BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **240,** 162–166 (1997)
ARTICLE NO. RC977628

Leukotriene B_4 Is the Functional Ligand Binding to and Activating the Cloned Chemoattractant Receptor, CMKRL1

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Received October 7, 1997

We recently described a novel chemoattractant receptor, provisionally named CMKRL1, which has turned out to be the first cloned leukotriene (LT) receptor. Present binding assays using tritiated LTB_4 and isolated membranes from COS-7 cells, transiently transfected with cDNA encoding this receptor, yielded a linear Scatchard plot showing expression of only a single, high-affinity receptor population with a mean K_d of 2.1 nM and B_{max} of 17.0 pmoles/mg protein. Sham-transfected cells exhibited no specific binding. LTB_4 elicited concentration-dependent increases in intracellular calcium measured with Fura-2 in individual CHO cells stably expressing CMKRL1. No response was seen with sham-transfected control cells, or in calcium-free medium which suggests that calcium mainly originates from extracellular sources. The LTB_4-induced cellular calcium increment was blocked in the presence of a monoclonal antibody, raised against a synthetic peptide corresponding to the extracellular tail of CMKRL1 and capable of visualizing the receptor by fluorescence immunocytochemistry. Taken together the analyses show that LTB_4 is the endogenous ligand for CMKRL1 which is, thus, identical to the LTB_4 receptor, designated BLTR according to the NC-IUPHAR nomenclature.

Using a homology screening strategy according to which we performed PCR amplification with degenerate primers in combination with southern blot hybridization, a novel seven-transmembrane receptor was recently cloned from a human B-cell lymphoblast cDNA library (1). Among previously cloned members in this superfamily of G-protein coupled receptors it showed highest overall sequence similarity with members in the subfamily of chemoattractant receptors. In accordance with the Genome Database Nomenclature we provisionally named this "orphan" receptor CMKRL1 (1) while subsequently attempting to identify the endogenous functional ligand for the receptor.

Shortly afterwards an identical cDNA sequence was cloned from human erythroleukemia cells and, mainly based on radioligand binding experiments with [^{35}S]-dATPαS, the corresponding receptor was proposed to be a new member of the P2Y group of purinoceptors (2). The interpretation of the binding data and of the functional assays on transfected cells, as well as the comparatively low relatedness of the deduced amino acid sequence to hitherto cloned P2Y receptors, warranted a re-evaluation of this conclusion. Thus, in more extensive studies of the binding and function of the receptor stably expressed in a human astrocytoma cell line, it was recently assessed that the purported $P2Y_7$ receptor (2) is not a member of the P2Y family of signalling molecules (3).

With the use of a subtraction strategy in retinoic acid-differentiated HL-60 cells a cDNA sequence identical to the coding sequence of CMKRL1 was again reported (4), and that study together with the present binding assays and functional experiments conclusively show that CMKRL1 is the receptor for leukotriene B_4 (LTB_4). This potent inflammatory chemoattractant derivative of arachidonic acid is a product of the 5-lipoxygenase pathway and is formed from LTA_4 by a specific hydrolase. The name leukotriene derives from the notion that leukotrienes can be produced by *leuko*cytes and that a conjugated *triene* forms a common structural entity. They are members of the group of eicosanoids which also contain, *e.g.*, prostaglandins and thromboxane.

MATERIALS AND METHODS

Cell transfection. Intact plasmid DNA (10-25 μg) of the cDNA clone, Lyme21-9 (1), was transfected with the calcium phosphate precipitation method. Chinese hamster ovary cells (CHO-K1) were used to establish stable expression of the corresponding receptor, CMKRL1. The pCD_{neo} vector was co-transfected to allow for selection with G-418 (Geneticin, 500 μg/ml). Single resistant colonies were isolated to obtain homogenous cell lines, and appropriate expression

0006-291X/97 $25.00

of the corresponding mRNA was verified by northern blot hybridization with cDNA probes. SV40 transformed African green monkey kidney cells (COS-7) were used for transient expression. These cells were harvested after 48 hrs for preparation and isolation of membranes. Sham-transfected cells were used as controls throughout.

