Homologous Desensitisation of the Mouse Leukotriene B₄ Receptor Involves Protein Kinase C-Mediated Phosphorylation of Serine 127

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Key Words

GPCR • BLT1 • LTB₄ • Desensitisation • PKC • *Xenopus laevis*

Abstract

Murine leukotriene B₄ (LTB₄) receptor (mBLT1) cDNA was identified by searching the EST database using human LTB₄ receptor as the query sequence. Expression of functional mBLT1 after injection of *in vitro* transcribed cRNA into *Xenopus laevis* oocytes was demonstrated as LTB₄-evoked, Ca²⁺-activated Cl⁻ currents recorded by two-electrode voltage clamp. From mBLT1-expressing oocytes, a dose-dependent relationship between the Ca²⁺-activated Cl⁻ current and LTB₄ concentration was demonstrated with an apparent EC₅₀ of 6.7 nM. Following LTB₄ stimulation of mBLT1, we observed two transient, spatially distinct Ca²⁺-activated, inwardly directed Cl⁻ currents in the oocytes: a fast peak current requiring relatively high LTB₄ concentrations, and a slowly progressing Cl⁻ current. Nucleotides, PGE₂, 12*R*-hydroxy-5, 8, 14-*cis*-10-*trans*-eicosatetraenoic acid, and LTD₄ did not

activate mBLT1. U75302, specifically targeting BLT1, significantly reduced LTB₄-evoked Cl⁻ currents. Repetitive LTB₄ administration desensitized the LTB₄-evoked currents. Activation of protein kinase C (PKC) by PMA addition completely eliminated the LTB₄-evoked currents, whereas down-regulation of PKC by prolonged PMA exposure (20 h) impaired mBLT1 desensitisation. In addition, Ser-127-Ala substitution of the PKC consensus phosphorylation site on the second intracellular loop prevented the mBLT1 desensitisation. These data indicate that PKC-mediated phosphorylation at Ser-127 leads to mBLT1 desensitisation.

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Introduction

Two G protein-coupled seven transmembrane domain receptors (GPCRs) for the lipid mediator LTB₄ have been molecularly identified and designated BLT1 and BLT2 [1, 2]. BLT1 has high affinity and specificity

for LTB₄, whereas BLT2 has lower affinity for LTB₄ and can bind other eicosanoids [3]. The LTB₄-mediated signalling by these GPCRs has been found to play important roles in recruitment and activation of leukocytes in host immune responses to pathogens as well as in tissue injury caused by different inflammatory diseases [3, 4]. Therefore, BLTs are potential targets for pharmacological intervention in inflammatory diseases such as asthma, rheumatoid arthritis, and arteriosclerosis [5].

Intracellular signalling from activated BLT1 has been found to involve phosphoinositide-specific phospholipase C (PLC) via pertussis toxin (PTX)-sensitive [6, 7] and PTX-insensitive G proteins [8]. The activation of PLC results in inositol 1, 4, 5-triphosphate- (IP₃) and diacylglycerol (DAG) synthesis. IP₃ stimulates the release of intracellular Ca²⁺ from IP₃-sensitive stores, leading to a transient increase in the intracellular Ca²⁺ concentration, and DAG potently activates PKC. Most GPCRs are subjected to homologous desensitisation [9] in a process involving Ser/Thr-targeted phosphorylation of cytoplasmic residues on the receptor by GPCR kinases (GRKs) and/or by PKC and PKA [10]. Desensitisation of BLT1 has previously been demonstrated in mouse leukocytes [11] and following expression of BLT1 in various cell types [12–14]. In these studies, PKC [13], GRK2 [12, 13], GRK5 [13], and GRK6 [11, 13] were shown to affect the downstream events of BLT1 activation. Activation of PKC in COS-7 cells transfected with hBLT1 or overexpression of GRK6 reduced the LTB₄-induced accumulation of inositol phosphates [13]. Mouse leukocytes lacking GRK6 exhibited increased chemokinetic and chemotactic responses towards LTB₄ and a prolonged increase in intracellular Ca²⁺ following stimulation with LTB₄ [11]. Interestingly, desensitisation of the leukocyte intracellular Ca²⁺ rise induced by repetitive addition of LTB₄ was found to take place in the absence and presence of GRK6, indicating that other signalling components are involved in agonist-mediated desensitisation of murine LTB₄ receptors.

To study the desensitisation process of the murine BLT1 receptor, we identified and cloned this receptor (Genbank accession no. AF110104) and expressed it in the *Xenopus laevis* expression system. We demonstrated that non-injected oocytes do not respond to LTB₄, whereas oocytes expressing mBLT1 respond to LTB₄ with high affinity. We subsequently tested mBLT1 for its apparent affinity for LTB₄ and its specificity and its sensitivity to PTX. Finally, we investigated whether substitution of Ser/Thr in putative PKC phosphorylation sites of the receptor leads to impairment of homologous desensitisation.

