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Solubilization and purification of the prostaglandin E_2 receptor from cardiac sarcolemma

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A prostaglandin E_2 (PGE_2) receptor was solubilized and isolated from cardiac sarcolemma membranes. Its binding characteristics are almost identical to those of the membrane bound receptor. [^{3}H]PGE_2 binding to solubilized and membrane bound receptor was sensitive to elevated temperature and no binding was observed in the absence of NaCl. No significant effects of DTT, ATP, Mg^{2+}, Ca^{2+} or of changes in buffer pH were observed on [^{3}H]PGE_2 binding to either solubilized or membrane-bound receptor. Unlabelled PGE_1 displaced over 90% of [^{3}H]PGE_2 from the CHAPS-solubilized receptor. PGD_2, PGI_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ were not effective in displacing [^{3}H]PGE_2 from the receptor. Scatchard analysis of [^{3}H]PGE_2 binding to CHAPS-solubilized receptor revealed the presence of two types of PGE_2 binding sites with K_d of 0.33 ± 0.05 nM and 3.00 ± 0.27 nM and B_{max} of 0.5 ± 0.04 and 2.0 ± 0.1 pmol/mg of protein. The functional PGE_2 receptor was isolated from CHAPS-solubilized SL membrane using two independent methods: first by a WGA-Sepharose chromatography and second by sucrose gradient density centrifugation. Receptor isolated by these two methods bound [^{3}H]PGE_2. Unlabelled PGE_1 and PGF_2 displaced [^{3}H]PGE_2 from the purified receptor. Scatchard analysis of [^{3}H]PGE_2 binding to purified receptor revealed the presence of the two binding sites as observed for the membrane bound and CHAPS-solubilized receptor. SDS-polyacrylamide gel electrophoresis of the purified receptor fractions revealed the presence of a protein band of M_r of approx. 100000. This 100-kDa was photolabelled with [^{3}H]azido-PGE_2, a photoactive derivative of PGE_2. We propose that this 100-kDa protein is a cardiac PGE_2 receptor.

Introduction

The prostaglandins (PG) of the E series induce a broad range of biological responses in variety of various tissues including heart [1–7]. We have recently demonstrated that bovine heart sarcolemmal (SL) vesicles contain high affinity binding sites for PGE_2 [5]. Two binding sites for PGE_2 were identified one with a K_d of 0.32 nM and a B_{max} of 0.4 pmol/mg of protein, and the second with a K_d of 3.41 nM and a B_{max} of 2.1 pmol/mg of protein [5]. Depending on conditions, PGE_2 either stimulates or inhibits cAMP metabolism [1–9] which suggests that the PGE_2 receptor is coupled to adenylate cyclase. PGE_2 binding to cardiac SL membrane markedly inhibits adenylate cyclase activity indicating that PGE_2 receptor in the heart is also coupled to adenylate cyclase [5]. In the adrenal medulla, however, PGE_2 neither activates nor inhibits adenylate cyclase [10], and therefore may not be coupled to adenylate cyclase. Until the PGE_2 receptor can be isolated and its structure determined, the mechanism of the varied effects of PGE_2, attributed to specific receptor stimulation, will remain in question.

Few attempts have been made to identify the prostaglandin receptor [9–14]. A putative PGE_2 receptor has been identified and partially purified from bovine adrenal medulla as a glycoprotein with M_r of approx. 110000 as determined by either size-exclusion gel chromatography [13] or protein cross-linking experiments [10]. In contrast, a PGE_2 receptor isolated from canine renal medulla was reported to be a glycoprotein with a M_r of 65000 [9]. PGE_2 binding proteins in rat

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Abbreviations: SL, sarcolemma; PG, prostaglandin; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; WGA, wheat germ agglutinin; GlcNAc, *N*-acetyl-D-glucosamine; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*',*N*'-tetraacetic acid.; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid; octyl glucoside, 1-*O*-n-octyl β-D-glucopyranoside; Tris, tris(hydroxymethyl)aminomethane.

liver plasma membrane [11] and murine macrophage-like cells [14] were postulated to be proteins of M_r of 105 000 and 95 000, respectively. The differences in M_r of these receptors may indicate either structural variants or differences in tissues used in those studies. One limitation of previous attempts to isolate the receptor was that the receptor was functionally inactivated during purification. Further characterization of these receptor proteins requires that the PGE_2 receptor be purified in a functionally intact state.

We have recently reported 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 [15]. Direct photolabelling of isolated cardiac SL with [^{3}H]azido-PGE_2 allowed us to identify a 100-kDa [^{3}H]azido-PGE_2 binding polypeptide, which we proposed was a cardiac PGE_2 receptor [15]. Here, we report, the first direct solubilization and purification of a PGE_2 receptor from cardiac SL membranes. The [^{3}H]PGE_2 binding characteristics of the solubilized receptor were found to be identical to those of the membrane-bound receptor [5]. The functional PGE_2 receptor was isolated from CHAPS-solubilized SL membrane by two independent methods: WGA-Sepharose affinity chromatography or sucrose density gradient centrifugation. The isolated receptor is a 100-kDa protein, which binds [^{3}H]PGE_2 and can be photolabelled with [^{3}H]azido-PGE_2.

