Prostaglandin E Receptors in Cardiac Sarcolemma
Identification and Coupling to Adenylate Cyclase

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Purified cardiac sarcolemmal membrane vesicles were used to determine if specific prostaglandin (PG) receptors are present on the myocyte. Two binding sites for PGE\textsubscript{2} were identified in isolated bovine sarcolemmal membranes: a high-affinity site with a dissociation constant ($K_d$) of 0.32 nM and a maximum binding ($B_{max}$) of 376 fmol/mg of protein and a lower-affinity site with a $K_d$ of 3.41 nM and a $B_{max}$ of 2,112 fmol/mg of protein. In competition experiments, unlabeled PGE\textsubscript{2} displaced $[^3H]$PGE\textsubscript{2} from its membrane receptor at concentrations similar to those of unlabeled PGE\textsubscript{2}. Both PGF\textsubscript{2}$\alpha$ and PGD\textsubscript{2} displaced $[^3H]$PGE\textsubscript{2} from the membrane, but only at high concentrations ($>10^{-6}$ M and $>10^{-5}$ M, respectively). Digestion of sarcolemmal membrane with trypsin resulted in a threefold decrease in specific $[^3H]$PGE\textsubscript{2} binding. Phosphorylation of the membrane with protein kinase A also decreased specific $[^3H]$PGE\textsubscript{2} binding. At concentrations of PGE\textsubscript{2} that occupy the high-affinity site, sarcolemmal adenylate cyclase activity was inhibited in the presence of 5'-guanylylimidodiphosphate [Gpp(NH)p]. We conclude that the isolated cardiac sarcolemmal membrane contains a high-affinity binding site for PGE\textsubscript{2} that is functionally coupled to adenylate cyclase. The binding site is stereospecific and probably recognizes the 9-keto,11-hydroxyl portion of the ring structure of these prostaglandins. (Circulation Research 1989;65:538–545)

Eicosanoids appear to play an important role in the heart physiologically and during pathological conditions such as ischemia.\textsuperscript{1-4} The prostaglandins (PGs) act directly on the myocardium as well as the coronary vasculature,\textsuperscript{1} while thromboxane (TX) A\textsubscript{2} is a potent coronary vasoconstrictor.\textsuperscript{5} During ischemia, eicosanoid production increases,\textsuperscript{1,5,6} possibly due to an increase in the release of arachidonic acid from myocardial phospholipids.\textsuperscript{7} In isolated coronary arteries, arachidonic acid has been shown to predominantly synthesize PGI\textsubscript{2}, PGE\textsubscript{2}, and TXA\textsubscript{2}.\textsuperscript{6} Despite the extensive work performed on myocardial eicosanoid release and synthesis, relatively little is known about their mechanism and site of action. In several tissues, the eicosanoids have been shown to exert their action via specific plasma membrane receptors.\textsuperscript{8-11} To our knowledge, no studies have determined the existence of eicosanoid receptors in the heart or the mechanism by which potential receptors are coupled to subcellular events. Unlike TXA\textsubscript{2} and PGI\textsubscript{2}, which are extremely labile compounds, PGE\textsubscript{2} is relatively stable. For this reason, we wanted to determine the presence of PGE\textsubscript{2} receptors in the myocardial membrane and the mechanism by which PGE\textsubscript{2} exerts its action on the heart.

The action of PGE\textsubscript{2} on various myocardial tissue preparations has been extensively investigated (for review, see Karmazyn and Dhalla\textsuperscript{1}). Addition of PGE\textsubscript{2} to in vitro perfused hearts has both a positive inotropic\textsuperscript{12-14} and a chronotropic\textsuperscript{15-17} effect. Direct coronary artery injection of PGE\textsubscript{2} in vivo immediately increases the force of contraction, although heart rate may increase or decrease, depending on the experimental model used.\textsuperscript{18,19} Several studies suggest a direct cellular action of prostaglandins on the heart, which includes alterations in glucose oxidation, myocardial O\textsubscript{2} consumption, myocardial
Preparation of Cardiac Sarcolemmal Vesicles Preparation

