Purification and Properties of Prostaglandin E₁/Prostacyclin Receptor of Human Blood Platelets*

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Activation of platelet adenylate cyclase by prostaglandin E₁ or prostacyclin is initiated through the interaction of the agonists with the same receptors on membrane. Prostaglandin E₁/prostacyclin receptors of human platelets were solubilized in buffer, containing 0.05% Triton X-100 and protease inhibitors. The soluble membrane protein was chromatographed on a DEAE-cellulose column and assayed by a microfiber filter by equilibrium binding technique. The active fractions eluted at 0.7 M KCl were pooled, and the receptors were purified to homogeneity by Sephadex G-200 gel filtration with an overall recovery of 30%. The isolated receptor was 2,200-fold purified over the starting platelets. As evidenced by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, the receptor showed a molecular mass of 190,000 daltons and is composed of two nonidentical subunits with molecular masses of 85,000 and 95,000 daltons. The interaction of prostaglandin E₁ with the purified receptor was rapid, saturable, reversible, and highly specific. Among all prostaglandins tested, only prostacyclin was capable of displacing [³H]prostaglandin E₁ bound to the receptor. Scatchard analysis of [³H]prostaglandin E₁ binding to the purified receptor suggested the presence of a single class of high affinity binding sites (Kd = 9.8 nM) and a second population of low affinity binding sites (Kd = 0.7 µM) in the same protein molecule. Incubation of the purified receptor with platelets stripped of the receptor by washing with low concentrations of Triton X-100 efficiently restored the ability of prostaglandin E₁ and prostacyclin to activate adenylate cyclase in these cells.

Aggregation of human blood platelets has been shown to be a critically important event in the process ranging from hemostasis to the extreme of thrombosis (1). The aggregation of platelets by various agonists such as ADP, l-epinephrine, or collagen is believed to be mediated in part through the intracellular synthesis of prostaglandin endoperoxide and thromboxane A₂ (2). In contrast, the inhibition of platelet aggregation is mediated primarily through the increase of cyclic AMP levels by the activation of adenylate cyclase by various autacoids including PGE₁, PGI₂, or PGD₂ (3-5). The hormone-responsive adenylate cyclase is believed to be composed of at least three subunits: an agonist-specific receptor, a guanine nucleotide binding regulatory protein, and a catalytic subunit (6). The receptors appear to be separate, mobile macromolecular protein entities residing on the outer surface of the membrane bilayer. Stimulation of adenylate cyclase is initiated through the interaction of activators including PGE₁ with the specific receptors on the membrane plane (7-9).

In the case of human platelets, the receptors of PGI₂, PGE₁, and PGD₂ have been identified and shown to be associated with the membrane structure (10, 11). However, these receptors have not yet been isolated nor have their structures been characterized. We describe here the purification and characterization of adenylate cyclase-linked PGE₁/PGI₂ receptors from human platelets and the reconstitution of PGE₁/PGI₂-responsive platelet adenylate cyclase activity by the purified receptors.

**EXPERIMENTAL PROCEDURES**

**Chemicals**

5,6-[³H]Prostaglandin E₁ (specific activity 50.3 Ci/mmol) and [α-³²P]ATP (specific activity 30 Ci/mmol) were obtained from New England Nuclear. [³H]PGE₁ was more than 98% radiochemically pure. Prostaglandins (PGA₁, PGA₂, PGB₁, PGB₂, PGE₁, PGE₂, and PGI₂), Lubrol PX, polyoxyethylene sorbitan monostearate (Tween 80), taurocholic acid, and CHAPS were obtained from Sigma; Triton X-100 and Bio-Beads SM-2 were the products of J. T. Baker Chemical Co. and Bio-Rad Laboratories, respectively. All other chemicals used were of analytical grade.