Experimental procedures

Materials

Hepes, histidine, Tris, lectins, trypsin, soyabean trypsin inhibitor, Triton X-100, CHAPS, WGA-Sepharose and GlcNAc were purchased from Sigma. [^{3}H]PGE_2 (185 Ci/mmol) and Protosol were obtained from New England Nuclear. Octyl glucoside was obtained from Boehringer Mannheim. Sephadex G-50 was from Pharmacia. $PGF_{2\alpha}$, 6-keto-$PGF_{1\alpha}$, and PGD_2 were from Cayman Chemicals, Ann Arbor, MI. SDS-PAGE reagent and molecular weight markers were purchased from Bio-Rad. Fresh bovine hearts were obtained from a local slaughterhouse.

Preparation of membrane vesicles

SL membranes were isolated by sucrose flotation essentially according to the method of Ref. 16, in the presence of a mixture of proteinase inhibitors as described by Ref. 15. Isolated vesicles were suspended at a concentration of 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. The SL vesicles were tightly sealed and possessed Ca^{2+}/calmodulin-dependent ATPase activity, electrogenic Na^+/Ca^{2+} exchange activity and adenylate cyclase activity, as described earlier [15–17]. Before use, samples of frozen membrane were thawed at 37°C. Protein was determined by the method of either Lowry et al. [18] or Bradford [19].

Membrane solubilization

SL vesicles (2 mg of protein/ml) were solubilized in a buffer containing 100 mM NaCl, 0.1 mM EGTA, 20 mM Hepes (pH 7.4) and different amounts of either CHAPS, Triton X-100 or octyl glucoside. Two concentrations (5 mg/mg of protein or 50 mg/mg of protein) of each detergent were tested (Table I). After incubation at 4°C for 30 min nonsoluble material was separated by centrifugation at 150 000 × g for 45 min at 4°C in a Ti70.1 rotor using a Beckman L8–70 centrifuge. The clear supernatant was removed and used as a source of solubilized PGE_2 receptor.

Binding assays

[^{3}H]PGE_2 binding to SL vesicles was carried out for 60 min at 37°C in 200 μl of medium containing 30 μg of SL protein, 100 mM NaCl, 20 mM Hepes (pH 7.4) and 2 nM [^{3}H]PGE_2. The reaction was terminated by filtration on to Whatman GF/C filters, followed by three 5-ml washes with ice-cold buffer containing 100 mM NaCl and 20 mM Hepes (pH 7.4). Filters were dried and counted using standard liquid scintillation techniques. [^{3}H]PGE_2 binding to solubilized samples was slightly modified as follows. The binding assay medium (200 μl) contained 0.4–80 μg of protein depending on the enrichment, 1 mM DTT, 0.1 mM EGTA, 100 mM NaCl, 5 mg CHAPS/ml and 20 mM Hepes (pH 7.4), and various concentrations of [^{3}H]PGE_2. The unbound PGE_2 was separated from the protein bound PGE_2 using one of two different methods: filtration (for nonsoluble residue or SL vesicles) or chromatography centrifugation (for solubilized and purified receptor) [20]. In the filtration method 160-μl aliquots were filtrated through Whatman GF/C filters and washed three times with 5 ml of cold washing buffer containing 100 mM NaCl and 20 mM Hepes (pH 7.4). The counts retained in the filters were determined using standard liquid scintillation counting techniques. In the chromatography-centrifugation procedure, 100 μl of sample was loaded onto a 1 ml packed Sephadex G-50 column equilibrated in binding buffer and centrifuged for 2 min at 2000 × g. Aliquots of the column effluent were assayed for both radioactivity and protein concentration. Specific binding of PGE_2 was defined as the difference between total binding (with [^{3}H]PGE_2 alone) and nonspecific binding (with [^{3}H]PGE_2 and 1 μM unlabeled PGE_2). Results are the average of duplicate or triplicate samples differing by less than 8%.

Scatchard analysis was carried out using the LIGAND program [21]. Preferance for one or two binding sites was determined both by the 'Runs' test (pass/fail)

and the partial F-test, with $P \leqq 0.05$ considered significant. Student's t-test was used to determine the difference between paired groups, with $P \leqq 0.05$ considered significant.