Production of monoclonal antibodies (mAb). A peptide was synthesized (Euro-Diagnostica, Sweden) corresponding to the first 15 of the N-terminal amino acid residues in CMKRL1, plus a C-terminal cysteine residue and then conjugated to BSA. Five mice were immunized i.p. 3 times at weekly intervals with peptide antigen mixed with the RIBI adjuvant system (Sigma). This was followed by a fourth injection 6 weeks afterwards. Ten days later the antibody response was checked in blood samples using ELISA (wells coated with peptide conjugated with KLH in bicarbonate buffer, pH 9.6) and indirect immunocytochemistry (CHO cells transfected with Lyme21-9, and sham-transfected controls). Mice with the strongest immune response were boosted by i.p. injection of the antigen mix and their spleen cells were used for fusion 3 days later. The spleen cells were fused with mouse myeloma SP 2/0 cells by the addition of PEG, followed by selection in HAT medium. Supernatants from wells with growing hybridomas were harvested and again tested by immunocytochemistry 9-20 days after fusion. Antibody-producing hybridomas were cloned out by limiting dilution.

Immunocytochemistry. CHO cells were grown on microscope coverslips in 6-well culturing plates and, following removal of the medium and washing in PBS, fixed in methanol at 4° C. The cells were preincubated in 10% goat serum for 20 min, the mouse mAb was added at varying concentrations, which was followed by a further incubation for 1 hr, all at RT. After thorough washing in PBS the cells were incubated in FITC-labelled goat anti-mouse IgG (Jackson ImmunoResearch) for 1 hr at RT. After another rinsing, the coverslips with cells were mounted in glycerol on top of microscope slides, and examined and photographed in a Leica Aristoplan fluorescence microscope equipped with appropriate excitation and barrier filters.

Intracellular calcium measurements. Transfected cells were seeded into 96-well plates (Corning-Costar) by dissociating confluent cultures using PBS containing 2 mM EDTA, diluting the resulting suspension 20× and adding 50 μl per well. Following over-night incubation at 37° C, the growth medium was replaced with cold (4° C) medium containing 1 μM Fura-2AM (Sigma) and 2.5 mM probenecid (Sigma), and the cells were incubated for one hour at 37° C. After 3 washes in extracellular medium buffer (135 mM NaCl, 4.6 mM KCl, 1.2 mM $MgCl_2$, 1.5 mM $CaCl_2$, 11 mM glucose, 11 mM Hepes, pH 7.4) the plates were mounted on the stage of a Nikon inverted fluorescence microscope attached to an SIT-camera and an image analysis system (Photon Technology International). Measurements were made using extracellular medium buffer at RT, and real-time digital background subtraction to compensate for the autofluorescence of the plastic plates. Agonists were added in 50 μl of extracellular medium buffer and mixed thoroughly with the original contents of the well. Fluorescence images were obtained by alternate excitation at 340 and 380 nm, and the emitted light was measured at 510 nm. The software configuration allowed for storage of fluorescence information from groups of cells, which could then be analyzed separately. Intracellular calcium concentrations were calibrated using the method described by Williams and Fay (5) and calculated from the aforementioned ratiometric images using the mathematical relationship described by Grynkiewicz *et al.* (6). For experiments involving calcium-free extracellular buffers, the buffer was prepared as described above but without calcium and magnesium and with 10 mM EGTA. In addition to the preincubation with Fura-2, the cells were subsequently incubated a further 30 min in either normal extracellular buffer or the calcium-free buffer. Agonists were then added in normal buffer solutions or calcium-free buffer solutions, respectively. For experiments with the mAb, cells were first incubated with Fura-2 and then for an additional 30 min in medium containing the mAb, or normal growth medium, followed by 3 washes with extracellular buffer (to avoid problems created by serum protein autofluorescence).

