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Prostaglandin D_2 receptor of mastocytoma P-815 cells – possible regulation by phosphorylation and dephosphorylation

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The ^{3}H-labeled prostaglandin D_2 ([^{3}H]PGD_2) binding protein in the membrane fraction of mastocytoma P-815 cells was characterized. The specific binding of [^{3}H]PGD_2 to the cells or the membranes reached a maximum at pH 5.6, and was saturable, displaceable and of high affinity when incubated at 0 or 37°C. The B_{max} values for [^{3}H]PGD_2 binding in the two preparations at pH 5.6 were much higher at 0°C than at 37°C, whereas the K_d values were almost equal (85.3 nM for the cells and 80.5 nM for the membranes, respectively). High specific [^{3}H]PGD_2 binding activity in the mildly acid-treated cells was still observed when the external pH was raised from 5.6 to 7.2. Furthermore, specific [^{3}H]PGD_2 binding to the membranes (at 0°C, pH 5.6) increased on addition of phosphatase inhibitors (NaF and molybdate) in the presence of 10 μM ATP, but practically disappeared on pretreatment of the membranes with phosphatase. On incubation of the membrane with [γ-^{32}P]ATP and molybdate, the stimulated incorporation of the [^{32}P]phosphate into several peptides, including ones having an M_r of around 100000–120000, was observed. These results suggest that [^{3}H]PGD_2 binding in the mastocytoma P-815 cell membrane is controlled through phosphorylation-dephosphorylation of the receptor itself.

Introduction

PGD_2 has been reported to perform various biological functions, such as neuromodulation in the brain [1], anti-aggregation in platelets [2], inhibition of luteinizing hormone release in the pituitary gland [3], and so on. In mast cells, PGD_2 increases cyclic AMP levels in the presence of a phosphodiesterase inhibitor, resulting in a marked inhibition of antigen-stimulated histamine release [4]. Although PGD_2 receptors have previously been demonstrated in rat brain synaptic membranes [5] and in human platelets [6], the characteristics, or even the existence, of PGD_2 receptors in mast cells remain unclear. The main reasons for this are the mast cell's high PGD_2 synthetase activity [7] and production of PGD_2 at the time of the release reaction [8]. In addition, the difficulty of preparing non-stimulated mast cells in large quantity compounds these problems.

On the other hand, neoplastic mouse mastocytoma P-815 cells have been a useful model in which the PGD_2 binding can be studied, because the cells proliferate rapidly in the peritoneal fluid with a number of properties characteristics of normal mast cells [9], but they scarcely produce any type of endogenous prostaglandin because of their complete lack of cyclooxygenase activity [10]. Addition of exogenous PGD_2 to mastocytoma P-815 cells has been previously reported to stimulate a low level of cAMP accumulation [11] and to suppress the growth rate [10].

In this study, we found the existence of high-affinity binding sites for PGD_2 on the plasma membranes of mastocytoma P-815 cells. Furthermore, the possible control of PGD_2 binding activity through phosphorylation and dephosphorylation of the receptors is discussed. A preliminary report has already appeared [12].

Materials and Methods

Cells

Mouse mastocytoma P-815 cells [9] were maintained in the ascitic form in BDF_1 male mice. The cells were

Abbreviations: PGD_2, prostaglandin D_2; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride; DFP, diisopropyl fluorophosphate, EGF, epidermal growth factor; SDS, sodium dodecylsulfate.

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harvested from the peritoneal fluid of the inoculated mice and then washed three times with PBS as described previously [13]. Cell viability was determined on the basis of nigrosin exclusion.

Preparation of the plasma membranes

All procedures were performed at 0–4°C. Mastocytoma P-815 cells ($1.5 \cdot 10^9$ cells) were washed, resuspended in 7 ml of TEMB buffer (10 mM Tris-HCl (pH 7.8)/1 mM EDTA/4 mM $MgCl_2$/6 mM 2-mercaptoethanol) and then homogenized with a Dounce-type homogenizer. The homogenate was centrifuged at $900 \times g$ for 10 min to sediment the nuclear fraction, which was in turn resuspended in 12 ml of TEMB and centrifuged at $900 \times g$ for 10 min. The two $900 \times g$ supernatant fractions obtained above were combined and centrifuged at $30000 \times g$ for 30 min to sediment plasma membranes and mitochondria. The precipitate was resuspended in 3.3 ml of 45% sucrose in 20 mM Tris-HCl (pH 7.8) and placed in a centrifuge tube (5CN; Hitachi, Tokyo, Japan). 0.5 ml each of 40% and 36% sucrose solutions were layered on top of the 45% sucrose solution, after which the tube was centrifuged at $66000 \times g$ for 60 min in a Hitachi SW-39 rotor. The membranes collected at the two interfaces, between 40 and 45% sucrose (heavy membranes, HM) and between 40 and 36% sucrose (light membranes, LM), and mitochondria were sedimented at the bottom of the tube. In most experiments, the two membrane fractions were combined, diluted 20-fold with PBS and centrifuged at $30000 \times g$ for 30 min. The sedimented membranes were resuspended in the same buffer and quickly frozen for storage, if necessary. The [^{3}H]PGD_2 binding activity of the membrane preparations remained stable for at least 4 weeks on storage at −80°C.