WGA affinity chromatography

[^{3}H]PGE_2-labelled SL membrane proteins were solubilized with CHAPS as described above and $MgCl_2$ and NaCl, final concentrations of 2 mM and 500 mM, respectively, were added to the solubilized membranes. The CHAPS-solubilized membrane was then loaded onto a WGA-Sepharose column as described by Michalak et al. [22]. The column was first washed with 40 ml of 20 mM Hepes (pH 7.4) buffer containing 500 mM NaCl, 0.1 mM EGTA, 2 mM $MgCl_2$, and 0.05 mg CHAPS/ml, followed by 40 ml of 20 mM Hepes (pH 7.4) buffer containing 100 mM NaCl, 0.1 mM EGTA, 2 mM $MgCl_2$, and 0.05 mg CHAPS/ml. The final 3 ml of wash was found never to contain more than background absorbance at 280 nm. Bound PGE_2 receptor was eluted with 10 ml of 0.02% SDS in 20 mM Hepes (pH 7.4) buffer containing 100 mM NaCl, 0.1 mM EGTA, 2 mM $MgCl_2$, and 0.05 mg CHAPS/ml. Approx. 90% of the bound counts eluted in the first 2 ml. Glycoproteins remaining in the column were eluted using 10 ml of 20 mM Hepes (pH 7.4) buffer containing 200 mM GlcNAc, 100 mM NaCl, 0.1 mM EGTA, 2 mM $MgCl_2$, and 0.05 mg CHAPS/ml. In control experiments free [^{3}H]PGE_2 was chromatographed on WGA-Sepharose column with no binding of [^{3}H]PGE_2 to the WGA-Sepharose column being observed (data not shown).

Fractions eluted from the WGA-Sepharose column (approx. 500 μl) were assayed by liquid scintillation counting, 280 nm absorbance determination, and SDS-PAGE [23]. Alternatively, unlabelled, CHAPS-solubilized SL proteins were applied to WGA-Sepharose and the PGE_2 receptor fractionated as described above. The 0.02% SDS fraction containing PGE_2 receptor was concentrated down to 500 μl using Aminco Ultrafiltration Cell Model 8200, and assayed for both protein by the Bradford method [19] and [^{3}H]PGE_2 binding by the chromatography-centrifugation method.

Sucrose density gradient centrifugation

[^{3}H]PGE_2-labelled SL membrane proteins were solubilized with CHAPS as described above loaded onto a 7.5%–37.5% linear sucrose gradient in a buffer containing 100 mM NaCl, 0.1 mM EGTA, 20 mM Hepes (pH 7.4) and 0.05 mg CHAPS/ml. Sucrose gradients were formed by repeated (three times) freezing (at −80°C) and thawing (at room temperature) of 30% (w/v) solution of sucrose. Gradients were then centrifuged for 18 h at 100000 × g in a Beckman SW 50.1 rotor and 300 μl fractions were collected using a Beckman Fraction Recovery System. Obtained fractions were analyzed for protein composition on SDS-PAGE, for radioactivity and for protein concentration. The fractions containing PGE_2 receptor were pooled, concentrated down using Aminco Ultrafiltration Cell Model 8200, and assayed for [^{3}H]PGE_2 binding by the chromatography-centrifugation method.

SDS-polyacrylamide gel electrophoresis

SDS-PAGE was performed on 10% SDS-PAGE in a discontinuous buffer system of Laemmli [23] employing a mini-slab gel electrophoresis apparatus (gel size, 1.5 mm thickness, 65 mm length). Prior to electrophoresis samples were solubilized in 2% SDS, 10% glycerol, and 50 mM Tris-HCl (pH 6.8), in the presence of either 5% 2-mercaptoethanol (reducing conditions) or 2 mM NEM (nonreducing conditions). Following electrophoresis, gels were stained with either Coomassie blue or silver. Molecular weight standards were: myosin, 200000; β-galactosidase, 116300; phosphorylase b, 97400; bovine serum albumin, 66000; and ovalbumin, 42000.

Photolabelling of PGE_2 receptor with [^{3}H]azido-PGE_2

[^{3}H]Azido-PGE_2 was synthesized asdescribed earlier [15]. For photolysis, the WGA-purified receptor was incubated with [^{3}H]azido-PGE_2 [15] 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, Middletown, CT) 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. SDS-PAGE was performed using 5–15% acrylamide gradient gels according to the procedure of Laemmli [23]. Gels were stained with Coomassie blue, and destained in a solution of 10% acetic acid and 5% methanol. [^{3}H]Azido-PGE_2 incorporation was detected by 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.

Analysis of lectin binding

For lectin binding analysis, proteins from SDS-PAGE were transferred electrophoretically onto nitrocellulose membrane according to the method of Towbin et al. [24] and incubated with appropriate peroxidase conjugated lectins (1 μg/ml) [22].

Results

Solubilization of the PGE_2 receptor

We tested three different detergents for their capacity to solubilize the PGE_2 receptor from cardiac SL

vesicles. The optimal CHAPS concentration which solubilized a functional PGE_2 receptor was approx. 8 mM or 5 mg/mg of protein. A single extraction of the SL membranes with 5 mg CHAPS/mg of protein solubilized 70–80% of the total proteins (range of 18 experiments) with recovery of over 90% of the total [^{3}H]PGE_2 binding sites (Table I).

Triton X-100 and octyl glucoside (5 mg/mg of protein) solubilized 50–60% of the total SL membrane proteins but [^{3}H]PGE_2 binding to the supernatant component was impaired (Table I). Membrane solubilization with increasing amounts of any one of the three detergents tested led to further loss of [^{3}H]PGE_2 binding activity (Table I). Increasing the ionic strength of the solubilization buffer had no effect on the efficiency of solubilization of the receptor (data not shown). All subsequent experiments presented in this paper were performed using 5 mg CHAPS per mg of protein (8 mM CHAPS).

