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SOLUBILIZATION AND CHARACTERIZATION OF LEUKOTRIENE B_4 RECEPTOR-GTP BINDING PROTEIN COMPLEX FROM PORCINE SPLEEN

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SUMMARY: A high amount of leukotriene B_4 (LTB_4) binding protein was observed in the porcine spleen. It was solubilized and partially purified from spleen membrane with 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS). Scatchard analysis indicated the presence of a single class of receptor with K_d and B_{max} values of 0.26 nM and 120 fmol/ mg protein, respectively. The receptor was specific for LTB_4, and K_i values for 20-hydroxy- and 20-carboxy-LTB_4, both inactive metabolites of LTB_4, were 1.7 nM and over 1,000 nM, respectively. By the addition of 10 μM GTPγS, a low affinity binding site appeared with a K_d value of 390 nM. A pretreatment of the receptor-GTP binding protein complex with islet-activating protein (IAP) increased the inhibitory effect of GTPγS on LTB_4 binding, indicating that the LTB_4 receptor is coupled with an IAP-sensitive GTP-binding protein in the porcine spleen.

Leukotrienes (LTs), a group of compounds derived from arachidonic acid, have a wide variety of biological activities (1,2). Arachidonic acid is converted to leukotriene A_4 (LTA_4) by the action of 5-lipoxygenase (3). LTA_4 is hydrolyzed to yield 5(*S*),12(*R*)-dihydroxy-6,14-

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Abbreviations: LTA_4, 5(*S*),6(*S*)-epoxy-7,9-*trans*-11,14-*cis*-eicosatetraenoic acid; LTB_4, 5(*S*),12(*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; GTPγS, guanosine 5'-O-(3-thiotriphosphate); and IAP, islet-activating protein.

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cis-8,10-*trans*-eicosatetraenoic acid (LTB_4) by LTA_4 hydrolase (4,5). LTB_4 is a potent stimulator of chemotaxis, aggregation, lysosomal enzyme release and superoxide anion generation of neutrophils (1,6). LTB_4 is reported to increase intracellular Ca^{2+} concentration (7,8), and induces phosphatidylinositol breakdown (7,9-11). Although a treatment of neutrophils with islet-activating protein (IAP) diminishes the LTB_4 action (7,9-12), and interactions between LTB_4 receptor and GTP-binding protein were proposed (13), direct evidence for the coupling of GTP-binding protein(s) and receptor has not been documented. We report here that the solubilized LTB_4 receptor is coupled with GTP-binding proteins in the porcine spleen.

MATERIALS AND METHODS

Materials---Commercial sources of materials and reagents were as follows. [^{3}H]LTB_4(174 Ci/mmol), from DuPont; 20-hydroxy-, 20-carboxy-LTB_4 and IAP (14), from Funakoshi (Tokyo); GTPγS and NAD, from Boehringer Mannheim; 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), from Wako Pure Chemicals (Osaka). LTB_4 was synthesized as described previously (15).

Solubilization and gel filtration of LTB_4 receptor---Porcine spleen (36 g) was homogenized in 5 volumes of 50 mM Tris-HCl buffer (pH 7.3)/1 mM EDTA with a Physcotron homogenizer (30 sec, 5 times). All procedures were carried out at 0-4°C unless stated otherwise. The homogenate was centrifuged at 1,000 x *g* for 10 min, followed by further centrifugation at 35,000 x *g* for 20 min. The precipitate was suspended in 28 ml of 50 mM Tris-HCl buffer (pH 7.3), and stored frozen at -80°C until use (the membrane fraction). To the membrane fraction (200 mg protein/14 ml), the buffer was added to give final concentrations of 50 mM Tris-HCl (pH 7.3), 20 %(w/v) glycerol, 10 mM $MgCl_2$ and 1 %(w/v) CHAPS. The mixture was stirred for 30 min, and centrifuged at 133,000 x *g* for 1 h. The supernatant fractions (77 mg protein/12 ml) were divided into half and applied (6 ml each) to a Superose 12 gel filtration column (2.6 x 60 cm, Pharmacia), which was equilibrated and eluted with 50 mM Tris-HCl buffer (pH 7.3)/1 mM EDTA/20 %(w/v) glycerol/0.1 %(w/v) CHAPS at a flow rate of 3 ml/min. The active fractions (60 mg protein/30 ml) were combined, and defined as the solubilized receptor.