[^{3}H]PGD_2 binding assay

[^{3}H]PGD_2 binding was assayed in essentially the same manner as described for [^{3}H]PGE binding [13,14]. The cells ($(5–6) \cdot 10^6$ cells) or plasma membranes (approx. 100–200 μg protein) were suspended in 100 μl of PBS, with or without acetic acid pH adjustment, and incubated with 6 nM [^{3}H]PGD_2 (1 μCi) for assaying the total PGD_2 binding and also in the presence of 50 μM PGD_2 for the nonspecific binding assay. Incubation was routinely followed for 60 min at 0°C or for 3 min at 37°C, because the specific binding of [^{3}H]PGD_2 to the cells and the membranes at both temperatures reached a plateau level at those respective times (see Fig. 1B). The reaction was terminated by the addition of 2 ml of cold saline and the reaction mixture was immediately filtered through glass microfilters (Whatman GF/C), which were in turn rinsed three times with 2 ml of cold saline. The filtration procedures were designed to be completed within 10 s. Radioactivities of the dried filters were measured in 8 ml of a toluene/Triton (2 : 1, v/v) solution containing 0.5% 2,5-diphenyloxazole and 0.8 ml H_2O.

Specific binding of [^{3}H]PGD_2 was defined as the total radioligand binding minus nonspecific binding which was not competed for by 6 μM of unlabeled PGD_2, and the data were then plotted according to Scatchard [15].

Phosphorylation assay

An aliquot of the membrane fraction (approx. 120 μg protein) suspended in 100 μl of pH-adjusted PBS containing 0.36 U/ml acid phosphatase or 5 mM molybdate, if necessary, was incubated with 10 μM [γ-^{32}P]ATP (20 μCi). The reaction was performed for 60 min at 0°C, or for 1–10 min at 37°C, and terminated by the addition of 10 μl of a stopping solution (20% SDS and 10 mM 2-mercaptoethanol). The mixture was boiled for 5 min, after which 0.01% Bromophenol blue and 10% glycerol were added.

SDS-polyacrylamide gel electrophoresis

Slab gel electrophoresis on a 12.5% polyacrylamide gel was performed according to the method of Laemmli [16]. Electrophoresis was usually conducted in a 12×14 cm slab gel for about 3–4 h. The gels were stained, destained and dried as described by Sandmeyer et al. [17]. The ^{32}P-labeled phosphorylated proteins were identified by autoradiographing the dried gels with Fuji X-ray film (Kanagawa, Japan) for 1 day at −80°C.

Protein

The protein content was determined by the method of Lowry et al. [18] with bovine serum albumin as the standard.

Materials

The prostaglandins were purchased from Funakoshi Pharmaceutical, Tokyo, Japan. They were dissolved in DMSO and diluted with PBS just prior to use. The final concentration of DMSO was adjusted so as to be less than 0.04% (v/v) in the incubation mixture. [^{3}H]PGD_2 (100 Ci/mmol) was obtained from New England Nuclear; $^{32}PO_4$ (888 MBq/ml) and [γ-^{32}P]ATP (3000 Ci/mmol) from Amersham-Japan, Tokyo, Japan; alkaline [EC 3.1.3.1] and acid [EC 3.1.3.2] phosphatases, PMSF, DFP and leupeptin were from Sigma, St. Louis, MO, U.S.A. Other chemicals of the highest available quality were obtained from commercial sources.

