Photolabelling of the prostaglandin E_2 receptor in cardiac sarcolemmal vesicles

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A [^{3}H]azidophenacyl ester of PGE_2 ([^{3}H]azido-PGE_2) was synthesized and used to photoaffinity label the protein component of the high affinity PGE_2 binding site in cardiac sarcolemma membrane. Photolysis of the isolated cardiac sarcolemmal vesicles in the presence of [^{3}H]azido-PGE_2 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 [^{3}H]azido-PGE_2 did not occur in the absence of photolysis. The photolabelling of the 100-kDa protein by [^{3}H]azido-PGE_2 was inhibited by excess unlabelled PGE_2 and azido-PGE_2. Specific binding of [^{3}H]azido-PGE_2 was displaced by excess unlabelled PGE_2 or azido-PGE_2, but not $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$ or PGD_2. These results indicate that the 100-kDa photoaffinity labelled [^{3}H]azido-PGE_2 binding protein contains the binding site for PGE_2 in isolated cardiac sarcolemma membranes.

Prostaglandin receptor; Cardiac sarcolemma; Photolabelling

1. INTRODUCTION

While prostaglandin E_2 (PGE_2) 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 PGE_2 by a specific cellular receptor. High affinity [^{3}H]PGE_2 binding sites have been extensively characterized in a variety of homogenates and isolated cells derived from tissues that are known targets for PGE_2 action [2]. In cardiac muscle direct action of PGE_2 involves alterations of glucose oxidation, myocardial O_2 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 PGE_2, which are functionally coupled to adenylate cyclase [8]. The receptor concentration is relatively high ($\sim$ 1 pmol/mg protein) in cardiac SL vesicles, and binding is specific, since PGE_2 and PGE_1 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 PGE_2 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 PGE_2 binding and the identification and subsequent isolation of the PGE_2 binding protein seems essential if the molecular basis of PGE_2 action in the heart and other tissues is to be understood.

We report here the synthesis of an azidophenacyl ester of PGE_2 (azido-PGE_2), which was used as a specific probe for the identification of the PGE_2 receptor in cardiac SL. Our results show that azido-PGE_2 is an excellent photoaffinity ligand. Direct photolabelling of isolated cardiac SL with [^{3}H]azido-PGE_2 has allowed us to identify a 100-kDa [^{3}H]azido-PGE_2 binding polypeptide.

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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-PGE_2, azidophenacyl ester of PGE_2

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 N_2 and stored at -85°C. Protein concentration was determined by the method of Lowry et al. [16].

Unlabelled and [^{3}H]azido-PGE_2 were synthesized as follows. Unlabelled PGE_2 (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 ($MgSO_4$), filtered and concentrated. The residue was purified by flash

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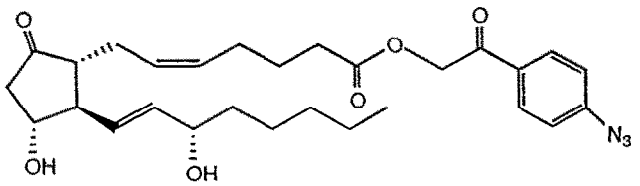
chromatography on silica gel eluting with a solution of chloroform:ethyl acetate:methanol (6:5:1) containing a small amount of acetic acid (3 drops/12 ml) and water (1 drop/12 ml) to give *p*-azidophenacyl ester of PGE_2 (azido-PGE_2): ir (CH_2Cl_2 cast) 3374 (OH), 2126 (N_3), 1744 (C=O), 1701 (C=O), and 1598 (aromatic) cm^{-1}; ^1H NMR ($CDCl_3$) 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, $-OCH_2-$ and 2 × -OH), 4.11 (m, 2H, 2×-CHOH), and 0.89 (t, 3H, J = 7 Hz, $-CH_3$). The same method was applied for the synthesis of *p*-azidophenacyl ester of [5, 6, 8, 11, 12, 14, 15 - ^3H (N)]PGE_2. 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]PGE_2 and [^3H]azido-PGE_2 binding was carried out as described by Lopaschuk et al. [8]. For photolysis, the SL vesicles were incubated with 18 nM [^3H]azido-PGE_2 in the presence or absence of unlabelled PGE_2 or azido-PGE_2, 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-PGE_2 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-PGE_2 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).



