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Leukotriene B_4 Induces Formation of Inositol Phosphates in Rat Peritoneal Polymorphonuclear Leukocytes

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SUMMARY

Leukotriene B_4 (LTB_4) induced rapid breakdown of prelabeled inositol phospholipids in rat peritoneal polymorphonuclear leukocytes (PMNs). Formation of [^{3}H]inositol trisphosphate ([^{3}H]IP_3) was rapid, with a peak of 250–300% of the control level, after 5–15 sec of exposure to LTB_4. Accumulation of [^{3}H]inositol bisphosphate was rapid, peaking after 30 sec of treatment. Accumulation of [^{3}H]inositol monophosphate was also rapid in the presence of LiCl. The kinetics of [^{3}H]IP_3, [^{3}H]inositol bisphosphate, and [^{3}H]inositol monophosphate accumulation suggest that LTB_4 may interact with receptors in PMNs and activate phospholipase C which in turn induces hydrolysis of inositol-phospholipids. The agonist activites of several LTB_4 analogs were employed to investigate the structure-activity relationships of LTB_4 receptor-mediated activation of phosphatidylinositol hydrolysis. Increases in [^{3}H]IP_3 formation were dependent upon the concentration of LTB_4 and the agonist analogs. The rank order potency of these analogs was equivalent to that of the pharmacological activity of LTB_4 agonists in the PMN chemotaxis assay. Furthermore, the islet activation protein isolated from *Bordetella pertussis* inhibited LTB_4-induced [^{3}H]IP_3 formation. The tumor-promoting phorbol myristate acetate also inhibited LTB_4-induced [^{3}H]IP_3 formation. The LTB_4 receptors on a partially purified PMN membrane were characterized. LTB_4 binding to the receptors was stereoselective and specific. The binding affinity (K_d) of [^{3}H]LTB_4 to the receptors was 1.3 ± 0.2 nM. The maximum density of binding was 5.5 ± 1.8 pmol/mg of protein. The rank order potency of binding affinities of several LTB_4 analogs was equivalent to that of the induction of IP_3 response induced by LTB_4 and analogs. These results suggest that LTB_4 may interact with receptors in rat PMNs, activate G protein-regulated phospholipase C, and induce [^{3}H]IP_3 formation.

LTB_4, a 5-lipoxygenase metabolite of arachidonic acid from neutrophils (1), mast cells, and other types of cells, has been demonstrated to have potent proinflammatory activities, e.g., chemotaxis, chemokinesis of PMNs, superoxide production, degranulation and lysosomal enzyme release from PMNs, leukocyte adhesion, and migration to and extravasation from the endothelium (2–7). Thus, LTB_4 is thought to be one of the key mediators of inflammation and may have an important role in hypersensitivity diseases. Using neutrophil chemotaxis and aggregation assays, several laboratories have demonstrated that LTB_4-induced neutrophil chemotaxis and aggregation were stereoselective and structurally specific, suggesting that these effects may be mediated via LTB_4 receptors in PMNs (2–6). Supporting this hypothesis, recent evidence has demonstrated that, in human, rabbit, and rat PMNs or the plasma membrane fraction of PMNs, there are stereoselective, saturable, and structurally specific binding sites for [^{3}H]LTB_4 (8–13). The [^{3}H]LTB_4 binding to the specific sites was modulated by guanine nucleotides and IAP from *Bordetella pertussis*, suggesting that the putative LTB_4 receptors in PMNs are coupled to the inhibitory guanine nucleotide binding protein (G_i) (14, 15).

The fact that the immunopharmacological responses of LTB_4 were regulated by GTP and IAP suggested that LTB_4 may inhibit membrane-bound adenylate cyclase. An inhibition of adenylate cyclase and subsequent decrease of intracellular cAMP may serve as the second messenger for LTB_4 in the PMNs. However, results contrary to this speculation have been reported. Gorman *et al.* (10) have reported that LTB_4 *increased* adenylate cyclase activity in membrane homogenate isolated from human PMNs and elevated the intracellular concentration of cAMP in human and rabbit PMNs. Recently, it has also been reported that LTB_4 may increase intracellular cal-

ABBREVIATIONS: LTB_4, leukotriene B_4; PMN, polymorphonuclear leukocyte; IAP, islet activation protein; G_i, inhibitory guanine nucleotide-binding protein; IP_3, inositol trisphosphate; PIP_2, phosphatidylinositol-bis-phosphate; racemic 5,12-DiHETE (LTB_4-epimer mixture), 5(*S*),12(*S*)-LTB_4 and 5(*R*),12(*R*)-LTB_4; racemic 6-*trans*-5,12-DiHETE (6-*trans*-LTB_4-epimer mixture), 5(*S*),12(*S*)-6-*trans*-LTB_4 and 5(*R*),12(*R*)-6-*trans*-LTB_4; 2-nor-LTB_4, 4(*S*),11(*R*)-dihydroxy-5,13-*cis*-7,9-*trans*-nonadecatetraenoic acid; HBS, HEPES-buffered saline; BSA, bovine serum albumin; IP_1, inositol monophosphate; IP_2, inositol bisphosphate; EDTA, ethylenediaminetetraacetic acid; HETE, hydroxy-eicosatetraenoic acid; TPA, 11-*O*-tetradecanoyl-phorbol 13-acetate; G_o, unknown function quanine nucleotide-binding protein; PMA, phorbol myristate acetate; PDD, αPDD, α-phorbol 12,13-didecanoate; quin2, {8-amino-2-[(2-amino-5-methylphenoxy)methyl]-6-methoxyquinoline-*N*,*N*,*N*′,*N*′-tetraacetic acid}.

cium (15–19). The calcium mobilization effect can be inhibited by IAP, suggesting that Ca^{2+} may serve as an intracellular second messenger for LTB_4 and its receptors. Thus, currently, there is a lack of consensus as to the mechanism of signal transduction for LTB_4 and its receptors.