Results

Effect of pH on the [^{3}H]PGD_2 binding to mastocytoma P-815 cells

As shown in Fig. 1A, the specific binding of [^{3}H]PGD_2 to mastocytoma P-815 cells was low at pH 7.2, but increased 6–7-fold to the maximum level as the

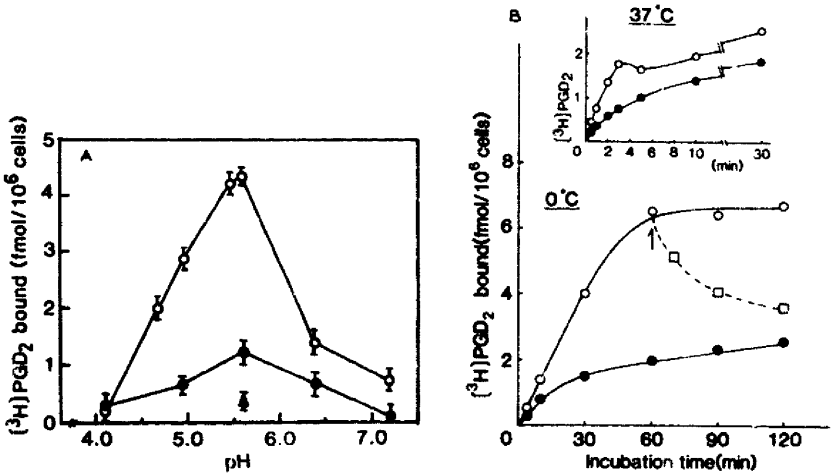

Fig. 1. (A) Effect of pH on [^{3}H]PGD_2 binding to mastocytoma P-815 cells. Mastocytoma P-815 cells ($6 \cdot 10^6$ cells) were incubated at 0 °C for 60 min (○) or at 37 °C for 3 min (●) or 10 min (▲) in 100 μl of PBS containing 6 nM [^{3}H]PGD_2 with or without 6 μM PGD_2. The pH of the incubation buffer was adjusted by adding aliquots of 0.5 M acetic acid. Each value represents the mean ± S.E. of four samples. (B) Time-course of binding and dissociation of [^{3}H]PGD_2 in mastocytoma P-815 cells. Mastocytoma P-815 cells ($5 \cdot 10^6$ cells) were incubated at 0 or 37 °C (inset) in a binding medium containing 5 nM [^{3}H]PGD_2. Total binding (○) was obtained as described in Materials and Methods. Nonspecific binding (●) was determined by including 6 μM PGD_2 in the incubation mixture. After 60 min incubation at 0 °C, 6 μM unlabeled PGD_2 was added (arrow) to initiate dissociation of [^{3}H]PGD_2, and the amount of [^{3}H]PGD_2 bound was determined after various time intervals (□). This figure is a typical record from one of three similar experiments. Each point represents the mean value of duplicate determinations.

pH was lowered to 5.6 through the addition of acetic acid to the reaction mixture incubated at 0 °C or 37 °C, and decreased again to negligible levels as the pH was further lowered to 4.0. The cell viability remained unchanged in the pH range tested, as judged on the basis of nigrosin exclusion (data not shown). Fig. 1B shows a typical time-dependency of [^{3}H]PGD_2 binding to mastocytoma P-815 cells incubated at pH 5.6 at different temperatures (0 °C or 37 °C). 60 min after the incubation at 0 °C, both the total and nonspecific binding of [^{3}H]PGD_2 to the cells increased only slightly with time, and the specific binding, i.e., the differences between total and nonspecific binding, apparently reached equilibrium. The addition of a large excess of unlabeled PGD_2 at 60 min caused displacement of more than 75% of specifically bound [^{3}H]PGD_2 within 60 min, indicating that the binding of [^{3}H]PGD_2 was reversible. At 37 °C, on the other hand, nonspecific binding of [^{3}H]PGD_2 increased linearly with the incubation time, whereas the specific binding exhibited a maximum in 3 min and the level was maintained for a further 30 min of incubation (inset in Fig. 1B).

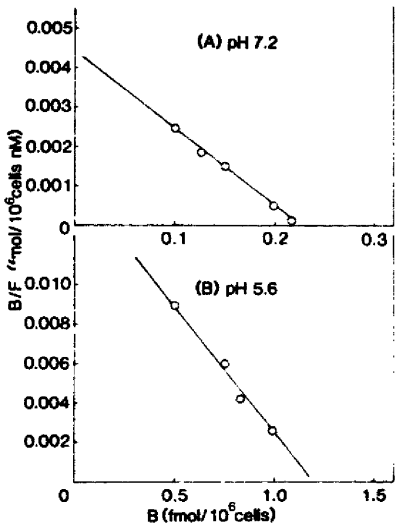

Fig. 2. Scatchard plots of [^{3}H]PGD_2 binding to mastocytoma P-815 cells at pH 7.2 and 5.6. Mastocytoma P-815 cells ($6 \cdot 10^6$ cells) were incubated at 0 °C for 60 min at pH 7.2 (A) or 5.6 (B) in PBS containing 6 nM [^{3}H]PGD_2 and 0–6 μM PGD_2. Each value is the mean of triplicate determinations.