Materials and Methods

Media and chemicals

ND96 solutions were prepared from 10X stocks, with pH adjusted to 7.65 with NaOH. ND96 contained (in mM): Na⁺ 96, Cl⁻ 104, K⁺ 2, Ca²⁺ 1.8, Mg²⁺ 1, and HEPES 5. ND96ps was further supplemented with sodium pyruvate (2.5 mM), streptomycin (0.01 mg/ml), and penicillin (0.01 mg/ml). The Ca²⁺-free ND96 contained (in mM): Na⁺ 96, Cl⁻ 100, K⁺ 2, Mg²⁺ 1, and HEPES 5. ND96Mg contained (in mM): Na⁺ 96, Cl⁻ 118, K⁺ 2, Mg²⁺ 10, EGTA 0.05, and HEPES 5. ND96Ca was the same as ND96Mg except for the replacement of Mg²⁺ with Ca²⁺. All chemicals were of analytical grade and from Sigma Chemical if not otherwise stated. *Bordetella pertussis* toxin (PTX, 100 µg/ml) from Calbiochem, collagenase 1A (50 mg/ml), tricaine [ethyl 3-aminobenzoate methanesulfonate, 0.15% (w/v)], sodium pyruvate (100 mM), and nucleotides (10.7 mM) were all dissolved in dH₂O. Prostaglandin E₂ (2 mM) and PMA (26.7 µM) were dissolved in ethanol. Stock solutions of LTB₄ (297 µM), LTD₄ (201 µM), and 12R-HETE (312 µM) from Cayman Chemical Company were all dissolved in ethanol. U75302 (1383 µM in ethanol) was from Biomol Research Laboratories. The *in vitro* transcription kit was obtained from Stratagene.

Cloning, sequencing, and site-directed mutagenesis

A TBLASTN search of the GenBank® database returned several EST sequences with similarity to the hBLT1. Alignment of one of the sequences with hBLT1 suggested that this mEST could encode a full-length or nearly full-length candidate LTB₄ receptor. The IMAGE clone was obtained from UK-HGMP (United Kingdom Human Genome Mapping Project, Hinxton, Cambridge), and the cDNA was sequenced bi-directionally by automated sequencing (Applied Biosystems Inc.) using Taq Polymerase (Ampli-Taq, Perkin-Elmer) and the dideoxy chain termination method with dye terminators. In the IMAGE clone 874601, we identified a 1056-bp open reading frame encoding a 351 amino acid residue protein identified as the murine leukotriene B₄ receptor 1 (mBLT1). This sequence has been deposited in GenBank® with the accession number AF110104. Mutant mBLT1s were generated by extension-overlap PCR using High Fidelity Platinum Polymerase (Invitrogen), with primers incorporating the desired nucleotide substitutions. PCR fragments encoding mutated mBLT1s were subsequently purified, digested with MluI and EcoRI, and ligated into the pT7T3D-mBLT1. Constructs were fully sequenced (MWG-AG Biotech, Ebersberg, Germany) to confirm mutations and receptor sequence.

In vitro transcription and injection of cRNA

Capped sense transcripts were made after linearization of pT7T3D-mBLT1 with NotI, using T7-RNA polymerase according to the manufacturer's protocol. cRNA quality was monitored by 1% agarose gel analysis, and the cRNA concentration was estimated by UV spectrometry. Aliquots of cRNA in water (0.50 ng/nl) were kept at -80°C. A total of 60 nl (30 ng) was injected into each oocyte using a Nanoliter Injector (World Precision Instruments) and borosilicate glass

Fig. 1. Dose-response relationship of mBLT1 and LTB₄. (a) A representative trace from an uninjected oocyte with 10 nM LTB₄ added to the perfusion medium, as indicated by the bar. (b) A representative dose-response experiment from one oocyte is shown. LTB₄ was added at four different concentrations (0.1, 1, 10, or 100 nM) to the perfusion medium (ND96) as indicated by the bars. A prolonged period of perfusion with ND96 between the LTB₄-evoked currents was necessary to omit desensitisation of the response. (c) LTB₄ dose-response curve. The increase in current after addition of LTB₄ was estimated as the difference between the peak values and the current before addition of LTB₄. The data are relative to the current elicited by 100 nM LTB₄. In all oocytes examined, the current induced by 100 nM LTB₄ was between 80 nA and 690 nA. The line represents a Hill-type non-linear least squares fit to the data points using the equation $\Delta I = \Delta I_{\max} [LTB_4]^n / ((EC_{50})^n + [LTB_4]^n)^{-1}$, giving an apparent EC_{50} and n at 6.7 nM and 1.15, respectively. Representative responses to 10 nM LTB₄ in the presence (d) or absence (e) of Ca²⁺ in the perfusing medium are shown. We observed no difference between the current induced by 10 nM LTB₄ in the presence or absence of Ca²⁺ from a total of 14 oocytes of which 8 were tested in the absence of Ca²⁺ ($P=0.84$, two tailed, unpaired Welch t -test).

micropipettes pulled on a DMZ-puller.

Preparation of oocytes

X. laevis frogs were anaesthetized in 0.15% (w/v) tricaine before partial removal of the ovary. During surgery, the frog was kept on ice. The partial ovary containing the oocytes was divided into small clumps in Ca^{2+} -free ND96, and the oocytes were defolliculated by collagenase 1A (2 mg/ml) treatment in Ca^{2+} -free ND96 (1 h, room temperature). The oocytes were extensively washed in Ca^{2+} -free ND96 then ND96, and finally kept in ND96ps. Healthy stage V or VI oocytes were removed and kept in 80-mm Petri dishes until the following day, at which time injection of cRNA was performed. After injection, the oocytes were maintained individually in 24-well dishes (Nunc, Nunc) in ND96ps at 17°C in an incubator. The medium was changed on the third day. Electrophysiological recordings (two-electrode voltage clamp) were performed on day 3-5 after cRNA injection.

