Biochimica et Biophysica Acta, 972 (1988) 45–53
Elsevier

BBA 12336

Inhibition of prostaglandin E_1-induced activation of adenylate cyclase in human blood platelet membrane

Nighat N. Kahn and Asru K. Sinha

Division of Cardiology, Department of Medicine, Montefiore Medical Center, Albert Einstein College of Medicine, Bronx, NY (U.S.A.)

(Received 24 February 1988)

Key words: Prostaglandin E_1; Adenylate cyclase activity; cyclic AMP; Platelet membrane; (Human blood)

Activation of human blood platelet adenylate cyclase is initiated through the binding of prostaglandin E_1 to the membrane receptors. Incubation of platelet membrane with [^{3}H]prostaglandin E_1 at pH 7.5 in the presence of 5 mM $MgCl_2$ showed that the binding of the autacoid was rapid, reversible and highly specific. The binding was linearly proportional to the activation of adenylate cyclase. Although the membrane-bound radioligand could not be removed either by GTP or its stable analogue 5′-guanylylimido diphosphate, 150 nM cyclic AMP displaced about 40% of the bound agonist from the membrane. Scatchard analyses of the binding of the prostanoid to the membrane in the presence or absence of cyclic AMP showed that the nucleotide specifically inhibited the high-affinity binding sites without affecting the low-affinity binding sites. Incubation of the membrane with 150 mM cyclic AMP and varying amounts of prostaglandin E_1 (25 nM to 1.0 μM) showed that the percent removal of the membrane-bound autacoid was similar to the percent inhibition of adenylate cyclase at each concentration of the agonist. At a concentration of 25 nM prostaglandin E_1, both the binding of the agonist and the activity of adenylate cyclase were maximally inhibited by 40%. With the increase of the agonist concentration in the assay mixture, the inhibitory effects of the nucleotide gradually decreased and at a concentration of 1.0 μM prostaglandin E_1 the effect of the nucleotide became negligible. These results show that cyclic AMP inhibits the activation of adenylate cyclase by low concentrations of prostaglandin E_1 through the inhibition of the binding of the agonist to high-affinity binding sites.

Introduction

Activation of adenylate cyclase in human blood platelets by prostanoids like prostacyclin, prostaglandin D_2, or prostaglandin E_1 is known to inhibit virtually all cellular functions through the increase of cyclic AMP level [1–3]. According to the currently accepted model, it is generally believed that the stimulation of the enzymic activity by various hormones and prostaglandins is initiated through the binding of these agonists to their specific receptors on the outer surface of the membrane bilayer [4–6]. The occupancy of the receptors by these agonists subsequently activates a GTP-binding regulatory protein which, in turn, activates the catalytic sites of the enzyme resulting

Abbreviations: Gpp[NH]p, 5′-guanylylimido diphosphate; SDS, sodium dodecyl sulfate.

Correspondence: A.K. Sinha, Division of Cardiology, Department of Medicine, Montefiore Medical Center, Albert Einstein College of Medicine, Bronx, NY 10467, U.S.A.

in the formation of cyclic AMP from ATP [7,8]. GTP is not only required for the activation of adenylate cyclase, but this nucleotide is also involved in the inhibition of the enzyme through the activation of an inhibitory regulatory protein. It has also been shown that this nucleotide regulates the interaction of agonists with the membrane receptors by displacing the bound agonists from the receptors and, thus, plays an important role in the desensitization of adenylate cyclase activity [5,9–11].

In frog erythrocyte membranes, which contain one high-affinity and one low-affinity prostaglandin E_1 receptor population, addition of GTP to the assay mixture rapidly released the bound prostanoid from the high-affinity binding sites, thus making the autacoid available for binding with the low-affinity binding site for subsequent activation of the enzyme [5]. The high-affinity prostaglandin E_1-binding sites which have no known function are thought to desensitize adenylate cyclase through the tight binding of the autacoid to the receptors [5].