Based on these results, the specific binding of [^{3}H]PGD_2 was routinely determined at 0 °C for 60 min, or at 37 °C for 3 min, if necessary.

Scatchard plot analysis of PGD_2 binding sites on mastocytoma P-815 cells

[^{3}H]PGD_2 binding in the presence of various amounts of PGD_2 at pH 7.2 (Fig. 2A) or at pH 5.6 (Fig. 2B) was determined by incubating the cells at 0 °C for 60 min. Scatchard analysis of the binding data gave a straight line, indicating the existence of a single class of high-affinity binding sites with equilibrium dissociation constants (K_d) of 78.0 nM (at pH 7.2) and 85.3 nM (at pH 5.6). B_{max} values were 0.23 fmol/10^6 cells (145 sites/cell) and 1.56 fmol/10^6 cells (983 sites/cell), at pH 7.2 and 5.6, respectively.

Subcellular distribution of [^{3}H]PGD_2 binding sites

Among the subcellular fractions of mastocytoma P-815 cells tested, the two membrane fractions (LM and

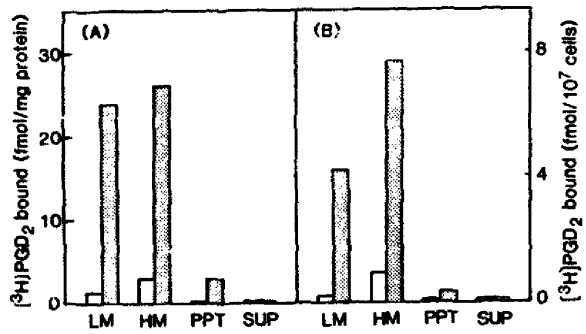

Fig. 3. [^{3}H]PGD_2 binding to subcellular fractions of mastocytoma P-815 cells. Aliquots of each subcellular fraction (approx. 130 μg protein) of mastocytoma P-815 cells were incubated at 0 °C for 60 min in PBS (100 μl) containing 6 nM [^{3}H]PGD_2 with or without 6 μM PGD_2 at pH 7.2 (open column) and 5.6 (hatched column). (A) [^{3}H]PGD_2 bound per mg protein, (B) [^{3}H]PGD_2 bound per $1 \cdot 10^7$ cells. LM, light membrane; HM, heavy membrane; PPT, 66000 × *g* precipitate on sucrose gradient centrifugation (containing mitochondria and lysosomes); SUP, 30000 × *g* supernatant (containing microsomes). Each value is the mean of duplicate determinations. Similar results were obtained for three more experiments of the same design.

HM) showed high [^{3}H]PGD_2 binding activity. Approx. 60 and 80% of the total amount of specific [^{3}H]PGD_2 binding was recovered in the combined LM and HM fractions at pH 5.6 and 7.2, respectively. The [^{3}H]PGD_2 binding to the membrane fractions was also higher at pH 5.6 (Fig. 3). At this pH, the relative abilities of several prostaglandins to displace the [^{3}H]PGD_2 bound to the membrane were in the order of $PGD_2 \gg PGE_2 \geq PGE_1 \gg PGF_{2\alpha}$ (Fig. 4), essentially the same order as the potencies of these prostaglandins in whole cells at pH 5.6, as well as in the membranes and whole cells at pH 7.2 (data not shown).

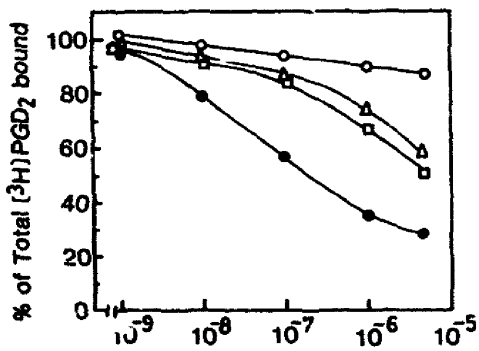

Fig. 4. Specificity of [^{3}H]PGD_2 binding to plasma membranes of mastocytoma P-815 cells. Plasma membranes (130 μg protein) were incubated at 0 °C for 60 min in PBS (100 μl) at pH 5.6 containing 6 nM [^{3}H]PGD_2 and 0–6 μM PGD_2 (●), PGE_1 (□), PGE_2 (△) or $PGF_{2\alpha}$ (○). The results are expressed as percentages of the total radioactivity (100% = 3137 dpm (14.1 fmol)/mg protein) recovered for the membranes incubated with 6 nM [^{3}H]PGD_2 alone. Each value is the mean of duplicate determinations. Similar results were obtained for two more experiments of the same design.