Vesicle Preparation

The buffers used in the preparation contained 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Vesicles were suspended at 3-5 mg protein/ml in 100 mM NaCl and 20 mM HEPES (pH 7.4), frozen in liquid nitrogen, and stored at -70°C. Subsequent PGE₂ binding studies demonstrated that these final wash procedures dissociated PGE₂ from the receptor (data not shown). Control sarcolemmal vesicles were subjected to an identical procedure, except PGE₂ was excluded from all isolation buffers. Protein was measured by the method of Lowry et al.²³

Preparation of Cardiac Sarcolemmal Membrane Vesicles

Sarcolemmal vesicles were isolated from bovine hearts (left ventricle) by a sucrose flotation method.²³ The buffers used in the preparation contained 0.1 mM phenylmethylsulfonyl fluoride (PMSF). Vesicles were suspended at 3-5 mg protein/ml in 100 mM NaCl and 20 mM HEPES (pH 7.4), frozen in liquid nitrogen, and stored at -70°C. The sarcolemmal vesicles used were tightly sealed and, as shown in Table 1, possess a Ca²⁺/calmodulin-dependent ATPase, an electrogenic Na⁺-Ca²⁺ exchanger, and an ATP-dependent Ca²⁺ uptake.²⁴ The sidedness of the sarcolemmal vesicles was estimated from ouabain inactivation of the Na⁺,K⁺-dependent ATPase activity assayed in the presence of monensin, a Na⁺ ionophore.²⁵ The sarcolemmal vesicle preparation used in this work consisted routinely of 15% "leaky" vesicles, 70% right-side-out vesicles, and 15% inside-out sealed vesicles. Sidedness was also confirmed by alamethicin activation of adenylate cyclase activity. High levels of [³H]quinuclidinyl benzilate binding and [³H]nitrendipine binding are also present in this cardiac sarcolemmal preparation.

In preparations used for measurement of the effects of PGE₂ on adenylate cyclase, 10⁻⁶ M PGE₂ was included in all buffers used to isolate the sarcolemmal vesicles. This was done because studies of Negishi et al.²⁶ suggest that binding of PGE₂ to its receptor maintains coupling between the receptor and other membrane proteins involved in the signal transduction. To remove membrane-bound PGE₂ after isolation, the vesicles (approximately 6 mg of protein) were washed in 50 ml of 100 mM NaCl and 20 mM HEPES (pH 7.4) and centrifuged at 200,000g for 35 minutes. This wash was repeated, and the vesicles were resuspended in the same medium, frozen in liquid nitrogen, and stored at -70°C. Subsequent PGE₂ binding studies demonstrated that these final wash procedures dissociated PGE₂ from the receptor (data not shown). Control sarcolemmal vesicles were subjected to an identical procedure, except PGE₂ was excluded from all the isolation buffers. Protein was measured by the method of Lowry et al.²³

Cultured aortic endothelial cells (a generous gift of C. Krueger and D. Cook, Department of Pharmacology, University of Alberta) were maintained in culture using Dulbecco’s medium, 10% fetal calf serum, and 1% penicillin-streptomycin. [³H]PGE₂ binding was performed in either intact cells suspended in Dulbecco’s medium or in a microsomal fraction obtained from cultured cells. A crude microsomal fraction of endothelial cell membrane was obtained by homogenizing cultured endothelial cells in 250 mM sucrose and 20 mM MOPS (pH 7.4) at one half maximal speed with a Tekmar tissumizer (Cincinnati, Ohio). The suspension was subsequently centrifuged at 1,000g. The resultant supernatant was centrifuged at 210,000g for 60 minutes. The crude microsomal pellet was resuspended in 250 mM sucrose and 20 mM MOPS (pH 7.4). Mitochondria were isolated from bovine ventricle by differential centrifugation. Ventricular muscle was homogenized in a sucrose-MOPS buffer, as described above, and centrifuged at 1,000g. The resultant supernatant was subsequently centrifuged at 10,000g. The pelleted mitochondria were then resuspended in 250 mM sucrose and 20 mM MOPS (pH 7.4), as used for [³H]PGE₂ binding. Cardiac sarcoplasmic reticulum and skeletal muscle sarcolemmal membranes, triads, and T tubules were obtained from M. Michalak.