In human platelets, the adenylate cyclase-linked receptors of prostacyclin, prostaglandin D_2 and prostaglandin E_1 have been identified and shown to be associated with the membrane structure [12,13]. The receptors of prostaglandin E_1 and prostacyclin, which are probably identical, have been purified from the platelet surface proteins [14]. The role of GTP in the activation of adenylate cyclase in these cells is, however, still not clear. Although it was reported earlier that GTP was needed for the activation of adenylate cyclase by prostaglandin E_1 in platelets [15], subsequent works showed that prostaglandins can activate the enzyme in the absence of the nucleotide [16–19]. In this paper, we report that the interaction of prostaglandin E_1 with the platelet membrane receptors is not regulated by GTP, but by cyclic AMP through the inhibition of the binding of the agonist specifically to the high-affinity binding sites. The removal of the autacoid from the high-affinity binding sites by cyclic AMP results in partial inhibition of the membrane adenylate cyclase which might be physiologically more relevant than the activation of the enzyme through the binding of the agonist to the low-affinity receptors in platelet membrane.

Materials and Methods

Chemicals. [5,6-^{3}H]Prostaglandin E_1 (spec. act 40.7 Ci/mmol), and [α-^{32}P]ATP (spec. act. 32.4 Ci/mmol) were obtained from New England Nuclear Research Products, Boston, MA. [^{3}H] Prostaglandin E_1 was more than 98% radiochemically pure. Prostaglandins (prostaglandin A_1 prostaglandin A_2, prostaglandin D_2, prostaglandin E_1, prostaglandin E_2 and prostacyclin) and Gpp[NH]p were purchased from Sigma Chemical Co., St. Louis MO. All other chemicals used were of analytical grade.

Platelet membrane preparation. All blood donors had abstained from medications for at least 2 weeks before blood donation. 200 ml of blood was collected and anticoagulated with trisodium citrate (final concentration 13 mM). Platelet-rich plasma was obtained by centrifugation of samples at 200 $\times g$ for 15 min at 23° C. The platelet-rich plasma was next centrifuged at 3000 $\times g$ for 15 min at 23° C. The platelet pellet thus obtained was resuspended in assay buffer, 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$ and disrupted by freezing in liquid nitrogen and thawing at 23° C. The platelet membranes were collected by centrifugation at 30000 $\times g$ for 15 min in a Beckman ultracentrifuge (model L 350) at 0° C. The supernatant was discarded and the membrane pellet was resuspended in the assay buffer at a protein concentration of 5–8 mg/ml.

Binding assay of [^{3}H]prostaglandin E_1 to platelet membrane. Unless otherwise stated, platelet membranes were incubated at 23° C for various times with the indicated concentrations of [^{3}H]prostaglandin E_1. Binding was performed by incubating 50–70 μg of protein in 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$ at 23° C in a total volume of 200 μl, with 3 nM of [^{3}H]prostaglandin E_1 (80000–90000 dpm). Parallel experiments were conducted containing excess 15 μM nonradioactive prostaglandin E_1 to determine nonspecific binding. The nonspecific binding was deducted from the total [^{3}H]prostaglandin E_1 binding to yield specific binding. At the end of the incubation 1.0 ml of the assay buffer (0° C) was added to each sample. The samples were vacuum filtered through a presoaked Whatman glass microfiber filter (GF/C; 2.9 cm diameter) [12,14].

Membrane-bound radioactivity which was retained on the filter was subsequently washed four times with 5 ml portions of ice-cold (0 °C) buffer. The filters were then dried, suspended in 10 ml scintillation fluid (Atomlight, New England Nuclear Research Product) and counted in a Searle scintillation counter (Isocap/300) with 60% efficiency for tritium.

Assay of adenylate cyclase. Except where otherwise indicated, adenylate cyclase activity of the platelet membrane preparation was determined by incubating 1.0 mM ATP containing [α-^{32}P]ATP of ($1.2 \cdot 10^6$ dpm), 2 mM $MgCl_2$, 10 mM theophylline, 1 mM creatine phosphate, 1 unit of creatine phosphokinase and 25 mM Tris-HCl buffer (pH 7.5) with varying amounts of prostaglandin E_1 in a total volume of 100 μl. Reactions were initiated by the addition of 20 μl of platelet membrane suspension containing 150 μg of protein and incubated at 23 °C for 10 min. Reactions were terminated by the addition of 0.1 ml 1% SDS. The radioactive cyclic AMP was separated according to Salomon et al. [20]. Unlabelled cyclic AMP (1.0 mM) was added to the reaction mixture at termination to facilitate the recovery of the radio nucleotide.

Determination of protein for the binding and adenylate cyclase assays was carried out by the method of Lowry et al. [21].