**Preparation of Platelets**

Typically, 10-12 g of fresh "bloody" platelets (obtained from the American Red Cross, Philadelphia) were mixed with 1.0 mM EDTA (final concentration) and centrifuged at 200 × g for 15 min at 23 °C to remove residual erythrocytes and leukocytes from the platelet suspension. The supernatant platelet-rich plasma was next centrifuged at 2000 × g for 20 min at 23 °C, and the platelet pellet thus obtained was washed three times by centrifugation after resuspending the pellet in 50 mM Tris-HCl buffer, pH 7.4 (1 g wet weight/ml of buffer) containing 1.0 mM EDTA and 0.15 M NaCl.

**Solubilization of PGE₁ Receptors**

To evaluate the efficacy of various detergents in solubilizing the PGE₁ receptors, the platelet suspension (1.0 ml) was treated separately with the following compounds in the above buffer containing 1.0 mM PMSF, 5 mM DTT, 5 mM MgCl₂, and 0.3 mM sucrose (final concentration shown in the parentheses): CHAPS (0.2%), Lubrol PX (0.2%), taurocholic acid (0.2%), Triton X-100 (0.05%), and Tween 60 (0.2%). After the addition of the detergents, the platelet suspension was incubated at 0 °C (in ice) for 30 min with occasional mild shaking. Care was taken to avoid frothing. After incubation, the detergent-treated platelet suspension was centrifuged at 35,000 × g for 30 min at 0 °C. The PGE₁ binding activities of both the supernatant and the pellet were subsequently determined.

Of all the detergents tested, Triton X-100 seemed to be the most...
TABLE I

| Detergent (final concentration, %) | Supernatant protein | Specific activity |
|------------------------------------|---------------------|------------------|
|   mg/ml                             | pmol PGE, bound/mg protein |
| Triton X-100 (0.05)                 | 2.0                 | 7.8              |
| CHAPS (0.2)                        | 9.0                 | 1.3              |
| Tween 60 (0.2)                     | 7.0                 | 1.2              |
| Lubrol PX (0.1)                     | 9.5                 | 0.6              |

efficient solubilizing agent (Table I). Except in the case of Triton X-100, the PGE, binding activities of the different extracts were determined without removing the detergents from the same, and the degree of solubilization of the receptor by individual agent may not be comparable. Thus, the data were useful only as a guideline for further purification. In the case of Triton X-100, the removal of the detergent was carried out by centrifugation at 35,000 x g at 0 °C. [3H]PGE, binding activity and the protein content of the supernatant were determined. Data shown are averages of three experiments.

Purification of PGE, Receptor

DEAE-cellulose Chromatography—All the chromatographic procedures described below were carried out at 4 °C. Typically, approximately 12 g of washed platelets suspended in 12 ml of the above buffer solution was used for Triton X-100 to solubilize PGE, receptor. The supernatant containing solubilized receptors (12 ml) was reduced to 6 ml by covering the samples in a dialysis bag with powdered polyethylene glycol (M, 15,000–20,000) at 0 °C and periodically removing the wet solid. The concentrated solution was clarified by centrifugation at 8,000 x g at 0 °C and immediately applied to a DEAE-cellulose column (2.5 x 60 cm) equilibrated with 10 mM Tris-HCl buffer, pH 7.4, containing 0.05% Triton X-100, 1.0 mM PMSF, 1.0 mM DTT, 5 mM MgCl2, and 0.3 M sucrose and fractionated by stepwise elution with increasing concentrations of KCl (0.1–0.7 M) in the same buffer except that the concentration of the buffer was increased to 50 mM. The eluates were collected at 75 ml each of 0.1, 0.2, and 0.5 M KCl in 50 mM Tris-HCl buffer but did not show the presence of any PGE, binding activity and were discarded (not shown). At 0.7 M KCl, the PGE, binding activity began to emerge from the column in a single peak (Fig. 1). Fractions (12–32) which contained the PGE, binding protein with highest specific activity were pooled and concentrated (6.0 ml). Further elution of the column with 1.0, 1.25, and 1.5 M KCl (75 ml each) did not show the presence of any PGE, binding activity in the eluates (not shown).

