Threonine 308 within a Putative Casein Kinase 2 Site of the Cytoplasmic Tail of Leukotriene B₄ Receptor (BLT1) Is Crucial for Ligand-induced, G-protein-coupled Receptor-specific Kinase 6-mediated Desensitization*

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Desensitization of G-protein-coupled receptors may involve phosphorylation of serine and threonine residues. The leukotriene B₄ (LTB₄) receptor (BLT1) contains 14 intracellular serines and threonines, 8 of which are part of consensus target sequences for protein kinase C (PKC) or casein kinase 2. In this study, we investigated the importance of PKC and GPCR-specific kinase (GRK) phosphorylation in BLT1 desensitization. Pretreatment of BLT1-transfected COS-7 cells with PKC activators caused a decrease of LTB₄-induced inositol phosphate (IP) accumulation. This reduction was prevented with the PKC inhibitor, staurosporine, and not observed in cells expressing a BLT1 deletion mutant (G291stop) lacking the cytoplasmic tail. Moreover LTB₄-induced IP accumulation was significantly inhibited by overexpression of GRK2, GRK5, and especially GRK6, in cells expressing wild type BLT1 but not in those expressing G291stop. GRK6-mediated desensitization correlated with increased phosphorylation of BLT1. The G319stop truncated BLT1 mutant displayed functional characteristics comparable with wild type BLT1 in terms of desensitization by GRK6, but not by PKC. Substitution of Thr308 within a putative casein kinase 2 site to proline or alanine in the full-length BLT1 receptor prevented most of GRK6-mediated inhibition of LTB₄-induced IP production but only partially affected LTB₄-induced BLT1 phosphorylation. Our findings thus suggest that Thr308 is a major residue involved in GRK6-mediated desensitization of BLT1 signaling.

Leukotriene B₄ (LTB₄) is a powerful inflammatory mediator derived from lipooxygenation of arachidonic acid. It is rapidly synthesized by phagocytic cells, principally neutrophils, upon challenge with a variety of stimuli (1). LTB₄ exerts a wide range of biological actions, such as neutrophil chemotaxis, chemokinesis, aggregation, degranulation, and induction of cation fluxes (reviewed in Ref. 2). We and others have also shown LTB₄ to modulate immune responses, including transcription of interleukin-2 receptor-α, interleukin-6, and c-fos in NK cells and monocytes (2–5).

In 1997, a high affinity human LTB₄ receptor (BLT1) was cloned (6, 7). It is a 352-amino acid protein with less than 36% homology with other receptors, suggesting that it belongs to a separate G protein-coupled receptor (GPCR) subfamily. Recently, a second human receptor for LTB₄ (BLT2) was cloned and shown to have lower affinity for LTB₄ and wider tissue distribution (8, 9). The recent development of mice with disrupted BLT1 suggests a major role for BLT1 in acute inflammation and immediate hypersensitivity as well as in leukocyte functions such as chemotaxis and firm adhesion to endothelium in response to LTB₄ (10, 11). Devchand and collaborators (12) have also shown that LTB₄ can bind to the peroxisome proliferator-activated receptor α with low affinity and may have a role in activating genes that terminate inflammatory processes.

The BLT1 signaling pathway involves the activation of phosphoinositide (PI)-specific phospholipase C (PLC)-β via pertussis toxin-sensitive (Gi/Go) and pertussis toxin-resistant (G16, G14) heterotrimeric guanine nucleotide-binding regulatory proteins (G-proteins) (6, 13). Hydrolysis of phosphatidylinositol 4,5-bisphosphate yields inositol phosphates (IP) and diacylglycerol; inositol 1,4,5-trisphosphate triggers the subsequent increase in intracellular Ca²⁺ concentration, a major player in the signaling pathway of BLT1 (6, 14–17).

After exposure to an agonist, cellular responses to subsequent stimuli are usually attenuated; this phenomenon is known as desensitization (18). Agonist stimulation through GPCRs is regulated at different levels; homologous desensitization is rapid and involves uncoupling from the G-protein, Ser/Thr phosphorylation of the ligand-occupied receptor by GPCR-specific kinases (GRKs), and binding of members of the arrestin family, which could act as adaptors between the receptor and components of the internalization machinery (18). Although receptor phosphorylation does not seem to be a prerequisite to arrestin binding, it has been shown to promote their association and receptor internalization via clathrin-coated vesicles (19, 20). The internalized receptors are then exposed to phosphatases and to the acidic environment of the early vesicles, which usually leads to ligand dissociation, receptor resensitization, and, eventually, recycling to the cell

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The abbreviations used are: LTB₄, leukotriene B₄; CK, casein kinase; G-protein, GTP-binding regulatory protein; GPCR, G-protein-coupled receptor; GRK, GPCR kinase; IP, inositol phosphate(s); PI, phosphoinositide; PKC, protein kinase C; PLC, phospholipase C; PMA, phorbol 12-myristate 13-acetate; WT, wild type.
surface. A percentage of the receptors, instead of recycling to the cell surface, can be targeted to lysosomes, which leads to down-regulation of the number of receptors and requires synthesis of new receptors for complete cellular resensitization (21).

Other kinases such as protein kinase C (PKC) and protein kinase A, activated during the signaling cascade, also play a role in the modulation of the cellular response by directly phosphorylating receptors and other components of signalization (22–26). These two messenger-dependent kinases mediate homologous as well as heterologous desensitization, since receptors that do not trigger their activation may also be targeted.GPCRs, like BLT1, can contain motifs of recognition by casein kinases (CKs) in their intracellular loops and cytoplasmic tail. The CK1α was shown to act as a GRK for the muscarinic M1 and M3 receptors (27, 28). Although Hanyaloglu and collaborators (29) reported that phosphorylation of CK2 sites of the thyrotropin-releasing hormone receptor is necessary for β-arrestin-dependent receptor internalization, there is no clear evidence, as yet, for a role for CK2 in either receptor activation or desensitization of other GPCRs.

