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SOLUBILIZATION OF PROSTAGLANDIN D_2 BINDING PROTEIN FROM PORCINE TEMPORAL CORTEX

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Prostaglandin (PG) D_2 binding activity was retained at the highest level in the P_2 fraction prepared from porcine temporal cortex with the use of buffer containing mannitol and quinacrine. Then, the activity in this fraction was solubilized with maximal recovery by 10 mM CHAPS. The specific PGD_2 binding time-dependently increased and was saturated at around 70 nM. Scatchard plots were fitted to a straight line with a Kd value of 20 nM and Bmax of 120 fmol/mg protein. The binding sites showed high specificity for PGD_2. In addition, heat and trypsin treatments remarkably decreased the binding activity. These results suggest that the specific binding protein for PGD_2 can be solubilized from these membranes.

Prostaglandin (PG) D_2 is one of the major prostanoids in the mammalian brain and exerts various central actions such as sleep induction, hypothermia, modulation of olfactory function, hormone release, and analgesia (1). During the course of study on its molecular mechanism, Shimizu *et al.* (2) detected a specific PGD_2 binding activity in the synaptic membrane fraction of rat brain. Using a variety of PGD_2 derivatives, we later showed this binding to be highly specific for PGD_2 (3). Further, using autoradiography and image analysis, we (4-7) demonstrated the binding sites to be located in discrete areas of the cerebral cortex, preoptic area, olfactory bulb, and hypothalamus, indicating good agreement with the

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Abbreviations: PG, prostaglandin; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate.

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above-mentioned central actions of PGD_2. In this study, we succeeded in solubilizing PGD_2 binding protein in an active form.

EXPERIMENTAL PROCEDURES

Materials - [5,6,8,9,12,14,15-^{3}H]PGD_2 was purchased from Dupont New England Nuclear. Quinacrine, deoxyribonuclease I, ribonuclease, trypsin, proteinase K (fungal), and phospholipase A_2 (*Naja naja* venom) were purchased from Sigma. CHAPS came from Dojindo Laboratories. Unlabeled PGs were generous gifts from Ono Pharmaceutical Company.

Preparation of P_2 fraction and solubilization with CHAPS - P_2 fraction was prepared from frozen porcine temporal cortex as described by Yumoto *et al.* (8), with the use of homogenizing buffer containing 0.3 M mannitol, 0.5 mM quinacrine, 1 mM PMSF, 2 mM 2-mercaptoethanol, 1 mM EDTA, and 10 mM HEPES at pH 7.5. CHAPS was dropwisely added with stirring to 5 mg protein/ml P_2 fraction in the homogenizing buffer to give a final concentration of 10 mM and the suspension was then stirred at 4 °C for 30 min. After centrifugation at 100,000g x 60 min, the supernatant was collected and referred to as the solubilized fraction.

Binding assay - PGD_2 binding was measured as described by Yumoto *et al.* (8). Unless stated otherwise, the reaction mixture contained a sample having 2 mg/ml protein, 20 nM [^{3}H]PGD_2, 1 mM EDTA, 2 mM 2-mercaptoethanol, 1 mM PMSF, 0.5 mM quinacrine, 0.1 M NaCl, 0.3 M mannitol, and 50 mM Tris-HCl (pH 7.5). In the case of the solubilized fraction, the CHAPS concentration in the reaction mixture was adjusted to 5 mM. The reaction was started by addition of [^{3}H]PGD_2 at 37 °C. Nonspecific binding was determined from the measurement of [^{3}H]PGD_2 binding in the presence of 100 μM unlabeled PGD_2. The specific binding was calculated by substraction of the nonspecific binding from the total binding. All values were expressed as the means of triplicate determinations.

Protein determination - Protein concentration was determined by the method of Bradford (9) with bovine gamma globulin as standard.