Sephadex G-200 Chromatography—The concentrated fractions from the DEAE-cellulose column were applied to a Sephadex G-200 column (1.0 x 40 cm) equilibrated with the buffer system used in the DEAE-cellulose chromatography except that KCl was omitted. The elution of the column was carried out with the same buffer. The eluates containing PGE, binding activity emerged in a single peak in fractions 27–35 (8 ml) (Fig. 2). The active fractions were pooled and concentrated to 0.4 ml as described above. The purified receptor was kept at −70 °C in 10 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose and 0.05% Triton X-100.

Removal of Triton X-100—Triton X-100 was removed from the protein sample using Bio-Beads SM-2 as described by Holloway (12). Optimum conditions for the removal of the detergents with minimum loss of protein were standardized by treating the samples (0.8 ml) with different amounts of moist beads for 30 min at 4 °C. The samples were then centrifuged at 35,000 x g for 30 min, and the supernatants were analyzed for protein and Triton X-100 content (13). Complete removal of the excess detergent was achieved using 1.1 ml of moist beads per 0.8 ml of sample. The removal of Triton X-100 from the receptor preparation increased the [3H]PGE, binding by 12-fold. However, the detergent has a protective action on the prostaglandin binding activity of the sample, and the samples were routinely stored with 0.05% Triton X-100.

Determination of Proteins—Proteins were determined according to Lowry et al. (14) with bovine serum albumin as the standard. The standard curve for the determination of the protein was constructed by adding 0–0.05% of Triton X-100 in the assay mixture. The presence of the above concentrations of the detergent does not interfere with the protein assay which was further verified by fluoroscamine assay (15).

Polyacrylamide Gel Electrophoresis—Homogeneity of the purified receptor protein was tested by polyacrylamide gel electrophoresis.
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under alkaline conditions (16). Molecular weight and the subunit composition of the purified receptor were determined by SDS-polyacrylamide (7.5%) gel electrophoresis with nonreduced and reduced protein using 0.1 M DTT (17). Typically, 20 \(\mu\)g of the purified protein were reduced by boiling the sample for 2 min in the presence of 2% SDS and 0.1 M DTT. In the case of the unreduced protein, the sample was similarly treated in the presence of the reducing agent. The gels were stained with 0.02% Coomassie Brilliant Blue in 10% acetic acid and destained in a 10% acetic acid, 10% isopropanol alcohol (1:1) mixture. The gels were also stained with AgNO\(_3\) as described by Merril et al. (18). The molecular weights of the PGE\(_1\) receptor and its subunits were determined by using marker proteins of known molecular weights (Bio-Rad).

In some phases of the work, it was necessary to elute the protein from the gel and assay its \([\text{H}]\)PGE\(_1\) binding activity after the electrophoresis. In these cases, the gels were cut into slices (approximately 1.0-mm thickness) and homogenized in 0.2 ml of 50 mM Tris-HCl buffer, pH 7.4, containing 5 mM MgCl\(_2\) for 5 min at 0 \({}^\circ\)C. The homogenate was centrifuged at 8000 \(\times g\) at 4 \({}^\circ\)C. The supernatant was collected, and the PGE\(_1\), binding activity was determined.