Measurement of Prostaglandin Binding

Receptor binding was assayed in 200 µl of medium containing 100 mM NaCl, 20 mM HEPES (pH 7.4), and varying concentrations of [³H]PGE₂ (30,000 dpm/tube). Sarcolemmal vesicles (2.5 µg protein)
were added, and the mixture was incubated for 60 minutes at 37°C. The reaction was terminated by pipetting an aliquot of the mixture onto Millipore filters (0.3-μm pore size, Bedford, Massachusetts). Filters were washed with 10 ml of buffer containing 100 mM NaCl and 20 mM HEPES (pH 7.4), dried, and counted with standard liquid scintillation techniques. Specific binding was determined by subtracting binding in the presence of an excess of the appropriate prostaglandin (10^-6 or 10^-5 M) from total binding. Preliminary studies were performed to determine optimal binding conditions. As shown in Figure 1A, binding of [3H]PGE_2 to sarcolemmal membrane was complete by 50 minutes of incubation and was also linearly dependent on sarcolemmal protein content (Figure 1B). Binding characteristics of [3H]PGE_2 to the membrane were identical between pH 7.0 and 8.5 (Figure 1C). Based on this data, all subsequent binding assays were carried out for 60 minutes at 37°C and pH 7.4 with 2.5 μg sarcolemmal protein. In the phosphorylation studies, prior to PGE_2 binding, membranes were preincubated for 5 minutes at room temperature in buffer containing 3 mM MgCl_2, 0.1 mM ethylene glycol-bis-(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 0.1 mM ATP, 10 μM cAMP, and 100 μg protein kinase A. Control experiments determined that MgCl_2, EGTA, ATP, and AMP-PNP alone did not affect [3H]PGE_2 binding (data not shown). In experiments involving protein digestion, prior to PGE_2 binding, sarcolemmal vesicles were preincubated for 30 minutes at 37°C with either trypsin or papain (1 mg/mg sarcolemmal protein). Proteolytic digestion was stopped by addition of 0.1 mM PMSF, followed by centrifugation for 60 minutes at 200,000g.

Measurement of Cardiac Sarcolemmal Adenylate Cyclase Activity

Sarcolemmal vesicles were preincubated for 15 minutes in the presence of 1 mg or 0.3 mg alamethicin/mg protein, then added to an incubation medium containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 6 mM MgCl_2, 8 mM KCl, 0.4 mg/ml bovine serum albumin, 0.4 mM dithiothreitol, 0.7 mM EGTA, 2.5 mM creatine, 0.095 mg creatine kinase, 1 mM isobutyl methylxanthine, and 1 mM [32P]ATP (300,000 dpm) in a final volume of 150 μl. Samples were incubated at 30°C for various time periods, and the reaction was terminated with 100 μl ice-cold 2% sodium dodecyl sulfate containing 45 mM ATP and 1.3 mM cAMP. The [32P]cAMP formed by the reaction was separated from [32P]ATP by sequential chromatography on Dowex-50w hydrogen cation exchange and neutral alumina columns.

Separation of Prostaglandins by High-Performance Liquid Chromatography

Stability of the labeled prostaglandins under the binding conditions was determined by freezing an aliquot of the incubation medium in liquid nitrogen before filtering and subsequently separating the prostaglandin by high-performance liquid chromatography (HPLC). Experiments were performed on a Waters model 840 HPLC (Milford, Massachusetts) with a Novapak 10-μm C-18 column (Milford, Massachusetts), with an on-line detector and Berthold HPLC radioactive monitor LB 505 (Nashua, New Hampshire).