Analysis of the equilibrium binding of [^{3}H]prostaglandin E_1 to platelet membrane. The interaction of prostaglandin E_1 with the platelet membrane receptors in the presence and absence of cyclic AMP was analyzed by the method of Scatchard [22]. The dissociation constant (K_d) and the number of binding sites (n) were obtained from nonlinear regression analysis of equilibrium binding by a nonweighted, iterative, least-squares algorithmic analysis using a Radio Shack TRS 80, model 4 microcomputer.

Results

Binding of [^{3}H]prostaglandin E_1 to platelet membrane

The binding of [^{3}H]prostaglandin E_1 to the platelet membrane preparation was rapid and attained equilibrium within 10 min at 23 °C (Fig. 1). The nonspecific binding of the radioligand to the membrane was increased to approx. 16% of the total binding during the incubation period. Addition of increasing concentrations of unlabelled prostaglandin E_1 at equilibrium rapidly displaced the bound radioligand from the membrane preparation, and at a concentration of 15 μM, the unlabelled autacoid displaced approx. 80% of the bound [^{3}H]prostaglandin E_1 (Fig. 2). The displacement of the membrane-bound radioligand by prostaglandin E_1 was found to be highly specific. Among all prostanoids tested, only prostacyclin and, to a lesser extent, prostaglandin E_2 were capable of displacing the bound [^{3}H]prostaglandin E_1 from the membrane. At a concentration of 15 μM, prostacyclin removed almost 65% of the bound [^{3}H]prostaglandin E_1, while prostaglandin E_2 removed approx. 25% of the membrane-bound radioligand at the same concentration. All other prostanoids including prostaglandins A_1, A_2, D_2 or 6-ketoprostaglandin $F_{1\alpha}$ were ineffective in removing the membrane-bound radioligand.

Correlation between the binding of prostaglandin E_1 and the activation of adenylate cyclase

To correlate the extent of prostaglandin E_1 binding to platelet membrane and the degree of

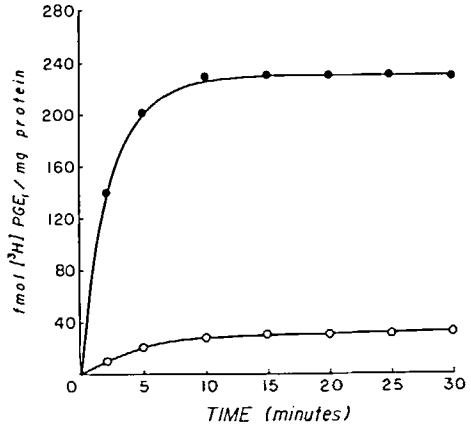

Fig. 1. Time course of [^{3}H]prostaglandin E_1 binding to platelet membrane. Platelet membrane was incubated with 3 nM [^{3}H]prostaglandin E_1 in 50 mM Tris-HCl buffer (pH 7.5) containing 5 mM $MgCl_2$. The total binding (●) was determined in the absence of any added unlabelled prostaglandin E_1. The nonspecific binding (○) was determined by adding 15 μM of the unlabelled prostaglandin to the assay mixture. Each point represents the mean four experiments. PG, prostaglandin.

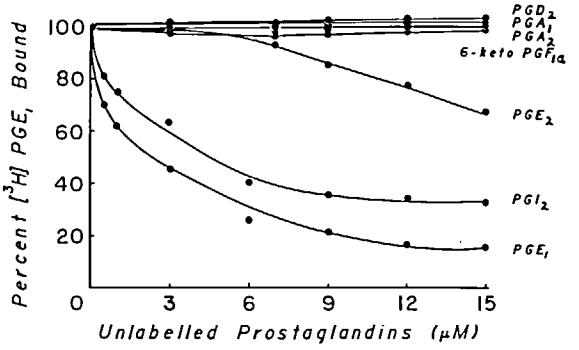

Fig. 2. Displacement of membrane-bound [^{3}H]prostaglandin E_1 by various prostaglandins. Platelet membranes were incubated (50–70 μg protein) with 3 nM [^{3}H]prostaglandin E_1 (80000–90000 dpm) and different concentrations of various prostaglandins as indicated at 23° C for 30 min. After incubation, membranes were separated by filtration as described. Results are mean of three experiments and are expressed as percent inhibition of the binding of [^{3}H]prostaglandin E_1 to the membrane in the absence of added unlabelled autacoids. PG, prostaglandin; PGI_2, prostacyclin.