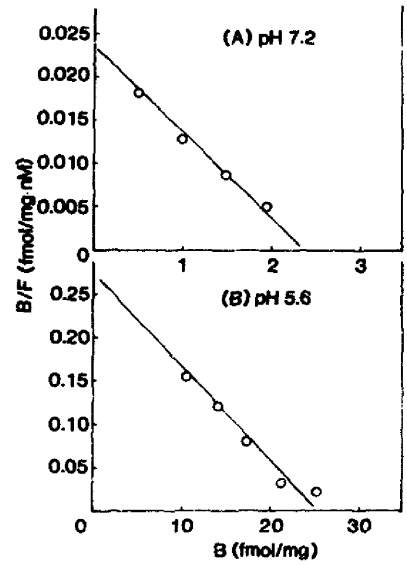

Fig. 5. Scatchard plots of [^{3}H]PGD_2 binding to plasma membranes at pH 7.2 and 5.6. Plasma membranes of mastocytoma P-815 cells (130 μg protein) were incubated at 0 °C for 60 min in 100 μl of PBS containing 6 nM [^{3}H]PGD_2 and 0–6 μM PGD_2 at pH 7.2 (A) or 5.6 (B). Each value is the mean of triplicate determinations.

The presence of a single class of binding sites for PGD_2 was also confirmed for the membrane fractions by Scatchard analysis (Figs. 5A and 5B). At pH 5.6, the PGD_2 binding site with a K_d value of 83.7 nM was calculated to have a B_{max} of 25.5 fmol/mg protein. On the other hand, at pH 7.2, the B_{max} value of the PGD_2 binding site (2.35 fmol/mg protein) was about one-eighth of that at pH 5.6, while the K_d value was similar (80.5 nM).

Effect of acid pretreatment on [^{3}H]PGD_2 binding to the cells

Mastocytoma P-815 cells, which had been washed after preincubation at pH 5.6 for 30 min at 0 °C, still retained higher [^{3}H]PGD_2 binding ability than did untreated cells when assayed at pH 7.2 (Table I). On the other hand, lowering the extracellular pH to 5.6, after the cells had been prelabeled with PGD_2 at pH 7.2 by incubation at 0 °C for 60 min following the removal of unbound PGD_2, still stimulated the cells to bind [^{3}H]PGD_2. In contrast to [^{3}H]PGD_2, the binding of [^{3}H]dihydroalprenolol and [^{3}H]cyclohexyladenosine to the membranes was not enhanced by the acid treatment, instead, that of DHA to the β-adrenoceptor was strongly inhibited (Table II). Thus, the stimulation of binding by acid treatment was shown to be specific for PGD_2 in mastocytoma P-815 cells.

Effects of NaF, molybdate and phosphatase on [^{3}H]PGD_2 binding to the membranes

[^{3}H]PGD_2 binding to the membranes at pH 5.6 or 7.2 increased on the addition of a phosphatase inhibitor

TABLE I

Effect of acid treatment on [^{3}H]PGD$_2$ binding to mastocytoma P-815 cells

The cells were preincubated at pH 7.2 or 5.6 in PBS at 0°C for 30 min. The pH was then adjusted to 5.6 or 7.2 by the addition of a small amount of acetic acid or NaOH. The cells were washed and resuspended in the second incubation buffer of the required pH, and [^{3}H]PGD$_2$ binding was determined after incubation at 0°C for 60 min. Each value is the mean of three determinations ± S.E.

pH of PBS		[^{3}H]PGD$_2$ bound (fmol/10^6 cells)
1st	2nd	
7.2	7.2	0.225 ± 0.033
	5.6	1.67 ± 0.14
5.6	5.6	2.65 ± 0.29
	7.2	1.68 ± 0.22

such as NaF or molybdate to the incubation medium. ATP (10 μM) was the only nucleotide effective in stimulating [^{3}H]PGD$_2$ binding to the membranes (Table III, Experiment I). On the other hand, pretreatment of the membranes with acid phosphatase for 10 min at 37°C almost completely inhibited the [^{3}H]PGD$_2$ binding ability of the membranes at both pH 5.6 and 7.2. However, the [^{3}H]PGD$_2$ binding activity did partially recover on incubation of the phosphatase-pretreated membrane in a phosphatase-free medium containing molybdate for 1 min at 37°C (Table III, Experiment II). The recovered binding sites had a K_d value of 35.2 nM and a lower B_{max} value (6.89 fmol/mg protein) (Fig. 6). The addition of various proteinase inhibitors, such as trypsin inhibitor, PMSF, DFP or leupeptin to the cells incubated with acid phosphatase at pH 5.6, did not modify the enzyme activity in inhibiting the PGD$_2$ binding, which indicates that the decrease of binding ability of the cells by incubation with acid phosphatase was not due to the contamination of proteinase activity in the preparation (data not shown).

