

Photolabelling of the prostaglandin E₂ receptor in cardiac sarcolemmal vesicles

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A [³H]azidophenacyl ester of PGE₂ ([³H]azido-PGE₂) was synthesized and used to photoaffinity label the protein component of the high affinity PGE₂ binding site in cardiac sarcolemma membrane. Photolysis of the isolated cardiac sarcolemmal vesicles in the presence of [³H]azido-PGE₂ resulted in the covalent labelling of a protein component that migrated on sodium dodecyl sulfate-polyacrylamide gels with an apparent molecular weight of 100 000. Incorporation of the [³H]azido-PGE₂ did not occur in the absence of photolysis. The photolabelling of the 100-kDa protein by [³H]azido-PGE₂ was inhibited by excess unlabelled PGE₂ and azido-PGE₂. Specific binding of [³H]azido-PGE₂ was displaced by excess unlabelled PGE₂ or azido-PGE₂, but not PGF_{2α}, 6-keto-PGF_{1α} or PGD₂. These results indicate that the 100-kDa photoaffinity labelled [³H]azido-PGE₂ binding protein contains the binding site for PGE₂ in isolated cardiac sarcolemma membranes.

Prostaglandin receptor; Cardiac sarcolemma; Photolabelling

1. INTRODUCTION

While prostaglandin E₂ (PGE₂) produces a broad range of biological responses [1-4], the exact mechanism of its actions remain unclear. The initiating process is believed to be the recognition of PGE₂ by a specific cellular receptor. High affinity [³H]PGE₂ binding sites have been extensively characterized in a variety of homogenates and isolated cells derived from tissues that are known targets for PGE₂ action [2]. In cardiac muscle direct action of PGE₂ involves alterations of glucose oxidation, myocardial O₂ consumption, myocardial triglyceride, and cAMP levels, and SL Na⁺/K⁺-dependent ATPase activity [3-8]. We have recently demonstrated that bovine heart SL vesicles contain high affinity stereo-specific binding sites for PGE₂, which are functionally coupled to adenylate cyclase [8]. The receptor concentration is relatively high (~1 pmol/mg protein) in cardiac SL vesicles, and binding is specific, since PGE₂ and PGE₁ bind to the receptor while other prostaglandins fail to bind [8]. SL vesicles may therefore be considered an excellent membrane source in which to identify and purify the PGE₂

receptor. Despite considerable interest in the function of prostaglandins, few attempts have been made to identify the membrane receptor [9-14]. Full delineation of both the properties of PGE₂ binding and the identification and subsequent isolation of the PGE₂ binding protein seems essential if the molecular basis of PGE₂ action in the heart and other tissues is to be understood.

We report here the synthesis of an azidophenacyl ester of PGE₂ (azido-PGE₂), which was used as a specific probe for the identification of the PGE₂ receptor in cardiac SL. Our results show that azido-PGE₂ is an excellent photoaffinity ligand. Direct photolabelling of isolated cardiac SL with [³H]azido-PGE₂ has allowed us to identify a 100-kDa [³H]azido-PGE₂ binding polypeptide.

2. EXPERIMENTAL

SL vesicles were isolated from bovine heart (left ventricle only) according to the procedures of Jones [15]. Vesicles were suspended in 3-5 mg/ml in 100 mM NaCl and 20 mM Hepes, pH 7.4, frozen in liquid N₂ and stored at -85°C. Protein concentration was determined by the method of Lowry et al. [16].

Unlabelled and [³H]azido-PGE₂ were synthesized as follows. Unlabelled PGE₂ (3.4 mg, 0.0097 mmol) in acetone (1 ml), was mixed with potassium carbonate (4 mg, 0.029 mmol), and heated at 40°C under an atmosphere of argon for 40 min. The resulting mixture was stirred with *p*-azidophenacyl bromide (ICN Biochemicals, Cleveland, OH) (2.5 mg, 0.01 mmol) at 40°C for 60 min. The mixture was subsequently cooled to room temperature, acidified with aqueous 1 N HCl, and extracted with methylene chloride. Extracts were dried (MgSO₄), filtered and concentrated. The residue was purified by flash