[3H]PGE_2 was separated from the incubation medium with octadecyltrichlorosilane Sep-Pak C-18 cartridges (Waters Instruments, Milford, Massachusetts) preequilibrated with 95% ethanol. Samples stored in liquid nitrogen were thawed, acidified with IN HCl, and passed through the cartridge. The cartridge was then washed with 6 ml 10% ethanol, 6 ml distilled H_2O, and 6 ml hexane. Prostaglandins were eluted from the cartridge with 6 ml acetone. The acetone was then evaporated under nitrogen, and the residue dissolved in 500 μl acetonitrile. Prostaglandins were separated with a 2-minute isocratic elution at a flow rate of 1.5 ml/min, a 5-minute gradient from 25-50% acetonitrile, and a 15-minute gradient from 50-100% acetonitrile. Under these conditions, PGE_2 eluted at 9.64 minutes, PGF_20 at 8.34 minutes, and PGD_2 at 8.97 minutes. Regardless of the radiolabeled prostaglandin used, 70-90% of the label coeluted with the corresponding unlabeled standard, thus ruling out significant degradation of the prostaglandins during the binding assay.
Results

Prostaglandin E₂ Binding

The specificity of PGE₂ binding was determined by incubation of sarcolemmal membranes with [³H]PGE₂ containing increasing concentrations of unlabeled PGE₂. As shown in Figure 2, unlabeled PGE₂ displaced labeled ligand at low concentrations (10⁻⁹ M). At high concentrations, unlabeled PGE₂ displaced greater than 90% of the label, indicating that nonspecific binding of [³H]PGE₂ was very low.

The ability of other prostaglandins to displace [³H]PGE₂ was also tested (Figure 2). Unlabeled PGE₁ displaced [³H]PGE₂ at concentrations similar to those for unlabeled PGE₂. PGF₂α (which differs from PGE₂ in that the 9-keto group is replaced by a 9-hydroxyl group) had a much lower affinity for the PGE₂ binding site. Similarly, PGD₂ also had a low affinity for the PGE₂ binding site. High concentrations of 6-keto-PGF₁α (the stable PGI₂ metabolite) were necessary to displace [³H]PGE₂ from its receptor. Using incubation conditions that stabilize PGI₂ (see "Materials and Methods"), we were unable to displace [³H]PGE₂ from its receptor with any concentration of Na · PGI₂.

To further characterize the binding of PGE₂ to sarcolemma membrane, a saturation analysis was performed, from which a Scatchard plot was constructed (Figure 3). A curvilinear plot for PGE₂ could be constructed that resolved into two distinct binding sites (Figure 3). Analysis by linear regression revealed a high-affinity site (Kᵦ of 0.32 nM, Bₘₐₓ of 376 fmol/mg protein) and a low-affinity site (Kᵦ of 3.41 nM, Bₘₐₓ of 2,112 fmol/mg protein).

To confirm that PGE₂ binding occurred at a membrane protein, sarcolemmal vesicles were digested with trypsin or papain (1 mg protease/mg protein). Trypsin pretreatment resulted in a threefold decrease in specific binding (from 423 to 144 fmol/mg protein). Papain pretreatment also resulted in a twofold decrease in specific binding (from 423 to 194 fmol/mg protein). [³H]PGE₂ binding was also determined in cardiac sarcolemmal membrane treated with various concentrations of detergents. Binding characteristics could be maintained in membrane solubilized with 8 mM 3-[[(3-cholamidopropyl)dimethylammonio]-l-propanesulfonate, 8 mM Triton X-100, or 17 mM octylglucoside (M. Michalak, unpublished observations).

Studies by Tomlins et al.²⁹ have demonstrated that cardiac sarcolemmal vesicle preparations obtained from intact heart contain some endothelial cell membrane contamination. The degree of endothelial membrane contamination was determined by measuring angiotensin-converting enzyme activity. Using this assay, we have measured endothelial membrane contamination in our preparation and found it to be less than 5% of the total membrane protein (J. Charuk, S. Howlett, and M. Michalak, unpublished observations). This raises the possibility, however, that the PGE₂ binding sites may reside on endothelial cell membrane as opposed to myocyte membrane. To test this possibility, specific binding of 2 nM [³H]PGE₂ was determined in both isolated aortic endothelial cells and in a microsomal membrane preparation from cultured endothelial cells. [³H]PGE₂ binding was found to be very low in
Prostaglandin E₂ Effects on Adenylate Cyclase Activity

The effects of PGE₂ on cardiac sarcolemmal adenylate cyclase activity were determined. To perform these experiments, sarcolemmal membranes were preincubated with the nonspecific ionophore alamethicin (0.3 mg/mg sarcolemmal protein). Alamethicin forms protein channels through the membrane and enables one to measure both prostaglandin binding localized on the extracellular surface of the membrane and adenylate cyclase activity localized on the intracellular surface of the membrane. As shown in Table 2, alamethicin pretreatment resulted in an approximately twofold increase in isoproterenol-stimulated adenylate cyclase activity. Addition of Gpp(NH)p (a nonhydrolyzable analogue of GTP) to intact or alamethin-permeabilized sarcolemma resulted in a 10-fold increase in isoproterenol-stimulated adenylate cyclase activity (Table 2).