**\([\text{H}]\)PGE\(_1\), Binding Assay**—Unless otherwise stated, the binding of \([\text{H}]\)PGE\(_1\), to the solubilized fractions from the platelets was performed by incubating 20–100 \(\mu\)g of protein for 30 min at 23 \({}^\circ\)C in 50 mM Tris-HCl buffer containing 5 mM MgCl\(_2\) in a total volume of 200 \(\mu\)l with 0.3 \(\mu\)M PGE\(_1\) containing 0.1 \(\mu\)Ci of \([\text{H}]\)PGE\(_1\). Since less than 1% of the PGE\(_1\), bound to the preparation, the concentration of the free ligand was essentially constant throughout the incubation. Unless otherwise indicated, parallel experiments were run using 50-fold excess labeled PGE\(_1\) in the above incubation mixture to determine the nonspecific binding. This value was subtracted from the total PGE\(_1\), bound to calculate the specific binding. At the end of the incubation, 1.0 ml of the above buffer (0 \({}^\circ\)C) was added to each tube, and the mixture was filtered under vacuum through a Whatman glass microfiber filter (GF/C, 2.0 \(\times\) 9.0 cm in diameter) which had been presoaked with the assay buffer. The solubilized receptor remained bound to the filter which was washed with 15 ml of the buffer at 0 \({}^\circ\)C. The filters were then dried and suspended in 10 ml of scintillation fluid (Amer sham, Sodium 1,4-Dioxane, 0.02% PPO, 0.005% POPOP, 1% Triton X-100) and counted in a Beckman Scintillation Spectrometer (LS-8000) with 45% efficiency for \(\text{H}\). The possibility that \([\text{H}]\)PGE\(_1\), might be degraded during the period of binding was tested by incubating the radiolabeled autacoid with solubilized proteins in the same incubation mixture as described above. After incubation, the suspension was acidified with 1% HClO\(_4\) and extracted with 3 volumes of a (CH\(_2\)Cl\(_2\)/CH\(_3\)OH (95:5) mixture three times at 0 \({}^\circ\)C. The extracted materials were then analyzed by high pressure liquid chromatography using a \(\mu\)Bondapak (particle size, 10 \(\mu\)m) reverse phase (C\(_18\)) column (Waters Associates), and H\(_2\)O/CH\(_3\)CN/CH\(_3\)COOH (75:23:2.0, v/v/v) solvent system as described earlier (13).

**Analysis of Equilibrium Binding of PGE\(_1\), to the Purified Receptors**

The interaction of PGE\(_1\), with the purified receptors was analyzed by the method of Scatchard (20). 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 algorithm analysis using a Radio Shack TRS 80, Model 4 microcomputer.

**Assay of Adenylate Cyclase**

Adenylate cyclase activity of platelet homogenate was determined by incubating 1.0 mM ATP containing 2 \(\mu\)Ci of \([\alpha\text{-32P}]\)ATP, 2 mM MgSO\(_4\), 10 mM theophylline, 1 mM creatine phosphate, 1 unit of creatine phosphokinase, 1.4 \(\mu\)M ETP, 50 mM Tris-HCl buffer, pH 7.4, in a total volume of 0.1 ml reaction mixture. After incubation at 37 \({}^\circ\)C for 5 min, the reactions were stopped by adding SDS (5 final concentration). The radioactive cyclic AMP was separated according to Salamon et al. (21). Unlabeled cyclic AMP (1.0 \(\mu\)M) was added to the reaction mixture to facilitate the recovery of the nucleotide. The post-inactivated enzyme preparation was made by heating the enzyme preparation with the buffer in a boiling water bath for 5 min. Platelet homogenate was prepared by the method described before (22).

**RESULTS**

**Purification of Human Blood Platelet PGE\(_1\), Receptor**—The purification protocol of the human platelet PGE\(_1\), receptor is summarized in Table II. PGE\(_1\), receptors were solubilized by treating fresh platelets with 0.05% Triton X-100. Over 90% of the PGE\(_1\), binding activity of the platelets was recovered in the detergent extract. The detergent extract was centrifuged, and the resultant supernatant was first fractionated on a DEAE-cellulose column followed by chromatography of the active fractions on a Sephadex G-200 column as described under "Experimental Procedures." PGE\(_1\), binding activity of the different fractions in each step was measured after the removal of the detergent by Bio-Beads SM-2. The procedure described above resulted in an increase of specific binding of \([\text{H}]\)PGE\(_1\), from 2.2 pmol/mg of protein to 4,900 pmol/mg of protein which showed over 2,200-fold purification of PGE\(_1\), receptor with 30% yield compared to the intact platelets.

