Selective Binding Site for \[^{3}\text{H}]\text{Prostacyclin on Platelets}

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**ABSTRACT**

Prostacyclin (PGI₂) is the most potent, naturally occurring inhibitor of platelet aggregation known. To determine whether PGI₂ is bound by platelets, high specific activity \([9-\text{H}]\text{PGI}_2\) was synthesized by iodination and subsequent base treatment of the labeled precursor \([9-\text{H}]\text{prostaglandin (PG)E}_2\) methyl ester. Binding experiments were performed at room temperature with normal citrated human platelet-rich plasma that contained \[^{14}\text{C}]\text{sucrose or [}^{\text{14}}\text{C}]\text{PGF}_{2\alpha}\) as an internal marker for the extracellular space. Binding of \([\text{H}]\text{PGI}_2\) plateaued within 2 min and this bond radioactivity could be displaced rapidly by excess nonradioactive PGI₂. Scatchard analysis of concentration-dependent binding yielded a hyperbolic plot which appeared to be caused by the existence of two classes of binding sites. The higher affinity class has a dissociation constant of 12.1±2.7 nM and a capacity of 93 (±21)sites per platelet. The lower affinity class had a dissociation constant of 0.909±2.36 μM and a capacity of 2,700±700 sites per platelet. The relative ability of PGI₂, PGE₁, PGE₃, and 6-keto-PGF₉α to displace \([\text{H}]\text{PGI}_2\) initially bound to the higher affinity class of sites was 100.5:<0.3:<0.3. These relative abilities parallel the relative potencies of these compounds as inhibitors of ADP-induced platelet aggregation in vitro. However, PGE₃, which is more potent than PGE₁, as an inhibitor of aggregation, did not displace bound \([\text{H}]\text{PGI}_2\). The higher affinity binding site for PGI₂ appears to be the specific receptor through which PGI₂ exerts its effect on platelets.

**INTRODUCTION**

Until recently, the most potent inhibitor of platelet aggregation known was prostaglandin (PG)E₁ (1). In 1974, PGD₂ was found to be more potent as an inhibitor of human platelet aggregation although it was relatively inactive on platelets obtained from other species (2). In 1976, Moncada et al. (3) discovered an even more potent but unstable inhibitor of platelet aggregation which was produced from prostaglandin endoperoxides by an enzyme present in blood vessels. They proposed that the continuous synthesis of this substance protects arterial walls against the deposition of platelet thrombi (4). The structure of this inhibitor has been determined and it is now known as prostacyclin (PGI₂) (5). Cultured endothelial cells possess the capacity to synthesize PGI₂ (6, 7). It inhibits the aggregation of platelets from all species examined (8, 9).

PGE₁, PGD₂ and PGI₂ have been shown to activate adenylate cyclase and increase cyclic (c)AMP levels in human platelets (1, 10–12). Tateoson et al. (12) demonstrated that PGI₂ at 10 nM caused an increase in cAMP levels equivalent to five times the basal level, whereas it required 1 μM PGD₂ to achieve this increase. The stimulation of adenylate cyclase by PGI₂ argues for its interaction with a specific membrane-bound receptor system similar to that involved in the β-adrenergic system (13). We describe here the properties of a binding site for PGI₂ on intact platelets, which appears to be its specific membrane receptor.

**METHODS**

PGI₂ was prepared by the method of Nicolaou et al. (14). For the preparation of \([\text{H}]\text{PGI}_2\), 8 μg of \([9-\text{H}]\text{PGF}_{2\alpha}\) methyl ester (10–12 Ci/mmol sp act) was dissolved in 70 μl methylene chloride and reacted overnight with 16 μg of iodine in the presence of 0.2 mg potassium carbonate at –10°C. The product, PGI₂methyl ester iodide, was purified by thin-layer chromatography (5% methanol in diethyl ether). Treatment of the purified intermediate with 60 μl sodium ethoxide in the presence of 160 μl 95% ethanol at 75°C for 1 h removed both the iodine atom and the ester group to yield \([9-\text{H}]\text{PGI}_2\), which was stable because of the basic conditions involved in its preparation. Thin-layer chromatography of \([9-\text{H}]\text{PGI}_2\) (chloroform: methanol; acetic acid; water; 90:9:1.0:0.6) indicated 95% purity. Other prostaglandins were a gift from Dr. John Pike, The Upjohn Co., Kalamazoo, Mich.
Blood from normal volunteers who had not taken drugs for 10 d was drawn into 0.1 vol 3.8% trisodium citrate and centrifuged at 180 g for 15 min. Platelet-rich plasma (PRP) was removed and the platelet count was determined with a Coulter Counter (Coulter Electronics Inc., Hialeah, Fla.). Platelet aggregation was measured at 37°C in an aggregometer (Chrono-log Corp., Havertown, Pa.) with 20 μM ADP as the aggregating agent. Prostaglandins were added in ethanol up to a maximum concentration of 0.5% vol/vol.