RESULTS AND DISCUSSION

PGD_2 binding to the P_2 fraction prepared in various homogenizing buffers - Specific PGD_2 binding activity varied among cortical specimens from different pigs, possibly because the binding activity was sensitive to proteases, glycosidases, and phospholipases as reported by Shimizu *et al.* (2). Porcine temporal cortex is one of the richest sources of PGD_2 binding activity among various mammalian brain regions (N. Yumoto, unpublished results) We therefore sought to improve the method for isolation of the P_2 fraction

Table 1. Effects of the addition of various substances to the homogenizing buffer on PGD_2 binding activity

	PGD_2 binding activity	
-sugars	7.3	fmol/mg protein
+sugars (0.3 M)		
sucrose	14.5	
mannitol	28.3	
α-methyl mannoside	13.8	
glucose	10.7	
galactose	16.9	
glycerol (10%(w/v))	15.2	
phospholipase inhibitor		
quinacrine (0.5 mM)	47.3	

P_2 membrane was prepared by the method of Yumoto *et al.* (8). The homogenizing buffer contained 1 mM EDTA, 2 mM β-mercaptoethanol, 1 mM PMSF, 20 μM indomethacin, 10 mM HEPES, and sugars or glycerol as listed in the table. The pH was adjusted to 7.5 at 4 °C. In the experiments using phospholipase inhibitor, 0.3 M mannitol was added to the homogenizing buffer.

from frozen porcine temporal cortex. PGD_2 binding to the P_2 fraction showed its highest values when the fraction was prepared in buffer containing mannitol among the sugars tested (Table 1). Mannitol has been preferred over sucrose to maintain osmotic pressure and to prevent the loss of soluble substances in the preparation of brain mitochondoria (11). Quinacrine, a potent inhibitor of phospholipase A_2 (10), further enhanced the binding activity in the presence of mannitol. However, various protease inhibitors tested (antipain, aprotinin, bestatin, leupeptin, PMSF, and phosphoramidon) had almost no effect (data not shown). Therefore, we prepared the P_2 fraction in the homogenizing buffer containing mannitol and quinacrine.

Solubilization of specific PGD_2 binding activity with CHAPS - Specific PGD_2 binding activity was solubilized from the P_2 fraction with various concentrations of CHAPS. Since CHAPS over 5 mM decreased the activity of the binding to the P_2 fraction in the binding assay, we kept its concentration in the reaction mixture at 5 mM in all cases (data not shown). The binding activity of the solubilized fraction increased dependent on the

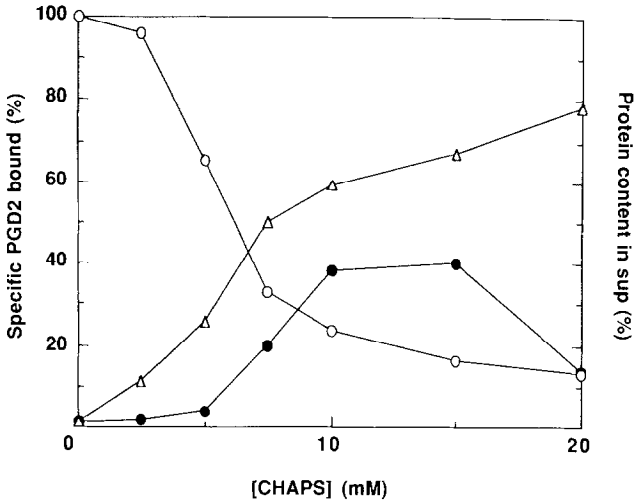

FIG.1. Solubilization of specific PGD_2 binding activity from P_2 fraction of porcine temporal cortex with CHAPS. P_2 fraction (5 mg protein/ml) was incubated for 30 min at 4 °C with CHAPS at the concentrations indicated in the figure. After centrifugation at 100,000g x 60 min, the supernatant was concentrated overnight with a CentriCell (nominal molecular weight limit=10 kDa, Polysciences Inc.) overnight. Subsequently, the binding activity of the supernatant (●) and of the resuspendal pellet (○) and the protein content of the supernatant (△) were determined. In the binding assay of the supernatant, the CHAPS concentration was kept at 5 mM.