activation of adenylate cyclase by the agonist, the membrane preparation was incubated with increasing concentrations of [^{3}H]prostaglandin E_1 at 23° C. After equilibrium was attained (10 min), the specific binding of the agonist to the membrane and the activation of adenylate cyclase of the same preparation were determined. The specific binding of the agonist to the membrane preparation which reached saturation at about 1.0 μM prostaglandin E_1 ran parallel to the formation of cyclic AMP through the activation of adenylate cyclase (Fig. 3, left panel). This relationship between the activation of the enzyme and the binding of the agonist was found to be true at least at concentrations of the autacoid between 25 nM and 1.0 μM. The amounts of cyclic AMP formed bore a linear relationship to the quantities of prostaglandin E_1 bound to the membrane (Fig. 3, right panel).

Displacement of membrane-bound [^{3}H]prostaglandin E_1 by cyclic AMP

Purine nucleotides, particularly GTP and its stable analogue Gpp[NH]p have been reported to displace both β-adrenergic agonists and prostaglandin E_1 from membranes by reducing the affinity of the binding sites of the high-affinity receptors [5,9–11]. However, when the platelet membrane preparation was incubated with [^{3}H]-prostaglandin E_1 for 15 min at 23° C to attain equilibrium, and then 0.1 mM GTP or Gpp[NH]p was added to the assay mixture, there was no displacement of the radioligand from the mem-

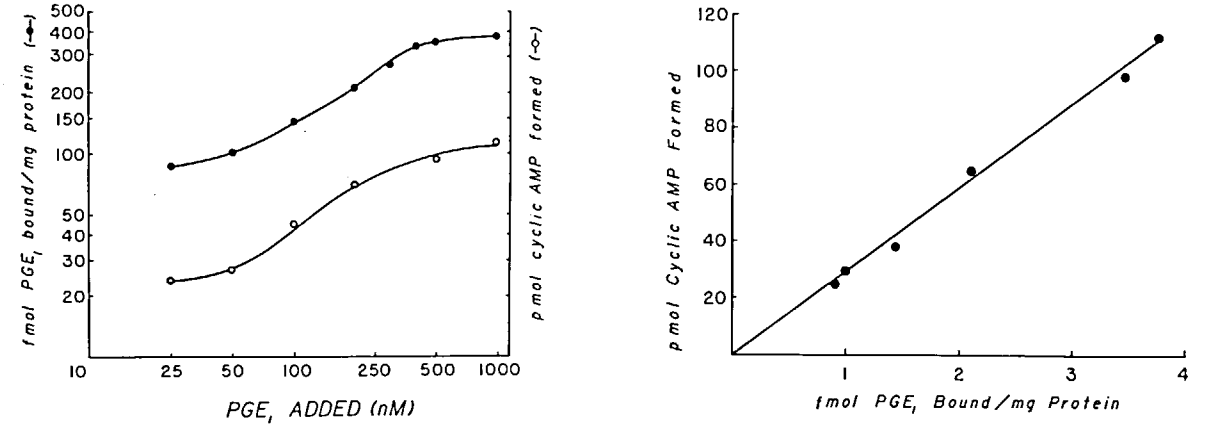

Fig. 3. Relationship between the binding of prostaglandin E_1 and the activation of adenylate cyclase in platelet membrane in the presence of various concentrations of the autacoid. Left panel: platelet membrane (150–200 μg protein) was incubated with different amounts of either [^{3}H]prostaglandin E_1 or unlabelled prostaglandin E_1 for 10 min at 23° C. The portion of the membrane preparation which was treated with the unlabelled prostaglandin was used for the assay of adenylate cyclase activity as described in Materials and Methods. The binding of the autacoid shown here represents the specific binding calculated by subtracting the non-specific binding from the total binding for each concentration of the labelled prostanoid. The results shown here are the mean of five experiments. Adenylate cyclase activity (○); [^{3}H]prostaglandin E_1 binding (●). Right panel: adenylate cyclase activity per mg membrane protein was plotted against the amount of prostaglandin E_1 bound to the preparation. PG, prostaglandin.