TABLE II

Effect of pH on the binding of [^{3}H]dihydroalprenolol ([^{3}H]DHA) and [^{3}H]cyclohexyladenosine ([^{3}H]CHA) to mastocytoma P-815 cells

The cells (5·10^6 cells) were incubated with 8 nM [^{3}H]DHA or 7 nM [^{3}H]CHA in the presence or absence of the respective nonradioactive ligand at a 1000-fold higher concentration in PBS medium of different pH values at 37°C for 30 min. Radioactivity was determined as described in the text. Each value represents the label bound to the cells and is the mean of three determinations ± S.E.

Incubation pH	[^{3}H]DHA bound (fmol/10^6 cells)	[^{3}H]CHA bound (fmol/10^6 cells)
7.17	42.3 ± 1.5	7.57 ± 0.58
6.62	10.6 ± 0.8	7.75 ± 0.64
5.54	5.42 ± 0.25	5.54 ± 0.15

TABLE II

Effects of NaF, molybdate, nucleotides and phosphatase on [^{3}H]PGD$_2$ binding to the membranes of mastocytoma P-815 cells

Experiment I. Plasma membranes (131 μg of protein) of mastocytoma P-815 cells were incubated at 37°C for 1 min in 100 μl of PBS at pH 7.2 and 5.6 in the presence of 6 nM [^{3}H]PGD$_2$ with or without 6 μM PGD$_2$ and each reagent: NaF (0.1 mM), molybdate (5 mM) or a nucleotide (10 μM). Experiment II. Plasma membranes (128 μg protein) of mastocytoma P-815 cells were incubated with acid phosphatase (0.36 U/ml) at 37°C for 10 min, centrifuged and washed with cold PBS to remove the residual enzyme. The phosphatase-pretreated membranes were further incubated with molybdate at 37°C for 1 min in 100 μl of PBS at pH 7.2 and 5.6 with or without phosphatase (0.36 U/ml) in the presence of 6 nM [^{3}H]PGD$_2$ with or without 6 μM PGD$_2$. Each value is the mean of three determinations ± S.E. * statistical significance $P < 0.05$.

Treatment	[^{3}H]PGD$_2$ bound (fmol/mg protein)	
	pH 7.2	pH 5.6
Experiment I		
Control	1.32 ± 0.23	4.72 ± 0.35
NaF	2.72 ± 0.28 *	14.1 ± 0.42 *
Molybdate	3.72 ± 0.36 *	13.6 ± 0.54 *
ATP	2.22 ± 0.26 *	10.7 ± 0.32 *
GTP	1.30 ± 0.35	3.28 ± 0.25
CTP	1.30 ± 0.24	3.56 ± 0.36
ITP	1.28 ± 0.22	2.89 ± 0.22
Experiment II		
Without phosphatase	1.10 ± 0.13	3.68 ± 0.15
With phosphatase	0.21 ± 0.05	0.56 ± 0.06

Effect of molybdate on phosphorylation of membrane proteins

To clarify whether the activation of [^{3}H]PGD$_2$ binding by molybdate correlates with the phosphorylation of protein(s), the membrane preparation was incubated in the presence of [γ-^{32}P]ATP at pH 5.6 and then analyzed

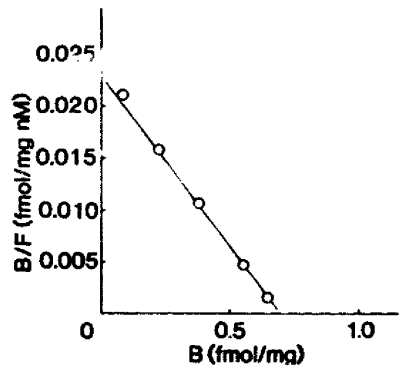

Fig. 6. Scatchard plots of [^{3}H]PGD$_2$ binding to acid phosphatase-pretreated plasma membranes. Plasma membranes (130 μg protein) were pretreated at pH 5.6 with acid phosphatase (0.36 U/ml) in 100 μl of PBS at 37°C for 20 min. The membranes were then washed with cold PBS and assayed for [^{3}H]PGD$_2$ binding at pH 5.6 without acid phosphatase (0.36 U/ml). Scatchard analysis was performed in a similar manner to that in Fig. 5. Each value is the mean of triplicate determinations.

