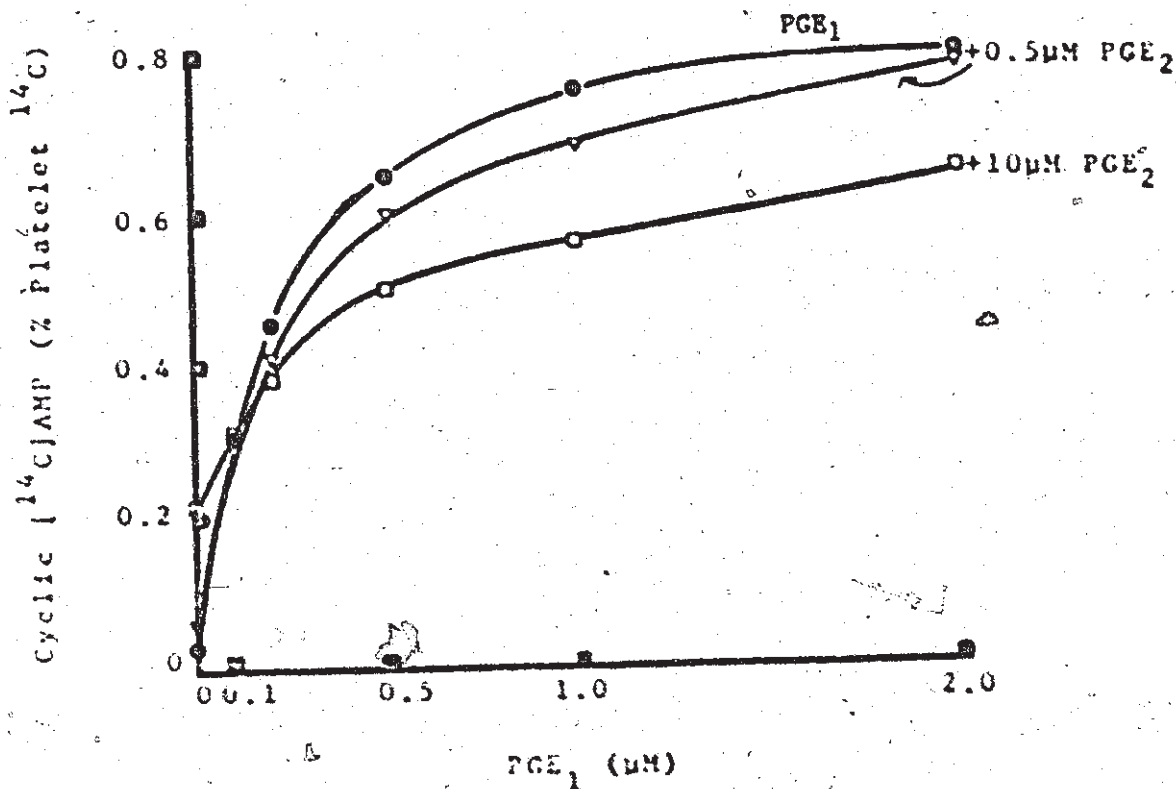


FIGURE 15. Effects of increasing concentrations, of PGE_1 with 0.5 μM PGE_2 or with 10 μM PGE_2 on cyclic [^{14}C]AMP levels in human platelets in the absence of a PDE inhibitor.

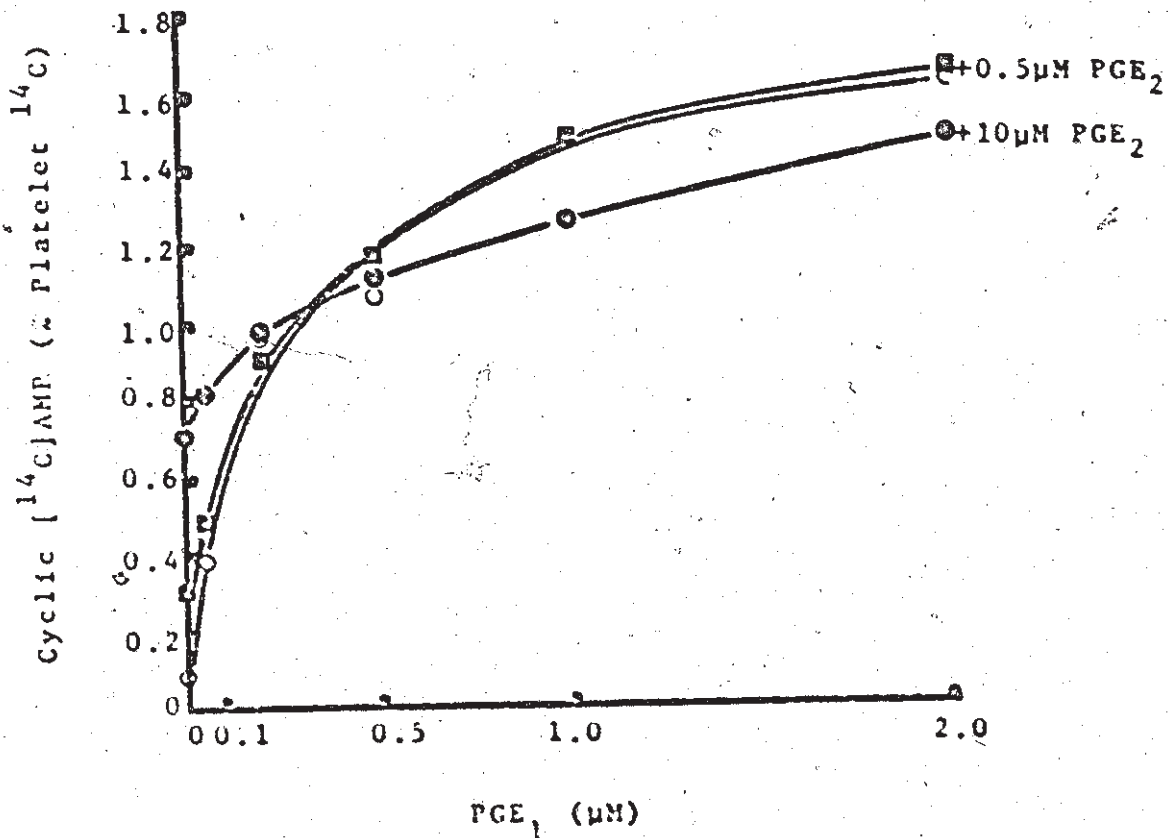


FIGURE 16. Effect of increasing concentrations of PGE₁ plus 20 mM caffeine (○) and of PGE₁ plus caffeine with 0.5 μM PGE₂ (■) or with 10 μM PGE₂ (●) on cyclic [¹⁴C]AMP levels in human platelets.

The kinetics of the activation of AC by PGE₂ suggest the existence of both low K_m and high K_m receptors for the activation of the enzyme by PGE₂ (Figure 17).

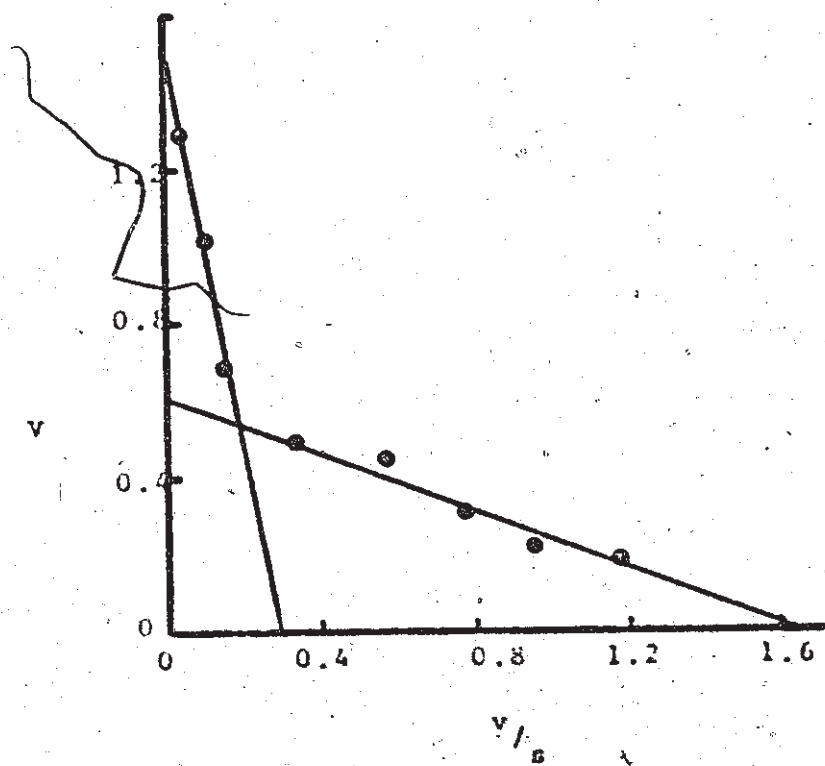


FIGURE 17. Eadie plot of the activation of AC by PGE₂ showing a K_m for PGE₂ of 5.07 μM ($V_{\text{max}}=1.42\%$ cyclic [¹⁴C]AMP/20 sec) and a K_m of 0.38 μM ($V_{\text{max}}=0.62\%$ cyclic [¹⁴C]AMP/20 sec).

Applying the kinetic constants for the high K_m effect of PGE_2 and for the activation of AC by PGE_1 ($K_m=0.31 \mu M$, $V_{max}=2.61\%$ cyclic [^{14}C]AMP/20 sec; obtained in a parallel experiment) to a treatment of the competition of two substrates for a single enzyme, theoretical curves were calculated showing the competitive effect of several PGE_1 concentrations in the presence of a range of PGE_2 concentrations on the activation of AC (Figure 18). No correction was made for the effect of the low K_m activation by PGE_2 . The same concentrations were used experimentally to determine the effect of PGE_1 on the activation of AC by PGE_2 and the data was included in the same figure. Although the fit was not close, the experimental curves showed the same pattern as the theoretical curves indicating the presence of mutual competitive inhibition.

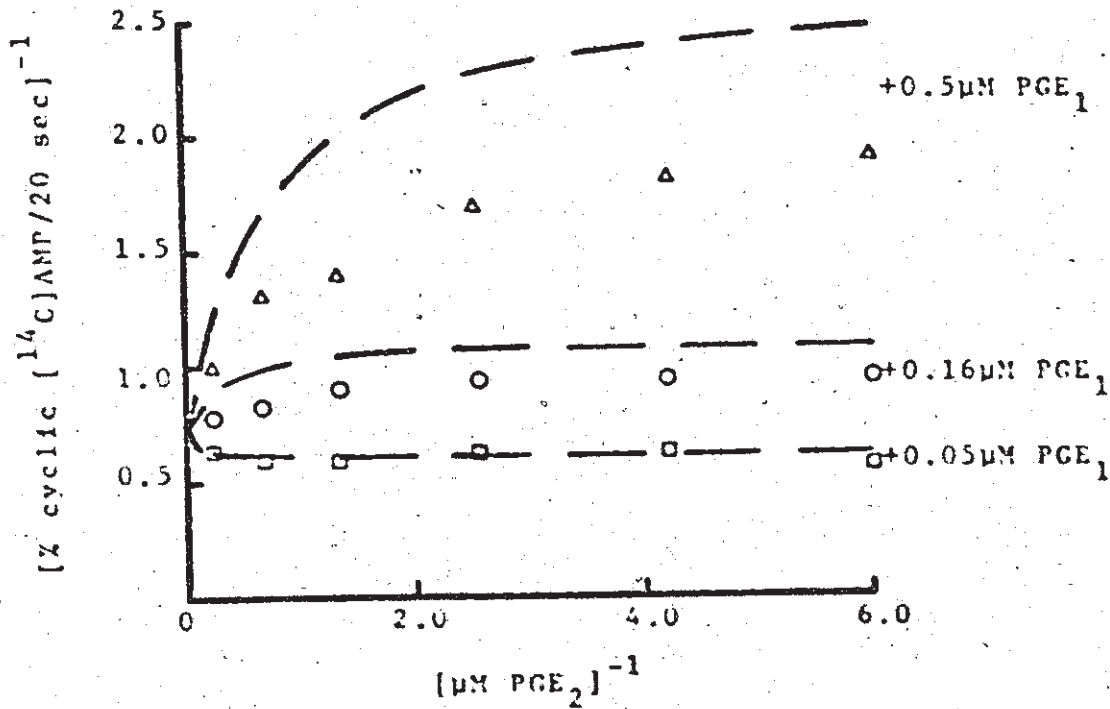


FIGURE-18. Double reciprocal plot of the effect of increasing PGE₂ concentrations on the activation of AC in the presence of selected concentrations of PGE₁. Theoretical curves using the same concentrations were calculated from kinetic constants that were determined in the same experiment using the equation:

$$\frac{1}{V(\text{total})} = \frac{1 + \frac{[PGE_2]}{K_m(PGE_2)} + \frac{[PGE_1]}{K_m(PGE_1)}}{\frac{V_{\max}(PGE_2)}{K_m(PGE_2)} [PGE_2] + \frac{V_{\max}(PGE_1)}{K_m(PGE_1)} [PGE_1]}$$

The effect of 7-oxa-13-prostynoic acid, reported by Fried et al. (1969) to be a prostaglandin antagonist, on the activation of AC by PGE₁ was examined and the effect was

competitive as shown in Figure 19.