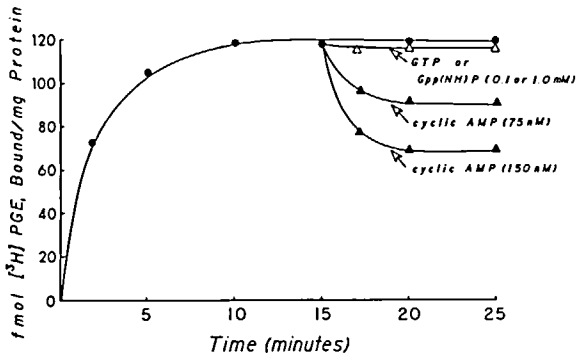

Fig. 4. Displacement of membrane-bound [^{3}H]prostaglandin E_1 by cyclic AMP. Platelet membrane (150–200 μg of protein) was incubated with 25 nM [^{3}H]prostaglandin E_1 for various times at 23° C. After equilibrium was attained (15 min), either GTP (0.1 mM or 1.0 mM) or Gpp[NH]p (0.1 mM or 1.0 mM) or cyclic AMP (75 nM or 150 nM) was added to the assay mixture (arrow). The incubation was continued as shown. At various intervals, the binding of the autacoid was determined. Each point represents the mean of six experiments and shows specific binding of the agonist. [^{3}H]Prostaglandin E_1 binding (●); GTP or Gpp[NH]p (△); cyclic AMP (▲). PG, prostaglandin.

brane (Fig. 4). Addition of even 1.0 mM of the above purine nucleotides failed to show any effect on the displacement of the agonist. On the other hand, addition of cyclic AMP to the assay mixture rapidly displaced the membrane-bound [^{3}H]prostaglandin E_1. At a concentration of 75 nM, cyclic AMP displaced about 25% of the membrane-bound ligand and at 150 nM, the nucleotide maximally displaced about 40% of [^{3}H]prostaglandin E_1 from the membrane. Incubation of the membrane preparation with 10 mM theophylline for 15 min at 23° C to inhibit phosphodiesterase activity before the addition of the radioligand resulted in 60% displacement of the bound [^{3}H]prostaglandin by 175 nM cyclic AMP. The inhibitor alone had little effect on the binding of the prostaglandin (95.5 ± 5.6 fmol/mg protein). The effect of cyclic AMP was found to be specific; no other nucleotide including ATP, ADP, AMP, GDP and GMP at a concentration of 10 mM or 200 nM cyclic GMP, had any effect on the displacement of the bound radioligand from the platelet membrane preparation (not shown). None of the cyclic AMP degradation products, such as AMP, adenosine or adenine, at 150 nM was effective in the displacement of the prostanoid from the membrane preparation (not shown).

Characteristics of [^{3}H]prostaglandin E_1 binding to platelet membrane in the presence of cyclic AMP

Since cyclic AMP displaces the bound prostaglandin E_1 from the platelet membrane, the binding characteristics of the autacoid to the membrane in the presence and absence of the nucleotide were determined by equilibrium binding of the radioligand by Scatchard analyses [22].

The Scatchard plot of the binding of [^{3}H]prostaglandin E_1 to the platelet membrane preparation was typically curvilinear in nature, indicating heterogeneity of the receptors population. However, the curvilinear nature of Scatchard plot could also arise from the negative cooperativity within a single class of receptors [23]. Our results do not distinguish between these two possibilities. Computer analysis of the binding data corresponded to one high-affinity ($K_{d,1}$ = 9.2 nM (ranged from 8.0 to 9.5 nM, n = 8); capacity (n) = 110 fmol/mg) receptor population and one low-affinity ($K_{d,2}$ = 0.95 μM (ranged from 0.85–1.1 μM, n = 8)) receptors population (Fig. 6). When the binding of [^{3}H]prostaglandin E_1 to the same membrane population was carried out in the presence of cyclic AMP, the Scatchard plot which was curvilinear in the absence of any added cyclic nucleotide became a straight line due to the loss of high-affinity binding sites (Fig. 5). The dissociation constant of the low-affinity binding ($K_{d,2}$) of prostaglandin E_1 to the membrane in the presence of cyclic AMP remained unaltered (1.0 μM). These results indicate that cyclic AMP specifically inhibited the binding of the autacoid to the high-affinity binding sites without affecting low-affinity binding.