**Electrophoretic Homogeneity: Molecular Weight and Subunit Composition of PGE\(_1\), Receptor Protein**—Alkaline gel electrophoresis under nonreducing conditions, the PGE\(_1\), binding protein from the Sephadex G-200 column showed that the isolated product was homogeneous when the gels were stained either with AgNO\(_3\) (Fig. 3A) or with Coomassie Brilliant Blue (Fig. 3B). In a parallel experiment, an identical gel which was not stained with the dye to avoid denaturation was cut into slices, the binding protein from the slices was eluted, and the \([\text{H}]\)PGE\(_1\), binding activity of the eluates was determined. The position of the gel slices showing the \([\text{H}]\)PGE\(_1\), binding activities corresponded exactly to the protein band in the gel stained by the dye except that in the case of the unstained gel, which was not fixed, the receptor protein apparently diffused more when compared to the dye-stained gel (Fig. 3B). Polyacrylamide gel electrophoresis of the purified protein, in the presence of SDS but in the absence of DTT, showed the molecular weight of the receptor to be 190,000 (Fig. 4). Upon reduction with DTT, the receptor protein resolved into two subunits migrating closely in the SDS-polyacrylamide gel. The \(M_s\) of the two subunits were estimated to be 85,000 and 95,000. These results indicate that the \([\text{H}]\)PGE\(_1\), binding protein of platelets is composed of two subunits with \(M_s\) of 85,000 and 95,000 possibly linked by disulfide bonds. The purified receptor was sensitive to trypsin digestion, suggesting its polypeptide nature. Incubation of 20 \(\mu\)g of receptor with 2 \(\mu\)g of trypsin in 50 mM Tris-HCl buffer, pH 8.1, containing 0.015 M CaCl\(_2\) completely destroyed the binding activity in 5 min at 23 \({}^\circ\)C.

**Binding of \([\text{H}]\)PGE\(_1\), to the Purified Receptors**—Binding of \([\text{H}]\)PGE\(_1\), to the purified receptor was rapid and attained equilibrium within 5–15 min at 23 \({}^\circ\)C. Addition of a large excess (15 \(\mu\)M) of unlabeled PGE\(_1\), to the reaction mixture at equilibrium promptly reduced the amount of \([\text{H}]\)PGE\(_1\), to less than 10% of the total binding in the absence of any added unlabeled PGE\(_1\), demonstrating reversibility (Fig. 5). When these values were corrected for the nonspecific binding, the displacement of bound \([\text{H}]\)PGE\(_1\), from the purified receptor by the unlabeled prostaglandin was nearly 100%. The binding of \([\text{H}]\)PGE\(_1\), was also specific. Only PGB\(_2\), at 15 \(\mu\)M concentration, displaced approximately 50% of the bound \([\text{H}]\)PGE\(_1\), from the purified receptor. Other prostaglandins including PGA\(_2\), PGB\(_3\), PGD\(_2\), PGE\(_2\), or PGE\(_3\), at 1–15 \(\mu\)M concentrations had little effect on the displacement of bound \([\text{H}]\)PGE\(_1\), from the receptor (Fig. 6). However, PGE\(_2\), at 25 and 30 \(\mu\)M concentrations inhibited \([\text{H}]\)PGE\(_1\), binding by approximately 25% and 38%, respectively (not shown). Other autacoids at these higher concentrations had no effect on the inhibition of \([\text{H}]\)PGE\(_1\), binding to the receptor.

There was apparently no degradation of \([\text{H}]\)PGE\(_1\), by the purified receptor when the radiolabeled compound was added to the binding assay mixture. The added \([\text{H}]\)PGE\(_1\), was quantitatively recovered by high performance liquid chromatogra-
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**TABLE II**