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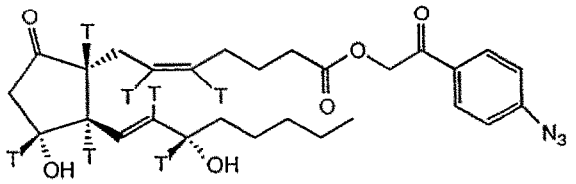

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Fig.1. Structure of azido-PGE_2 (azidophenacyl ester of PGE_2). Azido-PGE_2 (1) and [^3H]azido-PGE_2 (2) were synthesized as described under Experimental. T denotes positions of [^3H].

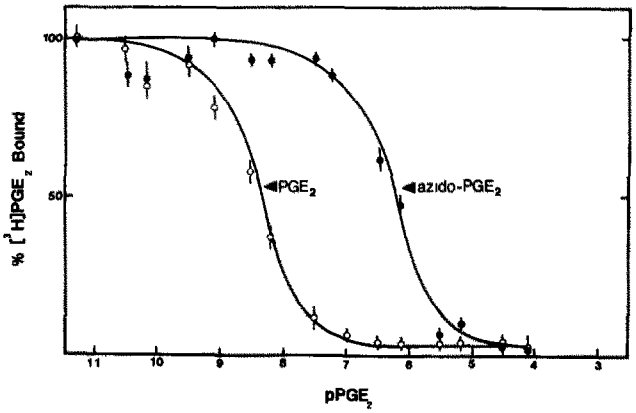

Fig.2. Competition of [^3H]PGE_2 binding to cardiac SL vesicles by unlabelled PGE_2 and azido-PGE_2. Competition of [^3H]PGE_2 binding was carried out in the presence of varying concentrations of unlabelled PGE_2 or azido-PGE_2 as described under Experimental. 100% represents total binding to the SL vesicles at 2 nM [^3H]PGE_2 (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-PGE_2 used in this study. To compare the properties of the azido-PGE_2 with those of PGE_2, we examined the effect of azido-PGE_2 on specific binding of [^3H]PGE_2 to the SL vesicles. [^3H]PGE_2 binding was inhibited by unlabelled PGE_2 with an IC_{50} value of approximately 5×10^{-9} M (Fig. 2). Azido-PGE_2 competed with [^3H]PGE_2 binding to SL vesicles although it was a less effective inhibitor of this binding than unlabelled PGE_2. The IC_{50} value for azido-PGE_2 was approximately 4×10^{-7} M (Fig. 2). Differences between the chemical structure of PGE_2 and azido-PGE_2 likely account for the reduced ability of azido-PGE_2 to compete for [^3H]PGE_2 bound to SL. This conclusion is supported by the relative ability of other PGs to displace [^3H]PGE_2 from SL vesicles [8]. In cardiac SL vesicles [^3H]PGE_2 binding is significantly inhibited by unlabelled PGE_1, but only weakly by $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and PGD_2 [8]. The inability of $PGF_{2\alpha}$ to compete with

Table I

Effects of different prostaglandins on azido-PGE_2 binding to cardiac SL vesicles

Prostaglandins	[^3H]azido-PGE_2 binding	
	(pmol/mg protein)	(% of control)
none	0.50	(100)
PGE_2	0.14	(28)
azido-PGE_2	0.08	(16)
$PGF_{2\alpha}$	0.46	(92)
6-keto-$PGF_{1\alpha}$	0.46	(92)
PGD_2	0.45	(90)

[^3H]azido-PGE_2 binding was carried out in the presence of 500 μM concentrations of unlabelled PGE_2, azido-PGE_2, $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and PGD_2, as described under Experimental.

[^{3}H]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 [^{3}H]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 [^{3}H]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 [^{3}H]azido-PGE$_2$ from SL vesicles (Table I). These results show that [^{3}H]azido-PGE$_2$ binding to SL occurred at a membrane protein that also binds PGE$_2$.