Effect of cyclic AMP on the activation of adenylate cyclase by prostaglandin E_1

It has been shown above that the formation of cyclic AMP due to the activation of adenylate cyclase increased linearly with the increased occupancy of prostaglandin E_1 on the platelet membrane receptors (Fig. 3). Since cyclic AMP displaced the bound autacoid from the platelet membrane, it was of interest to determine whether the presence of the nucleotide in the assay mixture

would prevent the autacoid-induced stimulation of adenylate cyclase activity by interfering with the binding of the ligand to the high-affinity receptors. In a series of experiments, the adenylate cyclase activity of platelet membrane was determined by adding 25, 50, 100 nM or 1 μM prostaglandin E_1 to the assay mixture in the presence of varying concentrations of cyclic AMP (0–200 nM). The activation of adenylate cyclase and the binding of the radiolabelled prostanoid to the membrane preparation for each of the above concentrations of prostaglandin E_1 in the assay mixture were simultaneously determined. It was found that the percent inhibition of the binding of [^{3}H]prostaglandin E_1 to the platelet membrane in the presence of various concentrations of cyclic AMP paralleled that of the inhibition of the activation of adenylate cyclase (Fig. 6). However, as the concentration of the agonist was increased gradually from 25 nM to 1.0 μM, both the inhibition of the enzymic activity and the displacement of the bound ligand by the cyclic nucleotide were gradually decreased. While at a concentration of 25 nM prostaglandin E_1 both the enzymic activity and the ligand binding were inhibited by 40% in

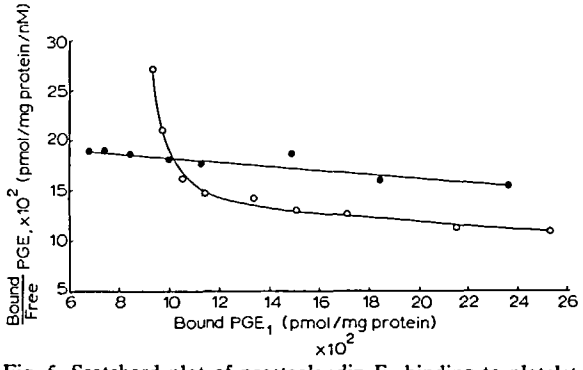

Fig. 5. Scatchard plot of prostaglandin E_1 binding to platelet membrane in the presence and absence of cyclic AMP. Platelet membrane (50–75 μg protein) was incubated with 3.0 nM [^{3}H]prostaglandin E_1 plus 0–15 μM of the unlabelled prostaglandin for 15 min at 23° C in the presence or absence of 150 nM cyclic AMP in the assay mixture. The binding was determined for each point by dividing the cpm by the calculated specific activity (cpm/mol) obtained by diluting [^{3}H]prostaglandin E_1 with a known concentration of the autacoid. Each point represents the mean of four experiments comparable to four other experiments. The binding of prostaglandin E_1 in the absence of cyclic AMP (○); the binding of the autacoid in the presence of cyclic AMP (●). PG, prostaglandin.

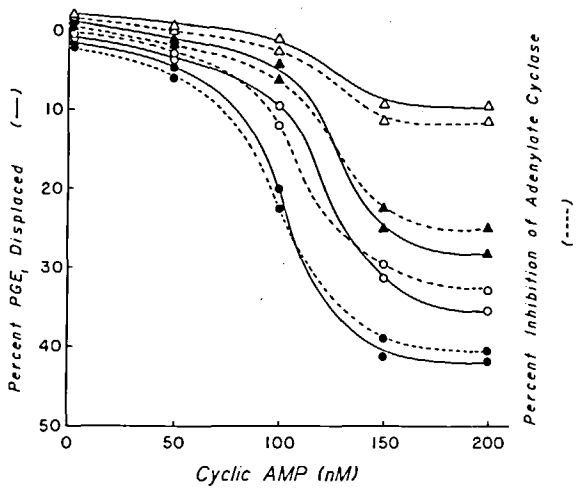

Fig. 6. Relationship between the displacement of prostaglandin E_1 from the platelet membrane and the inhibition of adenylate cyclase activity in the presence of increasing concentrations of cyclic AMP and prostaglandin E_1. The platelet membrane preparation was incubated with various amounts of radiolabelled or unlabelled prostaglandin E_1 for 10 min at 23° C to attain equilibrium. Cyclic AMP was then added to the assay mixture and the reaction mixture was incubated further for 5 min at 23° C. After the second incubation, the portion of the membrane preparation which was incubated with unlabelled prostaglandin E_1 was used for the determination of adenylate cyclase activity. Each point represents the mean of four experiments and, in the case of the binding of prostaglandin E_1, it shows specific binding. The continuous line (———) and the broken lines (------) represent activation of adenylate cyclase and the binding of prostaglandin E_1 to the membrane, respectively, in the presence of increasing amounts of cyclic AMP. The data presented here are expressed as percent inhibition compared to the activities in the absence of the cyclic nucleotide (100%). Prostaglandin E_1 concentration: 25 nM (●); 50 nM (○); 100 nM (▲); 1.0 μM (△). PG, prostaglandin.