In binding experiments, 0.2 μCi/ml [U-14C]glucose (3.6 Ci/mol sp act) or 0.05 μCi/ml [1-14C]PGF1α (45 Ci/mol sp act; New England Nuclear, Boston, Mass.) was added to the PRP as an internal marker for the extracellular space. Appropriate amounts of [3H]PG12 were added to the PRP and the sample was incubated at room temperature for a given time. Prostaglandins were added in ethanol up to a maximum concentration of 0.5% vol/vol. Incubation was terminated by centrifuging 1-ml samples in an Eppendorf microfuge (Brinkman Instruments, Inc., Westbury, N.Y.) at 15,000 g for 2 min. The supernate was removed rapidly with a Pasteur pipette. The tube was inverted, allowed to drain for 5 min, and the pellet was removed with a cotton swab. The pellets and samples of the supernates were oxidized in a Packard 306 Oxidizer (Packard Instrument Co., Inc., Downers Grove, Ill.). The [3H]H2O and [14C]CO2 were determined by liquid scintillation counting. Efficiency of combustion was 99% and counting efficiencies were determined by combustion and liquid scintillation counting of known amounts of radioactive standards. Bound [3H]PG12 per 106 platelets was determined after correction for background, extracellular space, and platelet count. In some experiments, bound [3H]PG12 was expressed as femtomoles per 106 platelets using its known specific activity after correction for counting efficiency.

RESULTS

Effect of PGI2 on platelet aggregation. Prostacyclin caused 50% inhibition of aggregation induced by 20 μM ADP at a final concentration of 3.4 nM. PGI2 was ≈ 7% as effective causing 50% inhibition of aggregation at 50 nM and PGE2 was only 3.5% as effective as PGI2. PGE2, PGF1α, and 6-keto-PGF1α were <0.15% as effective as PGI2 because they caused no detectable inhibition of aggregation at a concentration of 50 μM.

Time-course of binding and displacement. Incubation of PRP with a low concentration of [3H]PG12 (9 nM) for increasing lengths of time indicated that PG12 was bound by platelets. The amount of [3H]PG12 bound plateaued within 2 min and remained at approximately this level for the next 10 min (Fig. 1, solid line). Similar results were obtained with a high concentration of [3H]PG12 (118 nM) (data not shown). When a 100-fold excess of unlabeled PG12 was added 5 min after the 9 nM [3H]PG12, there was a rapid displacement of the bound radioactivity. The displacement was essentially complete after 2 min and the amount displaced accounted for 80% of the bound radioactivity (Fig. 1, open circles). In all subsequent experiments the PRP was incubated with [3H]PG12 for 5 min to ensure that equilibrium had been established.

Concentration dependence. PRP was incubated with concentrations of [3H]PG12 ranging from 4.5 to

![Figure 1](http://www.jci.org)
Therefore, the concentrations of PGI_2 and PGD_2 placement. The hormone, a secondary influence on platelet.

This can be a hyperbola, because a straight line (correlation coefficient, 0.996) was obtained when the same data was plotted on double log paper. The two asymptotes to this hyperbola (the product of which describes the hyperbola) were derived by iterative geometric construction (Fig. 2, dashed lines). It was assumed that the hyperbola reflected the binding of [3H]PG1_2 to two independent sites with different affinities and the asymptotes were used to derive the dissociation constants for each of these sites (15). This analysis yielded a higher affinity, low-capacity site with a dissociation constant (K_H) of 12.1±2.7 nM and a capacity of 93±21 sites per platelet. The lower affinity, high capacity site had a dissociation constant (K_L) of 0.909±0.236 μM and a capacity of 2,700±700 sites per platelet.