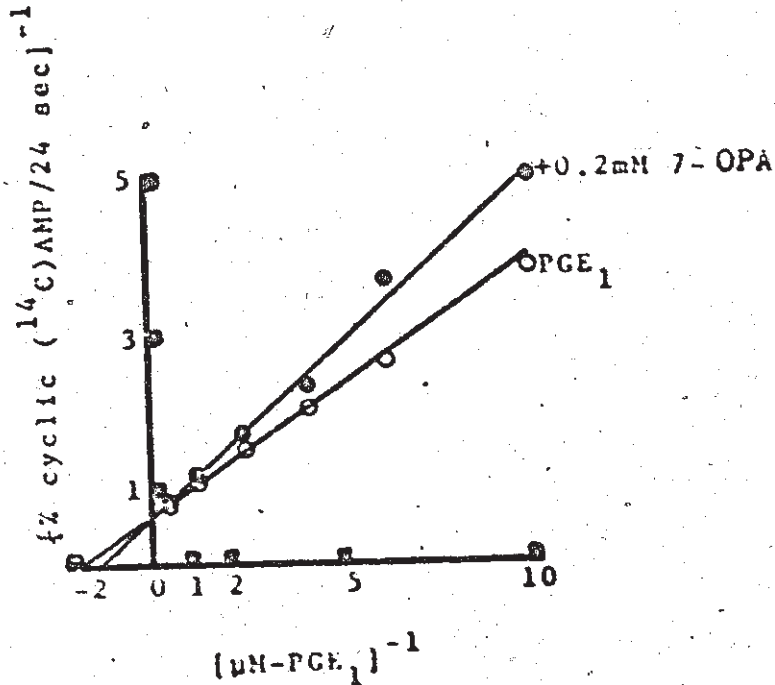


FIGURE 19. Lineweaver-Burk plot of the effect of 0.2 mM 7-oxa-13-prostynoic acid on the activation of AC by PGE₁.

The effects of PGE₁ and PGE₂ on the cyclic [¹⁴C]AMP levels with respect to time were examined (Figure 20). In the absence of a PDE inhibitor there was a rapid increase in cyclic [¹⁴C]AMP in the first 20-30 sec in response to PGE₁, PGE₂ or the two together. The level then fell rapidly to about one half the maximum and remained constant over at least the next eight minutes. In the presence of caffeine the initial rate of accumulation of cyclic [¹⁴C]AMP was faster and was maintained for 30 sec to one minute in

response to PGE_1 , PGE_2 or the two together, before reaching a plateau. When $0.5 \mu\text{M}$ PGE_1 and $10 \mu\text{M}$ PGE_2 were present together the effect of PGE_2 was to lower the level of cyclic [^{14}C]AMP about 16-25% in the presence of caffeine and about 40-55% in the absence of caffeine, depending on the time interval chosen.

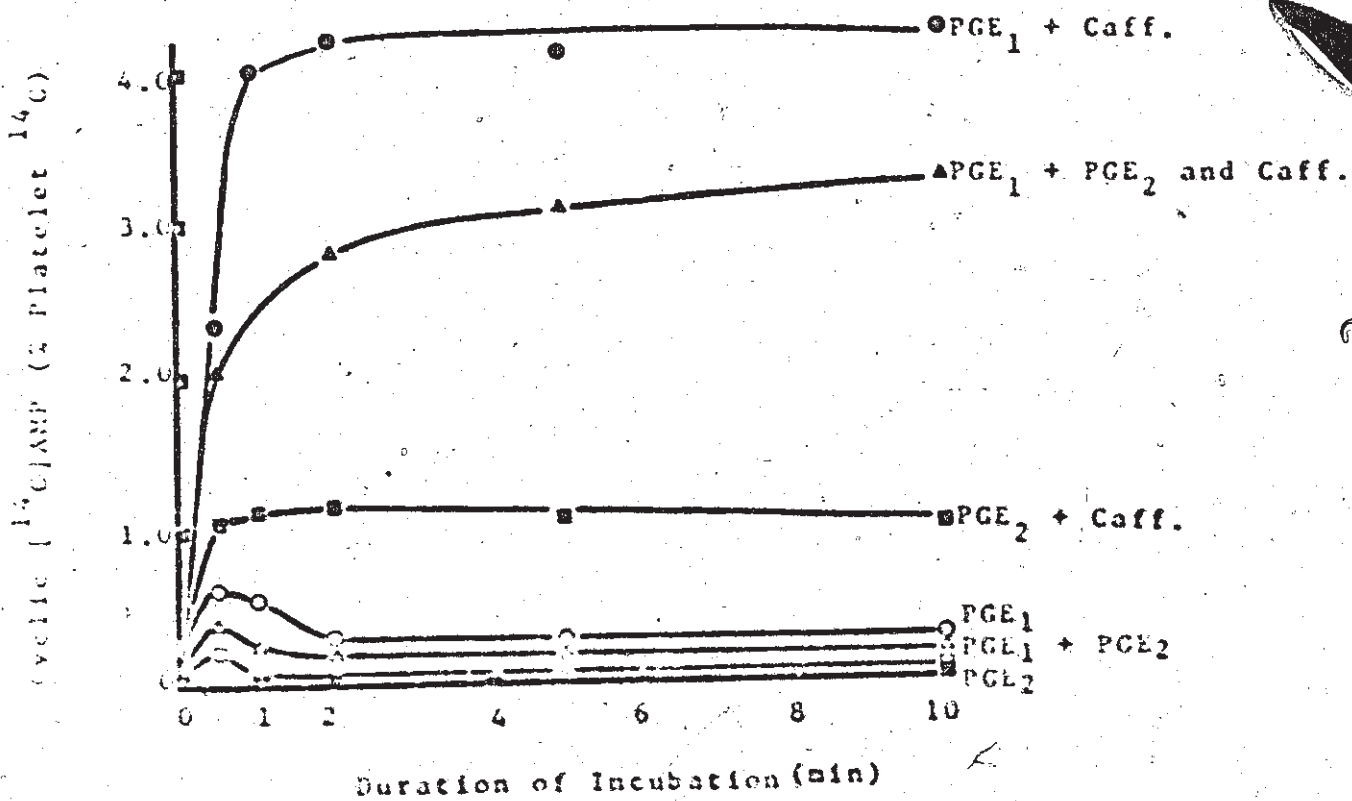


FIGURE 20. Time course of the effects of PGE_1 ($0.5 \mu\text{M}$), PGE_2 ($10 \mu\text{M}$) and of the two together in the presence (closed symbols) and absence (open symbols) of caffeine (20 mM) on cyclic [^{14}C]AMP levels in human platelets.

There are two possible explanations for the plateau seen in the presence of caffeine; either (a), if the PDE activity was completely inhibited by the caffeine then the AC activity had ceased, or (b), if the PDE activity was incompletely inhibited, then a steady state concentration of cyclic [^{14}C]AMP was established by the two enzymes.

In the absence of caffeine the decline in cyclic [^{14}C]AMP from the 30 sec maximum could be due to either a delayed activation of PDE or a gradual fall in AC activity, possibly due to a change in the function of the receptor-AC complex or to metabolism of PGE_1 . To investigate these effects, PGE_1 alone and caffeine alone were incubated for 4 min with PRP at which time caffeine was added to the former and PGE_1 to the latter (Figure 21).

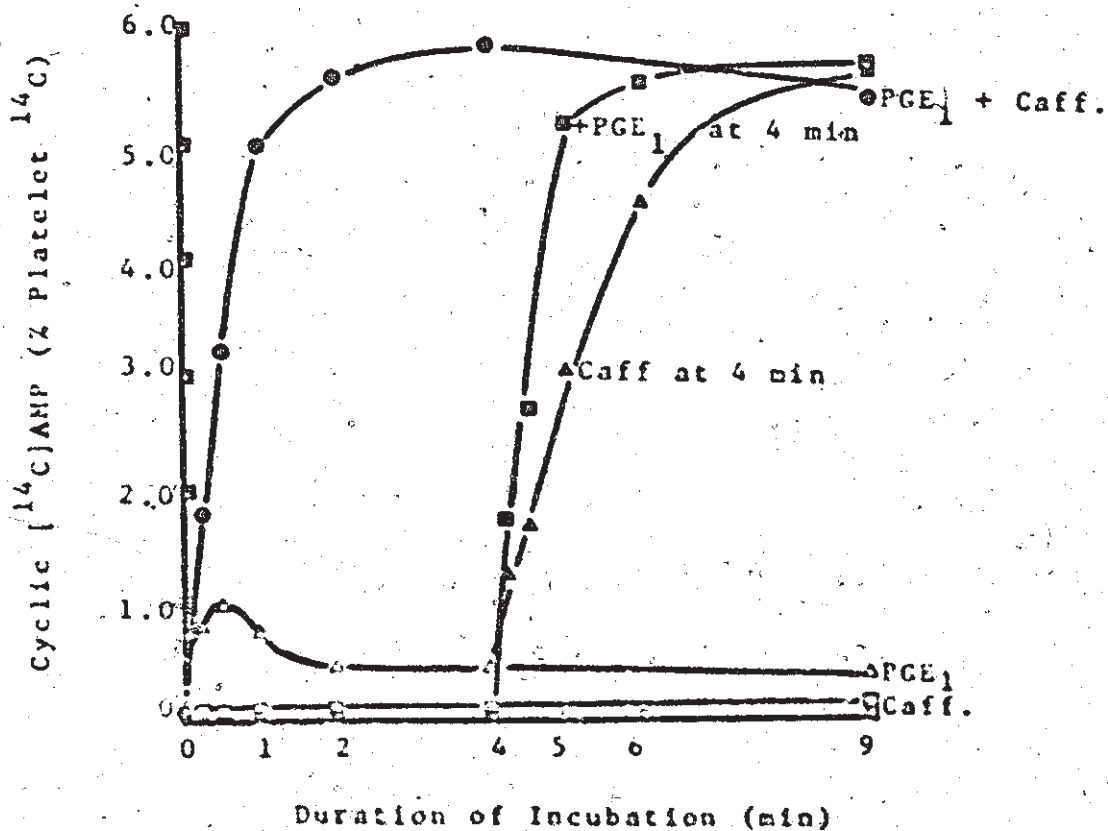


FIGURE 21. Time course of the effects of PGE₁ (0.5 μM) in the presence (●) and absence (△) of caffeine (20 μM) and of caffeine alone (◻) on cyclic [¹⁴C]AMP levels in human platelets. In addition, the effect of adding caffeine after preincubation with PGE₁ for 4 min (△) and of adding PGE₁ after preincubation with caffeine for 4 min (◻) was examined.

In both instances after 4 min, cyclic [¹⁴C]AMP levels rose to the same steady state level as when both PGE₁ and caffeine

were added simultaneously. The late addition of PGE_1 resulted in the same rate of increase in cyclic [^{14}C]AMP as in the 0 min control but the late addition of caffeine resulted in a much slower rise in the cyclic [^{14}C]AMP levels. These observations show clearly that some time after the exposure of platelets to PGE_1 the platelet AC-PDE system is reset so that a different steady state is established from that which would have been expected from the initial AC and PDE activities. Comparison of the differences in the initial rates between addition of PGE_1 or caffeine at 4 min and the initial control rate suggests that a decline in AC activity occurs after exposure of platelets to PGE_1 for more than 30 sec.

The actions of PGE_1 and of PGE_2 on PGE_1 were examined in a human washed platelet system. The effects of different concentrations of PGE_1 and the time course were similar to those encountered in PRP (Figure 22 and 23), except that the K_m of the PGE_1 activation of AC was only 0.05 μM . PGE_2 (0.05 μM) significantly inhibited the action of PGE_1 in the absence of a PDE inhibitor.

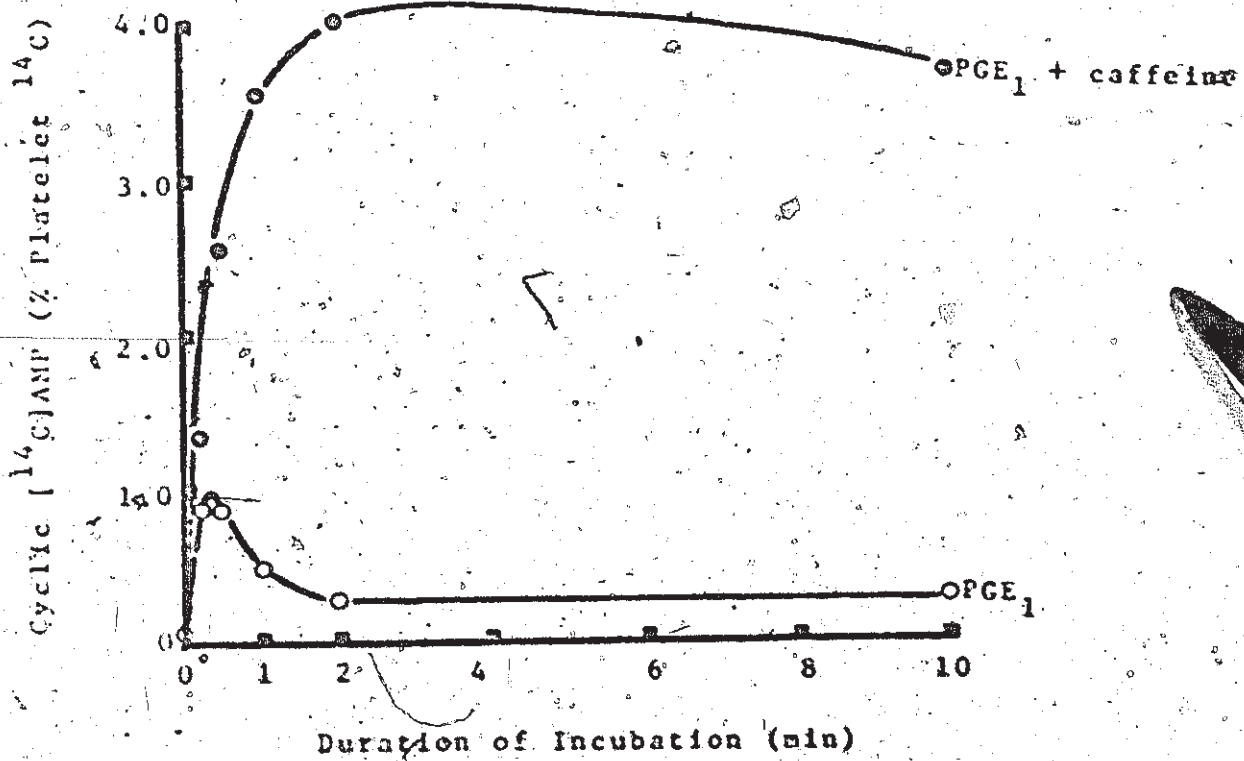


FIGURE 22. Time course of the effect of $0.5 \mu\text{M}$ PGE_1 and of $0.5 \mu\text{M}$ PGE_1 plus 20 mM caffeine on cyclic ^{14}C AMP levels in washed human platelets.