the presence of 150 nM cyclic AMP, at a 1.0 μM autacoid concentration, the activation and the binding were inhibited by only 10% by the same amount of the nucleotide under identical experimental conditions. The displacement curves of the membrane-bound [^{3}H]prostaglandin E_1 and the inhibition of adenylate cyclase activity by cyclic AMP were not linear but were 'S' shaped, indicating a possible cooperative nature of the effect of the nucleotide. The inhibition of adenylate cyclase activity of the membrane preparation by cyclic AMP was not mediated by cyclic AMP degradation products, since the addition of AMP, adenosine or adenine at a similar concentration (150

nM) produced no effect on the enzymic activity (not shown).

Discussion

These results show that unlike the case of frog erythrocyte membranes [5], the interaction of prostaglandin E_1 with the human platelet membrane is not controlled by GTP but by cyclic AMP. Although the failure of GTP to remove the bound prostaglandin E_1 from the platelet membrane could be related to the difference in species, the role of this nucleotide in the activation and inhibition of adenylate cyclase in the case of human platelet is, however, not clear [16–19]. Scatchard analysis of the binding of [^{3}H]prostaglandin E_1 to platelet membrane showed the presence of one high-affinity ($K_{d,1} = 9.2$ nM) binding site population and one low-affinity ($K_{d,2} = 0.95$ μM) binding site population. Similar results have been reported in purified prostaglandin E_1 receptors [14], membrane [12] and intact platelets [13]. In the presence of cyclic AMP, the usual curvilinear Scatchard plot of [^{3}H]prostaglandin E_1 binding became a straight line. The results indicated that in the presence of the cyclic nucleotide, the high-affinity prostaglandin E_1 binding sites of platelet membrane were specifically inhibited (Fig. 5). The decreased binding of [^{3}H]prostaglandin E_1 to the membrane preparation in the presence of cyclic AMP was due to the loss of high-affinity binding sites, probably through their conversion to low-affinity binding site. In the case of frog erythrocyte membranes, similar conversion of slowly dissociating high-affinity prostaglandin E_1 binding sites to rapidly dissociating low-affinity binding sites in the presence of GTP has been reported [5]. The binding of [^{3}H]prostaglandin E_1 to the platelet membrane and the activation of adenylate cyclase of the same preparation showed that the activation of the enzyme is linearly proportional to the occupancy of the agonist on the receptors, at least in the range of 25 nM to 1.0 μM. (Fig. 3). Since cyclic AMP removed the receptor-bound prostaglandin E_1, it is expected that removal of the agonist would result in a loss of activation of adenylate cyclase. Our results demonstrated that the cyclic nucleotide not only displaced the bound [^{3}H]prostaglandin E_1 from the membrane preparation, but the nucleotide also inhibited the enzyme by removing the agonist from the high-affinity receptors (Fig. 6).

Although the human platelet membrane contains both high- and low-affinity prostaglandin E_1 receptors [12–24], it is thought that the activation of adenylate cyclase by the autacoid is mediated through the low-affinity receptors only [12,13] and the high-affinity binding sites were probably involved in the desensitization reaction [5,13] or 'unspecific' interaction of the agonist with the low-affinity receptors needed for the activation of adenylate cyclase through the binding of prostaglandin E_1 to the high-affinity binding sites [24]. It should also be mentioned that high-affinity glucagon receptors on hepatocytes were shown to be linked to phospholipase C, whereas only low-affinity glucagon receptors were involved in the activation of adenylate cyclase [25]. However, our results showed that the removal of the agonist from the high-affinity binding sites by cyclic AMP resulted in the inhibition of adenylate cyclase (Fig. 6), and as such, it is apparent that the high-affinity binding sites for prostaglandin E_1 in platelet membrane, like the low-affinity binding sites, are also involved in the activation of adenylate cyclase. The inhibition of adenylate cyclase by cyclic AMP could only be demonstrated when the enzyme was activated by low concentrations of the agonist (Fig. 6).