CHAPS concentration used in the homogenizing buffer up to 10 mM and then decreased over 15 mM (Fig. 1). The maximal activity was obtained at 10 mM CHAPS with 40 fmol PGD_2 bound/mg protein. The recovery was 38%, a

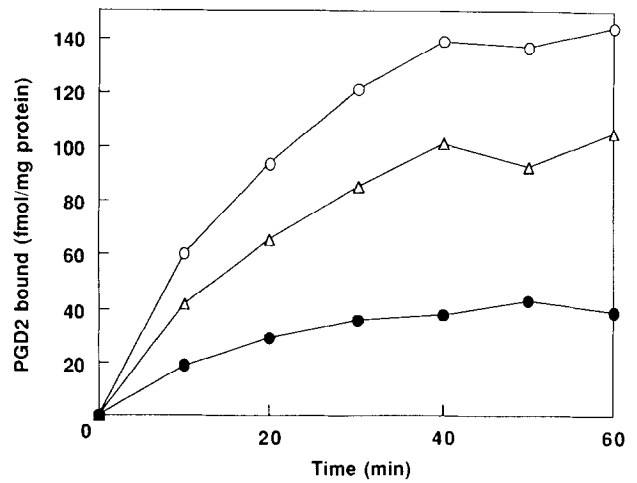

FIG.2. Time course of specific PGD_2 binding to the solubilized fraction. The solubilized fraction (2 mg protein /ml) was incubated with 20 nM [^{3}H]PGD_2 at 37 °C. Aliquots were removed at the indicated times and the binding activities were determined. The specific binding (●) was calculated by substraction of the nonspecific binding (△) from the total binding (○).

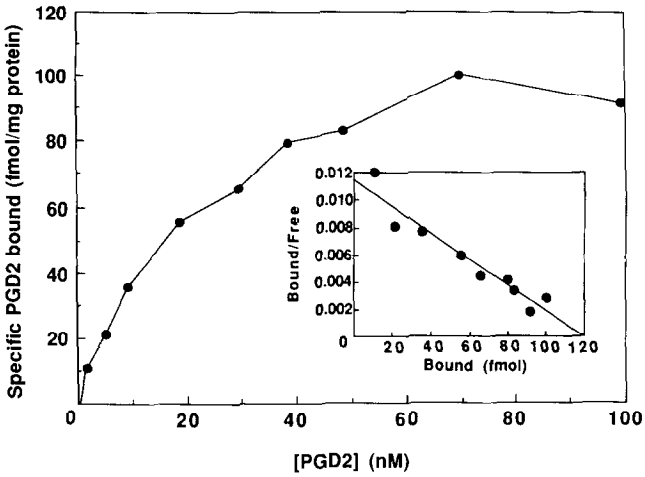

FIG.3. Specific PGD_2 binding to the solubilized fraction at various concentrations of PGD_2. The solubilized fraction (2 mg protein/ml) was incubated with PGD_2 at various concentrations from 2 to 100 nM. Inset: Specific binding activities were replotted as a Scatchard plot.

relatively low value but similar to that reported for the PGE_2 receptor (8).

Characteristics of $[^3H]PGD_2$ binding to the solubilized fraction - Fig. 2 shows the time course of specific $[^3H]PGD_2$ binding to the solubilized fraction. The specific binding slowly increased with time and reached equilibrium at around 50 min, whereas the specifc binding to the P_2 fraction reached its equilibrium within 5 min (data not shown). The nonspecific

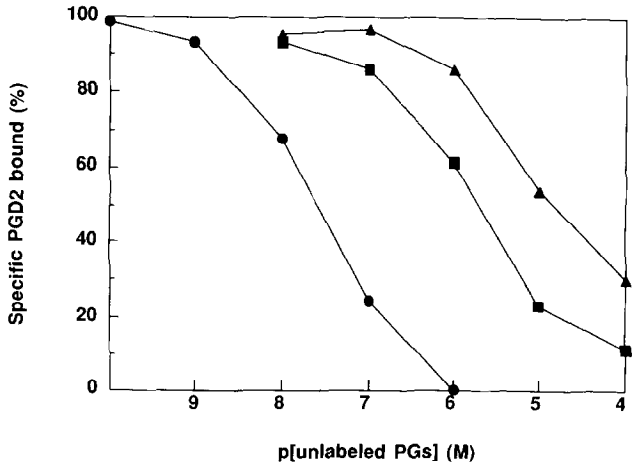

FIG.4. Specificity of $[^3H]PGD_2$ binding to the solubilized fraction. $[^3H]PGD_2$ binding at 10 nM was measured in the presence of unlabeled PGs [PGD_2 (●), $PGF_{2\alpha}$ (▲), PGE_2 (■)].