IP_3, generated from phosphatidylinositol-bis-phosphate (PIP_2), has recently been demonstrated to be an intracellular second messenger for membrane receptors (20). IP_3 has also been demonstrated to induce calcium mobilization in many types of cells (21–24). We have initiated [^{3}H]LTB_4 receptor-binding studies, and studies to investigate whether LTB_4 may induce phosphatidylinositol breakdown and formation of IP_3 in PMNs. Results presented in this paper show that LTB_4 and analogs may bind to membrane-localized receptors and induce IP_3 formation. IP_3 may be the intracellular second messenger for the LTB_4 receptor, and changes in phosphatidylinositol metabolism may precede the calcium mobilization effect of LTB_4 in rat PMNs.

Materials and Methods

Reagents and chemicals. LTB_4 [5(*S*), 12 (*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraeonic acid], 6-*trans*-LTB_4, racemic 5,12-DiHETE (LTB_4 epimer mixture), and racemic 6-*trans*-5,12-DiHETE (6-*trans*-LTB_4-epimer mixture) were prepared by total synthesis employing in part the methodology developed by Corey *et al.* (25). The 2-nor-LTB_4 was similarly prepared. Isolation of all isomers and analogs was accomplished by reverse phase high pressure liquid chromatography. Purity was determined by reverse phase high pressure liquid chromatography, by integrating the area under the peaks with a UV detector at 270 nm: LTB_4, 99.3%; LTB_4 epimeric mixture, 94%; 6-*trans*-LTB_4, 100%; 6-*trans*-LTB_4-epimeric mixture, 86%; 2-nor-LTB_4, 99.5%. 20-OH-LTB_4 was obtained from Biomol Co. (Philadelphia, PA). [^{3}H]LTB_4 (32 and 180 Ci/mmol) and L-*myo*-[1,2-^{3}H(N)]Inositol (40–60 Ci/mmol) were obtained from New England Nuclear (Boston, MA). Casein, bovine serum albumin (sodium form), ammonium formate, formic acid, and lithium chloride were purchased from Sigma Chemical Co. (St. Louis, MO).

Rat Peritoneal PMNs and labeling with [^{3}H]*myo*-inositol. Male inbred Lewis strain rats, body weight 250–350 g, were used. These animals were injected with 10 ml of sterilized casein solution (10%, w/v, in normal saline) intraperitoneally. The PMNs were lavaged from the peritoneal cavity 8–10 hr later with 50 ml of Ca^{2+}, Mg^{2+}-free 10mM HEPES-buffered saline (HBS) solution containing 0.1% BSA (solution A). Approximately 20–50 × 10^6 cells were obtained from each animal. Greater than 95% of the cells were identified as PMNs based on differential staining techniques. The PMNs obtained from each animal were washed twice in solution A, counted, and then resuspended in solution A containing 1.8 mM $MgCl_2$, and 0.2 mM $CaCl_2$ (solution B) to avoid self-aggregation. The density of cells in each tube was adjusted to 10 × 10^6/ml, [^{3}H]*myo*-Inositol was added to each tube at a concentration of 10 μCi/ml. The cells were labeled with [^{3}H]*myo*-inositol for 110 min at 37° with gentle shaking. A small aliquot of concentrated LiCl (2.0 M) was added into each tube to make the final LiCl concentration 10 mM. Incubation was continued for an additional 10 min. These cells were combined and washed twice with 20 ml of solution B containing 0.1% BSA and 10 mM LiCl (solution C). These cells were then resuspended in the prewarmed (37°) oxygenated (95% O_2, 5% CO_2) solution C at a concentration of 10 × 10^6/ml and used within 10 min. Using trypan blue staining technique, these cells remained viable (greater than 98%) after labeling and washing. Under the current conditions, approximately 4–7% of [^{3}H]*myo*-inositol was incorporated into PMNs.

Measurement of inositol phosphates. PMNs were labeled with [^{3}H]*myo*-inositol, washed, and resuspended in prewarmed solution C as described above. Three hundred μl of cell suspension were added into prewarmed (37°) test tubes, in triplicate, containing 100 nM LTB_4 or its analogs and incubated at 37° from 2 sec to 5 min. Alternatively, the cells (300 μl) were added into prewarmed triplicate test tubes containing varying concentrations of LTB_4 (0.1 nM–200 nM) or its analogs (1 nM–100 μM) and incubated for 20 sec. At the end of the incubation, 1.35 ml of chloroform: methanol (1:2) were added into each tube to stop the reaction. The test tubes were maintained at room temperature for 20 min and 450 μl of $CHCl_3$ were added to each tube followed by addition of 450 μl of H_2O to extract water-soluble inositol phosphates. The test tubes were vortexed vigorously for 10 sec and then centrifuged in a Beckman TJ-6 centrifuge at 2000 × *g* for 5 min to separate the aqueous phase from the organic phase solvents. One ml of the aqueous solvent was transferred to a test tube and mixed with 5 ml of H_2O. The [^{3}H]inositol phosphates in the aqueous solvents were separated by anion exchange column chromatography as described previously (26). Briefly, Dowex 1 × 8 anion exchange resin (formate form) was resuspended in H_2O at a weight/volume ratio of 1:1. One ml of the resin suspension was poured into a plastic column (0.5 × 5 cm) and washed with 5 ml of H_2O. The aqueous extract was then loaded onto the column and washed with 5 ml of H_2O four times. [^{3}H]IP_1 was eluted into scintillation counting vials with 1.5 ml of 0.2 M ammonium formate/0.1 M formic acid solution (solution D) twice. The column was then washed with 5 ml of solution D twice. [^{3}H]IP_2 was eluted with 1.5 ml of 0.5 M ammonium formate/0.1 M formic acid solution (solution E) twice. The column was washed with 5 ml of solution E twice. Finally, [^{3}H]IP_3 was eluted with 1.5 ml of 1.0 M ammonium formate/0.1 N formic acid twice. Fifteen ml of scintillation cocktail (Aquasol) were added to each vial, and the radioactivity in the vial was determined by scintillation spectrometry with efficiency of 25–32%. As the currently available methods are insufficient and inefficient to subfractionate the IP_3 fraction in the extract, the IP_3 fraction obtained with the current method probably contains a mixture of inositol-(1,3,4)-trisphosphate, inositol-(1,4,5)-trisphosphate, and possibly inositol-(1,3,4,5)-tetrakis-phosphate.