Summary of the purification protocol of PGE₁ receptor of human blood platelets

| Step                  | Total specific binding of PGE₁ pmol | Total protein mg | Specific activity pmol PGE₁ bound/mg protein | Yield /fold |
|-----------------------|-------------------------------------|-----------------|---------------------------------------------|-------------|
| Washed platelets      | 2,442                               | 1.11 g          | 2.2                                         | 100         |
| Triton X-100 supernatant | 2,357                              | 25 mg           | 94.3                                        | 96.2 42.8   |
| DEAE-cellulose        | 1,225                               | 0.5 mg          | 2,450                                       | 50 1135.6   |
| Sephadex G-200        | 792                                 | 0.15 mg         | 4,900                                       | 30 2227.2   |

**Fig. 3.** Electrophoretic homogeneity and the binding of [³H]PGE₁ to the protein eluted from polyacrylamide gel. A, polyacrylamide gel electrophoresis of Triton X-100 extract (100 µg of protein) and the purified receptor (10 µg of protein) were carried out in 1.5-mm slabs at pH 8.5 using Tris/borate buffer. The gels were stained with AgNO₃. Lane 1, Triton X-100 extract; Lane 2, the purified receptor. B, purified PGE₁ binding protein (10 µg) electrophoresed in 7.5% polyacrylamide gel and stained with Coomassie Brilliant Blue. In a parallel experiment, the gel was not stained for proteins but sliced (1 slice = 0.125 cm), and the proteins were eluted with 50 mM Tris-HCl buffer, pH 7.4, as described under “Experimental Procedures.” The figures shown here are typical of three different experiments.

**Fig. 4.** SDS-polyacrylamide gel electrophoresis of reduced and unreduced PGE₁ receptor. Reduction and SDS-polyacrylamide gel electrophoresis of the receptor protein were done as described under “Experimental Procedures.” Lanes 1 and 2 show unreduced and reduced PGE₁ receptor, respectively. Marker proteins used were: myosin (M, 200,000 (200K)), β-galactosidase (M, 116,250), phosphorylase b (M, 92,500 (92.5K)), bovine serum albumin (M, 66,200), and ovalbumin (M, 45,000).

**Fig. 5.** Time course of binding of [³H]PGE₁ to the purified PGE₁ receptor and the dissociation of the ligand by various prostaglandins. The purified receptor (5–10 µg) was incubated with [³H]PGE₁ (0.3 nM, 100,000 cpm) for various times at 23 °C as indicated. At specified times, the assay mixture was diluted, and the binding of the radioligand to the receptor was determined. At equilibrium, various prostaglandins (15 µM each) were added to the assay mixture (indicated by arrow), and the time course of dissociation of the [³H]PGE₁ from the receptor was determined. Data shown here are typical of six experiments.

binding (10–12%) was increased linearly with the increase of protein concentration in the binding assay mixture. The binding characteristics of [³H]PGE₁ to the purified receptor were analyzed by a Scatchard plot (20). The results suggested the presence of two classes of binding sites with widely different dissociation constants (Kₐ) and capacities...
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Inhibition of \([^{3}H]PGE_{1}\) binding to the purified receptor by various prostaglandins. Purified PGE\(_{1}\) receptor (10 \(\mu\)g) was incubated with \([^{3}H]PGE_{1}\) (0.3 \(\mu\)M, 100,000 cpm) with different unlabeled prostaglandins as indicated at 22 °C for 30 min. Results which are means of three experiments are expressed as percent inhibition by dividing counts/min by the calculated specific activity (cpm/mol) obtained by diluting 0.3 \(\mu\)M \([^{3}H]PGE_{1}\) with a known concentration of the unlabeled prostaglandin. Each point represents the mean of four experiments which are comparable to six other experiments.