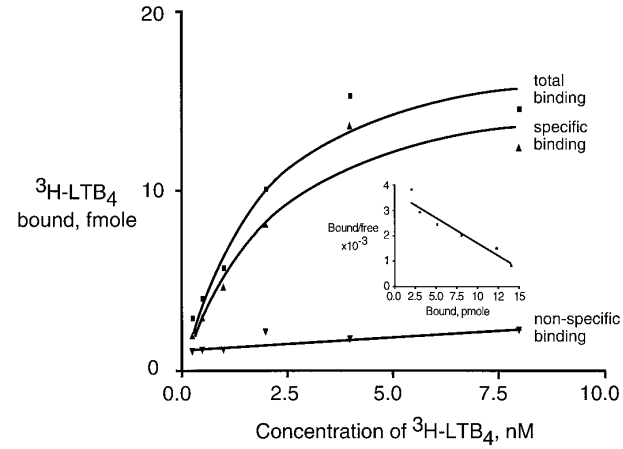

FIG. 1. Saturation and Scatchard (inset) analyses of [^{3}H]LTB$_4$ binding to membranes prepared from COS-7 cells transfected with Lyme21-9 cDNA to express CMKRL1. Results shown are the mean values of 3 experiments performed in triplicate.

Binding assays. The transiently transfected COS-7 cells were washed with PBS, which was replaced with 50 mM TRIS/HCl buffer (pH 7.5) containing 1 mM EGTA and 5 mM $MgCl_2$, and scraped off. After homogenization for 20 sec in a Polytron homogenizer (Brinkmann, setting 5.5) the material was centrifuged at 20,000 rpm for 30 min at 4° C, followed by washing through homogenization in the TRIS binding buffer and subsequent recentrifugation, which was repeated twice. The pellet was finally resuspended by homogenization and stored in liquid nitrogen until used. For the binding studies, membrane material corresponding to 30 μg protein (Bradbury assay) was blended with ice-cold binding buffer (0.02 M Hepes buffer containing 10 mM $CaCl_2$ and $MgCl_2$) to a final volume of 70 μl and added to wells in a 96-well polypropylene plate. [^{3}H]LTB$_4$ in varying concentrations and/or LTB$_4$ 1 μM were then added in an additional 70 μl of ice-cold binding buffer and mixed with the original well contents by briefly shaking the plate. The plate was then incubated at 4°C for 1 hr before bound and free [^{3}H]LTB$_4$ were separated by rapid filtration through Whatman glass fiber filters mounted in a cell harvester (Inotech). Each data point is the result of the mean of triplicate wells from three separate experiments, which have in turn been averaged.

RESULTS

In the assays of binding of [^{3}H]LTB$_4$ to isolated membrane preparations from COS-7 cells transfected with cDNA to express the receptor, CMKRL1, there was a concentration-dependent increase in both total and specific binding at 4° C until saturation was reached (Fig. 1). Non-specific binding, *i.e.*, the binding of tritiated ligand in the prescence of 1 μM non-labelled ligand, represented less than 15% of the total binding and did not vary significantly with radioligand concentration. There was a near-linear increase in the specific binding of [^{3}H]LTB$_4$ with increasing concentrations of membrane protein (Fig. 2), whereas membranes from sham-transfected cells exhibited no specific binding

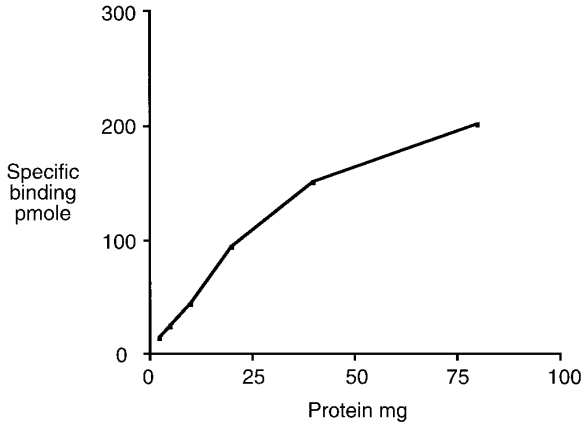

FIG. 2. Specific binding of [^{3}H]LTB$_4$ to membranes prepared from COS-7 cells transfected with the CMKRL1 expression plasmid, Lyme21-9, as a function of membrane concentration. Various (protein) concentrations of membranes were incubated with 2 nM tritiated LTB$_4$ for 60 min at 4° C, with or without 1 μM unlabelled LTB$_4$. Results are mean values of triplicate wells.