Preparation of plasma membrane-enriched fraction from rat PMNs. PMNs were obtained from 20 male rats. The cells were washed two times with HBS and resuspended in 40 ml of homogenization solution [20 mM Tris-HCl buffer, pH 7.5, containing 0.25 M sucrose and the following protease inhibitors: phenylmethylsulfonyl fluoride (0.5 mM), soybean trypsin inhibitor (10 μg/ml), bacitracin (100 μg/ml), benzamidine (0.1 mM), aprotinin (40 milliunits/ml), and EDTA (2 mM)]. All of the following procedures were performed at 0–4°. The cells were broken by 150–200 strokes of grinding in a homogenizer. The homogenate was centrifuged at 1,300 × *g* for 10 min to sediment the nuclei and unbroken cells. The supernatant was centrifuged at 100,000 × *g* for 60 min. The pellets were resuspended by homogenization in 80 ml of 10 mM Tris-HCl buffer (pH 7.5) containing 10% sucrose. The membrane suspension was then carefully layered onto 10 ml of 40% sucrose cushion in four nitrocellulose centrifuge tubes. The tubes were centrifuged at 100,000 × *g* for 60 min on an SW-27 swinging bucket rotor. The membranes settled at the boundary layers were collected and diluted with 5 volumes of 10 mM Tris-HCl (pH 7.5). The membranes were then sedimented by centrifugation at 100,000 × *g* for 60 min. The membrane pellets were quickly frozen in liquid nitrogen and stored at −70° for up to 4 weeks before used. The concentration of membrane protein was determined by the method of Bradford (27).

Binding of [^{3}H]LTB_4 to membrane receptors. [^{3}H]LTB_4 binding assays were performed at 22° in 20 mM Tris-HCl (pH 7.5) buffer containing 10 mM $CaCl_2$, 10 mM $MgCl_2$, [^{3}H]LTB_4, PMN membrane protein (standard conditions), and in the presence (or absence) of varying concentrations of LTB_4 or other LTB_4 analogs as noted in figure legends. Total and nonspecific binding of [^{3}H]LTB_4 were determined as mean ± standard error of triplicate assay samples performed in the absence or presence of 1000-fold excess of unlabeled LTB_4. Specific binding was calculated as the difference between total and nonspecific binding.

The kinetic experiments were performed under standard conditions,

using 2 nM [^{3}H]LTB_4, 50 μg/ml of PMN membrane protein in a volume of 3 ml of incubation mixture with or without 2 μM LTB_4, to determine the total and the nonspecific binding. Duplicate 100-μl aliquots of the incubation mixtures were taken at varying time points (from zero to 60 min) and analyzed. The saturation binding experiments were performed, under standard conditions, using 20 μg/ml of PMN membranes and increasing concentrations of [^{3}H]LTB_4 (0.2–15 nM) in a reaction volume of 0.5 ml and incubated for 30 min. LTB_4 (0.2–15 μM) was included in a separate set of incubation mixtures to determine the nonspecific binding. The data from the saturation binding experiments were subjected to a computer-assisted nonlinear least square curve-fitting analysis as described previously (28) and further analyzed by the method of Scatchard (29). The radioligand competition experiments were performed under standard conditions, using 2 nM [^{3}H]-LTB_4, 20 μg/ml of PMN membrane protein, and increasing concentrations of LTB_4 (0.1–10 μM) or other competing ligands (0.1–30 μM) in a reaction volume of 0.5 ml, and incubated for 30 min. The unbound radioligands and competing drugs were separated from the membrane-bound ligands by a vacuum filtration technique as described previously (30). Radioactivity on the filters was determined by liquid scintillation spectrometry. Data reported in each figure were taken from a single representative experiment (from three reproducible experiments) using triplicate assay tubes for the determination of each data point.

Results

Kinetics of LTB_4-induced inositol phosphate formation. When rat peritoneal PMNs were incubated with [^{3}H]*myo*-inositol under the experimental conditions, the uptake of [^{3}H]*myo*-inositol was directly dependent upon the concentration of [^{3}H]*myo*-inositol, the time of incubation, and the concentration of Ca^{2+} and Mg^{2+} (results not shown). Although divalent cations promoted the uptake of [^{3}H]*myo*-inositol, they also increased cellular secretion and self-aggregation. Consequently, the present experimental conditons (1.8 mM $MgCl_2$, 0.2 mM $CaCl_2$, and 0.1% BSA in 10 mM HBS solution for 2 hr at 37°) were chosen to allow maximal incorporation of [^{3}H]*myo*-inositol with minimal effects on PMN aggregation.