Two-electrode voltage clamp

The two-electrode voltage clamp experiments were performed using a lab-made recording chamber and perfusion system, an OOC-1 amplifier (World Precision Instruments), a Digidata 1200B digital recording system, and AxoScope software (Axon Instruments Inc.) for sampling to a PC, as previously described [15, 16]. Data were sampled at 1000 Hz or 500 Hz and low-pass filtered at 10 Hz. The microelectrodes were pulled from GC150F (Clark Electromedical Instruments) on a patch electrode puller (Hans Ochozki, Homburg) and were filled with 3 M KCl. The potential and the current microelectrodes had resistances between 0.6-2.0 MΩ. Only oocytes with a resting membrane potential more negative than -20 mV were used for voltage clamp. The oocytes were perfused throughout the recording session. The equilibrium potentials for Cl^- and K^+ in *X. laevis* oocytes are -28 mV and -95 mV [17], respectively, and an ideal holding potential for recording of Cl^- -currents would thus be -95 mV. However, clamping at -95 mV will often lead to saturation of the amplifier during Ca^{2+} -activated Cl^- currents; as a compromise, we used oocytes voltage clamped at either -50 or -60 mV for recordings of the Ca^{2+} -activated Cl^- currents [18]. Unless otherwise stated, the oocytes were clamped at -50 mV.

Results

LTB_4 dose response in mBLT1 expressing oocytes

X. laevis oocytes have endogenous Ca^{2+} -activated Cl^- channels that are dose-dependently activated by intracellular Ca^{2+} [19, 20]. Recording oocyte whole-cell Cl^- currents therefore allows detection of receptor activity elicited through intracellular Ca^{2+} signalling [21], and it has been used in cloning and characterisation of different leukotriene receptors [15, 22-24]. We used the Ca^{2+} -activated whole-cell Cl^- current recorded by the two-

electrode voltage-clamp technique to characterise activation and desensitisation of mBLT1. *In vitro* transcribed cRNA encoding the mBLT1 was injected into *X. laevis* oocytes, and whole-cell current recordings under two-electrode voltage clamp confirmed the expression of mBLT1 as measured by the activation of the Ca^{2+} -activated endogenous Cl^- channels in response to addition of LTB_4 . Non-injected oocytes did not respond to LTB_4 at a concentration of 10 nM (Fig 1a), indicating the absence of high-affinity endogenous leukotriene B_4 receptors in *X. laevis* oocytes.

The trace in Figure 1b shows a typical response to different concentrations of LTB_4 of the whole-cell, Ca^{2+} -activated Cl^- current in oocytes expressing mBLT1. The current induced by addition of 1 nM LTB_4 to the medium is a slowly activated inward current reflecting a minor increase in intracellular Ca^{2+} (Fig. 1b). This slowly activated inward current returns to baseline approximately 10-15 min after removal of LTB_4 . At 10 nM and 100 nM LTB_4 , there is an initial transient inward peak Cl^- current followed by a slowly progressing Cl^- current (Fig. 1b). To estimate an apparent EC_{50} value for LTB_4 -mediated activation of Cl^- currents in mBLT1-expressing oocytes, we estimated the increase in the initial current after addition of LTB_4 as the difference between the peak values and the current before addition of LTB_4 . Because the LTB_4 -evoked currents showed considerable variation between oocytes, we present the data relative to the current elicited by 100 nM LTB_4 (Fig. 1c). The solid line in Figure 1c represents a fit to the data points using the Hill equation (see Fig. 1 legend). From this fit, an apparent EC_{50} value for the LTB_4 -induced Ca^{2+} -activated Cl^- current is estimated at 6.7 nM in mBLT1-expressing oocytes. No difference in the magnitude of the fast transient current induced by 10 nM LTB_4 in the presence or absence of extracellular Ca^{2+} could be observed (Fig. 1d and e), indicating that this current results from Ca^{2+} release from intracellular stores.

Effect of U75302 and pertussis toxin on LTB_4 -mediated currents in mBLT1-expressing oocytes

U75302 is an LTB_4 analogue specific for BLT1 that has been shown to inhibit ^3H - LTB_4 binding to guinea pig lung membranes with a K_i estimated at 159 nM [25]. An agonistic effect of U75302 in human coronary artery smooth muscle cells has also been reported [26], indicating that U75302 could act as an antagonist and as a partial agonist depending on the cellular system. In mBLT1-expressing oocytes, 2 μM U75302 had no effect on the whole-cell current, but the inward Cl^- current induced by

Fig. 2. Effect of the LTB₄ receptor antagonist U75302 and pertussis toxin on the LTB₄-induced currents. (a) U75302 (2 μM) and LTB₄ (10 nM) were administered as indicated by the bars in the perfusion medium to mBLT1-expressing oocytes. The trace is representative of three similar experiments (two frogs). (b) mBLT1-expressing oocytes were incubated in PTX (0.5 or 1 μg/ml) for 20 h prior to stimulation with LTB₄ (10 nM). The LTB₄-evoked current (I) was estimated from a total of 17 oocytes (two frogs), of which 10 oocytes received PTX treatment. The effect of PTX was significant ($P < 0.05$, two tailed, unpaired Welch *t*-test).