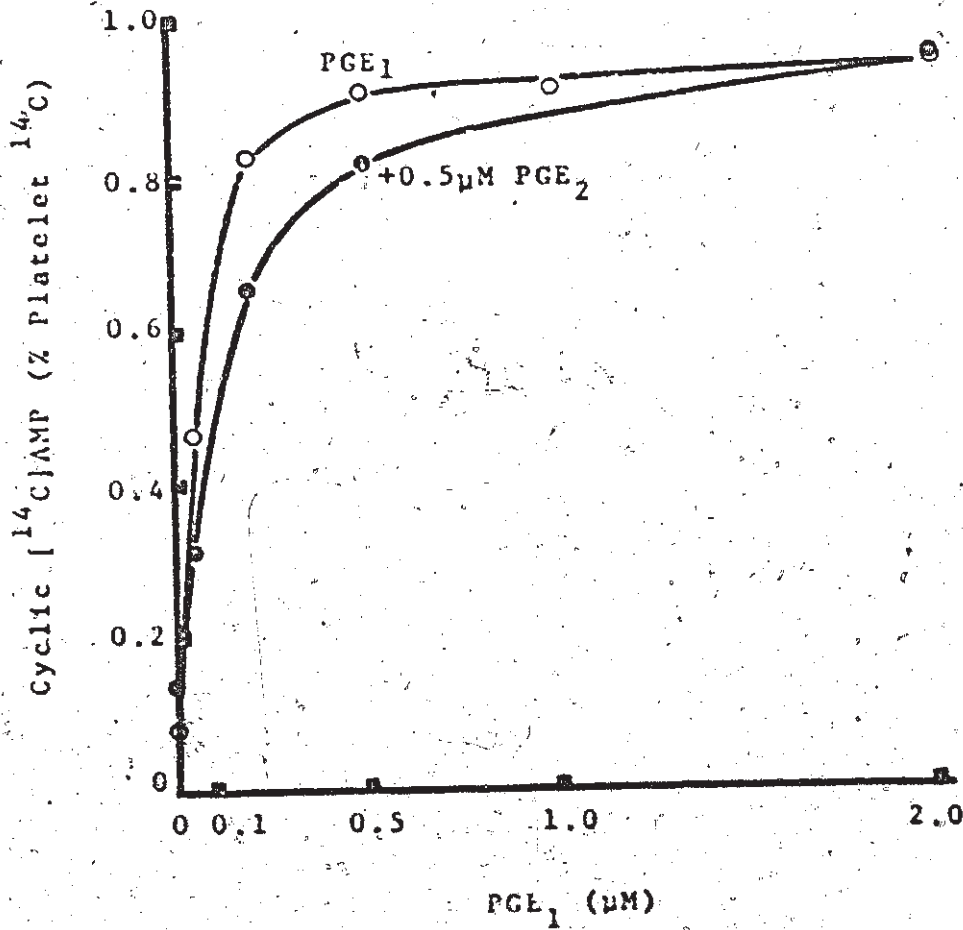


FIGURE 23. Effect of graded concentrations of PGE₁ with (●) and without (O) 0.5 μM PGE₂ on cyclic [¹⁴C]AMP levels in washed human platelets.

3. DISCUSSION

Prostaglandin E_1 caused a very marked concentration-dependent increase in the incorporation of radioactivity into cyclic [^{14}C]AMP in human platelets which had been pre-incubated with [^{14}C]adenine. Using a similar technique, Ball et al., (1970) reported that [^{14}C]ATP was present at 800 x the concentration of cyclic [^{14}C]AMP and was thus in abundant supply as substrate for AC. In the presence of PDE inhibitors (papaverine, RA233 and caffeine), PGE_1 caused a synergic increase in cyclic [^{14}C]AMP levels as has been reported by others (Ball et al., 1970; Mills & Smith, 1971). PGE_2 had a much weaker effect than PGE_1 in the absence of a PDE inhibitor, but also caused a synergic increase in cyclic [^{14}C]AMP in the presence of papaverine or caffeine. $PGF_{2\alpha}$ was virtually inactive in both the presence and absence of a PDE inhibitor.

Because the accumulation of cyclic [^{14}C]AMP in intact platelets in response to PGE_1 or PGE_2 in the presence of a PDE inhibitor was linear for at least 30 sec, the kinetics of the activation of AC could be studied. Lineweaver-Burk plots of the activation of AC by PGE_1 in the presence of various PDE inhibitors indicated a single PGE_1 receptor with a K_m value of about 0.3 μM in the case of caffeine. An

Eadie plot of the activation of AC by PGE₂ in the presence of caffeine suggested the presence of two PGE₂ receptors, one with a Km of 5.07 μM and the other with a Km of 0.38 μM. In the presence of caffeine the ratio of the Vmax obtained with PGE₁ to the Vmax obtained with the high Km PGE₂ receptor was 1.9 while with the low Km PGE₂ receptor it was 6.8.

Kinetic studies of the effect of PGE₁ on the activation of AC by PGE₂ suggest the presence of a mutual competitive inhibition of an AC receptor site. This finding is supported by the work of Kuehl and Humes (1972) on the binding of prostaglandins to receptor materials obtained from rat adipocytes, but in this system there is no associated activation of AC by prostaglandins. PGE₂ was observed to cause a decrease in cyclic [¹⁴C]AMP levels elevated by PGE₁ in the absence of PDE inhibitors, as Shio et al. (1972) have reported in rat platelets. Because the PGE₂ effect is greater in the absence than in the presence of a PDE inhibitor, its mode of action may partially be accounted for by an activation of PDE. Thus there appears to be two effects of PGE₂ on cyclic [¹⁴C]AMP levels in the presence of PGE₁, (a) in the presence of a PDE inhibitor a competition with PGE₁ for an AC receptor site and (b) in the absence of a PDE inhibitor an additional activation of PDE.

The effect of PGE_1 on the incorporation of radioactivity into cyclic AMP with respect to time was similar to that reported by other workers (Ball et al., 1970; Mills & Smith, 1972; Harwood et al., 1972). In the absence of a PDE inhibitor, PGE_1 increased cyclic [^{14}C]AMP levels to a maximum by 30 sec before they fell to a steady state level about half the maximum. In the presence of caffeine however, PGE_1 increased cyclic [^{14}C]AMP levels to a plateau steady state level. A comparison of the initial rates of cyclic [^{14}C]AMP accumulation when PGE_1 or caffeine was added at 4 min to 0 min caffeine or PGE_1 samples respectively, suggested that the 30 sec maximum seen in the absence of caffeine and that the swift transition to a plateau steady state seen in the presence of caffeine was due to a gradual decrease in AC activity. This could be due to protein binding of PGE_1 or to tachyphylaxis of the PGE_1 receptor.

7-Oxa-13-prostynoic acid, reported by Kuehl and Humes (1970) to have a competitive effect on prostaglandin action in mouse ery was in this study found to have a competitive effect on PGE_1 action in intact platelets.

4. SUMMARY

1. Formation of cyclic [^{14}C]AMP in platelets pre-

incubated with [14 C]adenine was linear for at least 30 sec in the presence of prostaglandins with a PDE inhibitor. This permitted the kinetics of the activation of AC to be studied in intact platelets.

2. The kinetics of the activation of AC by PGE₁ in the presence of various PDE inhibitors were studied and indicated the presence of a single receptor site.

3. The kinetics of the activation of AC by PGE₂ suggest the presence of high and low Km receptors for PGE₂.

4. The kinetics of the activation of AC by PGE₂ in the presence of PGE indicate a mutual competitive interaction.

5. In the absence of a PDE inhibitor PGE₁ increases cyclic [14 C]AMP levels to a maximum at 30 sec before they fall to a steady state level of about half the maximum. The results suggest that this is due to a decline in AC activity. In the presence of a PDE inhibitor cyclic [14 C]AMP levels rise to a steady state level after 1 min without going through a maximum.

6. In the absence of a PDE inhibitor PGE₂ may also reduce PGE₁ elevated levels of cyclic [14 C]AMP through an activation of PDE.

7. 7-Oxa-13-prostynoic acid competitively inhibits the activation of AC by PGE₁.

Chapter V

EFFECTS OF ADENOSINE DIPHOSPHATE AND OF EPINEPHRINE
ON PLATELET CYCLIC [14 C]AMP LEVELS IN THE PRESENCE
OF PROSTAGLANDIN E_1

1. INTRODUCTION

Epinephrine induces platelet aggregation by a process that appears to be mediated by an interaction with adrenergic α -receptors (O'Brien, 1963; Mitchell & Sharp, 1964).

Sutherland (1965) suggested that α -adrenergic effects might be associated with a fall in the level of cyclic AMP and indeed such an effect has now been demonstrated in several systems (Turtle et al., 1967; Robison et al., 1970). Salzman and Neri reported that epinephrine and ADR both produced a fall in the basal level of cyclic AMP in platelets and it has been suggested that platelet aggregation may always be associated with a fall in cyclic AMP levels (Salzman, 1972).

Other investigators however, (Cole et al., 1971; Robison et al., 1971; Haslam, 1971b; McDonald & Stuart, 1973) have found it difficult or impossible to demonstrate consistently a fall in basal cyclic AMP in response to either agent.

Epinephrine in the presence of papaverine has indeed been seen to stimulate platelet AC slightly (Haslam & Taylor, 1971a). Moreover, appreciable aggregation can occur with ADP in the presence of low PGE₁ concentrations when cyclic AMP levels are well above those in resting platelets (Haslam & Taylor, 1971b). Thus, it appears that the widely accepted hypothesis that aggregating agents may mediate

their effects through a decrease in platelet cyclic AMP levels is not supported.

The cyclic AMP-lowering effects of both epinephrine and ADP are very striking with respect both to the speed and magnitude of the response provided the level has first been raised by PGE_1 (Robison et al., 1969; Marquis et al., 1970; Haslam & Taylor, 1971a; Moskowitz et al., 1971; Harwood et al., 1972). In broken cell preparations epinephrine has been claimed to decrease platelet AC activity, an effect that is blocked by the α -adrenergic antagonist phentolamine (Zieve & Greenough, 1969; Marquis et al., 1970; Salzman et al., 1971b). Conversely, Harwood et al. (1972) suggested that epinephrine stimulates PDE because the epinephrine-induced decrease in cyclic AMP is reduced in the presence of a PDE inhibitor. In support of this Amer and Marquis (1972) have reported that epinephrine increased the proportion of low K_m PDE (PDE II) in platelet fractions.

No effect of ADP on AC in broken cell preparations from platelets has been observed (Salzman & Levine, 1971). However, it has been suggested that a reduction in platelet ATP may cause the decrease in platelet cyclic AMP formation in the presence of ADP (Haslam & Taylor, 1971b), possibly as a result of the conversion of extracellular ADP to ATP by nucleotide diphosphokinase (Guccione et al., 1971; Salzman,

1972). In an attempt to clarify this situation kinetic studies of the effects of ADP on changes in platelet cyclic [14 C]AMP levels in the presence of PGE₁ plus caffeine were carried out.

2. RESULTS

The effect of ADP on the activation of AC by PGE₁ in intact platelets was first studied. The results show a noncompetitive inhibition of the activation of AC measured in the presence of PGE₁ and caffeine and give a K_i for ADP of about 1 μ M (Figure 24).

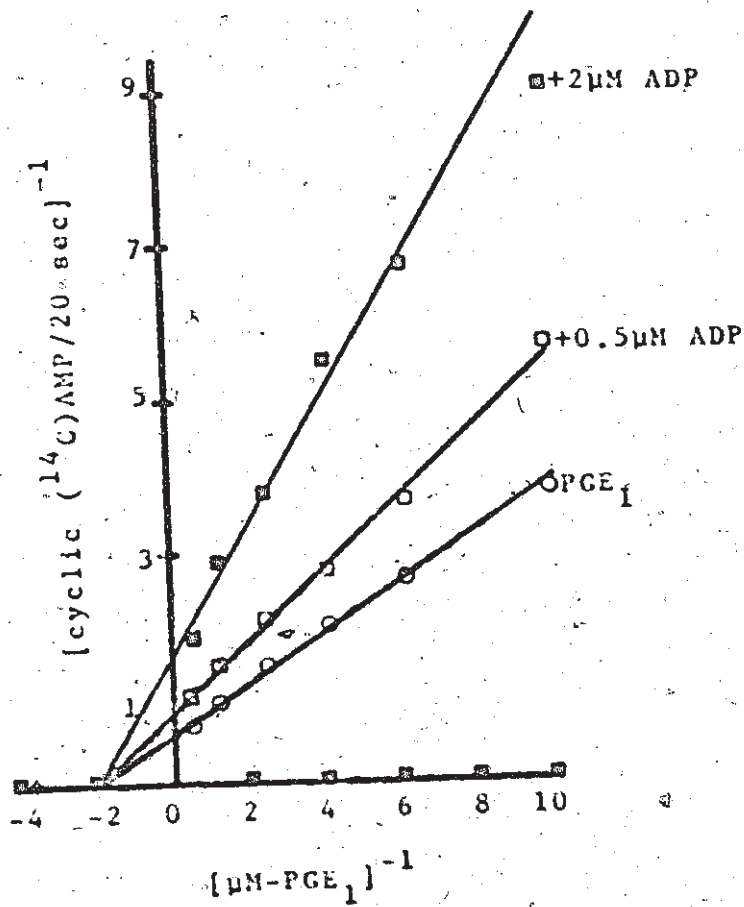


FIGURE 24. Lineweaver-Burk plot of the effect of ADP on the activation of AC in the presence of PGE₁ and caffeine (20 mM).

The effect of adding ADP or epinephrine to PGE₁-elevated steady state levels of cyclic [¹⁴C]AMP (Figure 25) was a rapid reduction of those levels to new steady states.