When the [^{3}H]*myo*-inositol-labeled PMNs were incubated with 100 nM LTB_4 at 37° for 5 min, rapid metabolism of phosphatidylinositol was observed. Fig. 1, A–C, shows the kinetics of [^{3}H]IP_1, [^{3}H]IP_2, and [^{3}H]IP_3 formation. In the presence of 10 mM LiCl, [^{3}H]IP_1 accumulated within 30 sec and increased during the incubation (Fig. 1A). Accumulation of [^{3}H]IP_1 was also observed but was reduced to 40–60% when LiCl was omitted in the incubation medium (results not shown). This observation is consistent with the generally accepted effects of LiCl in phosphatidylinosoitol metabolism. It has been proposed that LiCl inhibits the phosphatase that converts IP_1 to inositol and thus causes an accumulation of IP_1 (20). The accumulation of [^{3}H]IP_1 in the LTB_4-stimulated PMNs became significantly higher than that of the control samples 15 sec after stimulation, and the concentration of [^{3}H]IP_1 increased up to 5 min after stimulation.

The kinetics of [^{3}H]IP_2 and [^{3}H]IP_3 formation in the LTB_4-stimulated and control cells are shown in Fig. 1, B and C. Both [^{3}H]IP_2 and [^{3}H]IP_3 accumulated rapidly in response to LTB_4 with the peak of accumulation at 30 and 15 sec after addition, respectively. The concentration of [^{3}H]IP_2 reached a constant level 1 min after stimulation with LTB_4. The concentration of [^{3}H]IP_3 peaked at 10–15 sec and gradually decreased after 30 sec of stimulation with LTB_4. In other experiments (not shown), the peak response of [^{3}H]IP_3 accumulation was observed as early as 5 sec after addition of LTB_4. These results

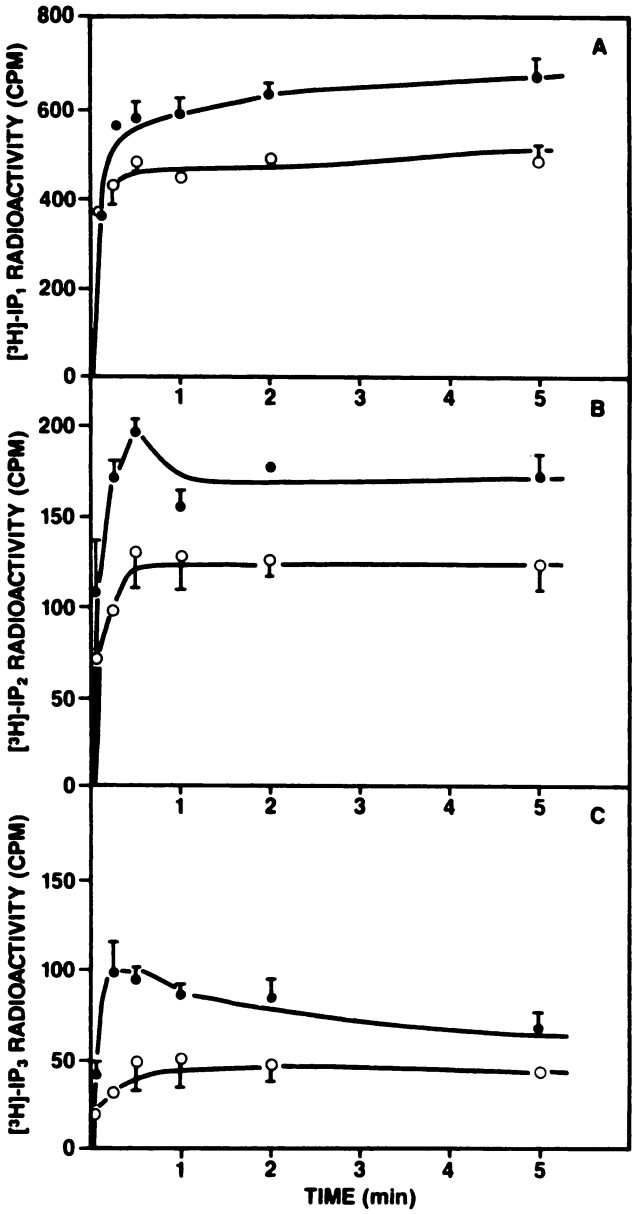

Fig. 1. Kinetic response of the formation of inositol phosphates in PMNs. Rat PMNs were labeled with [^{3}H]*myo*-inositol for 120 min. The cells were washed and resuspended at a concentration of 10 × 10^6 cells/ml and used within 8 min. LTB_4 (100 nM) (●) or saline control (○) was added to prewarmed cell suspensions from 0 to 5 min in triplicate. The aliquots from these incubations were removed at the indicated points of time. The IP_1 (A), IP_2 (B), and IP_3 (C) fractions of these samples were collected from the Dowex column as described in Materials and Methods. The radioactivity was determined by scintillation spectrometry. Results from a representative experiment are shown. The standard deviations, when not shown, were smaller than the symbols employed.

are consistent with the currently accepted mechanism of phosphatidylinositol breakdown, i.e., in response to agonist interaction with membrane-associated receptors, IP_3 is generated via C-type phospholipase(s) (20). IP_2 and IP_1 are the breakdown products of dephosphorylated IP_3 that, in the presence of LiCl, can accumulate to a significant level.