Table 2. Effects of various treatments on [^{3}H]PGD_2 binding to the solubilized fraction

treatment	Residual PGD_2 binding activity
heat (95 °C, 5 min)	0 %
deoxyribonuclease I (0.5 mg/ml)	95
ribonuclease (0.5 mg/ml)	105
trypsin (50 μg/ml)	10
proteinase K (50 μg/ml)	46
phospholipase A_2(50 μg/ml) +20 μM indomethacin	32
β-galactosidase (100 U/ml)	58

The solubilized fraction (4 mg protein/ml) was boiled for 5 min or preincubated with various enzymes listed above at 37 °C for 15 min. For the treatment with deoxyribonuclease I, 5 mM $MgCl_2$ was further added and for that with phopholipase A_2, 5 mM $CaCl_2$ was added. Results were expressed as percent of control. Control experiments were carried out in the absence of enzymes and the value of PGD_2 binding in the absence and the presence of Ca or Mg was 36, 34, or 39 fmol/mg protein, respectively.

binding showed relatively high level and consequently the ratio of the specific binding to the total binding exhibited a low value of approximately 30%. After the binding of [^{3}H]PGD_2 reached equilibrium, the [^{3}H]PGD_2-receptor complex did not dissociate to any significant extent during 60-min period following the addition of unlabeled PGD_2 (data not shown).

PGD_2 binding was measured at various concentrations of [^{3}H]PGD_2 from 2 to 100 nM (Fig. 3). Specific PGD_2 binding to the solubilized fraction increased dependent on [^{3}H]PGD_2 concentration and was saturated at around 70 nM. The Scatchard plots were well fitted to a straight line by the least squares method. The Kd value and Bmax were 21 nM and 119 fmol/mg protein, respectively. The Kd value showed almost the same value as that of rat brain synaptic membrane (2).

Various unlabeled PG's added to the reaction mixture in the [^{3}H]PGD_2 binding assay were markedly inhibitory, as shown in Fig. 4. The inhibitory potency was observed in the following order: $PGD_2 > PGF_{2\alpha} > PGE_2$. The IC_{50} values for PGD_2, $PGF_{2\alpha}$, and PGE_2 were obtained at 18, 708, and 8900 nM, respectively. This order of the relative affinities of PG's for the [^{3}H]PGD_2 binding sites is almost the same as that found with synaptic

membrane prepared from rat brain (2). Thus, the solubilized specific PGD_2 binding activity exhibited the following characteristics of the receptor: high affinity, high specificity, and saturablility.

Effects of various treatments on [^{3}H]PGD_2 binding to the solubilized fraction - In order to examine the nature of the molecule involved in PGD_2 binding, the solubilized fraction was treated with various enzymes or with heat (Table 2). The specific binding was completely abolished by boiling of the solubilized fraction at 95 °C for 5 min. The treatment with trypsin or proteinase K markedly decreased the binding activity by 90 or 54 %, respectively. However, deoxyribonuclease I or ribonuclease treatment had no effect. Therefore, PGD_2 binding activity appears to be due to the specific receptor protein. Furthermore, the treatment with phospholipase A_2 decreased the binding activity by 68% in the presence of indomethacin, a cyclooxygenase inhibitor. β-Galatosidase treatment also decreased the binding activity by 42%. These results suggest that the binding activity requires phospholipids and/or carbohydrates possibly to maintain its stable conformation. In support of this possibility, the addition of a phospholipase inhibitor to the homogenizing buffer enhanced the binding to the P_2 fraction (Table 1). By use of the conditions found in this study to afford receptor stability, the solubilization of active PGD_2 receptor protein will provide the basis for the purification of the protein and further investigation of its molecular properties.

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