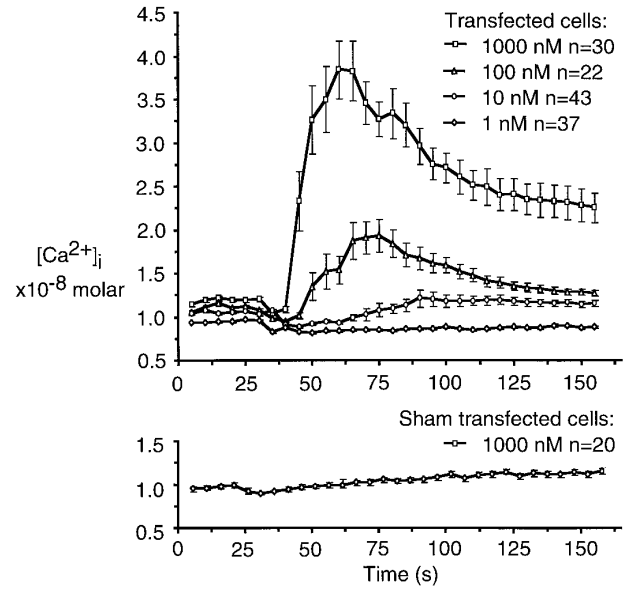

FIG. 3. Action of 4 different concentrations of LTB$_4$ (figures given are final concentrations in the test well) on intracellular calcium levels in CHO cells stably transfected to express CMKRL1 and preloaded with Fura-2 for monitoring of intracellular calcium concentration (fluorescence ratios). The lower curve illustrates the effect of the highest LTB$_4$ concentration tested on sham-transfected control cells. Values are means ± SEM (n).

(data not shown). The saturation binding studies yielded a linear Scatchard plot, shown as inset of Fig. 1. Thus, the radioligand identified a single class of high-affinity binding sites with an affinity constant (K_d) of 2.1 ± 0.5 nM (mean ± SEM, n = 3) and a B_{max} of 17.0 ± 2.0 pmoles/mg protein.

Addition of LTB$_4$ to CHO cells stably expressing CMKRL1 receptor message and preloaded with Fura-2 showed a rapid increase in the fluorescence ratio reflecting increased intracellular levels of calcium (Fig. 3). The effect was concentration-dependent between 1 and 1000 nM final concentrations in the test well, the onset being more rapid and the elevation steeper at the higher concentrations. After the maximum response had been attained the cellular calcium concentration slowly returned to baseline levels. LTB$_4$ was completely without effect at any dose level when added to sham-transfected control cells (shown for the 1000 nM concentration in Fig. 3, lower curve). When the regular cell medium was exchanged with calcium-free medium, LTB$_4$ no longer induced any significant increase in intracellular calcium levels (Fig. 4).

From a panel of 458 successfully growing hybridomas from mice immunized with a synthetic peptide corresponding to the N-terminus of CMKRL1, 48 supernatants were positive in the ELISA assay. Of these, 11 gave positive immunocytofluorescence reactions in transfected cells expressing CMKRL1, and following further cloning and growth of the individual hybridoma clones, 2 cell supernatants still showed positive immunoreactivity at the microscopic level. The immunocytochemical reaction of one mAb is illustrated in Fig. 5, which shows the distribution of a finely granular fluorescence in the periphery of CHO cells expressing CMKRL1 (Fig. 5a), whereas no fluorescence is seen in sham-transfected control cells (Fig. 5b). Also, no fluorescence was seen in control incubation with FITC-conjugated secondary antibody alone in either type of cell (not shown).

Preincubation of CMKRL1-expressing cells with the mAb illustrated in Fig. 5 blocked the LTB$_4$-induced calcium response almost completely (Fig. 6).