Reconstitution of PGE\(_{1}/PGI_{2}\)-responsive Platelet Adenylate Cyclase by the Purified Receptor—To test the biological activity of the purified receptor, attempts were made to reconstitute PGE\(_{1}\)-responsive adenylate cyclase of platelets with the purified receptors. When a platelet homogenate prepared from the cells washed with Triton X-100 to remove PGE\(_{1}\) receptors was examined, little stimulation of adenylate cyclase activity in response to the autacoid challenge was demonstrated when compared with the control (Table III). The purified receptors themselves did not show measurable adenylate cyclase activity. However, when the receptor was added to the detergent-washed platelets and subsequently the PGE\(_{1}\)-responsive adenylate cyclase activity of the homogenate was assayed, it was found that the purified receptor was capable of regenerating the response of the enzyme to PGE\(_{1}\). Addition of 10 \(\mu\)g of the purified receptor, the quantity approximately equivalent to the amount of the receptor present in 2 \(\times\) 10\(^{10}\) platelets, to the detergent-treated platelet restored the activation of adenylate cyclase of the homogenate by PGE\(_{1}\), by 57% compared to the control. PGI\(_{2}\), like PGE\(_{1}\), also stimulated the activity of platelet homogenate adenylate cyclase reconstituted with PGE\(_{1}\) receptor (Table III).

The effect of 0.05% Triton X-100 on the removal of PGE\(_{1}\) receptors from platelets was found to be highly specific, since the treatment of these cells with similar concentrations of the detergent under identical conditions did not reduce the stimulation of adenylate cyclase activity by either PGD\(_{2}\) or adenosine (Table III). Consequently, the addition of these agonists to the assay mixture containing the reconstituted adenylate cyclase produce no further stimulation of the enzyme over the control platelet homogenate (Table III).

Stability of the Purified PGE\(_{1}\) Receptor—The purified receptor (1.0 mg/ml), when kept at -70 °C in 50 mM Tris-HCl buffer, pH 7.4, containing 0.3 M sucrose and 0.05% Triton X-100, was found to have a half-life of approximately 3 months. The presence of detergent apparently had a protective action on the stability of the receptor. In the absence of the detergent, the purified receptor lost 50% of its binding activity in less than a month at -70 °C.

DISCUSSION

The interaction between the ligand and the receptor has been shown to be a prerequisite for the activation of adenylate
TABLE III
Reconstitution of PGE1/PGI2-responsive platelet adenylate cyclase by
the purified receptors

| Homogenate            | Agonist      | Cyclic AMP (nmol/mg protein/5 min ± S.E.) |
|-----------------------|--------------|------------------------------------------|
| Platelets             | None         | 52 ± 11                                  |
| Triton X-100-treated  | PGE1 (1.4 µM)| 1030 ± 40                                |
| Triton X-100-treated  | PGI1 (1.4 µM)| 1090 ± 50                                |
| Triton X-100-treated  | PGI2 (1.4 µM)| 451 ± 16                                 |
| Triton X-100-treated  | Adenosine (10 µM) | 260 ± 23                        |
| Triton X-100-treated  | PGE1 (1.4 µM)| 87 ± 12                                  |
| Triton X-100-treated  | PGI1 (1.4 µM)| 25 ± 15                                  |
| Triton X-100-treated  | PGI2 (1.4 µM)| 429 ± 20                                 |
| Triton X-100-treated  | Adenosine (10 µM)| 210 ± 18                     |
| Triton X-100-treated  | None         | 48 ± 10                                  |
| Triton X-100-treated  | PGE1 (1.4 µM)| 592 ± 25                                 |
| Triton X-100-treated  | PGI1 (1.4 µM)| 610 ± 20                                 |
| Triton X-100-treated  | PGI2 (1.4 µM)| 400 ± 38                                 |
| Triton X-100-treated  | Adenosine (1.4 µM)| 178 ± 29                     |
| Receptor only         | None         | <1                                       |
| Receptor only         | PGE1 (1.4 µM)| <1                                       |

The purified receptor had an apparent $M_r$ of 190,000 by SDS-polyacrylamide gel electrophoresis (Fig. 4). A similar weight estimate of the purified protein was also obtained by Sephadex G-200 gel filtration on a calibrated column (not shown).

SDS-polyacrylamide gel electrophoresis of the reduced protein showed that the receptor molecule is composed of two nonidentical subunits, with molecular weights of 85,000 and 95,000, held together by disulfide bonds(s).