10 nM LTB₄ was significantly reduced ($60 \pm 10\%$) (Fig. 2a). This indicates that U75302 is an mBLT1 antagonist in the *X. laevis* expression system.

The first step in the signalling cascade following activation of LTB₄ receptors in phagocytes and leukocytes is the stimulation of PTX-sensitive G protein(s) [6, 7]. To investigate whether mBLT1 couples to PTX-sensitive

Fig. 3. Effect of PMA on the response to LTB₄. LTB₄ (10 nM) was administered in the perfusion medium as indicated by the bar. (a) Representative current trace of an mBLT1-expressing oocyte preincubated in ND96ps supplemented with 100 or 200 nM PMA for 5–35 min. In the PMA-treated oocytes, no LTB₄-evoked current was induced in any of the oocytes tested (seven oocytes, three frogs). (b) Representative current trace of an mBLT1-expressing oocyte not preincubated with PMA showing a normal LTB₄-(10 nM) evoked current (11 oocytes, from the same 3 frogs).

endogenous G proteins in the *X. laevis* oocytes, mBLT1-expressing oocytes were pre-incubated in PTX before application of the two-electrode voltage clamp. Figure 2b shows the effect of pre-incubation of mBLT1-expressing oocytes for 20 h with PTX (0.5 or 1.0 μg/ml) on the current induced by 10 nM LTB₄. Figure 2b shows that PTX significantly reduced the current response to

10 nM LTB₄, indicating that the activation of the Ca²⁺ signal by LTB₄ in mBLT1-expressing oocytes is mediated in part by PTX-sensitive G proteins.

mBLT1-expressing oocytes do not respond to nucleotides, LTD₄, or 12R-HETE

Prior to identification of the human BLT1 by Yokomizo and coworkers [1], this GPCR was proposed to be a purinoceptor (P2Y7) [27]. Therefore, we investigated the effect of 20 μM of the nucleotides UTP, ATP, ADP, and AMP, respectively, on mBLT1-expressing oocytes. The compounds were added the perfusion medium or directly to the recording chamber in a small volume. None of these nucleotides elicited a Ca²⁺-activated Cl⁻ current in mBLT1-expressing oocytes (data not shown). The specificity of the receptor was also examined by perfusion with the cysteinyl leukotriene LTD₄ at 0.2 μM, the structural LTB₄ analogue 12R-HETE (12R-hydroxy-5, 8, 14-*cis*-10-*trans*-eicosatetraenoic acid) at different concentrations in the range 0.1-2 μM, and prostaglandin E₂ (PGE₂) at 10 μM. None of these eicosanoids elicited a Ca²⁺-activated Cl⁻ current in mBLT1-expressing oocytes. The fact that the oocytes expressed mBLT1 after exposure to the test compound was functionally verified; the addition of 10 nM LTB₄ evoked a typical Ca²⁺-activated Cl⁻ current in the oocytes. We observed that application of PGE₂ and the nucleotides led to small inward currents (< 20 nA, data not shown) in both mBLT1-expressing and non-injected oocytes, indicating that the current was independent of mBLT1 (data not shown). Thus, among the compounds tested, only LTB₄ could activate mBLT1 expressed in the oocytes.

Activation of PKC abolished LTB₄ activation of the Ca²⁺-activated Cl⁻ current

Because mBLT1 contains several putative phosphorylation sites for PKC, we investigated the effect of activation or inactivation of PKC on the LTB₄-mediated currents. PKC activity was stimulated by incubation of the oocytes expressing mBLT1 with 100 or 200 nM PMA for 5-35 min before addition of LTB₄. Figure 3a shows the effect of 10 nM LTB₄ on the Cl⁻ currents in mBLT1-expressing oocytes exposed to PMA. This figure shows that there is no effect of 10 nM LTB₄ on the Cl⁻ current response in PMA-treated, mBLT1-expressing oocytes, indicating that activation of PKC completely abolishes LTB₄-mediated Ca²⁺ signals in these oocytes. In contrast, control experiments performed on the same batch of oocytes on the same days but without PMA treatment

Fig. 4. Effect of prolonged PMA treatment on the currents elicited by repetitive administration of LTB₄. mBLT1-expressing oocytes perfused with ND96 were administered LTB₄ (10 nM), as indicated by the bars, at 5-min intervals. (a) Representative current trace from an mBLT1-expressing untreated oocyte showing agonist-induced desensitisation. (b) Representative current trace from an mBLT1-expressing oocyte preincubated for 20 h in ND96ps containing PMA (50 nM). A quantitative analysis of data corresponding to experiments presented here can be seen in Table 1.

showed normal responses to 10 nM LTB₄ (Fig. 3b). These data indicate that LTB₄-mediated Ca²⁺ signalling in these oocytes is abolished by activation of PKC and that the PMA-mediated activation of PKC leads to desensitisation of mBLT1 signalling. To test whether PKC directly inactivates the Ca²⁺-activated Cl⁻ channels in the oocytes, we recorded activation of the Ca²⁺-activated Cl⁻ channels in non-injected oocytes treated with the Ca²⁺ ionophore