The effects of PGE₂ on cardiac sarcolemmal adenylate cyclase activity as initially determined showed great variability. Possibly, interaction of the PGE receptor with G₁ (and G₅) proteins may become uncoupled during isolation of the cardiac sarcolemmal vesicles. Negishi et al.26 suggest that in adrenal medulla membranes, occupancy of the receptor with ligand can preserve receptor G-protein coupling. To test this possibility, sarcolemmal vesicles were prepared in the presence of 10⁻⁶ M PGE₂, then washed in the absence of PGE₂ before experimentation. PGE₂ binding experiments revealed that PGE₂ bound to the receptor prior to isolation was removed by the final washes (data not shown). Figure 5 shows the effects of PGE₂ on adenylate cyclase activity in permeabilized sarcolemmal vesicles isolated in the presence of PGE₂. PGE₂ consistently inhibited adenylate cyclase activity at concentrations of PGE₂ that occupy the high-affinity receptor.

Discussion

We have demonstrated that myocardial sarcolemmal vesicles contain high-affinity binding sites for PGE₂. Because PGE₂ predominates under physiological conditions, it is most likely this prostaglandin that binds to the sarcolemmal membrane in vivo. The source of PGE₂ remains undefined, but may originate from the heart itself. Coronary vessels are a major source of locally produced PGE₂.

**TABLE 2. Effect of Alamethicin and Gpp(NH)p on Isoproterenol-Stimulated Cardiac Sarcolemmal Adenylate Cyclase Activity**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Without isoproterenol</th>
<th>With isoproterenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Without alamethicin</td>
<td>56.3±3.7</td>
<td>155.6±12.1</td>
</tr>
<tr>
<td>+100 μM Gpp(NH)p</td>
<td>428.3±51.5</td>
<td>943.4±132.6</td>
</tr>
<tr>
<td>With alamethicin</td>
<td>132.8±4.8</td>
<td>237.2±6.3</td>
</tr>
<tr>
<td>+100 μM Gpp(NH)p</td>
<td>693.1±50.0</td>
<td>1643.5±55.1</td>
</tr>
</tbody>
</table>

Results are mean±SEM of four experiments.
that may interact with receptors on the myocyte. Scatchard analysis of PGE2 binding suggests that two distinct binding sites exist, one of high affinity and low capacity and the other of low affinity and high capacity. The two binding sites could be distinct receptors or a single receptor that changes affinity depending on the concentration of ligand present. The high-affinity binding sites for PGE2 on the sarcolemmal membrane have the same affinity as previously identified PGE2 receptors in other tissues.

PGE2 has been demonstrated to have marked effects on heart function in a number of species, including rat, cat, dog, rabbit, sheep, and humans (for review, see Karmazyn and Dhalla). Our results suggest that these effects are mediated by the binding of PGE2 to specific receptors on the myocyte itself. Species differences in the response of the heart to PGE2 may be mediated by differences in receptor binding characteristics. It is interesting to note that the existence of two classes of PGE2 binding sites appears to be conserved not only between species, but also between tissues. In recent studies, we demonstrated that PGE2 binds to bovine uterine smooth muscle with two classes of binding sites (R. Lerner, G.D. Lopaschuk, and P.M. Olley, unpublished observations). Two classes of PGE2 binding sites have also been identified in other tissue such as human platelets, hen oviduct, canine medullary membranes, and rabbit cortical collecting tubule.