Binding characteristics of solubilized receptor

Total [^{3}H]PGE_2 binding capacity of the CHAPS-solubilized receptor was slightly increased and nonspecific binding reduced compared to membrane bound receptor (Table II). [^{3}H]PGE_2 binding was sensitive to trypsin digestion of intact and solubilized SL membranes (Table II) indicating that [^{3}H]PGE_2 was binding to a membrane protein. Ligand binding to solubilized and membrane bound receptor was also sensitive to elevated temperature and no binding was observed in the absence of NaCl (Table II). In addition no significant effects of DTT, ATP, Mg^{2+}, Ca^{2+} and changes in pH of buffer on [^{3}H]PGE_2 binding to either CHAPS-solubilized or membrane bound receptor was seen (Table II).

The specificity of PGE_2 binding to the CHAPS-solubilized SL proteins was also determined by competition studies with unlabelled PGE_2 and PGE_1. As shown in Fig. 1, at high concentrations unlabelled PGE_1 and PGE_2 displaced greater than 90% of the label. Unlabelled PGE_1 displaced [^{3}H]PGE_2 at concentrations similar to those for unlabelled PGE_2. Unlabelled PGD_2, PGI_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$ were unable to displace [^{3}H]PGE_2 from the CHAPS-solubilized receptor (data not shown). These results suggest that PGE_1 and PGE_2 might be binding to the same receptor molecule and further confirmed our earlier observations that both PGE_1 and PGE_2 compete for [^{3}H]PGE_2 binding to intact cardiac SL vesicles [5].

SL vesicles have two types of PGE_2 binding sites (K_d = 0.3 nM and K_d = 3.4 nM) [5]. Scatchard analysis of PGE_2 binding to CHAPS-solubilized receptor revealed the presence of two types of PGE_2 binding sites with a K_d of 0.33 ± 0.05 nM and 3.00 ± 0.27 nM (mean ± S.E., n = 5) and a B_{max} of 0.50 ± 0.04 and 2.00 ± 0.10 pmoles PGE_2/mg of protein (mean ± S.E., n = 5), respectively. B_{max} and K_d values for both binding sites

TABLE I

Solubilization of PGE_2 receptor from cardiac SL membrane

SL vesicles were solubilized with appropriate detergent, centrifuged and the supernatant (S) and pellets (P) were assayed for [^{3}H]PGE_2 binding activity and protein content as described under Experimental procedures.

		Protein (mg)	[^{3}H]PGE_2 bound (pmol [^{3}H]PGE_2)	Recovery (%)
A. Intact SL vesicles		1.00	1.10	100
B. Solubilized SL vesicles				
1. Chaps				
(5 mg/mg protein)	P	0.18	0.025	2.2
	S	0.80	1.03	93.6
(50 mg/mg protein)	P	0.10	0.005	0.45
	S	0.80	0.89	80.9
2. Triton X-100				
(5 mg/mg protein)	P	0.46	0.018	1.6
	S	0.50	0.68	61.8
(50 mg/mg protein)	P	0.30	0.009	0.8
	S	0.60	0.39	35.4
3. Octyl glucoside				
(5 mg/mg protein)	P	0.35	0.11	10.0
	S	0.60	0.78	70.9
(50 mg/mg protein)	P	0.30	0.03	2.7
	S	0.65	0.40	36.3

TABLE II

Effects of incubation conditions on [^{3}H]PGE_2 binding to membrane-bound and CHAPS-solubilized PGE_2 receptor

[^{3}H]PGE_2 binding to SL vesicles and CHAPS-solubilized membrane (5 mg CHAPS/mg of protein) was carried out as described under Experimental procedures. Values are the means ± S.E. The numbers in parentheses denote the number of preparations analyzed.

Additions	Specific binding (pmol [^{3}H]PGE_2/mg protein)	
	SL vesicles	CHAPS-solubilized receptor
None [a]	1.00 ± 0.20 (10)	1.20 ± 0.15 (8)
Non NaCl [b]	0.20 ± 0.05 (4)	0.15 ± 0.05 (4)
55°C	0.0 (5)	0.0 (5)
Trypsin [c]	0.15 ± 0.05 (3)	0.0 (3)
DTT (1 mM)	1.15 ± 0.20 (4)	1.20 ± 0.30 (4)
ATP (5 mM)	1.20 ± 0.20 (3)	1.35 ± 0.20 (3)
$MgCl_2$	1.10 ± 0.20 (4)	1.30 ± 0.15 (4)
$CaCl_2$	1.10 ± 0.20 (4)	1.30 ± 0.15 (4)

[a] Standard binding conditions were used, i.e. 100 mM NaCl, 20 mM Hepes, pH 7.4, 37°C.

[b] 250 mM sucrose substituted for NaCl.

[c] SL vesicles or CHAPS-solubilized SL proteins were digested with 1 mg of trypsin/mg protein for 30 min at 37°C. The reaction was terminated by addition of 10-fold excess of soyabean trypsin inhibitor, followed by [^{3}H]PGE_2 binding as described under Experimental procedures.