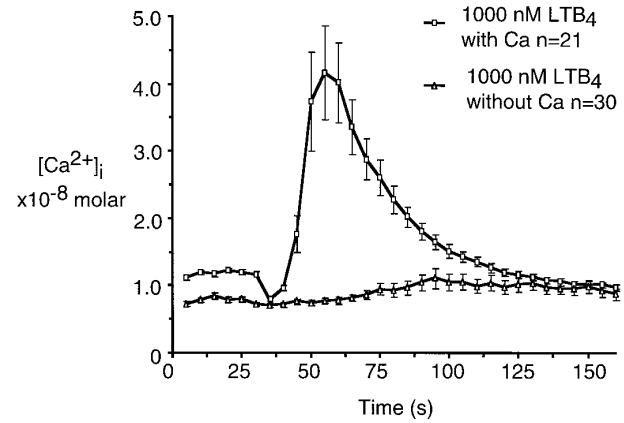

FIG. 4. Effect of 1000 nM (final concentration in test well) of LTB$_4$ on intracellular calcium levels in CHO cells transfected to express CMKRL1 (and preloaded with Fura-2 for monitoring of calcium fluorescence ratios). Effects of LTB$_4$ are compared in calcium (Ca) containing and calcium-free cell media. Values are means ± SEM (n).

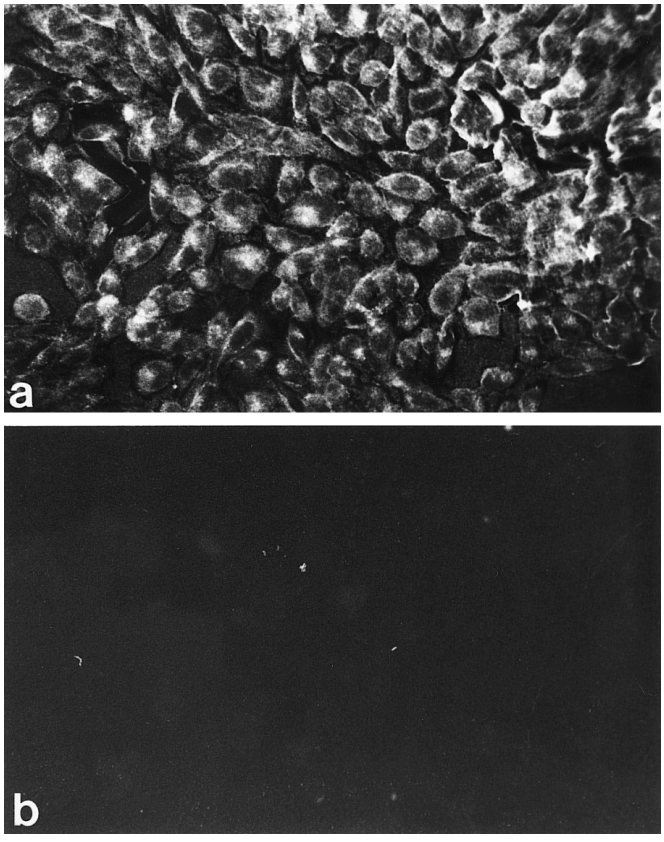

FIG. 5. Fluorescence immunocytochemistry of a monoclonal antibody (mAb), raised against a synthetic peptide corresponding to the first 15 amino acid residues in the extracellular tail of CMKRL1, showing (**a**) finely granular fluorescence in the periphery of CHO cells stably expressing CMKRL1, and (**b**) absence of fluorescence in sham-transfected control cells. Original magnification: 500×.

DISCUSSION

The present results show that tritiated LTB_4 binds with high affinity to a single receptor population in COS-7 cells transfected with recently cloned cDNA encoding a novel "orphan" receptor that we provisionally designated CMKRL1 (1) owing to the marked structural homology with members of the chemoattractant receptor group. Moreover, LTB_4 activates transfected CHO cells stably expressing CMKRL1 receptor message, resulting in a concentration-dependent increase in the cellular flux of calcium. The effect is blocked by a mAb obtained by immunizing mice with a synthetic peptide corresponding to the N-terminus of the deduced receptor protein. No binding or activation is seen with LTB_4 in sham-transfected control cells. This shows that LTB_4 is an endogenous functional ligand for CMKRL1 which is, thus, the first cloned leukotriene receptor. Subsequently cloned cDNA and receptor sequences are identical (2,4) whereas clone R2, obtained from a human genomic library (7), deviates in the 5′ end, which led to the identification of a different methionin initiation site in the proposed coding sequence.