The net and relative increases of [^{3}H]inositol phosphates are shown in Fig. 2. Accumulation of [^{3}H]inositol phosphates after LTB_4 stimulation was rapid (Fig. 2A). [^{3}H]IP_3 and [^{3}H]IP_2 formation, expressed as the ratio of [^{3}H]inositol phosphates

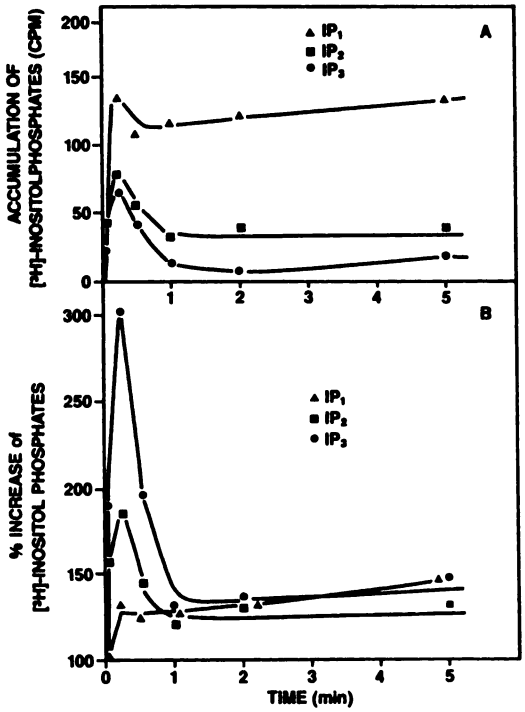

Fig. 2. The relative and net accumulation of [^{3}H]inositol phosphates in LTB_4-stimulated PMNs. The data shown in fig. 1 were recalculated to show the net accumulation (A) and percentage increase (B) of IP_1 (▲), IP_2 (■), and IP_3 (●) after LTB_4 stimulation. Data from a representative experiment are shown.

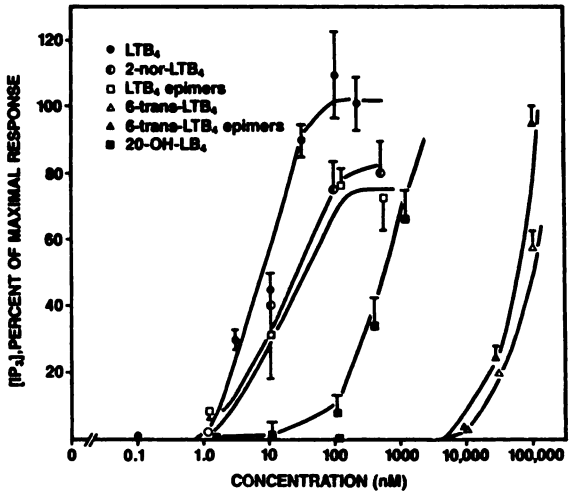

Fig. 3. Structure-activity relationship of [^{3}H]IP_3 synthesis induced by LTB_4 and its analogs. The PMNs were labeled with [^{3}H]*myo*-inositol and washed as described in Materials and Methods. The cells were treated with varying concentrations of LTB_4 (●), 2-nor-LTB_4 (◐), LTB_4 epimers (□), 6-*trans*-LTB_4 (△), 20-OH-LTB_4 (■), and 6-*trans*-LTB_4 epimers (▲) for 20 sec under conditions identical to those described in Fig. 1. Results were pooled from two or three experiments. One hundred per cent maximal response is arbitrarily defined as that amount of IP_3 formed (usually 70–90 cpm) in the presence of 100 nM LTB_4 for 20 sec in each experiment.

from LTB_4-stimulated cells to those of the control cells (Fig. 2B), increased to 300 and 180% approximately 15 sec after addition of LTB_4 and returned to a steady state level 1 min after the addition of LTB_4.

Structure-activity relationship of IP_3 formation induced by LTB_4 and its analogs. The [^{3}H]IP_3 synthesis induced by LTB_4 and its structural analogs is shown in Fig. 3. When the PMNs were incubated with varying concentrations of LTB_4 for 20 sec, a dose-dependent increase of [^{3}H]IP_3 formation was observed with a 50% effective concentration (EC_{50}) of 7 ± 1 nM. The minimal and maximal effective concentrations of LTB_4 were 1 and 100 nM, respectively. The mixture of LTB_4 epimers [5(*R*),12(*R*)-LTB_4 and 5(*S*),12(*S*)-LTB_4] was less active than LTB_4 in this assay. The EC_{50} was 40 ± 5 nM. This result indicated that the LTB_4-induced IP_3 formation is stereoselective because the stereoisomer was 5.8-fold less active than LTB_4. Another agonist, 2-nor-LTB_4, a C_{19} analog of LTB_4, also induced concentration-dependent [^{3}H]IP_3 formation. The EC_{50} of 2-nor-LTB_4 was 25 ± 3 nM, approximately 4-fold less active than LTB_4. 20-OH-LTB_4, an oxidative metabolite of LTB_4, induced [^{3}H]IP_3 formation with an EC_{50} at 350 nM, indicating that it is 50-fold less active than LTB_4. The specificity of IP_3 formation induced by LTB_4 and its analogs correlated with the chemotactic activities (2–6, 31, 32) and the LTB_4 receptor-binding activity (8–13) of these analogs.

The geometrical isomer of LTB_4 at the 6-*cis* double bond, i.e., 6-*trans*-LTB_4 and 6-*trans*-5,12-DiHETE [the epimeric mixture of 5(*R*),12(*R*)-6-*trans*-LTB_4 and 5(*S*)-12(*S*)-6-*trans*-LTB_4], were also studied. These analogs were active in this system; however, they were 3 orders of magnitude less active than LTB_4. The minimal effective concentrations inducing [^{3}H]IP_3 formation were 10–30 μM. 5-HETE, 12-HETE, 15-HETE, and LTD_4 were inactive at concentrations of 1–10 μM (results not shown). Thus, the rank order potency is LTB_4 > 2-nor-LTB_4 ≥ LTB_4 epimers > 20-OH-LTB_4 >> 6-*trans*-LTB_4 epimer ≥ 6-*trans*-LTB_4. These data demonstrate that the 6-*cis* double bond geometry is critical for the agonist activity as reported previously in rat, human, and rabbit chemotaxis and aggregation assay systems. Furthermore, the rank order potency of the LTB_4 analogs in the human and rabbit PMN chemotaxis and rat PMN aggregation assays is equivalent to that of the IP_3 biosynthesis response as demonstrated in the current study.