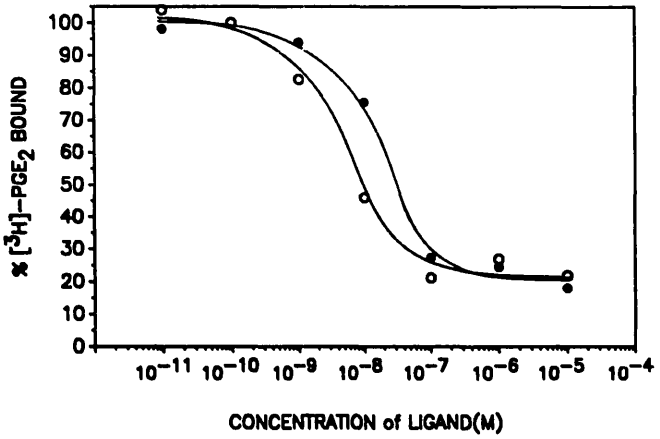

Fig. 1. Competition of the [^{3}H]PGE_2 binding to solubilized cardiac SL by unlabelled PGE_1 and PGE_2. SL vesicles were first solubilized with 5 mg CHAPS/mg of protein and [^{3}H]PGE_2 binding to the CHAPS-solubilized membrane proteins was performed as described under Experimental procedures. Closed circles, competition of binding by unlabelled PGE_1; open circles, competition of binding by PGE_2. Value of 100% represents total binding to the solubilized SL proteins at 2 nM [^{3}H]PGE_2.

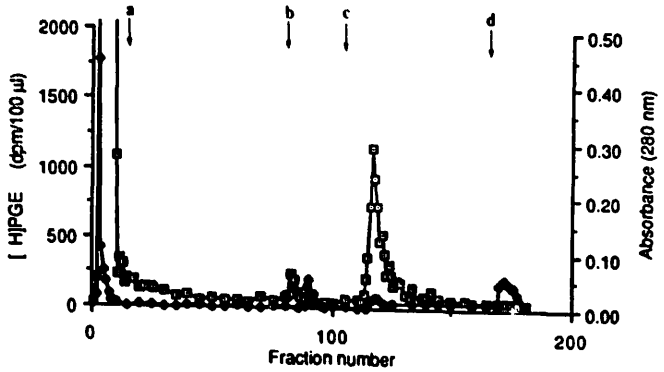

Fig. 2. WGA-Sepharose chromatography of PGE_2 receptor. SL vesicles were solubilized with CHAPS, centrifuged and CHAPS-solubilized fraction was labelled with [^{3}H]PGE_2 and separated on WGA-Sepharose according of the procedure described under Experimental procedures. WGA-Sepharose column was subsequently washed with different buffers as described under Experimental procedures; a, high salt buffer (500 mM NaCl, 0.1 mM EGTA, 2 mM $MgCl_2$, 0.05 mg CHAPS/ml, 20 mM Hepes, pH 7.4); b, low-salt buffer (100 mM NaCl, 0.1 mM EGTA, 2 mM $MgCl_2$, 0.05 mg CHAPS/ml, 20 mM Hepes, pH 7.4); c, low-salt buffer (100 mM NaCl, 0.1 mM EGTA, 2 mM $MgCl_2$, 0.05 mg CHAPS/ml, 20 mM Hepes, pH 7.4) containing 0.02% SDS; and d, low-salt buffer (100 mM NaCl, 0.1 mM EGTA, 2 mM $MgCl_2$, 0.05 mg CHAPS/ml, 20 mM Hepes, pH 7.4) containing 200 mM GlcNAc. Absorbance at 280 nm was monitored (◆). Aliquots (50 µl) of the 250 µl fractions were assayed for radioactivity by liquid scintillation counting (⊡).

in intact SL vesicles [5] and CHAPS-solubilized receptor (this study) are comparable.

Purification of PGE_2 receptor by WGA-Sepharose affinity chromatography

CHAPS-solubilized, [^{3}H]PGE_2-labelled SL proteins were fractionated on a WGA-Sepharose column. A representative chromatographic profile is shown in Fig. 2. More than 90% of the applied [^{3}H] radioactivity appeared in the column 'run-through' or in the initial washes and, hence failed to absorb to the WGA-Sepharose. About 10% of the applied radioactivity absorbed to the WGA-Sepharose column, and was eluted with 0.02% SDS in 100 mM NaCl, 0.1 mM EGTA, 2 mM $MgCl_2$, 0.05 mg CHAPS/ml, 20 mM Hepes (pH 7.4) (Fig. 2). Elution of label was accompanied by elution of > 0.5% of the total SL protein. The remaining protein (approx. 1%) could then be eluted with the specific sugar GlcNAc (Fig. 2). No additional radioactivity was eluted under these conditions. The specificity of PGE_2 receptor absorption to the WGA-Sepharose column was tested by repeating the chromatography experiments in the presence of GlcNAc. Under these conditions binding of PGE_2 receptor to WGA-Sepharose was not observed (data not shown). Free