LTB ₄ added	Untreated	P	PMA pretreated	P
First	1.00 (6)		1.00 (4)	
Second	0.32 ± 0.16 (6)	0.018	0.68 ± 0.13 (4)	0.102
Third	0.37 ± 0.21 (4)	0.057	0.57 ± 0.13 (4)	0.076
Fourth	0.29 ± 0.18 (4)	0.026	0.57 ± 0.15 (4)	0.100
Fifth	0.23 ± 0.15 (4)	0.010	0.55 ± 0.13 (4)	0.073

Table 1. Effect of PMA pretreatment on mBLT1 desensitisation following repetitive administration of LTB₄. mBLT1-expressing oocytes were left untreated or were incubated with 50 nM PMA for 20 h before analysis. For analysis of agonist-induced desensitisation, 10 nM LTB₄ was added at 5-min intervals to the perfusion medium as shown in Figure 4. The experiments were performed at a holding potential of -60 mV. To evaluate desensitisation of mBLT1, the currents induced at the second, third, fourth, and fifth LTB₄ exposures were divided with the current induced at the first LTB₄ exposure. The data are presented as mean ± SEM with the number of oocytes tested in the parentheses. P was determined by a paired two tailed t-test.

A23187 (1 µM). The oocytes were perfused with a Ca²⁺-free medium (ND96Mg), and pulses of Ca²⁺ were applied by changing the perfusion medium after a short period to Ca²⁺-containing medium (ND96Ca). During the pulses of ND96Ca, a fast initial Ca²⁺-activated Cl⁻-current spike was observed followed by a slower current that was sustained in the presence of Ca²⁺ medium (data not shown). Traces recorded from non-injected oocytes treated with PMA showed a reduction in the fast initial spike current, but the sustained current was still observed (data not shown). This finding indicates that the fast Ca²⁺-activated Cl⁻-current component was inhibited by PMA treatment, whereas the slowly progressing Cl⁻-current component was unaffected, in accordance with previously published data [28]. Thus, based on these data, it cannot be excluded that part of the effect of PMA could reflect an effect of PKC on the Cl⁻ channels responsible for the initial fast current; however, it is unlikely that the PMA treatment affects the Cl⁻ channels responsible for the prolonged Cl⁻ current.

Desensitisation of mBLT1: role of PKC

To investigate the mechanism of mBLT1 desensitisation, we exposed mBLT1-expressing oocytes to repetitive stimulation with LTB₄ (10 nM) at 5-min intervals. Figure 4a illustrates the effect of repetitive stimulations with LTB₄ on these oocytes. The oocytes were continuously perfused with ND96, and 10 nM LTB₄ was added via the perfusion medium, as indicated by the bars. At the first LTB₄ exposure, a large peak current was seen, followed by a more slowly evoked current

(Fig. 4a). To quantitatively determine the degree of LTB₄-mediated desensitisation, we divided the maximum current amplitudes following the second, third, fourth, and fifth LTB₄ exposures by that of the initial LTB₄ exposure. The degree of desensitisation is thereby reflected by a value approaching 1 when no desensitisation occurs and close to 0 when a high degree of desensitisation occurs. At the second, fourth, and fifth LTB₄ exposures, the current induced by LTB₄ was significantly reduced compared to the current induced by the first LTB₄ exposure (see Fig. 4a and Table 1). These data imply that the LTB₄-mediated currents in mBLT1-expressing oocytes are desensitised after application of the agonist, i.e., homologous desensitisation occurs.

To investigate if PKC could be involved in the desensitisation process, mBLT1-expressing oocytes were incubated overnight in a low concentration of PMA to inhibit PKC by down-regulation [29]. Figure 4b shows a typical example of a current trace obtained from an mBLT1-expressing oocyte that has been incubated for 20 h in PMA (50 nM) and subsequently exposed to repetitive stimulation with 10 nM LTB₄. In these PMA-treated oocytes, the response to LTB₄ was not desensitised following repetitive LTB₄ stimulation, and the currents induced at the first, second, third, fourth, and fifth exposures in PMA-treated oocytes are similar (see Fig. 4b and Table 1). These data indicate that down-regulation of PKC by prolonged incubation with PMA prevents the homologous desensitisation of the LTB₄-evoked currents in mBLT1-expressing oocytes. Therefore, PKC apparently plays an important role in

Fig. 5. Mutants of mBLT1. The top panel shows a schematic presentation of mBLT1 with 7 transmembrane regions and the helix 8 as proposed for the human BLT1 [30]. Several putative PKC sites were mutated, and the locations of these sites are shown. The lower panel shows part of a Clustal W alignment of BLT1 from mouse, rat, human, and guinea pig, and the mutation sites are also indicated.

the mechanisms leading to agonist-induced desensitisation of LTB₄-mediated signalling via mBLT1.