Fig. 3 shows the [^{3}H]azido-PGE$_2$ labelling profile obtained for the cardiac SL vesicles following photolysis. A major peak of [^{3}H]azido-PGE$_2$ incorporation was observed into a 100-kDa protein band, along with a broad peak of [^{3}H]azido-PGE$_2$ incorporation at the dye front (Fig. 3). This broad peak probably represents either free [^{3}H]azido-PGE$_2$ or nonspecific incorporation of [^{3}H]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 [^{3}H]azido-PGE$_2$ in the 100-kDa protein was decreased by approximately 70%. In the absence of photolysis, no incorporation of [^{3}H]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 [^{3}H]azido-PGE$_2$ found in the 100-kDa protein band was seen (data not shown). Based on the displacement

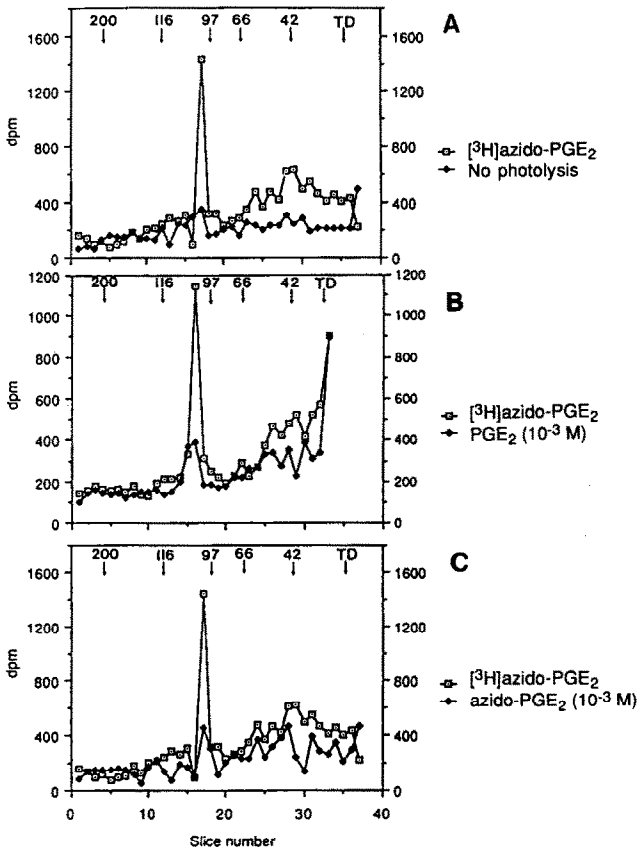

Fig.3. Photoaffinity labelling of cardiac SL membrane with [^{3}H]azido-PGE$_2$. Cardiac SL vesicles were photolabelled with [^{3}H]azido-PGE$_2$ in the absence or presence of unlabelled PGE$_2$ (10^{-3} M) (B) or azido-PGE$_2$ (10^{-3} M) (C) as described under Experimental. (A) no photolysis. Samples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

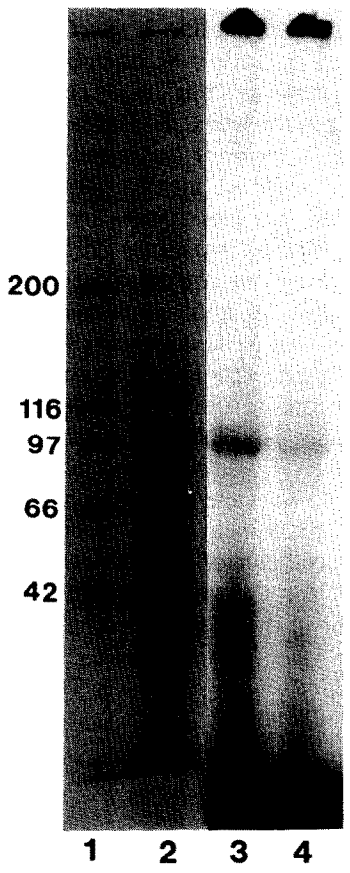

Fig.4. Photolabelling of cardiac sarcolemma membranes with [^{3}H]azido-PGE$_2$. Cardiac sarcolemmal vesicles (1 mg of protein/ml) were photolabelled with [^{3}H]azido-PGE$_2$ in the absence or presence of unlabelled PGE$_2$ (10^{-3} M) as described under Experimental. Samples (30 μg of protein). Following 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-PGE_2 in Fig. 3C we determined that photolabelling of the 100-kDa protein in SL membranes with [^{3}H]azido-PGE_2 was more than 70% specific. This is in agreement with photolabelling of β-adrenergic receptor molecules [18,19]. The autoradiogram of [^{3}H]azido-PGE_2 incorporation obtained for the same labelled membranes as used in Fig. 3 is shown in Fig. 4. [^{3}H]azido-PGE_2 was predominantly incorporated into one protein band of molecular weight of about 100 000 (Fig. 4, lane 3). Addition of unlabelled PGE_2 (10^{-3} M) to the incubation buffer before photolysis greatly reduces the incorporation of [^{3}H]azido-PGE_2 into the 100,000 dalton protein band (Fig. 4, lane 4).