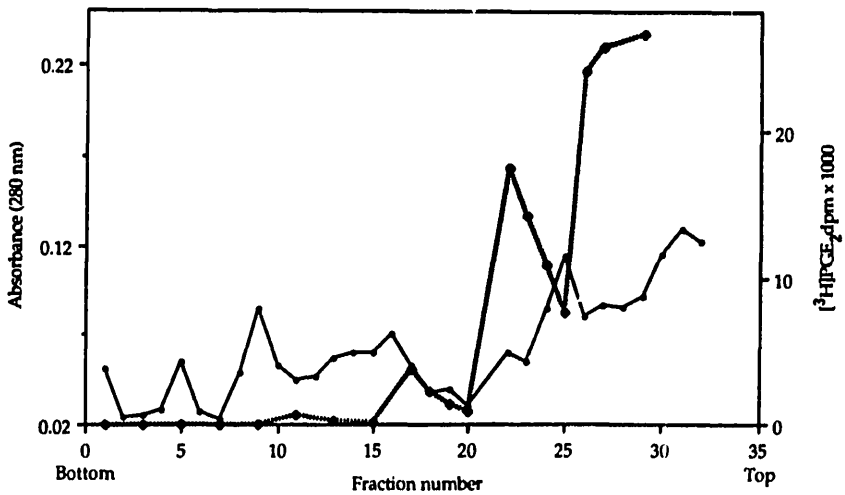

Fig. 3. Purification of the PGE_2 receptor by sucrose density gradient centrifugation. SL vesicles were solubilized with CHAPS, centrifuged and CHAPS-solubilized fraction was labelled with [^{3}H]PGE_2 and separated on a 7.5%–37.5% linear sucrose gradient as described under Experimental procedures. Absorbance at 280 nm was monitored (solid line). Aliquots (50 µl) of the 300 µl fractions were assayed for radioactivity by liquid scintillation counting (broken line).

A B

Fig. 4. SDS-PAGE of the purified PGE_2 receptor WGA-affinity chromatography purified PGE_2 receptor (A) and sucrose density gradient purified receptor (B) were separated on an SDS-PAGE and stained with Coomassie blue as described under Experimental procedures. The arrow head depicts the positions of the PGE_2 receptor.

[^{3}H]PGE_2 also did not bind to WGA-Sepharose column (data not shown). In addition, once the isolated receptor fraction was eluted it did not rebind to WGA-Sepharose either before or after elution of membrane glycoproteins with specific sugar GlcNAc (data not shown).

Purification of PGE_2 receptor by sucrose density gradient centrifugation

During the course of this study we have developed an alternative method for the purification of cardiac PGE_2 receptor. The receptor was purified using a simple one-step procedure based on molecular size separation (Fig. 3). On centrifugation through a linear sucrose gradient, a single PGE_2 receptor peak was observed in the upper half of the gradient (Fig. 3, broken line; fraction No. 22). All radioactivity in the top fractions of the gradient was found to be unbound [^{3}H]PGE_2.

Characterization of the isolated receptor

SDS-PAGE of the PGE_2 receptor purified by either WGA-Sepharose or sucrose gradient centrifugation revealed the presence of one protein band of M_r of approx. 100000 (Fig. 4A and B). The 100-kDa PGE_2 binding protein did not change its mobility on SDS-PAGE under either reducing or nonreducing conditions (data not shown). Since the isolated PGE_2 receptor failed to rebind to WGA-Sepharose and did not stain with peroxidase labelled WGA or concanavalin A (data not shown) we conclude that it is not glycosylated. However, it cannot be excluded that the protein is glycosylated but not able to bind WGA or concanavalin A.

Fig. 5 shows the [^{3}H]azido-PGE_2-labelling profile obtained for the purified PGE_2 receptor following photolysis in the presence of [^{3}H]azido-PGE_2. A major peak of [^{3}H]azido-PGE_2 incorporation was observed into a 100-kDa protein band. When aliquots of the same membranes were photolysed in the presence of unlabelled PGE_2 (10^{-3} M) or azido-PGE_2 (10^{-3} M) the amount of covalently bound [^{3}H]azido-PGE_2 in the

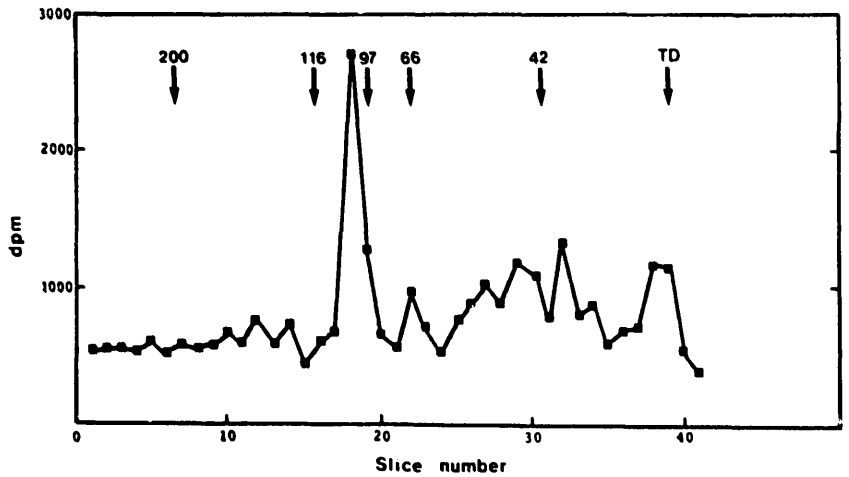

Fig. 5. Photolabelling of the purified PGE_2 receptor WGA-Sepharose purified receptor was photolabelled with [^{3}H]azido-PGE_2 as described as described by Michalak et al. [22], and separated on SDS-PAGE. Following staining and destaining, gel lanes were sliced in 2 mm sections and [^{3}H]azido-PGE_2 incorporation was measured by liquid scintillation counting. Arrows depict the location of molecular weight standards and tracking dye (TD). Numbers represent $M_r \times 10^{-3}$.