Inhibition of LTB_4-induced IP_3 formation by IAP and TPA. Radioligand binding studies reported recently have demonstrated that [^{3}H]LTB_4 binding to the membrane-bound receptor is regulated by G_i (or G_o) protein (19). A microbial toxin from *Bordetella pertussis*, IAP, has been shown to specifically inactivate the α-subunit of G_i (and G_o) protein (33, 34). As shown in Table 1, incubation of rat PMNs with IAP (from 0.1 to 50 ng/ml) significantly inhibited the LTB_4-induced [^{3}H]IP_3 accumulation. The incorporation of [^{3}H]*myo*-inositol into the PMNs during the treatment of IAP was not significantly inhibited (results not shown). These data suggest that the LTB_4-induced [^{3}H]IP_3 formation is mediated via G protein, possibly G_i or G_o protein. The inhibition of LTB_4-induced IP_3 formation was probably secondary to G_i inactivation. The inactivation of G_o by IAP is also possible, but not proven in the current study.

The mechanism of action of the tumor-promoting PMA (or TPA) has recently been characterized (35). PMN binds to the intracellular receptor, protein kinase C, and promotes protein phosphorylation. It has been demonstrated that activation of protein kinase C by PMA is rapid and can lead to an uncoupling of receptors from the phosphatidylinositol turnover mechanism (36). Table 1 shows that when the [^{3}H]*myo*-inositol-labeled PMNs were pretreated with TPA (2 min) and then stimulated with LTB_4, [^{3}H]IP_3 formation was inhibited. A structural ana-

TABLE 1

Regulation of LTB_4-induced IP_3 formation by IAP and tumor-promoting phorbol esters

PMNs were labeled with [^{3}H]*myo*-inositol in the presence of IAP for 2 hr. The cells were washed with solution C twice and used for the treatment of LTB_4. The cells remained viable after IAP treatment. The incorporation of [^{3}H]*myo*-inositol was not affected by the IAP under the current conditions.

LTB_4	IAP	Increase of [^{3}H]IP_3[a]	Percentage Inhibition
nM	*ng/ml*	*dpm*	
100	0	200 ± 21	0
100	0.1	135 ± 12	33
100	1.0	90 ± 6	55
100	50	73 ± 5	64
100	0	210 ± 25	0
100	TPA (100 nM)[b]	30 ± 4.5 ($p > 0.01$)	85
100	αPDD (100 nM)[b]	200 ± 22	5

[a] The increase of [^{3}H]IP_3 was determined by subtracting the level of [^{3}H]IP_3 in the basal state (control) from that of the LTB_4-stimulated state. IAP, TPA, and αPDD, at the concentrations used, have no measurable effects on the basal state of [^{3}H]IP_3 formation.

[b] The PMNs were labeled with [^{3}H]*myo*-inositol and then pretreated with 100 nM TPA or αPDD for 2 min at 37° before they were treated with 100 nM LTB_4. Under this condition, the cells remained viable (as determined by trypan blue staining) for up to 30 min.

log of TPA, αPDD, which does not have tumor-promoting or protein kinase C activity, did not inhibit LTB_4-induced [^{3}H]IP_3 formation.

Binding of [^{3}H]LTB_4 to PMN membrane receptors. Formation of IP_3 induced by LTB_4 appeared to be highly specific and suggested that the effects of LTB_4 on PMN phosphatidylinositol metabolism may be mediated through membrane receptors. We have thus characterized the binding of LTB_4 and its analogs to receptors in PMN membranes. When [^{3}H]LTB_4 was incubated with plasma membrane-enriched fraction prepared from PMNs, the specific binding was dependent upon the amount of membrane protein present. Divalent cations (Ca^{2+} and Mg^{2+}) enhanced specific binding of [^{3}H]LTB_4 (results not shown). [^{3}H]LTB_4 binding to the PMN membrane receptors increased in the first 5–8 min and reached a steady state level for up to 60 min (results not shown), thus confirming the data reported by Cheng *et al.* (8). The specific binding of [^{3}H]LTB_4 to PMN membranes increased, dependent upon the concentration of [^{3}H]LTB_4 (Fig. 4) and reached a plateau at 5–10 nM [^{3}H]LTB_4, indicating that the specific binding is saturable. The linear least squares best fit analysis (28) of the saturation binding results yielded a single class of specific binding sites with binding affinity (K_d) of 1.3 ± 0.2 nM and the maximum density of binding sites (B_{max}) of 5.5 ± 1.8 pmol/mg of protein. Conversion of the specific binding data by the method of Scatchard (29) yielded a linear plot confirming the single class of specific binding results. Using a computer-assisted nonlinear least squares program, we could not improve the fitting of the experimental data to the two-site LTB_4 receptor model. These data indicated that [^{3}H]LTB_4 binds to the specific sites on PMN membranes with high affinity and low capacity. The number of specific binding sites on a single PMN cell was approximately 3000–4000.

The effects of guanine nucleotides on [^{3}H]LTB_4 specific binding to PMN membranes were also evaluated. GTP and the nonhydrolyzable form, guanyl-5′-yl-imidodiphosphate, at concentrations of 100 nM–1 mM, inhibited [^{3}H]LTB_4 specific binding to PMN membranes by 30–40% (results not shown). Table 2 summarizes the characteristics of the LTB_4 receptors.