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Abbreviations: PG, prostaglandin; SL, sarcolemma; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; azido-PGE₂, azidophenacyl ester of PGE₂

chromatography on silica gel eluting with a solution of chloroform:ethyl acetate:methanol (6:5:1) containing a small amount of acetic acid (3 drops/12 ml) and water (1 drop/12 ml) to give *p*-azidophenacyl ester of PGE₂ (azido-PGE₂): ir (CH₂Cl₂ cast) 3374 (OH), 2126 (N₃), 1744 (C=O), 1701 (C=O), and 1598 (aromatic) cm⁻¹; ¹H NMR (CDCl₃) δ 7.92, 7.11 (both d, 2H each, *J* = 9 Hz each, aromatic), 5.72 (dd, 1H, *J* = 15, *J'* = 6 Hz, -CH=), 5.60 (dd, 1H, *J* = 15, *J'* = 8 Hz, -CH=), 5.40 (m, 2H, 2 × -CH=), 5.31, 5.30 (both s, 2H each, -OCH₂- and 2 × -OH), 4.11 (m, 2H, 2 × -CHOH), and 0.89 (t, 3H, *J* = 7 Hz, -CH₃). The same method was applied for the synthesis of *p*-azidophenacyl ester of [5, 6, 8, 11, 12, 14, 15 - ³H (N)]PGE₂. The fractions obtained from silica gel (0.7 ml) were monitored by liquid scintillation counting. Radioactive fractions were combined, concentrated in a Speed-Vac Concentrator (Sevant Instruments), dissolved in dimethylsulfoxide and used for photolysis experiments.

Competition of [³H]PGE₂ and [³H]azido-PGE₂ binding was carried out as described by Lopaschuk et al. [8]. For photolysis, the SL vesicles were incubated with 18 nM [³H]azido-PGE₂ in the presence or absence of unlabelled PGE₂ or azido-PGE₂, in a buffer containing 100 mM NaCl, 0.1 mM EGTA, and 20 mM Hepes, pH 7.4 at 37°C for 60 min. The binding reaction was carried out in the dark to prevent any undesired reactions from laboratory light when azido-PGE₂ was used. All photolysis experiments were done in an RPR 208 preparative reactor (Rayonet, The Southern New England Ultraviolet Co., Middletown, CN) equipped with RPR 3500-Å lamps. With the reactor at 4°C (cold room), the air temperature surrounding the sample was maintained at 4°C by an electric fan. In order to prevent the possible destruction of sensitive sulfur or aromatic amino acid in proteins during the photolysis, cross-linking was carried out for 10 min using an irradiation source at 350 nm light [16]. SDS-PAGE was performed using 5–15% acrylamide gradient gels according to the procedure of Laemmli [17]. Gels were stained with Coomassie Blue, and destained in a solution of 10% acetic acid and 5% methanol. [³H]azido-PGE₂ incorporation was detected by fluorography and/or liquid scintillation counting of gel slices. Gel slices (2 mm) were incubated overnight at 37°C in 0.2 ml of Protosol before liquid scintillation fluid was added. Gels used for fluorography were treated with Amplify before drying and then exposed at -85°C using Kodak XAR-5 film. Molecular weight standards used with size in parentheses were: myosin (200 kDa); β-galactosidase (116 kDa); phosphorylase b (97 kDa); bovine serum albumin (66 kDa); ovalbumin (42 kDa).

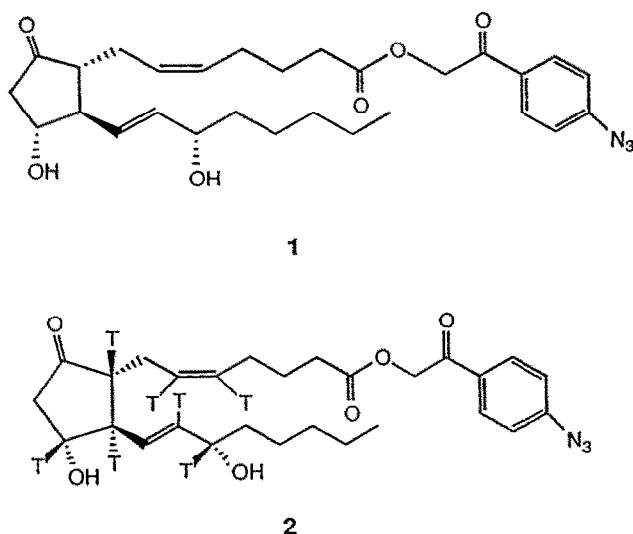


Fig.1. Structure of azido-PGE₂ (azidophenacyl ester of PGE₂). Azido-PGE₂ (1) and [³H]azido-PGE₂ (2) were synthesized as described under Experimental. T denotes positions of [³H].