Although the leukotrienes have been known as well-characterized chemical entities since the late seventies (8), and it has been known that their biological actions are mediated through high-affinity stereoselective membrane receptors, no sequence information has been available at either the amino acid or nucleotide level (9) until the cDNA encoding the corresponding receptor was cloned two decades later (1,4). Despite the longstanding absence of structural information the LTB_4 receptor has, nevertheless, been extensively investigated as a pharmacological entity (9) and is known under the BLT receptor designation (10). It can be expected that the cloning and identification of BLTR will now open up for cloning also of other structurally similar receptors in the leukotriene subfamily.

The affinity constant presently obtained in the binding of tritiated LTB_4 to transfected COS-7 cell membranes is close to the figure from guinea-pig eosinophil membranes (11), but higher than the value from retinoic-acid differentiated HL-60 cells or COS-7 cells (4). Differences in biological activity of the receptor may be related to how the receptor is expressed in the membrane of various cell types. This could, among other things, allow for a varying degree of dimerization that might affect receptor affinity (12). In this context it is notable that higher molecular weight receptor species have been reported for another chemoattractant receptor, namely C5a (13).

The interaction of LTB_4 with the cloned BLTR is counteracted by receptor antagonists as well as by various eicosanoids (4), and it is also blocked by the presently raised mAb against a synthetic N-terminal receptor peptide. The N-terminus is the most immunogenic domain of the seven-transmembrane receptor (14), and synthetic peptides corresponding to this region have

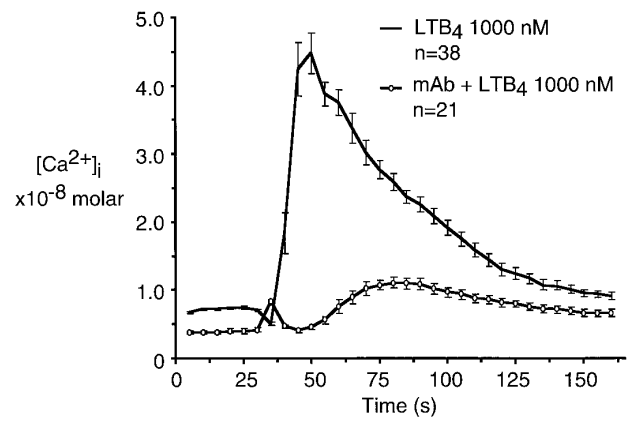

FIG. 6. Effect of the monoclonal antibody (mAb) that is illustrated in Fig. 5 on the cellular calcium influx induced by 1000 nM LTB_4 in CHO cells which stably express CMKRL1. Values are means ± SEM (n).

been used successfully for production of mAbs recognizing other related receptors, such as IL-8R (14) and CCR3 (15). This domain seems to be the primary ligand binding site for, *e.g.*, the chemoattractant receptor, C5a (16). It may be a mechanism through which a mAb recognizing this region blocks BLTR receptor function: by directly competing with the ligand binding, by occupying space indirectly preventing the access of LTB_4 to other (secondary) binding domains, or by inducing conformational changes in the receptor molecule leading to its temporary inactivation.

The G-protein mediated intracellular signalling activated by LTB_4 in transfected CHO cells seems to involve both an increase in intracellular calcium levels mediated by G_q, and an inhibition of adenylyl cyclase activity through G_i (4). The blockade of the intracellular calcium augmentation obtained during stimulation with LTB_4 in calcium-free medium indicates that the increased level of the ion in the CHO cells is derived mainly from extracellular sources, probably through activation of receptor-operated channels in the cell membrane. It is well recognized that signal transduction from a particular receptor may vary with the type of cell owing to the diversity in the effector system (17,18) and its availability in any given cell expressing that particular receptor.

ACKNOWLEDGMENTS

This research was supported by Grant 05680 from the Swedish Medical Research Council, and by grants from the Crafoord, Segerfalk, and Österlund Foundations and from the LEO Ltd. Research Foundation.

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