Pharmacological specificity of LTB_4 specific binding sites. To investigate the pharmacological specificity of [^{3}H]LTB_4 binding, radioligand competition studies were performed in the presence of increasing concentrations of LTB_4 and LTB_4 analogs. As shown in Fig. 5, LTB_4 competed with [^{3}H]LTB_4 binding to the specific sites in a concentration-dependent manner with an inhibition constant (K_i) of 2.5 nM. The structural analogs, LTB_4 epimers, 20-OH-LTB_4, 2-nor-LTB_4, 6-*trans*-LTB_4, and 6-trans-LTB_4 epimers, showed different degrees of affinity in competition with [^{3}H]LTB_4 binding to the receptors. The K_i values were 45, 30, 120, 1020, and 680 nM, respectively. Leukotriene C_4, D_4, E_4, and FPL 55712, an SRS-A antagonist, at concentrations greater than 10 μM, did not compete significantly with [^{3}H]LTB_4 binding to the receptors. These results demonstrate that binding of [^{3}H]LTB_4 to the membrane receptors was stereoselective since binding of the unnatural form of LTB_4 epimer mixtures was 20-fold less effective than the natural form, 5(*S*),12(*R*)-LTB_4. In addition, the metabolic product 20-OH-LTB_4 and the geometric isomer 5(*S*),12(*R*)-6-*trans*-LTB_4 were approximately 20- and 1000-fold less effective than LTB_4 in competition for the [^{3}H]LTB_4 receptors. These results also demonstrated that binding of LTB_4 and its analogs to PMN membranes is highly specific, and that the rank order potency of binding is equivalent to that for the phosphatidylinositol metabolism as evidenced by the induction of IP_3 formation (Fig. 3). Table 2 summarizes these results.

Discussion

Immunopharmacological studies have demonstrated that the chemotaxis, cellular aggregation, and lysosomal enzyme release induced by LTB_4 in PMNs may be mediated by specific receptors. Data obtained from radioligand studies have confirmed

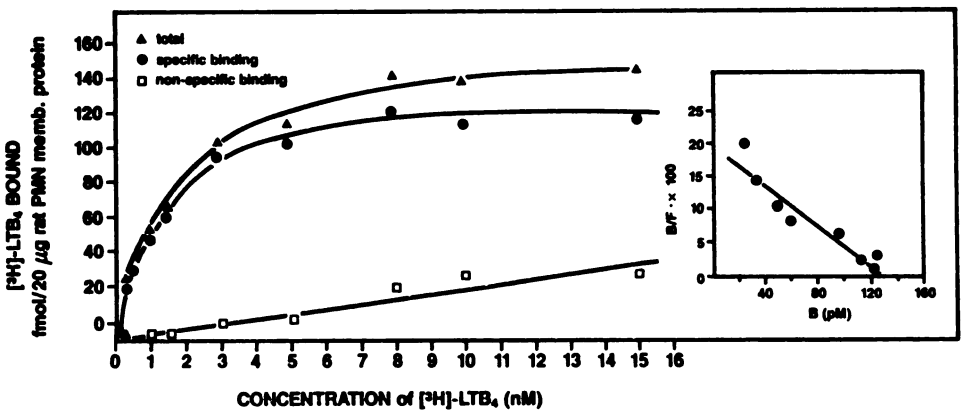

Fig. 4. Saturation binding of [^{3}H]LTB_4 to PMN membranes. The PMN membrane (20 μg/ml) was incubated with [^{3}H]LTB_4 (from 0.2 to 15 nM) under standard conditions, in the presence (□) or absence (▲) of 1000-fold LTB_4 for 30 min. The [^{3}H]LTB_4 specific binding (●) was calculated and plotted by the method of Scatchard (29) (*inset*). The standard error (not shown) for each *point* was approximately 8–10% of the indicated value.

TABLE 2

Comparative properties of LTB_4 receptor binding and phosphatidylinositol hydrolysis

	Binding affinity (K_i)[a]	IP_3 biosynthesis (EC_{50})
	nM	nM
A. LTB_4 analogs		
LTB_4	2.5 ± 0.3	7 ± 1
20-OH-LTB_4	30 ± 10	350
LTB_4-epimers	45 ± 8	40 ± 5
2-nor-LTB_4	120 ± 15	25 ± 3
6-*trans*-LTB_4	1,020 ± 150	60,000
6-*trans*-LTB_4-epimers	680 ± 80	90,000
B. [^{3}H]LTB_4 binding to membrane receptors		
a. Binding affinity (K_d)		1.5 ± 0.5 nM
b. Density (B_{max})		10 ± 2.2 pmol/mg of protein
c. Number of receptors/cell		3000–4000
d. Divalent cation (Ca^{2+}, Mg^{2+}) regulation		enhance receptor binding
e. Guanine nucleotide regulation		decrease [^{3}H]LTB_4 binding to receptor

[a] The binding affinities of LTB_4 analogs were determined by radioligand competition experiments as described in Fig. 5. The K_i values were determined using the equation:

$$K_i = \frac{IC_{50}}{1 + \frac{[^3H\text{-}LTB_4]}{K_d}}$$

where K_d is the equilibrium dissociation constant, IC_{50} is the concentration of the LTB analog which competed [^{3}H]LTB_4 binding to the receptors by 50%, and [^{3}H-LTB_4] is the concentration of [^{3}H]LTB_4 employed in these studies (39).