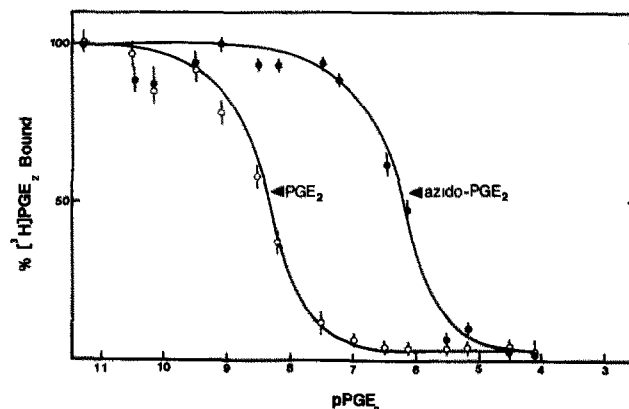


Fig.2. Competition of [³H]PGE₂ binding to cardiac SL vesicles by unlabelled PGE₂ and azido-PGE₂. Competition of [³H]PGE₂ binding was carried out in the presence of varying concentrations of unlabelled PGE₂ or azido-PGE₂ as described under Experimental. 100% represents total binding to the SL vesicles at 2 nM [³H]PGE₂ (1.2 pmol/mg protein). Average values ± SE of three experiments are presented.

3. RESULTS AND DISCUSSION

Fig. 1 shows the chemical structure of the azido-PGE₂ used in this study. To compare the properties of the azido-PGE₂ with those of PGE₂, we examined the effect of azido-PGE₂ on specific binding of [³H]PGE₂ to the SL vesicles. [³H]PGE₂ binding was inhibited by unlabelled PGE₂ with an IC₅₀ value of approximately 5 × 10⁻⁹ M (Fig. 2). Azido-PGE₂ competed with [³H]PGE₂ binding to SL vesicles although it was a less effective inhibitor of this binding than unlabelled PGE₂. The IC₅₀ value for azido-PGE₂ was approximately 4 × 10⁻⁷ M (Fig. 2). Differences between the chemical structure of PGE₂ and azido-PGE₂ likely account for the reduced ability of azido-PGE₂ to compete for [³H]PGE₂ bound to SL. This conclusion is supported by the relative ability of other PGs to displace [³H]PGE₂ from SL vesicles [8]. In cardiac SL vesicles [³H]PGE₂ binding is significantly inhibited by unlabelled PGE₁, but only weakly by PGF_{2α}, 6-keto-PGF_{1α}, and PGD₂ [8]. The inability of PGF_{2α} to compete with

Table I

Effects of different prostaglandins on azido-PGE₂ binding to cardiac SL vesicles

Prostaglandins	[³ H]azido-PGE ₂ binding	
	(pmol/mg protein)	(% of control)
none	0.50	(100)
PGE ₂	0.14	(28)
azido-PGE ₂	0.08	(16)
PGF _{2α}	0.46	(92)
6-keto-PGF _{1α}	0.46	(92)
PGD ₂	0.45	(90)

[³H]azido-PGE₂ binding was carried out in the presence of 500 μM concentrations of unlabelled PGE₂, azido-PGE₂, PGF_{2α}, 6-keto-PGF_{1α}, and PGD₂, as described under Experimental.

[^3H]PGE $_2$ binding sites in SL vesicles [8] is a good example of how a small change in chemical structure (substitution of the keto group at carbon-9 in PGE $_2$ with a hydroxyl group) significantly alters binding activity. In contrast, substitution of the COOH results in relatively much smaller change in affinity, probably due to steric hindrance, rather than an alteration in the binding site of PGE $_2$.