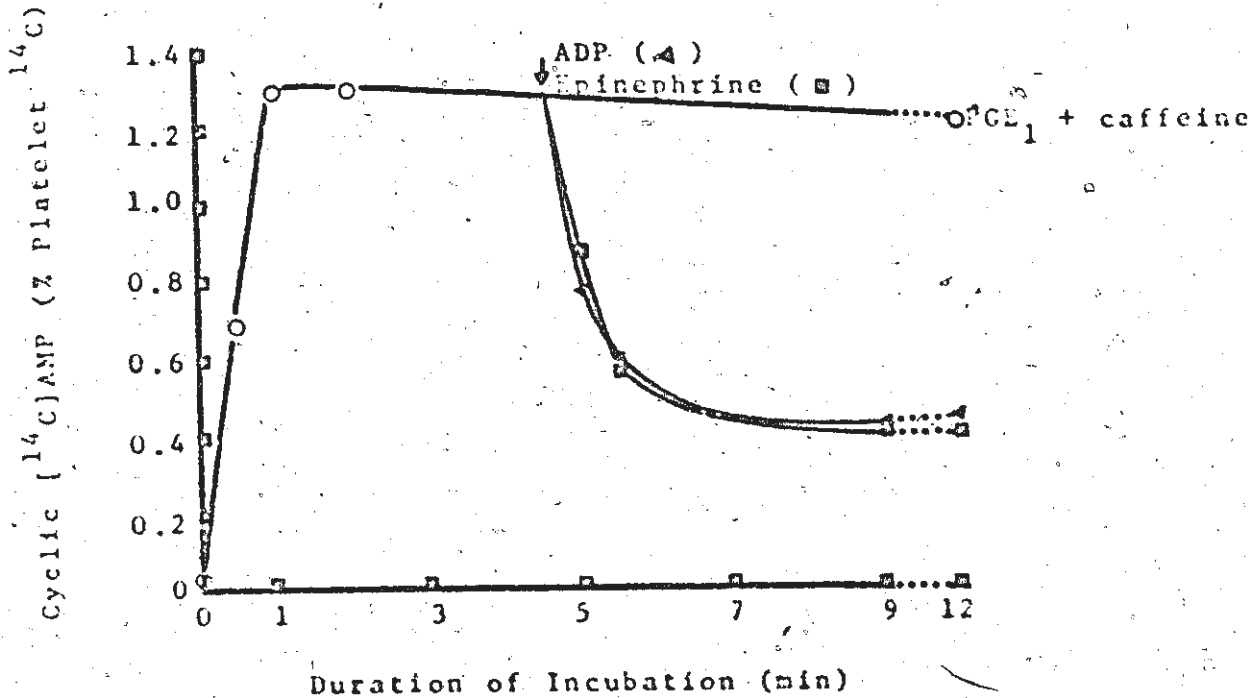


FIGURE 25. Effect of adding ADP (20 μ M) or epinephrine (10 μ M) at 4 min to PGE₁ (0.2 μ M) plus caffeine (20 mM) elevated levels of cyclic [¹⁴C]AMP in human platelets.

This reduction in cyclic [¹⁴C]AMP levels caused by ADP and by epinephrine must have been due to either an inhibition of AC or an activation of PDE. With the aid of the computer model the activities of these enzymes were altered either at 0 min or 4 min, such that new steady state levels, equal to half the control level of PGE₁ plus caffeine alone, were reached (Figure 26). This study showed that the rate of

approach to the new steady state level was more rapid when the PDE activity was increased than when the AC activity was decreased, both at 0 and at 4 minutes.

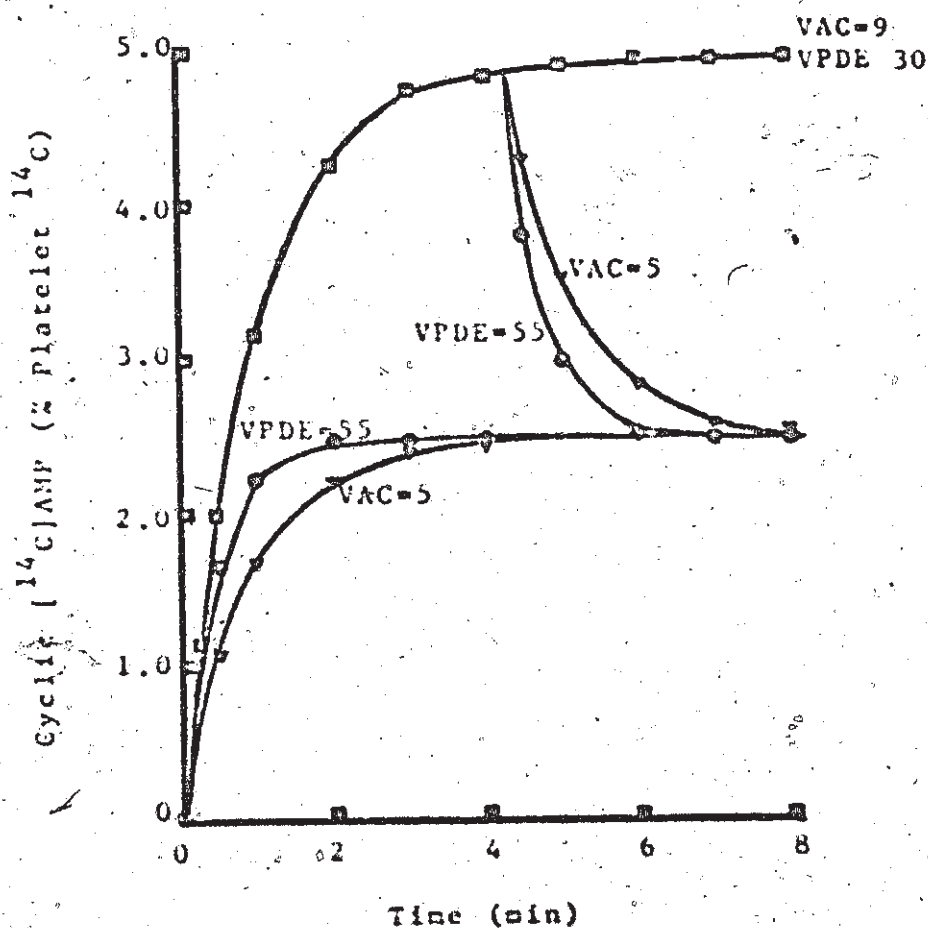


FIGURE 26. Computer model of the effect of PGE_1 ($0.5 \mu M$) plus caffeine ($20 \mu M$; \blacksquare) and of the effect of lowering the V_{max} of AC (VAC; \blacktriangledown) or raising the V_{max} of PDE (VPDE; \bullet) at 0 min and at 4 min on cyclic $[^{14}C]$ AMP levels.

This model situation was simulated experimentally. Thus from 0 min a decrease in AC activity was achieved by decreasing the PGE_1 concentration and an increase in the PDE activity was achieved by decreasing the caffeine concentration (Figure 27). The relationship between the two effects was exactly that predicted by the computer model. The effects of ADP and epinephrine in the same situation were then observed to determine the curve to which they approximated best (Figure 27).

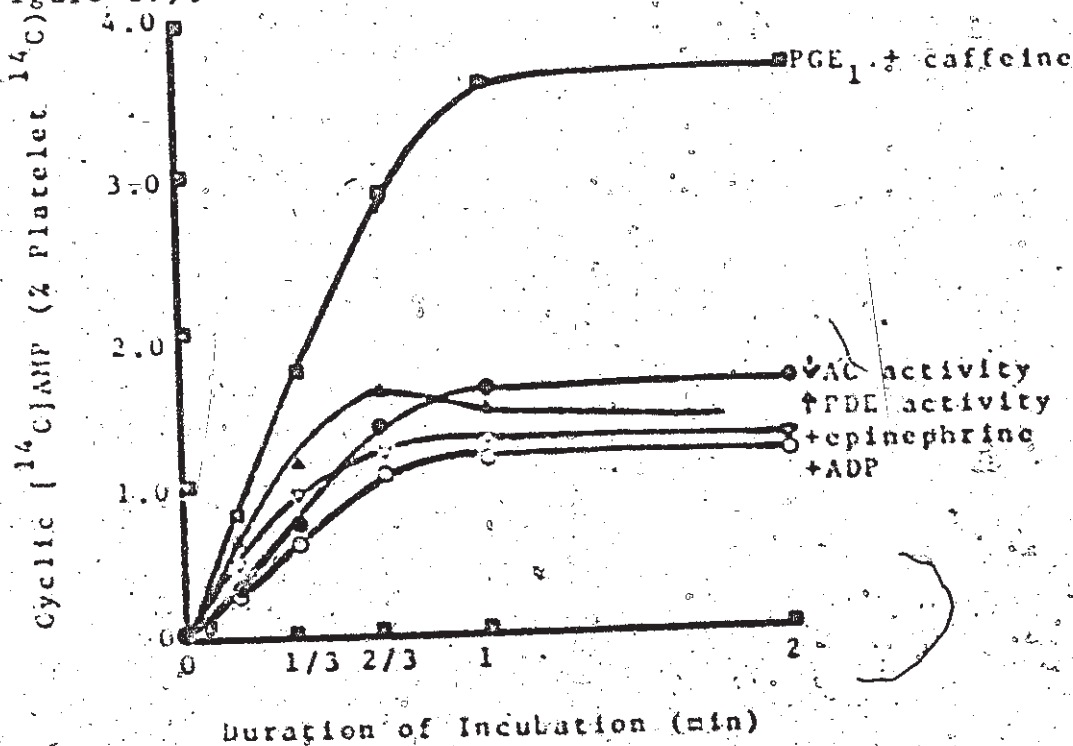


FIGURE 27. Effects of ADP and of epinephrine on the approach to steady state cyclic $[^{14}C]$ AMP levels in the presence of PGE_1 and caffeine. The effects of $0.5 \mu M PGE_1 + 20 mM$ caffeine (■), $0.12 \mu M PGE_1 + 20 mM$ caffeine (●), $0.5 \mu M PGE_1 + 4 mM$ caffeine (▲), $0.5 \mu M PGE_1 + 20 mM$ caffeine + $1.5 \mu M$ ADP (○) and of $0.5 \mu M PGE_1 + 20 mM$ caffeine + $1.5 \mu M$ epinephrine (▼) on cyclic $[^{14}C]$ AMP levels in human platelets.

The results suggested that the predominant effect of ADP was on AC while that of epinephrine was on the residual PDE. Thus, the crossover between the epinephrine and low PGE₁ curves suggests that epinephrine probably does not act principally by inhibiting the AC activity.

In another experiment a decrease in the PGE₁ concentration (i.e. a decrease in AC activity) or in the caffeine concentration (i.e. an increase in PDE activity) after a 4 min exposure to PGE₁ + caffeine was achieved by transferring 0.2 ml of each sample to 0.8 ml PPP containing either PGE₁ or caffeine but not both, thus diluting the PGE₁ or caffeine and hence lowering their effective concentrations (Figure 28). The resultant decreases in cyclic [¹⁴C]AMP to new steady state values agreed very well with the computer prediction. When under the same conditions PPP containing PGE₁ plus caffeine was diluted into PPP containing PGE plus caffeine with ADP or epinephrine, the ADP-induced decrease in cyclic [¹⁴C]AMP levels was faster than that caused by epinephrine (Figure 28). This suggests that in this case ADP has its main effect on the residual PDE while epinephrine has an effect on AC. Thus, when studying the effects of ADP and epinephrine on the increase to and the decrease from the steady-state level of cyclic [¹⁴C]AMP in the presence of PGE₁ and caffeine, conflicting results were obtained as to their possible respective nodes of action.

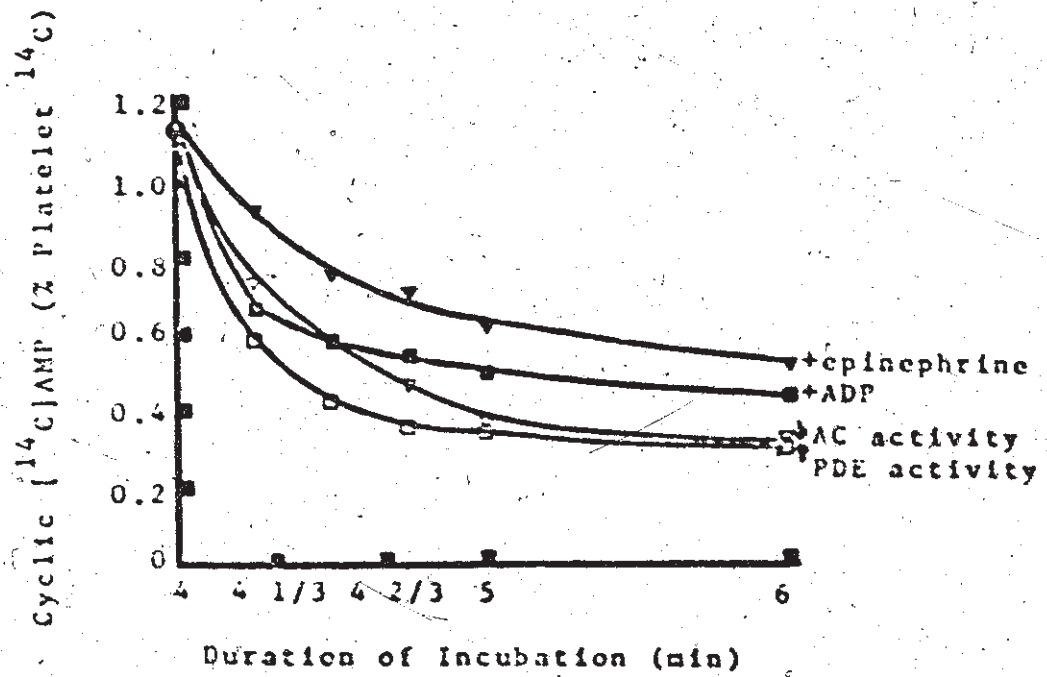


FIGURE 28. Effects of ADP and of epinephrine on the steady state level of cyclic [¹⁴C]AMP in the presence of PGE₁ + caffeine. All samples were incubated for 4 min with 0.5 μM PGE₁ + 10 mM caffeine before 0.2 ml portions of each were diluted with 0.8 ml PPP + additions to give the following final concentrations: 0.1 μM PGE₁ + 10 mM caffeine (▽), 0.5 μM PGE₁ + 2 mM caffeine (□), 0.5 μM PGE₁ + 10 mM caffeine + 1 μM ADP (■) and 0.5 μM PGE₁ + 10 mM caffeine + 0.5 μM epinephrine (▼).