Specificity of prostacyclin binding. Several prostaglandins were tested for their ability to displace [3H]PG1_2 from platelets. In Fig. 3 it can be seen that unlabeled PGI_2 causes displacement of radioactivity at low concentrations. PGE_1, was the next most potent compound, whereas PGE_2 produced only a slight amount of displacement and PGD_2 caused essentially no displacement. The concentrations of PGI_2, PGE_1, PGE_2, and PGD_2 required to displace 50% of the bound [3H]PG1_2 were 0.3, 6, and <100 μM, respectively. Therefore, the relative affinities were 100:5:<0.3:<0.3 for PGI_2, PGE_1, PGE_2, and PGD_2. The ability of 6-keto-PGF_1α, PGF_2α, or PGF_1α to displace bound [3H]-PG1_2 was also determined (Fig. 3). Slight displacement was observed with PGF_1α and 6-keto-PGF_1α, whereas essentially no displacement was observed with PGF_2α. The affinity for 6-keto-PGF_1α was <0.3% relative to PGI_2.

Comparison of extracellular space determinations with [14C]sucrose or [14C]PGF_1α. Table I illustrates an experiment in which the binding of [3H]PG1_2 was determined with either [14C]sucrose or [14C]PGF_1α to correct for the extracellular space. It can be seen that the values obtained are identical. Furthermore, an essentially identical rate of displacement of [3H]PG1_2 by nonradioactive PG1_2 was obtained in one experiment with either [14C]sucrose or [14C]PGF_1α as the internal marker. In another experiment, with equilibrium conditions and [14C]PGF_1α as the internal marker, two binding sites were obtained on Scatchard analysis. These had dissociation constants of 12.4 nM and 0.93 μM almost identical to those obtained in the seven experiments with [14C]sucrose shown in Fig. 2.

DISCUSSION

To be relevant to the action of a hormone the binding of a radioactive ligand should be saturable, reversible, and specific, and should be comparable in time-course and concentration to the effect produced (16).

To determine the binding of a hormone to intact cells

**FIGURE 2.** Scatchard analysis of concentration-dependent binding. PRP was incubated with [3H]PG1_2 in concentrations from 4.5 to 518 nM and binding was measured with [14C]sucrose as the internal marker. Each point is plotted ± SEM. The smooth curve represents the hyperbolic plot and the dotted lines were used to obtain K_H and K_L. Each point represents the mean of 14 determinations on seven subjects. With an unpaired Student t test each point was significantly different from its neighbors at a P level of from <0.05 to <0.001.
it is usual to employ a second radioactive compound which neither penetrates the cells nor is bound to them as a marker for extracellular space. In most of our experiments we used \(^{14}\text{C}\)sucrose as this marker. However, we found that \(^{14}\text{C}\)PGF\(_{10}\) could be used equally well as the marker (Table I) and therefore we justified all conclusions reached with sucrose as the marker by also using \(^{14}\text{C}\)PGF\(_{10}\).

As shown in Fig. 1, the binding of PGI\(_2\) by platelets was rapid and 80% of the binding was complete by 1 min. The effects of PGI\(_2\) on aggregation and increases in platelet cAMP occur within this same time (11, 12), suggesting that binding is associated with almost immediate activation of adenylate cyclase. The rapid displacement of bound \(^{3}\text{H}\)PGI\(_2\) by unlabeled PGI\(_2\) (Fig. I) shows that binding was reversible and suggests that PGI\(_2\) is bound at an extracellular site. It previously has been shown that E- and F-type prostaglandins do not equilibrate across membranes in the absence of an active transport system (17). This is consistent with our findings that the plasma spaces determined with \(^{14}\text{C}\)sucrose and \(^{14}\text{C}\)PGF\(_{10}\) were identical (Table I).

The concentration-dependent aspects of PGI\(_2\) binding were complex as a hyperbolic plot was obtained on Scatchard analysis (Fig. 2). We assumed that this data reflected the binding of \(^{3}\text{H}\)PGI\(_2\) to two independent sites with different affinities. An alternative explanation could be that negative cooperativity is involved (18).

The dissociation constant (12.1 nM) of the higher affinity binding site for PGI\(_2\) is of the same order of magnitude as that found by other investigators studying prostaglandin binding in several other biological systems (16, 19) although it is fourfold higher than the value obtained for 50% inhibition of platelet aggregation (3.4 nM). However, a direct constant is a measure of the direct physical interaction of PGI\(_2\) with its receptor, whereas the effective dose for 50% inhibition of aggregation reflects complex interactions involv-
In Table I is shown the binding of \([1^H]PGI_2\) to different markers for plasma space.