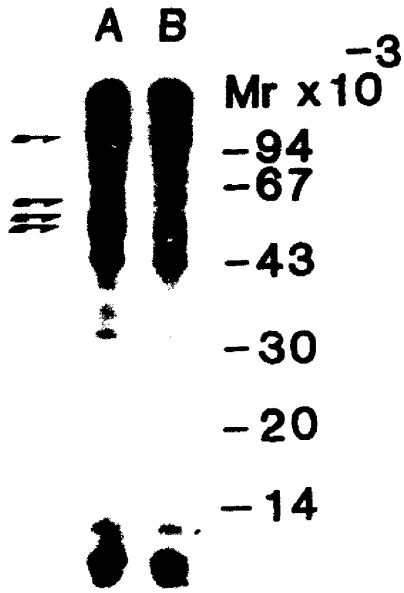

Fig. 7. Autoradiogram of the SDS-polyacrylamide gel after electrophoresis of ^{32}P-labeled plasma membranes. Plasma membranes (125 μg protein) were incubated with 10 μM [γ-^{32}P]ATP at 0 °C for 60 min in the presence (A) or absence (B) of 5 mM molybdate. The phosphorylation reaction was terminated by the addition of a stopping solution containing SDS. Equal aliquots of the reaction mixtures were subjected to electrophoresis on a 12.5% polyacrylamide gel and then autoradiographed with X-ray film at −80 °C. The numbers on the right side are the molecular weights of standard marker proteins. Arrows indicate the peptides strongly phosphorylated in the presence of molybdate.

using SDS-polyacrylamide gel electrophoresis. As shown in Fig. 7, several peptides (shown as arrows) were strongly labeled in the presence of molybdate. When the plasma membrane, even when pretreated with phosphatase and washed, was incubated in the presence of [^{32}P]ATP, molybdate strongly stimulated the incorporation of [^{32}P]phosphate into the peptides (data not shown).

Discussion

In the present study, we have demonstrated that mastocytoma P-815 cells have specific binding sites for PGD_2 on the plasma membrane, in addition to those for PGE_1 [13]. We have also demonstrated that the binding activity is much higher at an acidic pH than under neutral conditions. The stimulatory effect of acid treatment on [^{3}H]PGD_2 binding to whole cells and to plasma membranes was due to the increases in B_{max} values but not of K_d values. In addition, the stimulation at pH 5.6 was observed even at 0 °C, and even after raising the pH to 7.2. These results suggest that the higher binding activity under acidic conditions is not due to the new synthesis of PGD_2-receptor components or to the alteration of membrane fluidity. One possible explanation for higher PGD_2 binding at pH 5.6 and at 0 °C is that, in this condition, more binding sites are accessible because at pH 7.2 and at 37 °C these sites are endocytosed. However, this is unlikely because, as shown in Table I, [^{3}H]PGD_2 binding was increased in acidic pH at 0 °C, whether the cells were washed at pH 5.6 or at pH 7.2 at 0 °C, under which condition, internalization of receptors cannot take place. It is known that exposure of ligand-receptor complexes to a mildly acidic condition causes dissociation of several ligands, including EGF [19], insulin [20], α_2-macroglobulin [21], lysosomal enzymes [22] and glucagon [23], from their receptors on the surface of cell membranes. [^{3}H]PGD_2 binding was stimulated by lowering the pH in cells which had been prelabeled with PGD_2 at pH 7.2. In addition, [^{3}H]PGD_2 binding was stimulated at pH 5.6 in the membrane preparation. The endocytic uptake of the [^{3}H]PGD_2-receptor complex into the cytoplasm seems unlikely to be the cause of the increase in [^{3}H]PGD_2 binding sites in mastocytoma P-815 cells when exposed to acidic pH at 0 °C. This is because the endocytic reaction of ligand-receptor complexes is well-known to be temperature- and energy-dependent and also to reduce the number of cell surface receptors [24]. Although an acid-induced increase in prostaglandin binding was also reported for PGE_2 receptors on human peripheral monocytes [24], fat cells [25], and bovine adrenal membranes [26], and for PGE_1 receptors on bovine thyroid membranes [27], it is not clear whether the pH-dependency for prostaglandin binding is a property of the ligands, their receptors, or both. In addition to the exact mechanism of the pH-dependency, the physiological significance of these phenomena remains unclear. The acid stimulation of PGD_2 binding may reflection the reaction under the acidic condition of inflammatory tissues.