With the increase of the amount of prostaglandin E_1 in the assay mixture, the inhibitory effect of the nucleotide gradually decreased and at a 1.0 μM prostaglandin E_1 concentration, the effect of cyclic AMP was minimal. This failure of cyclic AMP to inhibit adenylate cyclase at higher concentrations of prostaglandin E_1 is probably related to the differential effects of the nucleotide on the high- and low-affinity binding sites. Since at low concentrations, the ligand would bind primarily to the high-affinity receptors, the presence of cyclic AMP which affects only the high-affinity binding sites would be expected to inhibit the enzyme through the removal of the agonist from these receptors (Fig. 5). However, as the concentration of prostaglandin E_1 in the reaction mixture was gradually increased, the binding of the autacoid to the low-affinity receptors would gradually increase and result in the increasing

activation of adenylate cyclase through these binding sites. Since cyclic AMP does not inhibit the activities of low-affinity receptors, the activation of adenylate cyclase at high concentrations of prostaglandin E_1 through the binding of the autacoid to the low-affinity receptors would be sufficiently high to overcome the inhibitory effect of cyclic AMP on the high-affinity receptors. These results showed that the high affinity prostaglandin E_1 binding sites on platelet membrane are not necessarily involved in the desensitization of adenylate cyclase, and prostaglandin E_1 can activate the enzyme through its interaction with both high- and low-affinity receptors. However, the activation of adenylate cyclase in human platelets through high-affinity binding sites is probably physiologically more important than the activation of the enzyme through the low-affinity binding sites, because the concentrations of prostaglandins in circulation never reach a sufficient level (micromolar ranges) to activate adenylate cyclase through the binding of the agonist to the low-affinity receptors for the inhibition of platelet aggregation. On the other hand, the physiological concentrations (nanomolar ranges) of prostacyclin, which interacts with the same prostaglandin E_1 receptors of platelets [12,14], are known to inhibit platelet aggregation under in vivo conditions by increasing the cyclic AMP level through the activation of adenylate cyclase [26]. The activation of adenylate cyclase in intact platelets by nanomolar concentrations of prostacyclin also indicated that the high-affinity autacoid binding sites are more likely to be involved in the activation of the enzyme in these cells than is the desensitization phenomenon described above [5,13]. We reported earlier that the stimulation of intact platelets with 25 nM prostaglandin E_1 increased the basal level of cyclic AMP from $2.0 + 0.5$ pmol/10^8 cells to 4.5 ± 0.8 pmol/10^8 cells with a half-life of approx. 1 min [6]. The transitory increase of the cyclic AMP level could probably be partly explained by the cellular increase of the cyclic AMP level that would displace the agonist from the high-affinity receptors and, consequently, inhibit the formation of cyclic AMP. Indeed, we recently found that the addition of NaF (1.0 mM) to intact platelets not only increase the basal cyclic AMP level to 3.5 ± 0.6 pmol/10^8 cells, but displaced 40% of the cell-bound [^{3}H]prostaglandin E_1 (unpublished results). Thus, the inhibition of the formation of cyclic AMP through the removal of the agonist from the high-affinity binding sites indicates that the nucleotide could control its own synthesis through a feed-back mechanism. However, in human erythrocyte membranes which do not have any adenylate cyclase, but contain both high- and low-affinity prostaglandin E_1 binding sites, a similar effect of cyclic AMP on the displacement of the bound ligand from the high-affinity binding sites has been reported [27].

Although the mechanism of inhibition of the binding of prostaglandin E_1 to the high-affinity receptors by cyclic AMP is not known at present, the phosphorylation of prostaglandin E_1 receptors by cyclic AMP-dependent protein kinase in the inhibitory process might be involved. Phosphorylation of β-adrenergic receptors by cyclic AMP-dependent protein kinase in the desensitization of adenylate cyclase has been reported [28]. However, in a preliminary study we have found that the addition of purified bovine protein kinase inhibitor to the assay mixture did not influence the displacement of bound [^{3}H]prostaglandin E_1 by cyclic AMP (unpublished results).

Acknowledgements

This work was partly supported by grant HL-41386 from the National Institutes of Health. The authors thank Drs. J. Scheuer and H.S. Mueller for their interest and Ms. Janice Brewton for her secretarial assistance.

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