The effect of a range of epinephrine concentrations on cyclic [¹⁴C]AMP levels in the presence and absence of a PDE inhibitor was next examined. In the presence of caffeine, epinephrine reduced the levels of cyclic [¹⁴C]AMP stimulated

by PGE₁, less than in the absence of caffeine, particularly at low epinephrine concentrations. This suggests an activation of PDE by epinephrine as a possible means by which epinephrine may cause a decrease in cyclic AMP levels in intact platelets (Table 5). Both in the presence of PGE₁ or of PGE₁ + papaverine (Table 6), the epinephrine-induced decrease in cyclic AMP levels is blocked by phentolazine but not by (-)-propranolol, indicating that it is an α -adrenergic effect.

ADDITIONS	EPINEPHRINE (μ M)			
	0	1	2	10
0.5 μ M PGE ₁ -- 1 cyclic [¹⁴ C]AMP -- 2 inhibition	0.411 --	0.107 74	0.073 81	0.063 85
0.5 μ M PGE ₁ + 20 mM caffeine -- 1 cyclic [¹⁴ C]AMP -- 2 inhibition	1.443 --	1.178 15	1.992 --	0.542 62

TABLE 5. Effect of epinephrine on the elevation of cyclic [¹⁴C]AMP levels by PGE₁ in the presence and absence of caffeine. Duration of incubations, 20 sec.

ADDITIONS	Cyclic [¹⁴ C]AMP (% Platelet ¹⁴ C)			
	EPINEPHRINE (μM)			
	0	1	2	10
0.5 μM PGE ₁	0.411	0.107	0.078	1.063
0.5 μM PGE ₁ + 10 μM Phentolamine	0.442	0.421	0.423	0.340
0.5 μM PGE ₁ + 10 μM (-)-Propranolol	0.420	0.423	0.164	0.070
0.5 μM PGE ₁ + 2 mM Papaverine	0.361	0.287	0.244	0.159
0.5 μM PGE ₁ + 2 mM Papaverine + 10 μM Phentolamine	0.367	0.379	0.353	0.308
0.5 μM PGE ₁ + 2 mM Papaverine + 10 μM (-)-Propranolol	0.364	0.291	0.231	0.154

Table 6. Effects of epinephrine ± antagonists on the elevation of cyclic [¹⁴C]AMP levels by PGE₁ ± papaverine. Duration of incubations, 20 sec.

3. DISCUSSION

Kinetic studies indicated a noncompetitive inhibition by ADP of the activation of AC by PGE₁. This is consistent

with the hypothesis that the action of ADP may be able to deplete pool of ATP utilized by AC. However, the measurable decrease in metabolic ATP during aggregation is very small, so it would be necessary to postulate that the ATP used by AC was compartmentalized (Mills & Smith, 1972). Other indirect mechanisms of action ranging from direct receptor-AC interactions to non-specific effects of changes in membrane structure are equally possible.

ADP or epinephrine markedly reduced the accumulation of cyclic [^{14}C]AMP caused by PGE_1 + caffeine when added with these agents, as well as when added 4 min later. In the latter case, the rate of loss of cyclic [^{14}C]AMP, calculated from Figure 25 ($0.2 \mu\text{M } \text{PGE}_1 + 20 \text{ mM caffeine}$) due to the addition of ADP was approximately $0.064 \mu\text{moles/min}/10^{11}$ platelets. This provides a minimum value for the turnover of cyclic [^{14}C]AMP in that PGE_1 -elevated steady state situation. This value is less than that obtained in a similar calculation by Mills and Smith (1972) but the concentration of PGE_1 they used was greater.

When the AC activity was decreased or the PDE activity increased in the computer model, such that the same new steady state level of cyclic AMP was achieved, the rate of approach to that level was more rapid, from both 0 min and 4 min after addition of PGE_1 + caffeine when the PDE activity was increased than when the AC activity was decreased.

Experimentally, the reduction of the PGE₁ concentration (i.e. a reduction in the AC activity) or of the caffeine concentration (i.e. an increase in the PDE activity) simulated the computer predictions, i.e. a reduction of the caffeine concentration resulted in a faster approach to the new steady state level of cyclic [¹⁴C]AMP from both 0 min and 4 min than a reduction in the PGE₁ concentration.

When the effects of ADP and of epinephrine, added at 0 min or 4 min were compared, opposite conclusions as to their modes of action were suggested. At 0 min it appeared that ADP mainly inhibited AC activity while epinephrine mainly increased PDE activity. At 4 min the reverse seemed to apply.

However, it should be noted that the condition of the platelets at 0 min and at 4 min was not the same: in the latter case the prolonged elevation of platelet cyclic AMP levels could have affected the reactions of the platelet to ~~addition of ADP~~ or to epinephrine. At 0 min, probably the more natural situation, the epinephrine kinetics suggested an action on PDE. Azer and Marquis (1972) demonstrated an increased PDE II (low K_m enzyme) activity in the presence of epinephrine in membrane-supplemented whole homogenates of human platelets. In addition, Harwood et al. (1972) reported that the epinephrine effect was reduced in the presence of a PDE inhibitor as was shown in Table 3. A

possible mode of action of ADP at 0 min concerning a depletion of platelet ATP was mentioned in the introduction to this chapter. At 4 min, the effect of epinephrine on PDE activity may have been inhibited by the PGE_1 due to the fact that PGE_1 itself may have an inhibitory effect on PDE II activity. (Amer & Marquis, 1972). These authors could demonstrate no effect of ADP on PDE activity. Although the results show an apparent effect of epinephrine on AC activity as would support the observations of Zieve and Greenough (1969), Marquis et al. (1970) and Salzman and Levine (1971), the possibility of an activation of residual PDE activity was not completely removed in these observations. In the case of epinephrine, its effects appear to be mediated through platelet α -adrenergic receptors, as reported by others (Robison et al., 1969; Marquis et al., 1970; Moskowitz et al., 1971).

6. SUMMARY

1. ADP causes a noncompetitive inhibition of the activation of AC by PGE_1 with a K_i of about 1 μM .
2. Alteration of AC or PDE activities at 0 min or 4 min after addition of PGE_1 + caffeine, either experimentally or

in a computer simulation resulted in different rates of approach to new lower steady state levels of cyclic [^{14}C]AMP.

3. Addition of ADP or of epinephrine at 0 min or at 4 min to samples of PRP containing PGE_1 + caffeine, resulted in a different rate of approach to the new lower steady state levels of cyclic [^{14}C]AMP. The significance of this is discussed.

4. Low concentrations of epinephrine inhibit the increase in cyclic [^{14}C]AMP caused by PGE_1 more powerfully in the absence than in the presence of caffeine, suggesting that part of the effect of epinephrine was due to activation of PDE.

5. The effects of epinephrine on platelet cyclic [^{14}C]AMP levels were mediated by α -adrenergic receptors.

Chapter VI

THE RELATIONSHIP BETWEEN PLATELET CYCLIC [14 C]AMP LEVELS
AND THE INHIBITION OF ADP-INDUCED AGGREGATION BY
PROSTAGLANDIN E_1

1. INTRODUCTION

Exogenous cyclic AMP itself inhibits platelet aggregation (Marcus & Zucker, 1965), but the dibutyryl derivative is more effective because of its more lipophilic nature (Marquis et al., 1969; Salzman & Levine, 1971). Caffeine and PGE₁ act synergically in increasing the accumulation of cyclic AMP in platelets and at the functional level, the same synergism is seen in the inhibition of platelet aggregation (Cole et al., 1971; Vigdahl et al., 1971; Mills & Smith, 1972). The PGE₁-induced increase in cyclic AMP was seen to occur before the inhibition of aggregation (Vigdahl et al., 1969; Marquis et al., 1970; Ball et al., 1970). The order of potency of the prostaglandins in stimulating platelet AC and in increasing the level of cyclic AMP has been found to be the same as their order of potency as inhibitors of platelet aggregation (Cole et al., 1971; Robison et al., 1971). However the cyclic AMP level observed in platelets at different times after addition of PGE₁ does not correlate with the inhibition of the aggregation caused by subsequent addition of ADP. Thus cyclic AMP levels peak after about 30 sec but the inhibition of aggregation is maintained or increases further (Ball et al., 1970; McDonald & Stuart, 1973).

Mills & Smith (1971) found three different relationships between the increase in cyclic [14 C]AMP and the inhibition of subsequent aggregation in the presence of a PDE inhibitor, PGE₁ and PGE₁ plus a PDE inhibitor. It has been suggested that these discrepancies may be due to the inhibition of cyclic AMP formation by ADP and that inhibition of aggregation may relate better to the cyclic AMP level after addition of ADP than to the level before (Haslam & Taylor, 1971; Haslam, 1973). The purpose of this study was to help clarify this issue.

2. RESULTS.

The relationship between the inhibition of ADP-induced aggregation by different concentrations of PGE₁ and the associated increases in cyclic [14 C]AMP measured 30 sec after the simultaneous addition of ADP and PGE₁ is constant whether the extent of that inhibition is measured after 30, 45 or 60 sec (Figure 29). However in the presence of a range of PGE₁ concentrations plus papaverine different relationships are obtained when the extent of the inhibition is measured after 30, 45 or 60 sec (Figure 29). These two relationships between the inhibition of aggregation and the increase in cyclic [14 C]AMP levels coincided if a lag of

about 15 sec was allowed between the time at which the cyclic [14 C]AMP level was measured and the time at which the inhibition of aggregation was measured. A specific instance is illustrated in Figure 30. In this case PGE₁ (2.0 μ M) and PGE₁ (0.05 μ M) plus papaverine (0.2 mM) caused the same increase in cyclic [14 C]AMP 30 sec after addition of ADP. The same inhibition of extent of aggregation however, is only seen at the time at which the aggregation curves cross each other (i.e. 45 sec).

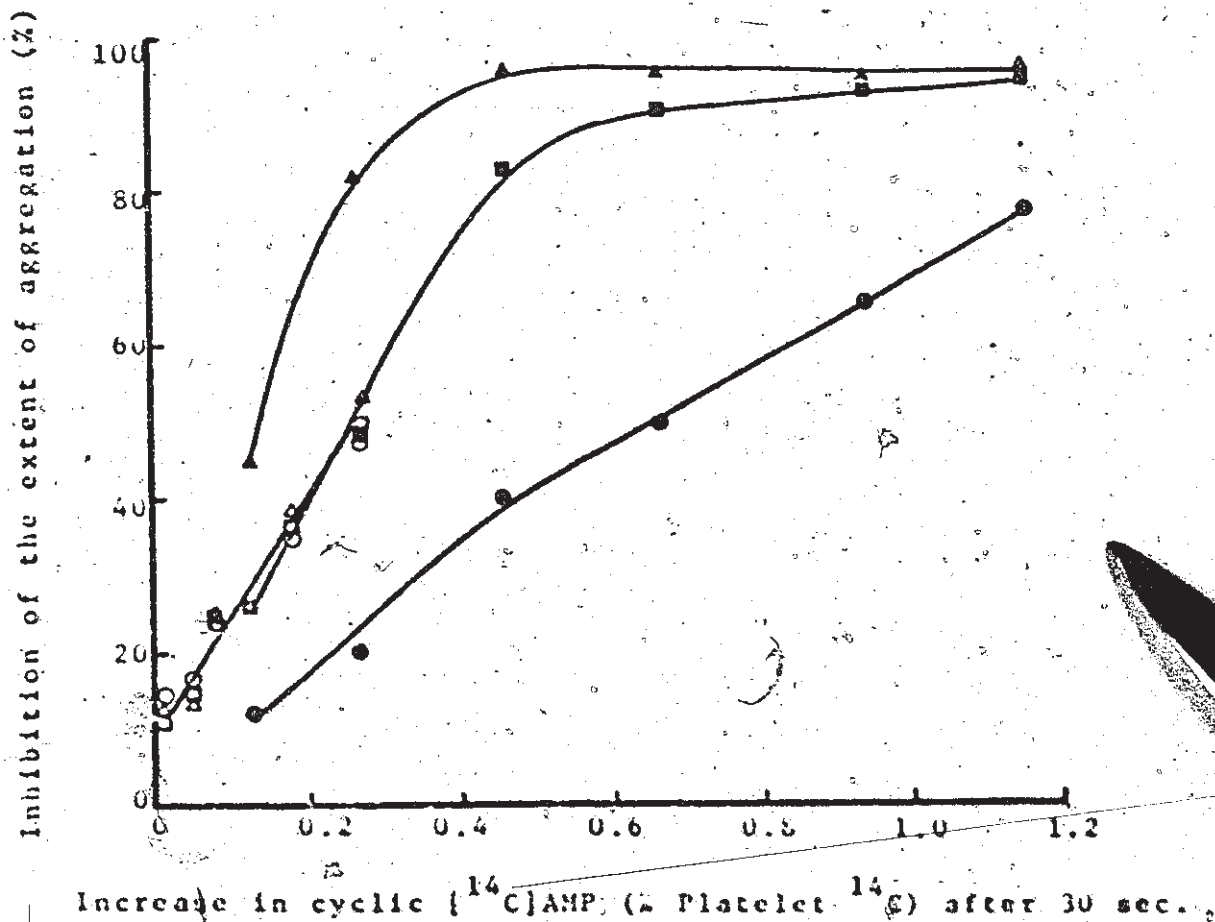


FIGURE 29. The relationships between the inhibitions of ADP-induced aggregation, by a range of PGE₁ concentrations (open symbols) and by a range of PGE₁ concentrations plus 0.2 mM papaverine (closed symbols), and the increases in cyclic [¹⁴C]AMP levels measured 30 sec after the simultaneous additions of 2 μM ADP and the inhibitors. The inhibition of the extent of aggregation was measured 60 sec (▲, ▲), 45 sec (■, □) and 30 sec (●, ○) after addition of ADP.