<table>
<thead>
<tr>
<th>Plasma space marker</th>
<th>[^{14}C]PGF_2\alpha</th>
<th>[^{14}C]sucrose</th>
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<tr>
<td>[^{14}C]measurements</td>
<td>[^{14}C]PGF_2\alpha</td>
<td>[^{14}C]sucrose</td>
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<td>In 20 (\mu)l supernate, (cpm)</td>
<td>1,375±0.70</td>
<td>3,447±1.80</td>
</tr>
<tr>
<td>In pellet, (cpm)</td>
<td>200±27</td>
<td>524±45</td>
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<tr>
<td>[^{14}C]measurements</td>
<td>[^{14}C]PGF_2\alpha</td>
<td>[^{14}C]sucrose</td>
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<tr>
<td>In 20 (\mu)l supernate, (cpm)</td>
<td>811±40</td>
<td>881±40</td>
</tr>
<tr>
<td>In pellet, (cpm)</td>
<td>241±25</td>
<td>250±18</td>
</tr>
<tr>
<td>Bound by platelets, (cpm)</td>
<td>121±4</td>
<td>113±12</td>
</tr>
</tbody>
</table>

Each number represents the mean of three values±SD corrected for background. These were obtained in a single experiment in which the equilibrium binding of \([1^H]PGI_2\) at 6 \(nM\) was tested with either \[^{14}C\]PGF_2\alpha or \[^{14}C\]sucrose as the marker for the extracellular space. There were \(4.99 \times 10^8\) platelets/ml. Other details are given in Methods.

TABLE I

Determination of \([1^H]PGI_2\) Binding with Two Different Markers for Plasma Space

The structural specificity of prostaglandin binding was investigated by testing the ability of several unlabeled prostaglandins to displace \([1^H]PGI_2\) initially bound to the higher affinity site on platelets (Fig. 3). The concentration of \(PGI_2\) required to displace 50% of this bound radioactivity was 0.3 \(\mu M\). As expected, this value lay between the dissociation constants for the lower and higher affinity sites because some of the radioactivity displaced from the higher affinity sites reequilibrated with the large number of lower affinity sites during the 10-min incubation period. \(PGE_1\) also displaced bound \([1^H]PGI_2\) although it was only 5% as active as \(PGI_2\) itself. The remainder of prostaglandins, including PGD_2 were <0.3% as effective as \(PGI_2\). \(PGE_1\) is unstable at neutral pH and spontaneously converts to 6-keto-PGF_2\alpha. Because 6-keto-PGF_2\alpha was almost incapable of displacing bound radioactivity, it is unlikely that the binding of 6-keto-PGF_2\alpha was a factor in our measurements.

With the exception of PGD_2, the results of these displacement experiments correlate well with the relative potencies obtained in the aggregation experiments. PGD_2 did not displace bound \(PGE_1\) but was approximately twice as active as \(PGE_1\) as an inhibitor of aggregation (7% as effective as \(PGI_2\)). PGD_2 also increases cAMP in platelets and its potency, relative to \(PGE_1\) and \(PGI_2\), correlates well with its ability to inhibit aggregation (12). Previous to the discovery of \(PGI_2\), Mills and Macfarlane (10) suggested that \(PGE_1\) and \(PGD_2\) act on different receptors on platelets because of subtle differences noted in the time-course of increases in cAMP produced by these two prostaglandins. Subsequently, based on measurements of platelet cAMP, they suggested that \(PGE_1\) and \(PGL_2\) act on the same receptor, whereas PGD_2 acts on another (21, 22). The results of the binding experiments, therefore, support the conclusions drawn from these biochemical studies.

MacDonald and Stuart (23) originally reported that \(PGE_1\) is bound by intact platelets. This work recently has been extended by Schafer et al. (24) who showed that \([1^H]PGE_1\) is bound by human platelet membranes. Scatchard analysis of their data also indicated the existence of two binding sites. Furthermore, consistent with the idea of a common receptor, they found that either \(PGE_1\), \(PGI_2\), or \(PGE_2\) could displace bound \([1^H]PGE_1\).

In conclusion, we have described a high-affinity binding site for \(PGI_2\) on platelets that fulfills most of the necessary criteria for a specific receptor. Our results indicate that PGD_2 does not occupy this receptor. It will be of interest to determine whether platelets of patients with thrombotic tendencies have a diminished number of binding sites for \(PGL_2\).

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REFERENCES