Binding Assay---LTB_4 receptor (0.3 mg protein for the solubilized receptor or 0.5 mg protein for the membrane fraction) was preincubated with 50 mM Tris-HCl buffer (pH 7.3) containing 10 mM $MgCl_2$ and reagents (total volume of 0.5 ml) for 5 min at 25°C. Addition of 10 mM $MgCl_2$ increased the binding activity by 78 %. [^{3}H]LTB_4 (0.114 nM, 22,200 dpm) was added to start the reaction, and the incubation was carried out for 30 min at 25°C. Ice-cold 50 mM Tris-HCl buffer (pH 7.3) containing 10 mM $MgCl_2$ (4 ml) was added to reaction tubes, and the contents were passed through a Whatman glass filter, which was washed three times with 4 ml each of ice-cold buffer. GF/B filter (25 mm diameter) soaked in 0.3 % (w/v) polyethyleneimine over 24 h (16) was used for the solubilized receptor, and Whatman GF/C filter for the membrane fraction. The filter was dried, and the radioactivity was determined. Nonspecific binding was measured in the presence of 2 μM unlabeled LTB_4. The specific binding was defined as the difference between total and nonspecific binding. The specific binding constituted more than 90 % of the total binding. LTB_4 was not metabolized under the present assay conditions, as analyzed by

high performance-liquid chromatography (4). Computer analysis methods (EBDA and LIGAND) (17) were used to evaluate the results. The protein concentration was determined by the method of Lowry *et al.* with bovine serum albumin as a standard (18).

IAP-treatment of solubilized receptor-GTP binding protein complex---The solubilized receptor was treated with the preactivated A-protomer of IAP, essentially according to the method described by Okajima *et al.* (19). The solubilized receptor (4 mg) was incubated with activated IAP (50 μg) at 30°C for 20 min in a 2.5 ml of the mixture containing 10 mM Tris-HCl buffer (pH 7.3), 10 mM NAD, 1 mM ATP, 1 mM EGTA, 10 mM thymidine, 2 mM dithiothreitol, 2.5 mM $MgCl_2$, 3 mM phosphoenolpyruvate, and 10 μg/ml pyruvate kinase. The control incubation was done in the reaction mixture without IAP. After incubation, the reaction mixture was applied to a PD-10 column (Pharmacia), which had been equilibrated with 50 mM Tris-HCl buffer (pH 7.3)/1 mM EDTA/20% glycerol/0.1 % CHAPS to remove small-size molecules.

RESULTS

Distribution of LTB_4 binding activity---The LTB_4 binding activity was high in porcine leukocytes (33,000 dpm/mg protein, n=3), and spleen (14,000 dpm/mg, n=3). In the guinea pig, the specific binding activity was high in the spleen (8,730 dpm/mg, n=3) and lung (4,790 dpm/mg, n=2), followed by brain (2,800 dpm/mg, n=2), and large and small intestines (1,900 and 1,400 dpm/mg, n=2, respectively). There were no evidence of binding in heart, stomach, liver and kidney of the guinea pig. Among various species, the porcine spleen had the highest activity (14,000 dpm/mg), while rat spleen had a negligible amount of binding activity (460 dpm/mg protein, n=3). An intermediate result was obtained in the bovine spleen (4,110 dpm/mg protein, n=3).