TABLE III

[^{3}H]PGE_2 binding to purified receptor

PGE_2 receptor was purified from CHAPS-solubilized SL membrane (5 mg CHAPS/mg of protein) either by WGA-affinity chromatography or by sucrose density gradient centrifugation as described under Experimental procedures. [^{3}H]PGE_2 binding was performed as described under Experimental procedures in the absence or presence of 1 μM PGE_1 or PGE_2.

	Specific binding (nmol [^{3}H]PGE_2/mg protein)
WGA-Sepharose purified receptor	0.19
In the presence of 1 μM PGE_2	0.01
In the presence of 1 μM PGE_1	0.01
Sucrose gradient purified receptor	630.00
In the presence of 1 μM PGE_2	150.00
In the presence of 1 μM PGE_1	220.00

100-kDa purified receptor protein was decreased by approximately 80% in the presence of PGE_2 (10^{-3} M) or azido-PGE_2 (10^{-3} M)(data not shown).

Table III shows PGE_2 binding to the isolated PGE_2 receptor. Specific binding of [^{3}H]PGE_2 to the WGA-Sepharose purified receptor was 190 ± 15 pmol/mg proteins (mean $\pm$ S.E., $n = 4$). This corresponds to approx. 200-fold purification of PGE_2 receptor by this procedure and yielded about 30–40 ng/mg of SL protein of isolated receptor. Silver stained SDS-PAGE revealed the presence of only one protein band in the purified receptor preparation suggesting that the relatively low PGE_2 binding to this receptor might be due to the denaturating of this molecule during purification and elution from the WGA-Sepharose with a low concentration of SDS. Sucrose gradient purified receptor, however, bound 630.00 nmol of PGE_2/mg of protein. This procedure leads to the isolation of relatively active receptor, as compared to the WGA-Sepharose method, and corresponded to 6300-fold purification. Scatchard analysis of PGE_2 binding to purified receptor revealed the presence of two types of PGE_2 binding sites with a K_d of 0.49 nM and 5.40 nM. These values are comparable to those found in in intact SL vesicles [5] and CHAPS-solubilized receptor (this study).

Discussion

We have isolated a PGE_2 receptor from cardiac SL membranes by two independent methods. The first includes a one step WGA-Sepharose affinity chromatography procedure involving specific elution with a 0.02% SDS solution. Purified PG receptor was also obtained by a simple one-step procedure based on molecular size separation by the sucrose gradient centrifugation of solubilized SL proteins. As judged from its mobility in SDS-PAGE the purified receptor is a polypeptide of M_r of approx. 100000. The same polypeptide was obtained when WGA-Sepharose or sucrose gradient centrifugation methods were used further supporting our hypothesis that this molecule corresponds to the cardiac PGE_2 receptor.

WGA-Sepharose yields PGE_2 receptor with a relatively low binding capacity. Based on the [^{3}H]PGE_2 binding to the isolated receptor (190 pmoles of [^{3}H]PGE_2/mg of protein) it may be concluded that the purified receptor preparation would not be 100% pure. Coomassie blue or silver staining of SDS-PAGE of the isolated receptor preparation, however, revealed the presence of only one protein band at 100 kDa. The WGA-Sepharose procedure developed by us to isolate the PGE_2 receptor may have resulted in the purification of partially inactivated receptor. This may be due to the use of a low concentration of SDS for final elution of the receptor polypeptide from WGA-Sepharose. Based on the M_r of the isolated receptor and PGE_2 binding to the intact and solubilized SL a purification fold of 10000 would be required in order to achieve homogeneity. Using the WGA-Sepharose procedure only 200-fold purification is achieved based on the specific binding of PGE_2 to the purified receptor. This is most likely due to a partial inactivation of the receptor molecule during the WGA-Sepharose chromatography followed by an SDS elution. Sucrose gradient centrifugation method offers an alternative procedure for the purification of PGE_2 receptor. PGE_2 receptor purified by sucrose gradient centrifugation bound 0.63 μmol of [^{3}H]PGE_2/mg of protein. This corresponds to 6300-fold purification and indicates that highly active, functional PGE_2 receptor can be purified by this procedure.