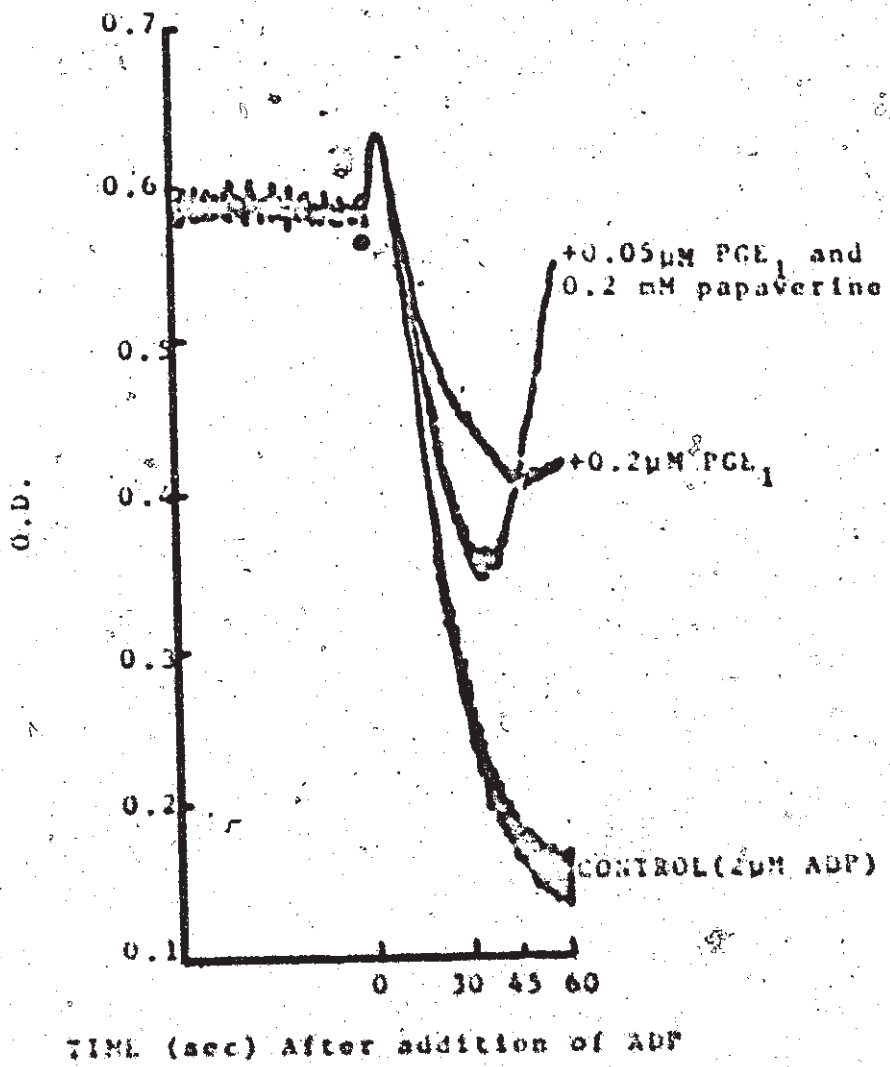


FIGURE 10. Inhibition of ADP-induced aggregation by 2.0 μM PGE₁ and by 0.05 μM PGE₁ plus 0.2 mM papaverine. All additions were made at (●).

The relationship between the inhibition of aggregation, measured either 30 or 45 sec after addition of ADP, by PGE_1 plus papaverine and the increases in cyclic [^{14}C]AMP levels was dependent on the ADP concentration (Figure 31). At higher ADP concentrations a given increase in cyclic [^{14}C]AMP was associated with less inhibition of aggregation.

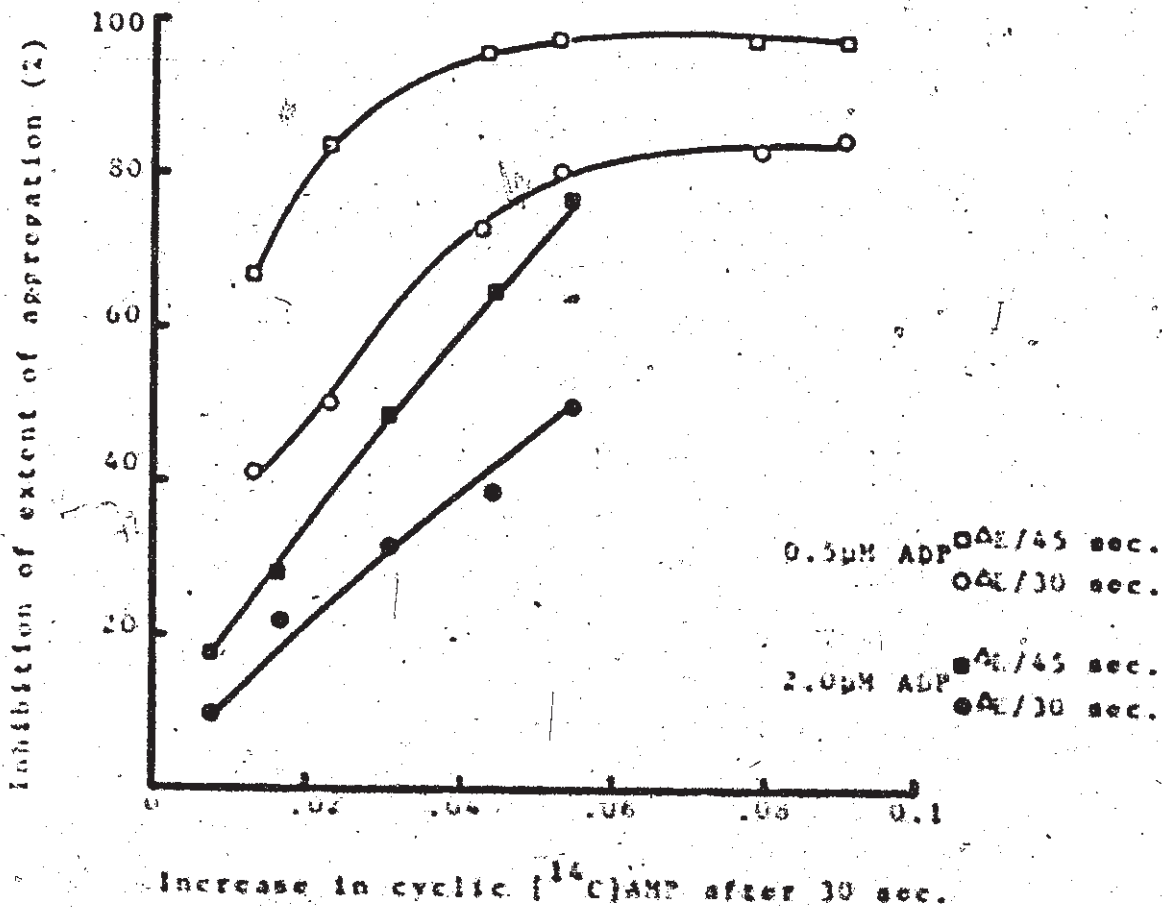


FIGURE 31. The relationships between the extent of inhibition of aggregation by a range of PGE_1 concentrations plus 0.2 μM papaverine and the increase in cyclic [^{14}C]AMP levels for ADP concentrations of 0.5 μM (open symbols) and 2.0 μM (closed symbols).

The relationships between the inhibitions of aggregation by the addition of compounds 3 minutes before ADP and the increases in cyclic [14 C]AMP measured before or after the addition of ADP are shown in Table 7. Two concentrations of ADP were examined as well as the effects of PGE₁, papaverine and PGE₁ plus papaverine. The results show that (a) 3 minute preincubations with the inhibitors resulted in an increased inhibition of aggregation when compared with the effects of simultaneous addition of inhibitors and ADP, (b) ADP decreased cyclic [14 C]AMP levels in a concentration dependent manner with all the inhibitors and (c) inhibition of aggregation was more closely related to cyclic [14 C]AMP levels measured after the addition of ADP than before.

	Addition of ADP		2 cyclic [¹⁴ C]AMP Measured at Increase Time (min) over control	Inhibition of Extent of Aggregation at:		
	Time (min)	Concentration (μM)		30 sec	45 sec	60 sec
PGE ₁ Papaverine PGE ₁ + Papaverine	0 0 0	0.5 0.5 0.5	1/2 1/2 1/2	47 31 27	61 49 48	70 74 70
PGE ₁ Papaverine PGE ₁ + Papaverine	0 0 0	4 4 4	1/2 1/2 1/2	-- 6 --	2 6 5	4 12 10
PGE ₁ Papaverine PGE ₁ + Papaverine			3 3 3			
PGE ₁ Papaverine PGE ₁ + Papaverine	3 3 3	0.5 0.5 0.5	3 1/2 3 1/2 3 1/2	75 86 92	74 87 92	84 87 88
PGE ₁ Papaverine PGE ₁ + Papaverine	3 3 3	4 4 4	3 1/2 3 1/2 3 1/2	13 70 81	14 80 72	15 78 72

TABLE 7. The relationship between the inhibition of aggregation and the increase in cyclic [¹⁴C] AMP levels: effects of two concentrations of ADP and of a 3 minute preincubation with inhibitors. Inhibitors were: 0.05μM PGE₁, 0.2 mM papaverine and 0.01μM PGE₁ plus 0.06 mM papaverine.

3. DISCUSSION

The extent of the inhibition of ADP-induced aggregation by PGE_1 , whether measured at 30, 45 or 60 sec after the addition of ADP, correlated with the increase in cyclic [^{14}C]AMP measured 30 sec after the addition of ADP. Under the same conditions PGE_1 plus papaverine gave the same correlation only if the inhibition was measured 15 sec after the cyclic [^{14}C]AMP level was measured. In the case of PGE_1 plus ADP, cyclic [^{14}C]AMP levels have probably reached their maximum level by 30 sec, while with PGE_1 plus papaverine plus ADP, cyclic [^{14}C]AMP levels probably continue to rise over the first minute. This could explain why the inhibition continues to rise in the latter case but not in the former. The results also suggest that cyclic AMP may take up to 15 sec to exert its effects on platelet aggregation.

Despite the correlations seen between the effects of PGE_1 with or without papaverine on ADP-induced aggregation and platelet cyclic [^{14}C]AMP levels for a particular ADP concentration, intracellular cyclic AMP appeared to be less effective in mediating inhibition at higher ADP concentrations. No definite explanation of this phenomenon can be offered, but it is at least possible that ADP interacts not only with

AC but also with the mechanism by which cyclic AMP causes inhibition of aggregation e.g. by causing dephosphorylation of a hypothetical inhibiting phosphoprotein.

Cyclic [14 C]AMP levels measured after a 3 min pre-incubation just prior to the addition of ADP did not correlate with the inhibition of aggregation as well as those measured 30 sec after the addition of ADP. Thus, as has been suggested by Haslam and Taylor (1971) the cyclic AMP level that is important as regards aggregation may be that which is present after the addition of the aggregating agent rather than the level that is present immediately prior to the addition of the aggregating agent as implied by several other workers (Mills & Smith, 1971; McDonald & Stuart, 1973).

4. SUMMARY

1. The inhibition of ADP-induced aggregation by PGE_1 correlated better with cyclic [14 C]AMP levels measured after the addition of ADP rather than those levels present immediately prior to the aggregation.

2. In the case of the inhibition of aggregation by PGE_1 plus papaverine the results suggest that cyclic AMP (measured after the addition of ADP) may take up to 15 sec to exert

its effects on that aggregation,

3. A given intracellular level of cyclic [14 C]AMP appears to be more effective against aggregation caused by a low rather than a high concentration of ADP.

Chapter VII

EFFECTS OF PROSTAGLANDIN PRECURSORS ON PLATELET FUNCTION
AND ON CYCLIC [14 C]AMP LEVELS IN PLATELETS

1. INTRODUCTION

Great structural specificity of prostaglandin action, rarely seen in other biological systems, is demonstrated in rat platelets where PGE_1 inhibits aggregation and PGE_2 enhances aggregation (Kloetzer, 1966). With human platelets PGE_2 inhibits the first wave and enhances only the second wave of ADP-induced aggregation in citrated PRP (Shio & Ranwell, 1972). In this regard it is interesting to note that recent observations (Smith et al., 1970; Silver et al., 1972) have revealed the presence of prostaglandin synthetase in the platelet itself. The prostaglandin content of unstimulated platelets is low, but considerable amounts of PGE_2 and $PGF_{2\alpha}$ were found after treatment with thrombin and lesser amounts after treatment with collagen, epinephrine or ADP (Smith et al., 1973). It is possible that the intracellular regulation of platelet function may be affected by prostaglandin formation. In this regard the effect of aspirin is notable. Aspirin and indomethacin inhibit prostaglandin formation in human platelets (Smith et al., 1971) and inhibit the second wave of aggregation in human platelets as well as the associated release reaction (Zucker & Peterson, 1970; Weiss et al., 1968).

Smith and Willis (1971) reported that although the production of prostaglandins in platelets is inhibited by aspirin, the release of phospholipase A_1 is not, suggesting that the action of aspirin on platelets is to inhibit the conversion of arachidonic acid into PGE_2 and $PGF_{2\alpha}$ rather than the liberation of arachidonic acid from the membrane phospholipids. They suggest that the PGE_2 and $PGF_{2\alpha}$ formed by platelets act by increasing vascular permeability (Smith & Willis, 1970) as do other substances that are released at the same time as the prostaglandins.

The possibility that prostaglandins may play a role in the regulation of platelet function suggests that blood prostaglandin concentrations may be important as they are released by various physiological stimuli from many tissues especially in pathological conditions. Ferreira (1967) however, demonstrated that prostaglandins are rapidly metabolized in the blood stream and that physiological concentrations of prostaglandins had no effect on platelet morphology.

As human platelets possess prostaglandin synthetase the possible formation of PGE_1 from its precursor, 8,11,14-eicosatrienoic acid was examined using the activation of platelet AC as a very sensitive method of detecting PGE_1 . Recently Ingeman et al. (1973) reported that arachidonic acid, the fatty acid precursor

of PGE₂, induced aggregation as well as the formation of PGE₂ in human citrated PRP. The possible role of the synthesized PGE₂ in the arachidonic acid-induced aggregation was investigated as well as the possibility that arachidonic acid may affect basal or elevated levels of cyclic AMP through the formation of PGE₂.

2. RESULTS

The ability of human platelets to synthesize PGE₁ from its fatty acid precursor, di-homo- γ -linolenic acid was examined under several conditions (Table 8 and Table 9). Even in the presence of hydroquinone and glutathione (which would be expected to potentiate prostaglandin synthesis; Leonardi et al., 1972) and papaverine, only a very small increase in cyclic [¹⁴C]AMP attributable to the fatty acid was found, presumably in response to synthesized PGE₁. Because the effect of di-homo- γ -linolenic acid on cyclic [¹⁴C]AMP formation was very small no further work was done with this fatty acid.