Characterization of the solubilized LTB_4 receptor---On gel filtration, the solubilized receptor was eluted at a retention volume corresponding to a molecular weight around 650 kDa as estimated by Superose 6 (1.6 x 50 cm, Pharmacia) gel filtration. While 10 mM divalent cations ($MgCl_2$, $MnCl_2$, $CaCl_2$) were stimulatory by about two-fold for the receptor binding, NaCl, KCl and LiCl at 1 M were rather inhibitory by 63 %, 31 % and 83 %, respectively. The pH optima for the binding was between 6 and 8. The specific binding reached to a plateau in 20 min at 25°C, and the bound [^{3}H]LTB_4 was reversibly dissociated by the addition of 2 μM unlabeled LTB_4. By Scatchard analysis, a single entity of the binding site was observed with K_d and B_{max} values of 0.26 nM and 120 fmol/mg protein, respectively (Table I). The binding was specific for LTB_4. K_i values for LTB_4, 20-hydroxy-, and 20-carboxy-LTB_4 were 0.33, 1.7 and over 1,000 nM, respectively (Fig. 1). The LTB_4 receptor became unstable after ion exchange chromatography such as DEAE-5PW column (Tosoh, Tokyo). After 24 h at 4°C, no binding activity was detected. Further purification, therefore, has not been successful so far.

Table I

K_d and B_{max} values of $[^3H]LTB_4$ binding sites of porcine spleen membrane fraction and solubilized receptor. $[^3H]LTB_4$ (0.114 nM, 22,200 dpm) was incubated with spleen membrane fraction (0.5 mg protein), or solubilized receptor (0.3 mg protein) in 50 mM Tris-HCl buffer, pH 7.3 containing 10 mM $MgCl_2$ and increasing concentrations of unlabeled LTB_4. The data were analyzed as described (EBDA and LIGAND) (17).

Receptors	Addition of GTPγS	High affinity site		Low affinity site	
		K_d	B_{max}	K_d	B_{max}
	μM	nM	fmol/mg protein	nM	fmol/mg protein
Membrane fraction	0	1.0	230	ND[a]	
	10	1.7	230	630	22,000
Solubilized receptor	0	0.26	120	ND[a]	
	10	1.6	230	390	13,000

[a] ND, not detected.

Effects of GTPγS and IAP-treatment---Addition of 10 μM GTPγS changed the $[^3H]LTB_4$ binding parameters as shown in Fig. 1. The K_d value of the high affinity sites increased from 0.26 nM to 1.6 nM. In addition, the low affinity site with a K_d value of 390 nM appeared (Table I). This phenomenon was also observed in the crude membrane fraction (Table I). $[^3H]LTB_4$ binding was slightly inhibited by IAP treatment. The low affinity site as observed in case of GTPγS addition (Table I and Fig. 1) did not appear. However, the inhibitory effect of GTPγS was enhanced by IAP treatment; namely, at 10^{-8} M concentration of GTPγS, $[^3H]LTB_4$ binding was inhibited by 29 % or 68 % either in the absence or presence

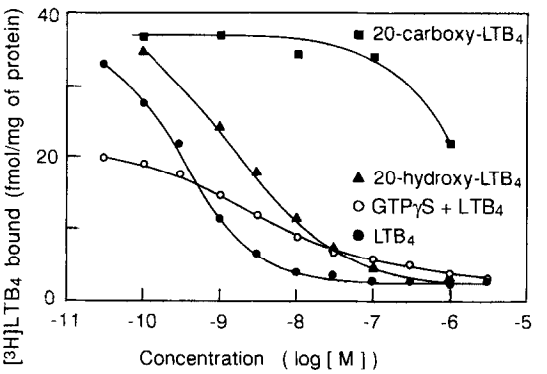

Fig. 1. Inhibition of $[^3H]LTB_4$ binding to porcine spleen receptor by GTPγS, unlabeled LTB_4, and its ω-oxidized metabolites. The solubilized receptor (0.3 mg protein) was preincubated with varying concentrations of LTB_4 (●), 10 μM GTPγS plus LTB_4 (○), 20-hydroxy-LTB_4 (▲) or 20-carboxy-LTB_4(■) for 5 min at 25°C. $[^3H]LTB_4$ was added to initiate the reaction, and the incubation was done for 30 min at 25°C.