Binding of PGE2 to the high-affinity sites on the sarcolemmal membrane results in an inhibition of adenylate cyclase. PGE2 probably exerts its effects on myocardial contractility and heart rate through receptors coupled to adenylate cyclase. Inhibition of adenylate cyclase by PGE2 has also been demonstrated in other tissues. Kather and Simon demonstrated that fat cell adenylate cyclase is inhibited by low concentrations of PGE2, yet is stimulated by high concentrations of PGE2. Similarly, in isolated renal cortical–collecting tubule cells, PGE2 inhibits cAMP release at low concentrations (10^-8 to 10^-6) but stimulates cAMP release at high concentrations (>10^-8). Recently, Melien et al demonstrated that PGE2 also inhibits hormone-induced cAMP accumulation in hepatocytes. The actual mechanism by which prostaglandins mediate cAMP levels, however, has not been measured directly. One reason for this is the difficulty in simultaneously accessing the prostaglandin receptor on one side of the membrane and the catalytic subunit of adenylate cyclase on the other side. In this study, we demonstrate that alamethicin-permeabilized cardiac sarcolemmal vesicles are a useful tool for studying prostaglandin receptor–response coupling. In initial studies with this preparation, the effect of PGE2 on adenylate cyclase activity varied. In a number of preparations, we found that PGE2 inhibited adenylate cyclase at low concentrations, whereas in other preparations, no effect of PGE2 was seen. The reason for this variability may involve uncoupling of the PGE receptor from the GTP binding protein(s) during isolation of the sarcolemmal vesicles. Recently, Negishi et al demonstrated that occupancy of the PGE2 receptor on renal medullar membranes by PGE2 promotes the interaction of the receptor with the GTP binding protein(s). In our studies, initial preparation of cardiac sarcolemmal vesicles in the presence of PGE2 appears to maintain the coupling between the PGE2-inhibited adenylate cyclase at concentrations that occupy the high-affinity receptor.

The suggestion that the PGE2 receptor is coupled to adenylate cyclase activity via G proteins in cardiac sarcolemmal membranes is supported by studies in other tissues. In cultured hepatocytes, the inhibitory effect of PGE2 on cAMP accumulation is abolished by pertussis toxin, which suggests the involvement of a G protein. In renal medulla, Watanabe et al demonstrated that the PGE2 receptor is coupled to pertussis toxin–sensitive G regulatory protein. GTP analogues added to a digitoninsolubilized microsomal preparation altered PGE2
binding kinetics, an effect that was reversed by pertussis toxin. Adenylate cyclase was not measured in this experimental system. In adrenal medulla, Negishi et al\(^7\) found that the PGE\(_2\) receptor can be covalently cross-linked to a GTP-binding protein, and that it is a pertussis toxin–insensitive G protein that is not coupled to adenylate cyclase. They speculate that in adrenal medulla, the PGE receptor may initiate its response via phospholipase C stimulation. In the heart, our data suggest that PGE\(_2\) (and possibly PGE\(_3\)) acts through a receptor coupled to G\(_i\), which results in inhibition of adenylate cyclase. The possibility also exists that PGE\(_2\) and PGE\(_3\) interact with other hormones by acting on common catalytic subunits of adenylate cyclase via these G proteins. This possibility remains to be determined.

PGE\(_2\) attenuates the response of adipocytes, fibroblasts, and the renal medulla to \(\beta\)-adrenergic agonists\(^3\)–\(^5\) as well as renal tissue to vasopressin.\(^1\) Strasser et al\(^6\) demonstrate that in smooth muscle cells, PGE\(_1\) promotes the translocation of a \(\beta\)-adrenergic receptor kinase from the cytosol to the plasma membrane. This translocation occurs concomitantly with homologous desensitization of the PGE\(_1\) receptor. Whether PGE receptor desensitization occurs through phosphorylation has yet to be determined. The alamethicin-permeabilized cardiac sarcolemmal preparation used in this study should prove a useful tool in investigating this.

In summary, we demonstrate that high-affinity, stereospecific binding sites for PGE\(_2\) exist on the cardiac sarcolemmal membrane. Binding of PGE\(_2\) to its receptors is coupled to adenylate cyclase, possibly via guanine regulatory proteins. Use of isolated cardiac sarcolemmal vesicles should prove useful in further characterizing the direct actions of eicosanoids on the heart.

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References


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