Ser-127 is important for agonist-induced desensitisation of mBLT1

Sequence analysis of mBLT1 revealed that it has several putative phosphorylation sites for PKC. Among these Ser-86, Ser-127, Ser-217/Thr-220, and Ser-314/Thr-315 were chosen for further analysis because these sites are highly conserved among mouse, rat, human, and guinea pig BLT1s (see Fig. 5). Ser-127 is located in the proposed

second intracellular loop, and Ser-314 and Thr-315 are located in the proposed C-terminal intracellular part of the receptor close to the predicted helix 8 of BLT1 (Fig. 5) [30]. Ser-86 is located in the first extracellular loop, and phosphorylation of this serine should therefore not take place. By site-directed mutagenesis, these putative PKC phosphorylation sites were changed to Ala and the mutants tested for LTB₄-mediated desensitisation. Figure 6a shows a typical recording of an oocyte expressing the mutant mBLT1-S127A. Perfusion with 100 nM LTB₄ induced a fast transient current (Fig 6a), as was also seen

	Second/first	P	Third/first	P
WT	0.0019 ± 0.0006 (27)		0.0014 ± 0.0004 (27)	
S86A	0.0023 ± 0.0011 (5)	0.69	0.0028 ± 0.0008 (5)	0.50
S127A	0.3900 ± 0.1141 (9)	0.0002	0.2000 ± 0.1011 (9)	0.017
S217A/T220A	0.0026 ± 0.0001 (2)	0.11	0.0004 ± 0.0002 (2)	0.28
S314A/T315A	0.0011 ± 0.0003 (4)	0.09	0.0011 ± 0.0005 (4)	0.95

Table 2. Desensitisation following repetitive administration of LTB₄ to oocytes expressing wild-type or mutant mBLT1. Oocytes expressing wild-type or mutant mBLT1 were exposed to 100 nM LTB₄ in the perfusion medium at 5-min intervals. The experiments were performed at a holding potential of -60 mV. To evaluate desensitisation of mBLT1, the currents induced at the second and third LTB₄ exposures were divided with the current induced at the first exposure. The data are presented as mean ± SEM with the number of oocytes tested in the parentheses. P was determined by an unpaired two tailed t-test.

Fig. 6. Reduced agonist-induced desensitisation of the mutant mBLT1-S127A receptor. Mutant mBLT1-expressing oocytes perfused with ND96 were administered LTB₄ (100 nM), as indicated by the bars, at 5-min intervals. The experiments were performed at a clamping potential of -60 mV. (a) Representative current trace from an mBLT1-S127A-expressing oocyte with significantly reduced agonist-induced desensitisation. (b) Representative current trace from an mBLT1-S217A/T220A-expressing oocyte showing agonist-induced desensitisation. The degree of agonist-induced desensitisation for each mutant receptor is shown in Table 2.

for the wild-type receptor (Fig. 4a), indicating that the receptor was expressed and functional. To investigate whether mBLT1-S127A desensitizes as the wild-type receptor does, oocytes expressing this receptor were repetitively administered 100 nM LTB₄ (indicated by the bars) in the perfusion medium (Fig. 6a). The mBLT1-S127A responded with a fast transient current upon repeated LTB₄ exposures, indicating that it was not desensitized as wild-type receptors were (Fig. 6a and Table 2). We observed a significant difference in

desensitisation of mBLT1-S127A compared to wild-type mBLT1 following repetitive stimulation of LTB₄ (Table 2). Furthermore, the lack of desensitisation of mBLT1-S127A was comparable to the response of wild-type receptors when PKC was down-regulated by prolonged incubation with PMA, as seen in Figure 4b. These data strongly indicate that Ser-127 of mBLT1 is needed for agonist-mediated desensitisation, and it is likely that PKC mediates this desensitisation by direct phosphorylation of Ser-127. However, we cannot exclude that PKC

phosphorylates another kinase that then phosphorylates the receptor at Ser-127. We also performed desensitisation experiments with the mutant mBLT1-S86A and with the two double mutants mBLT1-S217A/T220A and mBLT1-S314A/T315A. Figure 6b shows a typical current trace of the mBLT1-S217A/T220A double mutant that was desensitized similarly to the wild-type receptor upon repetitive stimulations with LTB₄. The data for all mutant receptors have been quantified as described above (Table 2), and these data show that agonist-induced desensitisation of the mBLT1 mutants was comparable to that of wild-type receptors for all mutants except mBLT1-127A. These data indicate that the residues Ser-86, Ser-217/Thr-220, and Ser-314/Thr-315 are not involved in agonist-mediated desensitisation of mBLT1, whereas phosphorylation of Ser-127 by PKC very likely mediates agonist-induced desensitisation of mBLT1.

Discussion

Activation of mBLT1 expressed in oocytes by LTB₄ results in the release of intracellular Ca²⁺ and subsequent stimulation of Ca²⁺-activated Cl⁻ channels. The time course of the Cl⁻ currents in mBLT1-expressing oocytes activated by LTB₄ (10 nM and 100 nM) shows a fast initial peak current followed by a slow and more prolonged current. The prolonged current was also evoked by 1 nM LTB₄, whereas the fast, transient peak was absent at this low LTB₄ concentration. These observations indicate that there are two phases of intracellular Ca²⁺ mobilisation following mBLT1 activation in the oocytes. A fast, transient peak current, dependent on Ca²⁺ release from intracellular stores, and a prolonged current, dependent on Ca²⁺ influx have previously been demonstrated upon injection of IP₃ in the oocytes [18]. These currents seem to be mediated by two different Cl⁻ channels, designated I_{Cl-1} and I_{Cl-2}, and it has been proposed by Hartzell that IP₃-induced release of Ca²⁺ from intracellular stores leads to activation of I_{Cl-1} and that store-opened Ca²⁺-channels lead to stimulation of both I_{Cl-1} and I_{Cl-2} [31]. In agreement with this, we find in the present investigation that the initial LTB₄-induced peak current reflects release of Ca²⁺ from intracellular stores because it is unaffected by the presence/absence of extracellular Ca²⁺. Because a low LTB₄ concentration in the present study evokes a prolonged current in mBLT1-expressing oocytes (see Fig. 1b, 1 nM) without activation of the initial fast peak current, we cannot conclude whether the initial spike-i.e., release of Ca²⁺ from the intracellular stores-is a prerequisite for