In order to characterize the specificity of the binding of azido-PGE $_2$ to SL the effects of PGF $_{2\alpha}$, 6-keto-PGF $_{1\alpha}$, and PGD $_2$ on [^3H]azido-PGE $_2$ binding to the membrane was studied. Table I shows that both unlabelled PGE $_2$ and azido-PGE $_2$ produced significant inhibition of [^3H]azido-PGE $_2$ binding to the membrane. PGF $_{2\alpha}$, 6-keto-PGF $_{1\alpha}$, and PGD $_2$ failed to displace more than 10% of [^3H]azido-PGE $_2$ from SL vesicles (Table I). These results show that [^3H]azido-PGE $_2$ binding to SL occurred at a membrane protein that also binds PGE $_2$.

Fig. 3 shows the [^3H]azido-PGE $_2$ labelling profile obtained for the cardiac SL vesicles following photolysis. A major peak of [^3H]azido-PGE $_2$ incorporation was observed into a 100-kDa protein band, along with a broad peak of [^3H]azido-PGE $_2$ incorporation at the dye front (Fig. 3). This broad peak probably represents either free [^3H]azido-PGE $_2$ or nonspecific incorporation of [^3H]azido-PGE $_2$ into membrane phospholipids. When aliquots of the same membranes were photolysed in the presence of unlabelled PGE $_2$ (10^{-3} M) (Fig. 3B) or azido-PGE $_2$ (10^{-3} M) (Fig. 3C) the amount of covalently bound [^3H]azido-PGE $_2$ in the 100-kDa protein was decreased by approximately 70%. In the absence of photolysis, no incorporation of [^3H]azido-PGE $_2$ was observed (Fig. 3A). If excess unlabelled PGE $_2$ was added after photolysis, as opposed to being present during photolysis, no alteration in the amount of [^3H]azido-PGE $_2$ found in the 100-kDa protein band was seen (data not shown). Based on the displacement

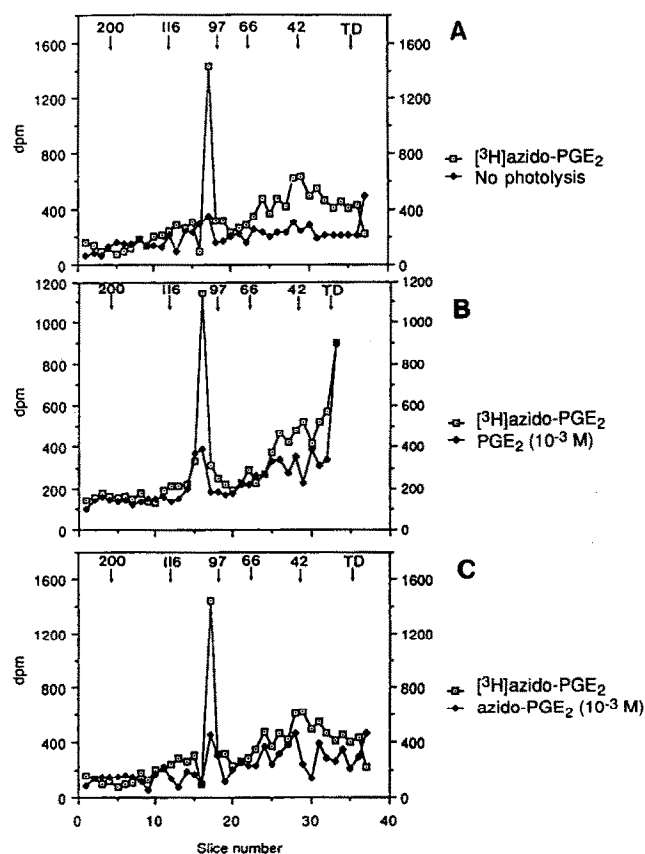


Fig. 3. Photoaffinity labelling of cardiac SL membrane with [^3H]azido-PGE $_2$. Cardiac SL vesicles were photolabelled with [^3H]azido-PGE $_2$ in the absence or presence of unlabelled PGE $_2$ (10^{-3} M) (B) or azido-PGE $_2$ (10^{-3} M) (C) as described under Experimental. (A) no photolysis. Samples (30 μg of protein) were subjected to SDS-PAGE. Following staining and destaining, gel lanes were sliced in 2 mm sections and [^3H]azido-PGE $_2$ incorporation was measured by liquid scintillation counting. Arrows depict the location of molecular weight standards and tracking dye (TD). Numbers represent $M_r \times 10^3$.