Incubation Time (min)	Additions			
	Papaverine (2 mM)	Papaverine (2 mM) + di-homo- γ -linolenic acid		
		1 μ M	10 μ M	40 μ M
1	.059	.059	.061	.071
4	.113	.112	.119	.139
16	.126	.131	.131	.145

TABLE 8. Effect of the concentration of the PGE₂ precursor, di-homo- γ -linolenic acid and of the time of incubation on cyclic [¹⁴C]AMP (2 of the platelet ¹⁴C) in the presence of papaverine (2.0 mM) in human platelets.

Incubation Time (min)	Additions		
	Papaverine (2 mM)	Papaverine (2 mM) + di-homo- γ -linolenic acid	
		10 μ M	40 μ M
1	.073	.081	.095
4	.125	.134	.139
16	.164	.174	.206

TABLE 9. Effect of the concentration of the PGE₂ precursor, di-homo- γ -linolenic acid and of the time of incubation of cyclic [¹⁴C]AMP levels (2 of the platelet ¹⁴C) in the presence of papaverine (2.0 mM), hydroquinone (0.1 mM) and glutathione (1.0 mM) in human platelets.

The effect of PGE_2 on ADP-induced aggregation was then examined (Figure 32). PGE_2 when added simultaneously with ADP caused an inhibition of the first wave and an enhancement of the second wave of aggregation, confirming the work of Shio and Ramwell (1972).

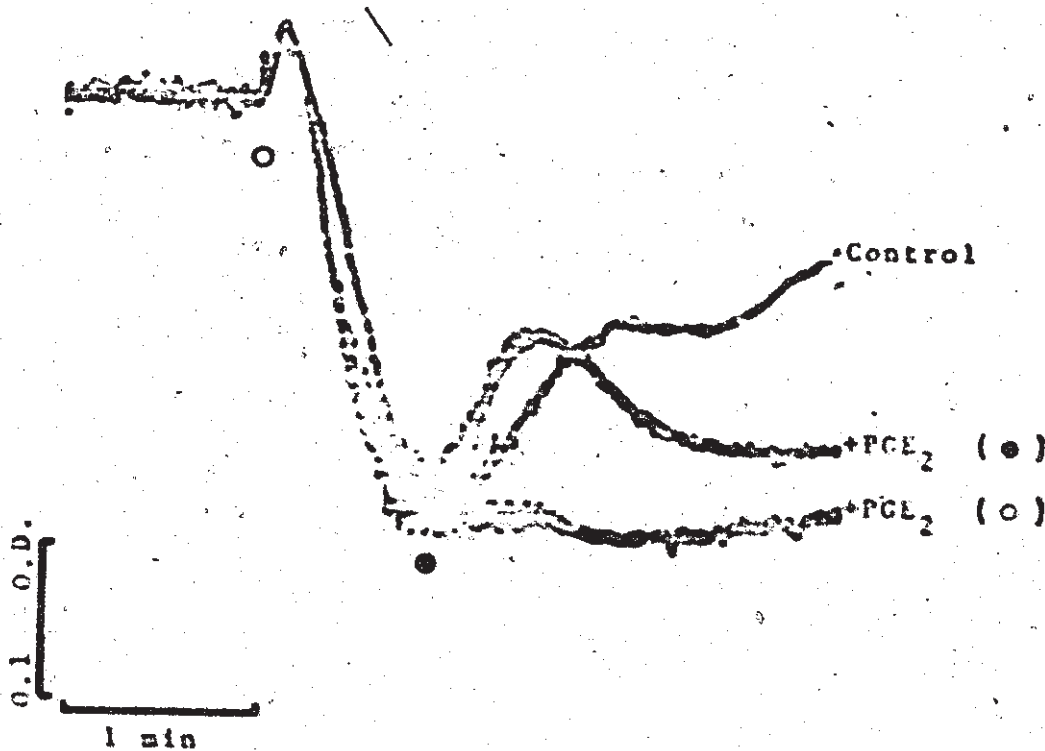


FIGURE 32. Effects of PGE_2 ($2.5 \mu\text{M}$) on ADP ($0.3 \mu\text{M}$)-induced aggregation.

The effect of aspirin on ADP-induced aggregation (Figure 33), also confirms previous work (Zucker et al., 1968; Weiss et al., 1968).

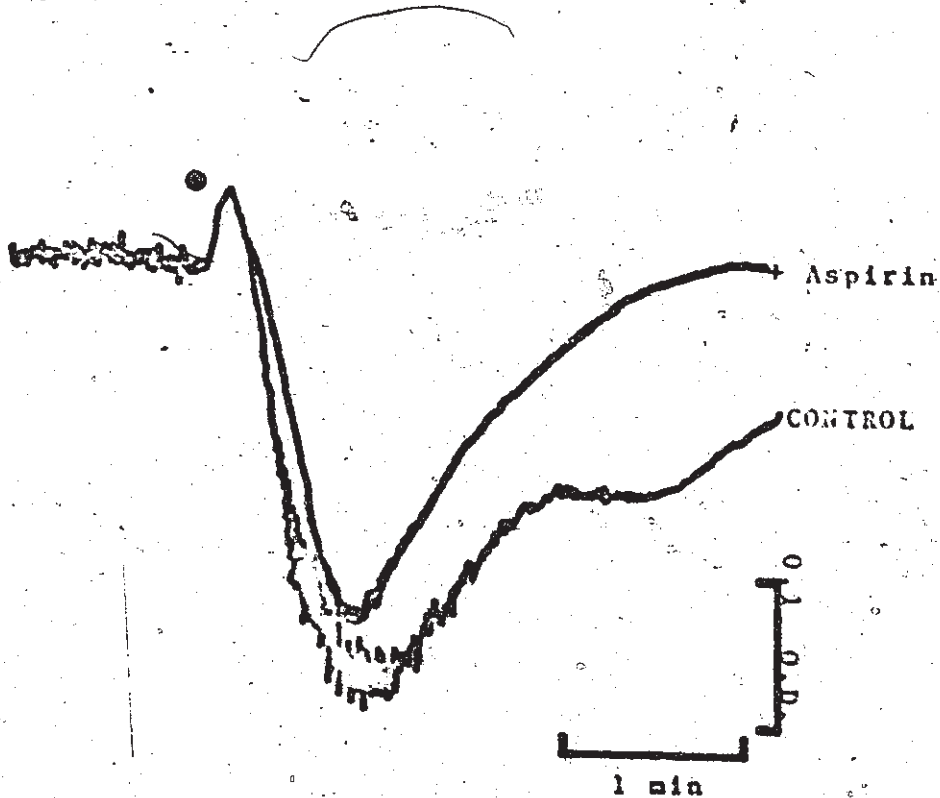


FIGURE 33. Effect of 0.5 mM aspirin on ADP-induced aggregation. Aspirin was added simultaneously with 0.5 μ M ADP at (●).

Arachidonic acid (5,8,11,14-eicosatetraenoic acid) caused complete and irreversible aggregation in human PRP (Figure 34). There was a delay in aggregation, but not in the shape change, which was inversely related to the concentration of arachidonic acid. If the aggregation was

the result of synthesis of PGE_2 or some other material from arachidonic acid the delay would suggest that a critical level of this compound had to be reached before the aggregation could occur. In a control study 8,11-eicosadienoic acid showed no aggregating activity.

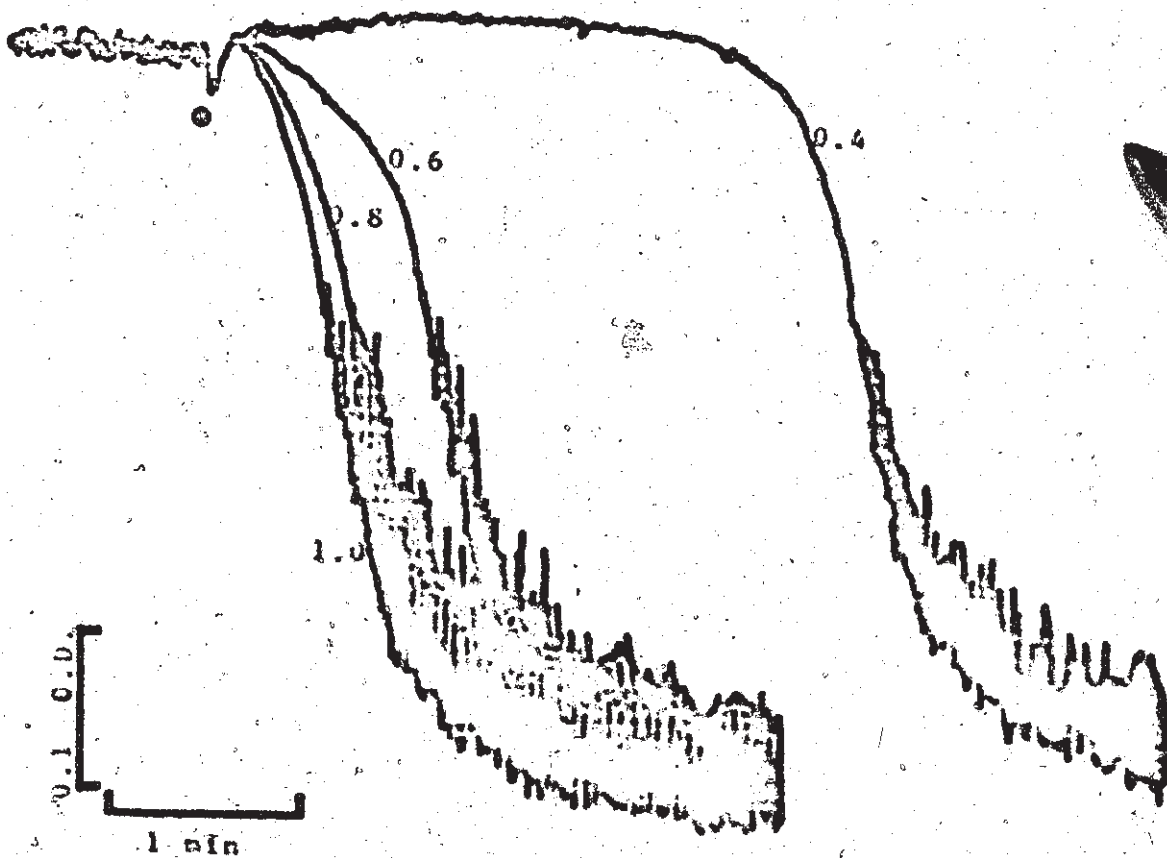


FIGURE 34. Effect on human PRP of arachidonic acid added at (●) in the μM concentrations shown in the figure. Arachidonic acid was neutralized with NaHCO_3 and diluted with saline.

To examine the roles of prostaglandin synthetase and the release reaction in the aggregation induced by arachidonic acid the effect of aspirin on the aggregation was examined (Figure 35). Aspirin (0.4 mM), added to the PRP 1 min before the fatty acid, completely inhibited arachidonic acid-induced aggregation. This indicates that arachidonic acid is not acting by causing a non-specific lysis of the platelets and implicates prostaglandin synthetase at some stage in its action.

Aggregation induced by arachidonic acid was associated with the release of 5-HT from the platelets and this was completely inhibited by aspirin (Table II). This result is consistent with the hypothesis that arachidonic acid is converted by prostaglandin synthetase to a metabolite which triggers the release reaction.

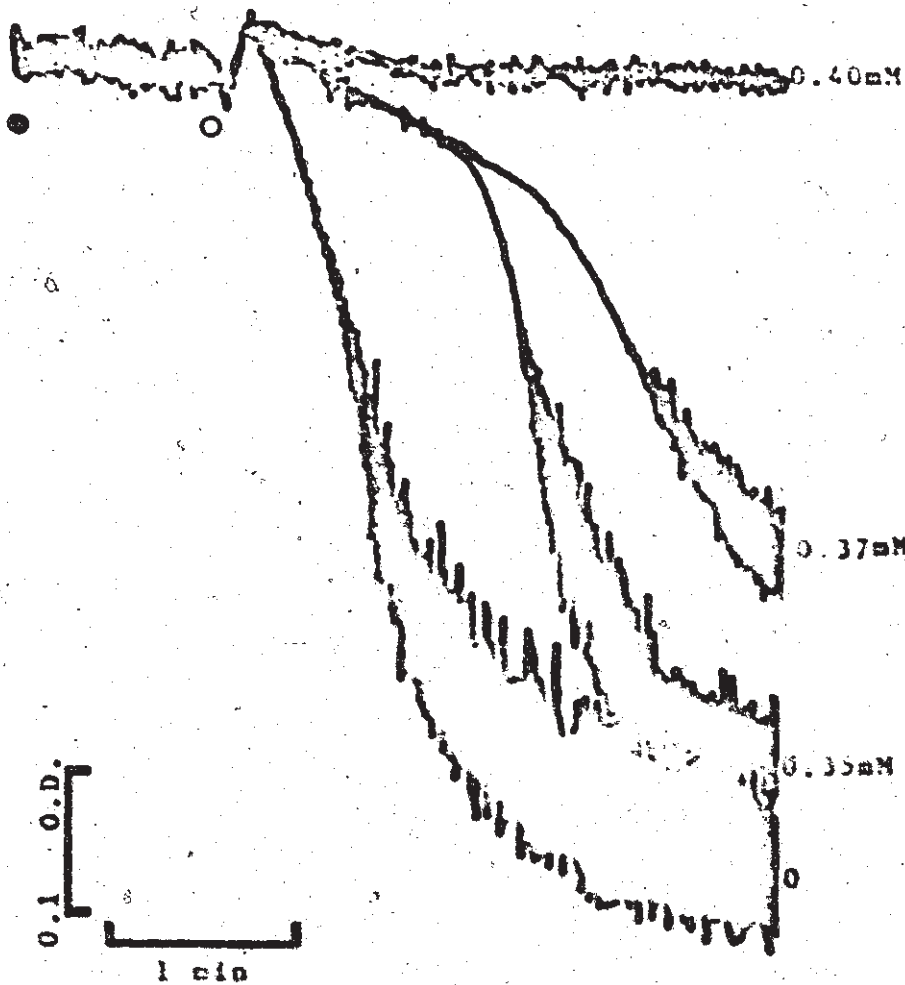


FIGURE 35. Effect of aspirin added at (●) in the concentrations shown in the figure, on arachidonic acid-induced aggregation. Arachidonic acid (1 mM) was added at (○).