K_d values for the [^{3}H]PGD_2 binding sites of mastocytoma P-815 cells (85.3 nM) and their membranes (86.4 nM) were similar to that reported for rat brain synaptic membranes (28 nM) [5]. This binding site is highly specific to PGD_2, whereas PGE_1, PGE_2, $PGF_{2\alpha}$ displaced [^{3}H]PGD_2 binding only at considerably higher concentrations. On the other hand, the binding capacity of mastocytoma P-815 cells for PGD_2 was only about 1/30 to 1/100 of that for PGE_1 (B_{max} values of the high- and low-affinity binding sites for PGE_1 were 12 and 31 fmol/10^6 cells [13], versus those for PGD_2, 0.23 fmol/10^6 cells, at pH 7.2). In addition, the K_d value for PGD_2 is similar to the value for the low-affinity sites of PGE_2 (22 nM), but not to the high-affinity sites (1.1 nM) [13]. These differences reflect the much lower ability of PGD_2 compared to that of PGE_1 in stimulating cAMP synthesis in mastocytoma P-815 cells. Therefore, the potent growth inhibition of mastocytoma P-815

cells by PGD_2 [11] may not be mediated by cAMP. In various leukemia cell lines, PGD_2 and Δ^{12}-PGJ_2, the ultimate PGD_2 metabolite which has a growth inhibitory effect on these cells, were reported to be virtually inactive with respect to raising the intracellular cAMP level [28]. At present, it is unclear whether or by what means PGD_2 receptors of mastocytoma P-815 cells mediate PGD_2-induced growth inhibition.

NaF [29] and molybdate [30], which are inhibitors of acid phosphatase, were reported to stimulate hormone binding, as in the case of estradiol receptors from rat [31] and calf uteri [32], and glucocorticoid receptors from rat thymocytes [33]. Similarly to these findings, NaF or molybdate significantly activated [^{3}H]PGD_2 binding of mastocytoma P-815 cells. Furthermore, the increase in [^{3}H]PGD_2 binding by molybdate was comparable to the increased phosphorylation of several peptides observed in SDS-polyacrylamide gel electrophoresis. Among them, some peptides having the suspected molecular weights between 100000 and 120000 nearly correspond to the molecular weights for PGE receptors reported in various tissues. The PGE_2 receptors from murine macrophage [34], rat liver plasma membranes [35], and bovine adrenal membranes [26] were shown to have molecular weights of 98000, 105000 and 110000, respectively. However, none of these receptor peptides has been purified. Thus the exact relationship between phosphorylated peptides and prostaglandin receptors is at present unclear.

Although the characteristics of the phosphorylated peptides have not yet been identified, it is possible that the binding capacity of PGD_2 receptors is regulated through phosphorylation and dephosphorylation of the receptor itself. Treatment of the membranes with phosphatase caused a loss of PGD_2 binding sites. However, incubation of the phosphatase-pretreated membranes in a phosphatase-free medium containing ATP, divalent cations and molybdate at 37°C resulted in the partial recovery of these binding sites. These results suggest that reversible phosphorylation and dephosphorylation of the [^{3}H]PGD_2 protein might occur as a result of the action of an unidentified protein kinase and a phosphatase present in the membranes. We have detected in our preliminary experiment that the binding of [^{3}H]PGE_1 to mastocytoma membranes was also stimulated in the presence of ATP and molybdate, and disappeared by treating cells with phosphatase (data not shown). The insulin receptor from rat liver plasma membranes [36] and the epidermal growth factor receptor from A-431 human epidermoid carcinoma cells [37] have been partially purified as receptor proteins containing kinase activity. We cannot rule out the possibility that the target protein for phosphorylation is not the receptor itself but other protein(s), the phosphorylation of which leads to the activation of [^{3}H]PGD_2 binding. Further purification and characterization of the binding protein of mastocytoma P-815 cell membrane is required to clarify these points.

Neoplastic mouse mastocytoma P-815 cells have a number of properties characteristic of normal differentiated mast cells containing histamine, serotonin and other components, though in much lower levels compared with normal mast cells [9,38]. In addition, the stimulatory effect of PGD_2 on cAMP synthesis to normal mast cells was observed to be great in the presence of theophylline [38], resulting in a marked inhibition of antigen-stimulated histamine release from mast cells [4]. This specificity of the cells in the response to the stimulation by PGD_2 is likely to arise from the difference in the number of PGD_2 receptors between the two cell types. Therefore, the PGD_2 receptor, the presence of which has been clearly demonstrated in neoplastic mastocytoma P-815 cells, may play a role in the physiological activity of normal mast cells.

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