I_{Cl-2} under the present conditions. The simplest interpretation of our data is that mBLT1 receptor activation gives rise to production of IP₃, leading to a transient increase in intracellular Ca²⁺ during which I_{Cl-1} is activated, followed by a prolonged period with slightly elevated intracellular Ca²⁺ during which I_{Cl-2} is activated. This interpretation is in agreement with the previous observation that the slow, Ca²⁺-mediated Cl⁻-current component has the lowest threshold for intracellular Ca²⁺ [32]. The kinetics of the Ca²⁺-activated Cl⁻ channels in *X. laevis* oocytes are complex and have recently been studied extensively in terms of the source of the activating Ca²⁺, number of Cl⁻ channels involved, and the diffusion time for Ca²⁺ (see [33]).

Using the initial peak current, we found that activation of the Ca²⁺-dependent Cl⁻ current depends on the concentration of LTB₄, with an EC₅₀ estimated at 6.7 nM. This finding is in accordance with previous studies of LTB₄-mediated responses having EC₅₀ values in the low nanomolar range, e.g., 2.5 nM for the chemotactic response, and 20 nM for upregulation of the integrin complement receptor type 3 in murine neutrophils [34]. For U-937 cells, HL-60 cells, and human neutrophils, EC₅₀ values for LTB₄-mediated responses on endogenous receptors were observed between 0.2 nM and 30 nM [35-43].

Mobilisation of intracellular Ca²⁺ by LTB₄ has previously been shown to be dependent on PTX-sensitive G proteins [6, 7, 40], whereas, Ca²⁺-mobilisation in response to LTB₄ in CHO cells transfected with the human LTB₄ receptor was mostly insensitive to PTX [1]. In the present study, the Cl⁻ current responses evoked by activation of expressed mBLT1 were significantly but not completely inhibited by treatment with PTX. These observations indicate that LTB₄ signalling involves both PTX-sensitive and -insensitive G proteins and that species differences in G protein coupling to BLT1 receptors might exist. We did not examine the time course of PTX-mediated inhibition on the LTB₄-evoked currents and therefore cannot exclude that complete inhibition of the response could occur at some earlier time point. However, both PTX-sensitive (G_o) and PTX-insensitive (G_q) G proteins, activated by a single GPCR, were previously shown to couple with PLC in oocytes [44]; it is therefore not surprising that PTX treatment of the oocytes did not completely block the Cl⁻ currents evoked by LTB₄ activation of mBLT1.

The hBLT1 sequence [1] has 100% identity to a sequence previously reported to be a purinoceptor (P2Y7) [27]. However, Yokomizo et al. [1] demonstrated that

the Cos-7 cells possess intrinsic purinoceptors and that these cells responded with Ca^{2+} transients following exposure to ATP (1 μM). Furthermore, expression of hBLT1 in cells that had negligible amounts of intrinsic purinoceptors failed to demonstrate Ca^{2+} responses to ATP. Instead, these cells responded significantly to 10 nM LTB_4 [1]. Because our experiments clearly show that mBLT1 expressed in the oocytes does not respond to 20 μM UTP, ATP, ADP, and AMP, it seems reasonable to exclude mBLT1 as a candidate purinergic receptor. We also investigated the specificity of mBLT1 by exposing mBLT1-expressing oocytes to 12R-HETE, LTD_4 , and PGE_2 . Addition of 12R-HETE and LTD_4 to mBLT1-expressing oocytes did not evoke any currents. Previous studies have revealed that 12R-HETE displaced $^3\text{H-LTB}_4$ binding to hBLT1 (K_i 30 nM) [1] and that its isomer 12S-HETE displaced $^3\text{H-LTB}_4$ binding to mBLT1 (K_i 34 nM) [45]. Because 2 μM 12R-HETE did not evoke Cl^- currents in the mBLT1-expressing oocytes in the current work, it is likely that mBLT1 has a lower affinity for 12R-HETE compared to hBLT1, or that 12R-HETE is an antagonist of mBLT1. It should be noted that with exposure of oocytes to PGE_2 , a small inward current was observed; however, the same magnitude of this current was observed in non-expressing and mBLT1-expressing oocytes, indicating that PGE_2 does not provoke inward Cl^- currents by activation of mBLT1.