Experiment 1

	cpm	% release
PRP control	1955	100
PPP control	104	0
Arachidonic acid (1 mM)	966	44
Arachidonic acid + 0.6 mM aspirin	98	0

Experiment 2

	cpm	% release
PRP control	1913	100
PPP control	135	0
Arachidonic acid (1 mM)	789	34
Arachidonic acid + 0.6 mM aspirin	124	0

TABLE 10. Effect of preincubation with 0.6 mM aspirin for 1 minute on the arachidonic acid-induced release of [14 C]5-HT. 200 μ l of PRP, preincubated with [14 C]5-HT (0.4 μ M, 0.02 μ Ci/ml) for 1 hr, was taken from samples in the aggregometer and centrifuged for 30 sec with 20 μ l of 0.077 M EDTA in the Lppendorf centrifuge. 100 μ l of the supernatant was taken for counting. Controls were 100 μ l PRP (corrected for dilution by EDTA) and 100 μ l PPP from a sample incubated with saline. These samples represented 100% and 0% release, respectively.

The effects of arachidonic acid on basal and PGE_1 -elevated levels of cyclic [^{14}C]AMP were examined. Arachidonic acid had no effect on basal levels but caused a marked decrease in PGE_1 -elevated levels similar to the effect of PGE_2 in the absence of a PDE inhibitor (Figure 36). This decrease however, was not abolished by aspirin in a concentration that did inhibit the aggregation and was therefore presumably not due to released ADP. 8,11-eicosadienoic acid had no effect on the PGE_1 -elevated levels of cyclic [^{14}C]AMP.

In an attempt to determine the nature of the arachidonic acid-induced decrease in PGE_1 -elevated levels of cyclic [^{14}C]AMP, the two were added simultaneously to PRP and incubated for 30 sec in the presence of a PDE inhibitor. A Lineweaver-Burk plot (Figure 37) of the results indicate that arachidonic acid competitively inhibits the activation of AC by PGE_1 .

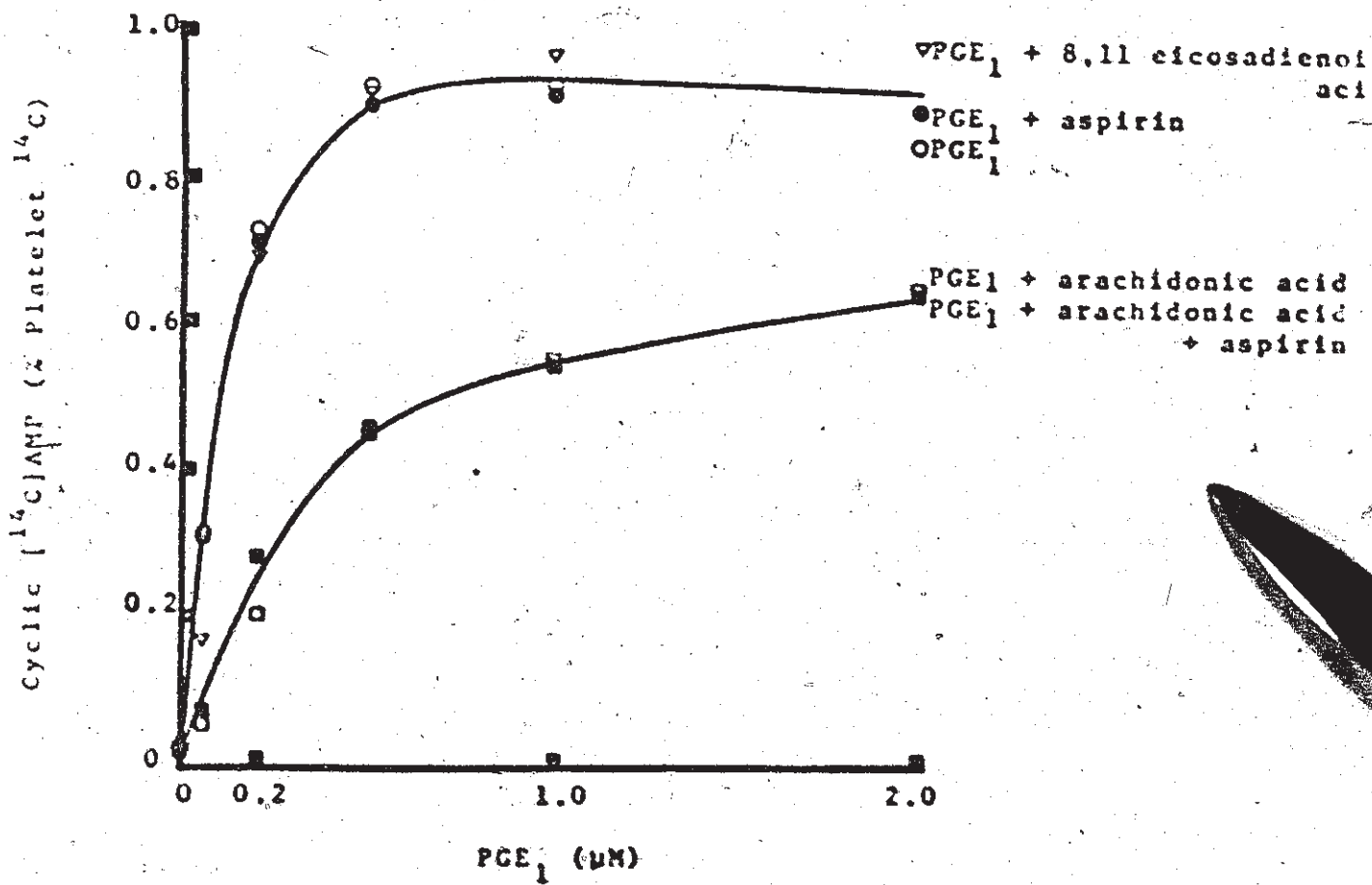


FIGURE 36. Effect of arachidonic acid (0.3 μM) with (■) or without (□) aspirin (0.4 μM) on the elevation of cyclic [^{14}C]AMP levels by PGE₁ (○), in the absence of a PDE inhibitor. Controls were PGE₁ + 8,11-eicosadienoic acid (0.8 μM) (▽) and PGE₁ + aspirin (●). PRP was preincubated with the fatty acids for 5 min and with aspirin for 6 min before addition of PGE₁. In a parallel experiment in the aggregometer arachidonic acid caused aggregation which was completely inhibited by aspirin. Cyclic [^{14}C]AMP was determined 30 sec after addition of PGE₁.

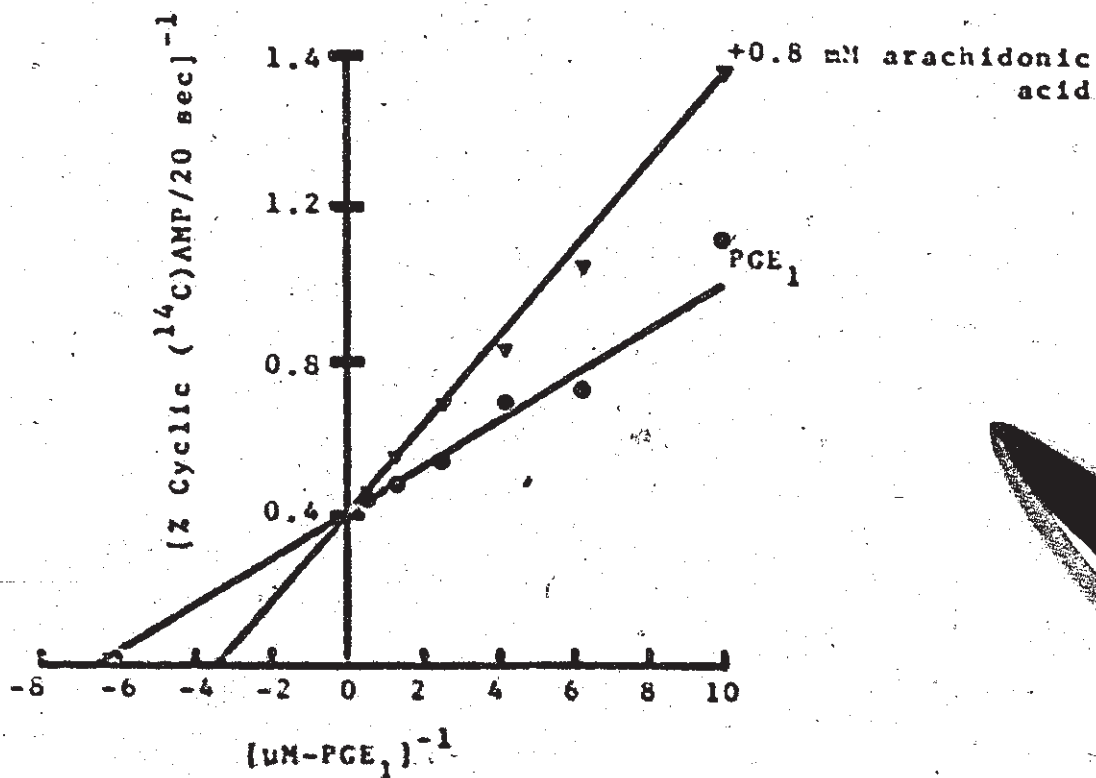


FIGURE 37. Lineweaver-Burk plot of the effect of arachidonic acid (0.8 mM) on the activation of AC by PGE₁. Caffeine (20 mM) was present as a PDE inhibitor.

3. DISCUSSION

In the present study human platelets appeared to have only a very limited capacity to synthesize PGE₁ from its precursor di-homo- γ -linolenic acid. The concentration of the fatty acid that was used (1-40 μ M) may have been too low

however, to see a marked effect. Clausen and Srivastava (1972) have demonstrated PGE_1 formation in human washed platelets from [^{14}C]acetate.

PGE_2 inhibited the first and enhanced the second wave of ADP-induced aggregation. Presumably the weak stimulation of AC by PGE_2 was the cause of the inhibition; while the enhancement of the second wave of aggregation, which was inhibited by aspirin, would suggest a causal or regulatory role for PGE_2 in this phase of ADP-induced aggregation.

Arachidonic acid, the precursor of PGE_2 present in platelet membrane phospholipids (Marcus et al., 1969), caused aggregation as recently reported by Ingeman et al. (1973). Schoene et al. (1972) reported that [$1\text{-}^{14}\text{C}$] PGE_2 was synthesized in human platelets from [$1\text{-}^{14}\text{C}$]arachidonic acid. Aspirin inhibited the aggregation caused by arachidonic acid as well as the associated release reaction. The lack of any aggregation induced by the control fatty acid suggests that the action of arachidonic acid is specific. In this case the results suggest that a product of the action of prostaglandin synthetase on arachidonic acid may cause the release reaction. As PGE_2 potentiates but does not cause aggregation, intermediates or other products may also be involved. A recent report by Willis (1973) has confirmed this last suggestion.

In an effort to demonstrate PGE₂ formation from arachidonic acid, the possibility of similar effects of the fatty acids to those shown by PGE₂ (Chapter IV) on basal and PGE₁-elevated levels of cyclic [¹⁴C]AMP were examined. No significant effect of arachidonic acid on basal cyclic [¹⁴C]AMP levels was demonstrated.

Although arachidonic acid did reduce PGE₁-elevated levels of cyclic [¹⁴C]AMP in a manner similar to, but more pronounced than PGE₂, the effect was not inhibited by aspirin at a concentration that did inhibit the aggregation induced by arachidonic acid. In a kinetic study of the effect of arachidonic acid on the PGE₁ stimulation of AC, arachidonic acid competitively inhibited the PGE₁ stimulation. It would appear that arachidonic acid itself may have an affinity for the prostaglandin receptor, as Kuchi (1972) suggests in his studies on rat adipocytes. 8,11-Eicosadienoic acid, used as a fatty acid control, neither caused aggregation nor lowered PGE₁-elevated cyclic [¹⁴C]AMP levels.

4. SUMMARY

1. Incubation of PRP with the PGE₁ precursor di-homo-γ-linolenic acid resulted in very little production of cyclic

[¹⁴C]AMP and hence, presumably very little PGE₁.

2. Arachidonic acid caused complete and irreversible platelet aggregation. This aggregation as well as its associated release reaction were inhibited by aspirin.

3. Arachidonic acid inhibited the increase in cyclic [¹⁴C]AMP caused by PGE₁, an effect that was not blocked by aspirin. Arachidonic acid competitively inhibited the activation of platelet AC by PGE₁.

GENERAL SUMMARY

Because platelets can be obtained as a homogenous preparation and because they are responsive to many pharmacologically active agents, they provide an excellent system for pharmacological analysis. Using a radioisotope incorporation technique as the most sensitive means of detecting changes in intracellular levels of cyclic AMP, it was possible to examine the effects of a variety of agents on those levels. Prostaglandins, known for their potent biological activities affect platelet function, e.g. PGE_1 inhibits platelet aggregation and PGE_2 can potentiate platelet aggregation. The effects of PGE_1 are mediated by cyclic AMP and modified by the presence of aggregating agents. It proved possible to perform a kinetic analysis of the effects of PGE_1 and of PGE_2 on AC in intact platelets as well as the interactions between the effects of PGE_1 , ADP and epinephrine. A computer simulation of these effects on the intracellular cyclic AMP concentrations was achieved, thus making it possible to postulate specific mechanisms of action for these agents.

Correlations were defined between the cyclic AMP levels in platelets and the inhibition of platelet aggregation by PGE_1 or PGE_2 with a PDE inhibitor. It was confirmed that the inhibition of aggregation is more closely

related to the level of cyclic AMP which results from the interaction of the aggregating agent and PGE_1 with AC, than to the level produced by PGE_1 alone. The effects of fatty acid precursors of prostaglandins on platelet aggregation and cyclic AMP levels were investigated. Arachidonic acid caused platelet aggregation and inhibited the activation of AC by PGE_1 .

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