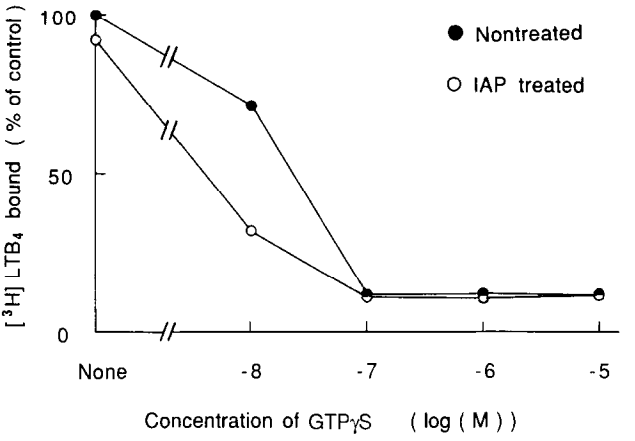

Fig 2. Effect of GTPγS on IAP-treated receptor. Aliquots of IAP-treated receptor (○) or nontreated receptor (●) were preincubated with varying concentrations of GTPγS for 5 min, and [^{3}H]LTB_4 was added to initiate the binding reaction. Data were expressed as the percentage of binding on nontreated sample without GTPγS (100 %, 5,643 dpm).

of IAP treatment (Fig. 2). The maximal reducing effect on LTB_4 binding by GTPγS was the same as observed in the nontreated receptor (Fig. 2).

DISCUSSION

The LTB_4 binding activity was high in leukocytes and spleen in various animals, but the species differences were prominent. A high binding activity of LTB_4 to the guinea pig spleen was reported by Cheng *et al.* (20). It was rich in porcine and guinea pig, but scarcely detected in rat. In the present study, LTB_4 receptor was solubilized and partially purified from porcine spleen in an active form. In contrast to previous reports showing 2 binding sites in neutrophils (21) or HL-60 cells (22), the Scatchard analysis indicates only a single class of receptor in the porcine spleen. The high affinity constant (0.26 nM) is in good agreement with the potent effect of this compound on chemotaxis (< 1 nM (6)). The solubilized receptor bound specifically to LTB_4, and K_i values of the two biologically inactive metabolites were much higher than that of LTB_4, again indicating that the solubilized protein is a functional receptor.

We have also shown for the first time that the solubilized receptor is coupled to the GTP-binding protein(s). The addition of GTPγS, a non-hydrolyzable analogue of GTP, decreased the affinity of LTB_4 to the receptor and induced low affinity binding sites (Table I). Goldman *et al.* implied that the low affinity sites play roles in degranulation and superoxide generation (13) of neutrophils, but the physiological significance of these low-affinity sites in the spleen remains unclear.

Although several groups have reported that biological activities of LTB_4 were blocked by IAP treatment of the cells (7,9-12), direct evidence for the coupling of an IAP-sensitive GTP-binding protein and LTB_4 receptor has not yet been described. IAP-treatment by itself slightly decreased the LTB_4 binding (data not shown), but it augmented the inhibitory effect of GTPγS on LTB_4 binding (Fig. 2). Maximal reducing effects of both treatments were the same. These results can be interpreted either by the presence of two GTP-binding proteins (IAP-sensitive and insensitive) or a partial decrease of the affinity of GTP-binding protein to LTB_4 receptor by ADP-ribosylation. Further studies are necessary including purification, and characterization of functional receptor for LTB_4 and GTP-binding proteins in order to elucidate the signal transduction mechanism of LTB_4 in the spleen.

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