The human platelet adenylate cyclase is activated by PGI2, PGE1, and PGD2 (3–5, 9, 10). Studies with platelet membrane indicated that, while PGI2 and PGE1 receptors might be identical, PGD2 has its own specific receptors linked to adenylate cyclase (10). The specificity of displacement of the bound [3H]PGE1 from the purified receptors by various prostaglandins indicated that only PGI2 is capable of dissociating [3H]PGE1 from the protein and PGD2 at similar concentrations was totally ineffective (Figs. 5 and 6). Although lower concentrations (1–15 µM) of PGE1 could not displace bound [3H]PGE1 from the purified receptor, higher concentrations (25 and 30 µM) of the prostaglandin dissociated the radioligand from the receptor. These results are in agreement with previous studies (10, 11). However, it should be noted that the PGI2 level in circulation is much higher than that of PGE1, which occurs in a very small quantity, and, among all the prostaglandins, only PGI2 displaced the bound [3H]PGE1 from the receptor. It is, therefore, possible that the purified PGE1 receptor is actually the PGI2 receptor.

Other investigators have reported that the binding of [3H]PGE1 to platelet membrane or intact plates involves one high affinity-low capacity binding sites population and one affinity-high capacity binding sites population (10, 11). The estimated $K_d$ values of the purified receptors ($K_{d1} = 9.8 \times 10^{-9}$ M; $K_{d2} = 0.7 \times 10^{-8}$ M) compared well with the $K_d$ values of platelet membrane (10) ($K_{d1} = 6.6 \times 10^{-9}$ M; $K_{d2} = 1.9 \times 10^{-8}$ M) and intact plates (11) ($K_{d1} = 9.5 \times 10^{-9}$ M; $K_{d2} = 1.6 \times 10^{-8}$ M). Our results (Fig. 7) not only confirm these observations, but also established that these two binding sites are located in the same protein molecule. However, the data do not allow us to conclude whether the difference in affinity for the binding of PGE1 to the receptor is due to the presence of two different classes of binding sites in the same receptor molecule or to the negative cooperativity created in the single class of receptors due to increased ligand concentration (23).

The purified receptor, for its optimal binding to [3H]PGE1, needed Mg2+ in the assay mixture, and the metal ions could not be substituted by Ca2+ (Fig. 8). These results suggest that Mg2+ is not only needed for the catalytic activity of adenylate cyclase, but it is also required for the ligand receptor interaction.

The purified receptor not only binds to its ligand with a high degree of specificity, but also showed biological activity by reconstituting a PGE1- or PGI2-responsive adenylate cyclase in platelets (Table III). Since PGE1 and PGI2 were found to interact with the same receptor, it is expected that the reconstituted enzyme would be activated by both the autacoids. Incubation of the detergent-washed platelets with the receptor prior to the homogenization of the cells efficiently reconstituted (57%) the stimulation of the enzymic activity by both the agonists. Use of polyethylene glycol or other agents which are normally necessary for the reconstitution of a β-adrenergic responsive adenylate cyclase (30) was unnecessary in the case of purified PGE1/PGI2 receptor. Use of polyethylene glycol 6000 in the reconstitution mixture did not improve the efficiency of the process (not shown).
The platelet adenylate cyclase is also activated by PGD₂ and adenosine (4, 10). However, the receptors of these agonists, unlike PGE₁/PGI₂ receptors, cannot be removed by treating these cells with 0.05% Triton X-100. The ease of removal of PGE₁/PGI₂ receptors from platelet membrane suggests that receptor protein is probably attached peripherally to the outer surface of the membrane bilayer. In contrast, the receptors of PGD₂ and adenosine might be more deeply embedded on the membrane. Although PGE₁/PGI₂ receptors might be only peripherally attached to the membranes, the macromolecules, nevertheless, are platelet proteins. Since platelet-free plasma does not contain any PGE₁/PGI₂ receptor protein, the possibility that platelets could pick up these receptors from the plasma was ruled out.

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