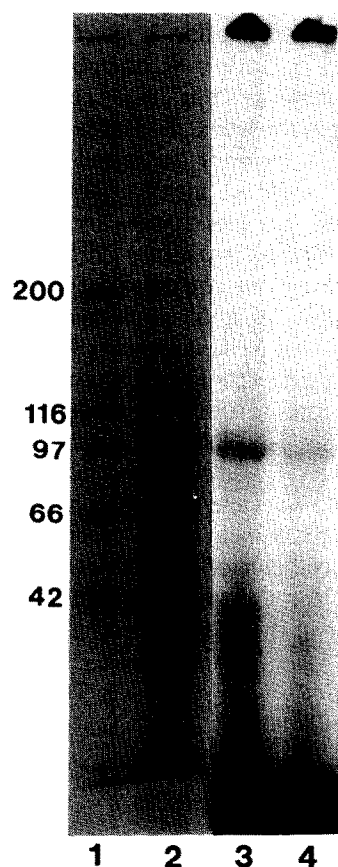


Fig. 4. Photolabelling of cardiac sarcolemma membranes with [^3H]azido-PGE $_2$. Cardiac sarcolemmal vesicles (1 mg of protein/ml) were photolabelled with [^3H]azido-PGE $_2$ in the absence or presence of unlabelled PGE $_2$ (10^{-3} M) as described under Experimental. Samples (30 μg of protein). Following staining and destaining, the slab gel was then treated with Amplify and exposed to X-ray film. Lanes 1 and 2 are Coomassie blue stained molecular weight standards and proteins of cardiac sarcolemmal vesicles, respectively. Numbers represent $M_r \times 10^3$.

of labelled azido-PGE₂ in Fig. 3C we determined that photolabelling of the 100-kDa protein in SL membranes with [³H]azido-PGE₂ was more than 70% specific. This is in agreement with photolabelling of β -adrenergic receptor molecules [18,19]. The autoradiogram of [³H]azido-PGE₂ incorporation obtained for the same labelled membranes as used in Fig. 3 is shown in Fig. 4. [³H]azido-PGE₂ was predominantly incorporated into one protein band of molecular weight of about 100 000 (Fig. 4, lane 3). Addition of unlabelled PGE₂ (10⁻³ M) to the incubation buffer before photolysis greatly reduces the incorporation of [³H]azido-PGE₂ into the 100,000 dalton protein band (Fig. 4, lane 4).

The data presented here are the first direct identification of the PGE₂ binding protein (receptor) using a photoaffinity derivative of PGE₂. The results of our experiments demonstrate that [³H]azido-PGE₂ is an excellent photoaffinity ligand for the high-affinity PGE₂ binding sites in isolated cardiac SL membranes, and therefore should be useful in identifying PGE₂ receptors in other tissues. Using [³H]azido-PGE₂, we show that a 100-kDa polypeptide is specifically labelled by this compound in cardiac SL membranes. We believe that this 100-kDa protein band corresponds to the PGE₂ receptor in the heart. The molecular weight of the cardiac PGE₂ receptor assessed by the direct photolabelling method in this study resembles the values obtained in other tissues by indirect methods [9–12]. PGE₂ binding protein in rat liver plasma membrane [9], murine macrophage-like cells [10], bovine adrenal medulla [11] and canine renal outer medulla [12] was postulated to be a protein of molecular weight between 65 000 and 110 000. We have recently isolated the 100-kDa protein from bovine heart SL membranes and showed that it specifically binds both [³H]PGE₂ and [³H]azido-PGE₂ (unpublished observations).

In summary, we have identified a 100-kDa protein from cardiac SL which binds specifically [³H]azido-PGE₂. The specificity of [³H]azido-PGE₂ provides an excellent tool for the identification and isolation of PGE₂ receptor in the heart and other tissues.

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