The purified PGE_2 receptor does not stain with peroxidase labelled lectin, indicating that it is not glycosylated and therefore, must be retained by WGA-Sepharose as a result of association with a glyco-mediator which survives solubilization of the membranes. This characteristic is similar to the dihydropyridine-binding subunit of the skeletal muscle dihydropyridine receptor, as well as skeletal muscle dystrophin. Both of these proteins are retained on WGA-Sepharose and are eluted by a detergent solution rather than a specific sugar [25,26]. At present the nature of the mediator involved in the interaction of the PGE_2 receptor with the lectin column is not clear, however, a complex of SL membrane glycoproteins (120 kDa and 100 kDa) containing WGA and Concanavalin A binding sites can be eluted from lectin column by a specific sugar [22]. Thus, low concentrations of SDS are able to dissociate the PGE_2 receptor-glycoproteins complex. The 120-kDa and 100-kDa glycoproteins are the major Ca^{2+} binding proteins in the SL membranes [22]. The association between PGE_2 receptor and these membrane

glycoproteins is not clear at the present. PGE_2 receptor molecules either interact with these glycoproteins the native membrane or this association is related to the type of micelles formed when SL vesicles are solubilized with CHAPS. This subject will require further study.

In order to purify the PGE_2 receptor, we first determined the conditions which optimally solubilize the SL membrane, while retaining PGE_2 binding activity in the solubilized sample. Amongst the detergents tested, CHAPS was found to solubilize the cardiac SL membranes without loss of PGE_2 binding activity in the solubilized fractions. PGE_2 binding to the soluble receptor is slightly enhanced, but like intact membrane, requires high ionic strength (100 mM NaCl). The CHAPS-solubilized receptor has two types of ligand binding sites with a K_d of 0.33 and 3.00 nM and a B_{max} of 0.50 and 2.00 pmol/mg of protein, respectively. This agrees well with K_d and B_{max} values observed for the membrane-bound receptor [5]. PG binding to CHAPS-solubilized receptor is displaced by PGE_2 and PGE_1 but not by PGD_2, PGI_2, $PGF_{2\alpha}$ and 6-keto-$PGF_{1\alpha}$. This is also in a good agreement with our earlier observations for intact SL vesicles [5].

In previous studies a number of detergents have been used in attempts to solubilize different eicosanoid receptors including digitonin, CHAPS and Triton X-100 [9,11,14,27–29]. Unfortunately, PG high-affinity binding properties have frequently been lost upon solubilization of the receptor. Recently, Tsai et al. [28] reported solubilization of the PGI_2 receptor in platelet membrane and showed that CHAPS was also far superior in releasing the PGI_2 receptor compared to any other detergent tested. Watanabe et al. [9] used digitonin and CHAPS to solubilize a PGE_2 binding protein from renal medulla [9]. These investigators noted that the digitonin solubilized receptor is associated with a guanine nucleotide regulatory protein which can be dissociated from the receptor by solubilization with CHAPS [9]. Based on these studies and our own results, CHAPS may be consider the detergent of choice for the isolation of the pure PGE_2 receptor molecule.

The purified PGE_2 receptor from heart SL reveals one polypeptide band of M_r 100000 (Fig. 5), which can be photolabelled with [^{3}H]azido-PGE_2, a photoactive derivative of PGE_2 [15]. Using [^{3}H]azido-PGE_2, we showed recently that a 100-kDa polypeptide is specifically labelled by this compound in cardiac SL membranes [15]. We propose that this 100-kDa protein corresponds to the PGE_2 receptor in the heart. This molecular weight resembles values reported for PGE_2 binding proteins from other tissues [9–14]. Using the sucrose-density gradient sedimentation method the PGE_2 receptor in rat liver plasma membrane was estimated to be a 105-kDa polypeptide [11]. Fernandez-Botran and Suzuki [14] using PGE_2 coupled to Sepharose 4B followed by gel filtration and isoelectric focusing isolated a PGE_2 binding protein of M_r 95000 from murine macrophage-like cells. Negishi et al. [10] used the technique of cross-linking bovine adrenal membrane with dithiobis(succinimidyl propionate) followed by GTP-Sepharose or WGA-Sepharose affinity chromatography in order to isolate a complex of PGE_2 receptor and GTP-binding protein. The cross-linked complex eluted as a 200-kDa oligomer, with the M_r of the PGE_2 binding protein estimated at 110000 (200-kDa minus 90-kDa GTP-binding protein). With the exception of one study, where the canine renal medulla PGE_2 receptor was reported to be a glycoprotein with a M_r of 65000 [9], molecular weights of PGE_2 receptors have been estimated to be 100000, similar to the cardiac receptor isolated in this study. The size of the isolated PGE_2 receptor is in contrast to estimated sizes for the platelet PGI_2/PGE_1 receptor (two subunits 85-kDa and 95-kDa [30]), the thromboxane A_2/PGH_2 receptor (140–180-kDa polypeptide [30] or 57-kDa polypeptide [31]) or the leukotrienes B_4 receptor (60-kDa [32]). Kyldin and Hammerstrom [12], however, by size-exclusion chromatography estimated a 107-kDa for $PGF_{2\alpha}$ receptor in sheep or bovine corpora lutea. Molecular cloning of the cDNA encoding the receptor polypeptide will provide an exact molecular weight of the PGE_2 receptor in the heart.

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