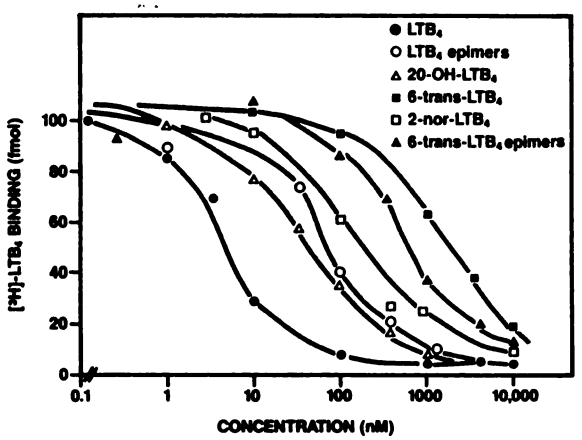

Fig. 5. Competition of [^{3}H]LTB_4 binding to PMN membrane receptors. The PMN membranes (20 μg/ml) were incubated with 2 nM [^{3}H]LTB_4 and increasing concentration of LTB_4 (●), 2-nor-LTB_4 (□), LTB_4-epimer mixture (○), 20-OH-LTB_4 (△), 6-*trans*-LTB_4 (■) and 6-*trans*-LTB_4 epimers (▲) for 30 min under standard conditions. The nonspecific binding of [^{3}H]LTB_4 was usually 6–8% of the specific binding. The standard error for each *point* was approximately 5–8% of the average value.

that these highly lipophilic ligands bind to high affinity, low capacity, stereoselective, and G protein-regulated receptors. These results suggested that an inhibition of adenylate cyclase and/or a decrease of intracellular cAMP may serve as the second messenger for LTB_4 and its receptors. This hypothesis, however, was not supported by experimental evidence, since Gorman *et al.* (10) have shown that, when PMNs were stimulated with LTB_4, the intracellular cAMP concentration was elevated 2–5-fold.

Recently, Goldman *et al.* (19), using the calcium fluorescent dye quin2, have demonstrated that LTB_4 can elevate the calcium concentration in the PMNs. The LTB_4-induced quin2 fluorescence was stereospecific and was inhibited by IAP treatment. This suggests that (a) calcium mobilization is mediated by the membrane-bound LTB_4 receptors and (b) calcium may serve as an intracellular mediator for the immunopharmacological effects such as chemotaxis, aggregation, and enzyme release.

The results of the current study support the hypothesis proposed by Goldman *et al.* (15, 19) and, furthermore, demonstrate that the critical intracellular calcium mobilization agent, IP_3, can be identified and quantitated in rat PMNs. IP_3, Ca^{2+}, and possibly diacylglycerol may serve as the LTB_4 receptor-mediated second messengers in PMNs. This is supported by the following observations. IP_3 was formed in the PMNs within 5–15 sec when the cells were stimulated with LTB_4, preceding most of the measurable effects induced by LTB_4 such as chemotaxis, cellular aggregation, enzyme release, and superoxide formation (3). LTB_4 was highly effective in inducing IP_3 formation with an EC_{50} at 7 nM, close to the K_d concentration of LTB_4 binding to the PMNs (8–13, 15) or PMN membranes (Ref. 9 and current study). The maximal effective concentration of LTB_4 which induced IP_3 was 50–100 nM, close to or equivalent to the maximal effective concentration of LTB_4 required for chemotaxis and aggregation activities. Furthermore, the rank order potency of the LTB_4 agonists inducing IP_3 formation was equivalent to that reported earlier from human PMN chemotactic activities. Most importantly, the rank order potency of LTB_4 agonist binding to the PMN membrane receptors was equivalent to that of the IP_3 formation (Table 2), the rat PMN aggregation response (6, 31), the human peripheral blood PMN chemotaxis response (2, 3, 32), and the calcium mobilization effect (15, 19) induced by LTB_4 and analogs. Thus, these results clearly demonstrated that LTB_4-induced IP_3 formation is highly sensitive, stereoselective, and structurally specific, indicating that it is mediated via the LTB_4 receptors. IAP, which specifically inactivates the G_i or G_o protein, inhibited LTB_4-induced PMN chemotaxis (14); calcium mobilization (15, 19) also significantly inhibited the LTB_4-induced IP_3 formation. These results clearly demonstrated that the G_i or G_o protein is critically involved in regulating the LTB_4 receptor-mediated signal transduction and formation of intracellular messengers. The intracellular messengers IP_3 and diacylglycerol may induce Ca^{2+} mobilization (20–23) and activation of protein kinase C (35), respectively, and resulted in many different cellular and immunological responses. Protein kinase C can be directly activated by TPA. In many receptor systems,

this leads to an attenuation of the receptor-mediated phosphatidylinositol hydrolysis response (36). In the current study, the LTB_4 receptor-induced phosphatidylinositol hydrolysis was specifically and significantly inhibited by TPA treatment. This result demonstrates that activation of protein kinase C can attenuate or uncouple the LTB_4 receptor-mediated signal transduction processes.

Volpi *et al.* (37) have demonstrated recently that, in PMNs isolated from rabbits, the chemotactic peptide, formylmethionyleucylphenylalanine, can induce a rapid and extensive hydrolysis of PIP_2. The PIP_2 hydrolysis effect of LTB_4 in this system was not pronounced. A recent report, however, demonstrated that the water-soluble [^{3}H]IP_3 in rabbit PMNs can be identified only transiently (15 sec) after stimulation of LTB_4 (38). Thus, the mechanisms of signal transduction for LTB_4 may be similar in rabbit and rat peritoneal PMNs. In human peripheral blood purified PMNs, it has not been reported that LTB_4 can induce the formation of IP_3 or its metabolites despite the fact that LTB_4 has been shown to induce Ca^{2+} mobilization (15, 17–19). Thus, it is still possible that LTB_4 receptor-mediated Ca^{2+} mobilization in human peripheral blood PMNs is independent of phosphatidylinositol metabolic effects and distinctly different from that observed in rat or rabbit peritoneal PMNs.

The similarity of LTB_4-induced calcium mobilization and the membrane receptor-regulatory mechanism, and the similarity of the receptor specificity in rabbit, rat, and human PMNs, suggests that the IP_3, diacylglycerol, and intracellular calcium, rather than cAMP or cGMP, may be the intracellular messengers that are coupled to the G_i (or a related G protein) and membrane receptors in PMNs.

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