The data presented here are the first direct identification of the PGE_2 binding protein (receptor) using a photoaffinity derivative of PGE_2. The results of our experiments demonstrate that [^{3}H]azido-PGE_2 is an excellent photoaffinity ligand for the high-affinity PGE_2 binding sites in isolated cardiac SL membranes, and therefore should be useful in identifying PGE_2 receptors in other tissues. Using [^{3}H]azido-PGE_2, 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 PGE_2 receptor in the heart. The molecular weight of the cardiac PGE_2 receptor assessed by the direct photolabelling method in this study resembles the values obtained in other tissues by indirect methods [9–12]. PGE_2 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 [^{3}H]PGE_2 and [^{3}H]azido-PGE_2 (unpublished observations).

In summary, we have identified a 100-kDa protein from cardiac SL which binds specifically [^{3}H]azido-PGE_2. The specificity of [^{3}H]azido-PGE_2 provides an excellent tool for the identification and isolation of PGE_2 receptor in the heart and other tissues.

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REFERENCES

[1] Samuelsson, B., Goldyne, M., Greenstrom, E., Hamberg, M., Hammarstrom, S. and Malmsten, C. (1978) Annu. Rev. Biochem. 47, 997–1029.
[2] Robertson, R.P. (1986) Prostaglandins 31, 395–411.
[3] Karamazyn, M. and Dhalla, N.S. (1983) Can. J. Physiol. Pharmacol. 61, 1207–1225.
[4] Moncada, S., Flower, R.J. and Vane, J.R. (1985) in: The Pharmacological Basis of Therapeutics (Gilman, A.G., Goodman, L.S., Rall, T.W. and Murad, F., eds) MacMillan Publishing Co., New York, pp. 660–673.
[5] Glaviano, V. and Masters, T. (1971) Am. J. Physiol. 220, 1187–1193.
[6] Klein, I. and Levey, G. (1971) Metabolism 29, 890–896.
[7] Buxtan, I.L.O. and Brunton, L.L. (1983) J. Biol. Chem. 258, 10233–10239.
[8] Lopaschuk, G.D., Michalak, M., Wandler, E., Lerner, R.W., Piscione, T.D., Coceani, F. and Olley, P.M. (1988) Circ. Res. 65, 538–545.
[9] Smigel, M. and Fleischer, S. (1977) J. Biol. Chem. 252, 3689–3696.
[10] Fernandez-Botran, R. and Suzuki, T. (1984) J. Immunol. 133, 2662–2667.
[11] Negishi, M., Ito, S., Tanaka, T., Yokohama, H., Hayaishi, H., Katada, T., Ui, M. and Hayashi, O. (1987) J. Biol. Chem. 262, 12077–12084.
[12] Watanabe, T., Umega, K. and Smith, W.L. (1986) J. Biol. Chem. 261, 13430–13439.
[13] Kylden, U. and Hammarstrom, S. (1980) Eur. J. Biochem. 109, 489–494.
[14] Dutta-Roy, A.K. and Singha, A.K. (1987) J. Biol. Chem. 262, 12685–12691.
[15] Jones, L.R. (1988) Methods Enzymol. 157, 85–91.
[16] Chong, P.C.S. and Hodges, R.S. (1981) J. Biol. Chem. 256, 5064–5070.
[17] Laemmli, U.K. (1970) Nature (Lond.) 227, 680–685.
[18] Rashidbaigi, A. and Ruoho, A.E. (1981) Proc. Natl. Acad. Sci. USA 78, 1609–1613.
[19] Rashidbaigi, A. and Ruoho, A.E. (1982) Biochem. Biophys. Res. Commun. 106, 139–148.