Agonist-induced desensitisation of GPCR activity is elicited by phosphorylation via GRKs followed by β -arrestin binding [9]. GPCR desensitisation by PKA and PKC has been shown to result from phosphorylation of a Ser/Thr residue on the C-terminal part of the GPCR or at the intracellular loops [46]. The chemoattractant receptor C5a-R has some sequence identity to mBLT1 (27%) and has been shown to be desensitised by phosphorylation of its C-terminal region by PKC [47]. Furthermore, lipoxin A_4 was shown to mediate downregulation of the number of LTB_4 receptors in guinea pig T lymphocytes by a PKC-dependent mechanism [48]. We found that short-term treatment of the oocytes with PMA completely abolished activation of mBLT1 by LTB_4 , indicating heterologous desensitisation of mBLT1 by PKC. Furthermore, because the short-term PMA treatment of non-injected, A23187-treated oocytes did not inactivate the prolonged Ca^{2+} -activated Cl^- currents induced by perfusion with a Ca^{2+} -containing medium, PKC seemed not to inactivate the Ca^{2+} -activated Cl^- channels by direct or indirect phosphorylation of the channels themselves. Furthermore, PMA-induced phosphorylation of hBLT1 expressed in RBL-2H3 cells increased significantly

following a short-term exposure of the cells to PMA [14]. These data point to a role for PKC in heterologous desensitisation of mBLT1, and they indicate that direct phosphorylation of mBLT1 by PKC could be mediating this heterologous desensitisation. However, we cannot exclude that activation of PKC by PMA could have an effect on PLC, IP_3 receptors, or other components affecting mBLT1 signalling in the oocytes.

Our data also show that the agonist-induced desensitisation (homologous desensitisation) of mBLT1 occurs immediately after the initial exposure to LTB_4 . Although the agonist-induced desensitisation is thought to be mediated by GRKs, we observe that this desensitisation is abolished by prolonged incubation with PMA, which could indicate that PKC also has a role in agonist-induced desensitisation. Because BLT1 receptor phosphorylation has been shown to be induced following activation of PKC [14], we speculate that heterologous and homologous desensitisation of mBLT1 signalling is mediated by PKC by direct phosphorylation of the receptor.

Using site-directed mutagenesis of PKC consensus sites in mBLT1 and analysis of these mutant receptors following expression in oocytes, we identified Ser-127 as a requirement for agonist-induced desensitisation. In all other tested mutants, i.e., mBLT1-S86A, mBLT1-S217A/T220A, and mBLT1-S314A/T315A, we observed agonist-induced desensitisation comparable to that which occurs with the wild-type receptor. The desensitisation behaviour of mBLT1-S127A was similar to that of wild-type receptors after prolonged incubation with PMA, suggesting that the initial steps in agonist-induced desensitisation of mBLT1 are mediated by PKC-mediated phosphorylation at Ser-127. In many cases, GPCR desensitisation has been shown to depend on phosphorylation events on the intracellular C-terminal part of the protein or in the third intracellular loop (see [49]). Attempts to identify crucial sites for desensitisation in BLT1 have revealed that substitution of Thr-308 with Pro or Ala within a putative casein kinase II site in the cytoplasmic tail prevented GRK6-mediated inhibition of BLT1 activity; however, it had only a partial effect on agonist-induced desensitisation of BLT1 [13]. Based on C-terminal truncations of hBLT1 at positions 291 or 319, it has been argued that PKC sites in the C-terminal part of hBLT1 were required for heterologous desensitisation, whereas PKC sites in the intracellular loops were not [13]. This conclusion contrasts with that suggested by our data, which identify Ser-127 in the second intracellular loop as required for desensitisation of mBLT1.

In conclusion, we have identified the murine BLT1 and functionally expressed it in *X. laevis* oocytes, demonstrating a dose-dependent relationship between the Ca^{2+} -activated Cl^- current and LTB_4 concentration. The receptor showed homologous desensitisation, but substitution of the PKC consensus phosphorylation site Ser-127 with Ala prevented this desensitisation. Thus, homologous desensitisation of mBLT1 seems to be mediated by PKC-mediated phosphorylation at Ser-127 in the predicted second intracellular loop of the receptor.

Abbreviations

12R-HETE (12R-hydroxy-5,8,14-*cis*-10-*trans*-eicosatetraenoic acid); 12S-HETE (12S-hydroxy-5,8,14-*cis*-10-*trans*-eicosatetraenoic acid); C5a (anaphylatoxin complement 5a fragment); C5a-R (C5a receptor); CHO cell (Chinese Hamster Ovary cell); Cos-7 cell (African Green Monkey kidney cells, transformed by simian virus 40); DAG (diacylglycerol); EST (expressed sequence

tag); fMLP (*N*-formylmethionyl-leucylphenylalanine); fMLP-R (*N*-formylmethionyl-leucylphenylalanine receptor); GPCR (G protein-coupled receptor); GRK (G protein-coupled receptor kinase); hBLT1 (human leukotriene B_4 receptor); IP_3 (inositol 1,4,5-triphosphate); LTB_4 (leukotriene B_4 , 5(*S*),12(*R*)-dihydroxy-6,14-*cis*-8,10-*trans*-eicosatetraenoic acid); LTD_4 (leukotriene D_4); mBLT1 (murine leukotriene B_4 receptor); $\text{NF-}\kappa\text{B}$ (interleukin-8 nuclear factor); P2Y7 (purinergic receptor 2Y7); PGE_2 (prostaglandin E_2); PKC (protein kinase C); PLC (phospholipase C); PMNL (polymorphonuclear leukocyte); PPAR α (peroxisome proliferator activated receptor α); PTX (*Bordetella pertussis* toxin); Tricaine (ethyl 3-aminobenzoate methanesulfonate); *X* (*Xenopus*).

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