Rapid desensitization of BLT1 signaling is observed after stimulation with LTB₄. This may involve early uncoupling of the receptor from its transductional elements, followed, in some cells, by down-regulation of high affinity LTB₄ receptors; this latter stage may involve phosphorylation of the receptor or associated proteins, since PKC activation by other routes also down-regulates LTB₄ binding sites (30). Reexpression of receptors on the cell surface is rapid and appears to be associated with recycling of internalized receptors in certain cell types (31). LTB₄ receptors can undergo both agonist- and phorbol ester-induced desensitization (31–34). In vivo desensitization has also been demonstrated in rabbit neutrophils after LTB₄ exposure (32).

As deduced from its amino acid sequence, BLT1 contains a GPCR signature, no consensus tyrosine kinase phosphorylation site, two conserved CK2 phosphorylation sites at positions Ser²⁰⁰ and Ser²⁰² and Thr³⁰⁸/Ser³¹⁰, and multiple serine and threonine residues in the cytoplasmic loops and C-terminal tail. Six of them (Ser¹²⁷, Ser¹²⁶, Thr¹⁹⁷, Thr¹⁹⁴, Thr¹³⁵, and Thr¹²⁴) are within consensus phosphorylation sites for PKC. Whereas accumulating data have allowed definition of a consensus recognition site for CK, none has been established for GRKs. However, both types of kinases share a preference for Ser/Thr residues within an acidic environment (reviewed in Refs. 18 and 35).

Little is known about the structure/function elements of BLT1. In the present study, we examined the potential contribution of PKC and GRK in heterologous and homologous desensitization of BLT1. We identified a structural determinant essential for LTB₄-induced BLT1 desensitization mediated by GRK6.

MATERIALS AND METHODS

Reagents—cDNAs encoding Gα₁₅, Gα₁₆, and PLCβ₅ were generous gifts from Dr. M. I. Simon (California Institute of Technology, Pasadena, CA); the pJ3M expression vector (36) was a generous gift from Dr. J. Chernoff (Fox Chase Cancer Center, Philadelphia, PA). cDNAs encoding GRK2, -3, -5, and -6 were generous gifts from Dr. Jeffrey Benovic (Thomas Jefferson University, Philadelphia, PA). Other materials and their sources were as follows: Lipofectamine, Genetecin, and all culture media from Invitrogen; 4-(2-aminoethyl)-benzenesulfon- nyl fluoride, apotinin, bovine serum albumin, calyculin A, leupeptin, phosphoramidon, phosphol 12-myristate 13-acetate (MMA), Protein A-Sepha- rose, soybean trypsin inhibitor, staurosporine, 1-chloro-3-tosylamido-7- amino-2-heptanone, and Triton X-100 from Sigma; broad range molecular weight markers and Bio-Rad protein assay from Bio-Rad; casein kinase 2 inhibitor 5,6-dichloro-1-b-β-bifuranylosylbenzimidazole, and PKC inhibitor GF109203X from Calbiochem; fetuin bovine serum from BIO MEDIA Canada Inc., Drummondville, Quebec, Canada; FuGENE-6 Transfection reagent, Pico polymerase from Roche Molecular Biochemicals; restriction endonuclease from Promega (Madison, WI); T4 DNA ligase, [³²P]Phosphate, and [²⁵³I]Hmbio-inositol from Amersham Biosciences; LTB₄ from Cayman Chemical, Ann Arbor, MI; perchloric acid from VWR Canlab, Ville Mont-Royal, Quebec, Canada; fluorescein isothio- cyanate-conjugated goat anti-mouse antibody from BIO/SCAN Scientific, Mississauga, Ontario, Canada; gentamicin sulfate from Schering- Canada Inc., Pointe-Claire, Quebec, Canada; CK1 inhibitor N-(2-aminoethyl)-5-chloroisouquinoline-8-sulfonamide from Toronto Research Chemicals Inc. (North York, Ontario, Canada).

Construction of Myt-tagged Wild Type (WT) and Mutant Receptors—The value of the pJ3M expression vector (36) was previously described (13). In this construction, the N-terminal initiator methionine was replaced by the Myc sequence MEQLIISEEDLSRG, resulting in a Myc epitope-tagged BLT1 protein. Each mutant BLT1 was identified by the original amino acid followed by the residue number, which is replaced by a stop codon or substituted for another amino acid. The C-terminus deletion mutant G291stop was constructed using a cassette formed by two oligonucleotides: G291stop-FWD and G291stop-RVS, resulting in the Myc and BglII restriction sites of pJ3M-WT BLT1, eliminating the cytoplasmic tail.

The G319stop mutant receptor was constructed using PCR amplification with the BLT1-FWD (13) and the G319stop-RVS (5′-GG-GCTGGAGGCCCCTCCTGGGACC GGCCGGC-3′) primer resulting in the SST/JA-RVS (5′-GCTGCCCGGCCTGCCGGC-3′) primer. This product was subcloned into the MIuI and BglII restriction sites of pJ3M-WT BLT1, eliminating the cytoplasmic tail. The G319stop mutant receptor was constructed using PCR amplification with the BLT1-FWD and the G319stop-RVS (5′-GG-GCTGGAGGCCCCTCCTGGGACC GGCCGGC-3′) primer resulting in the SST/JA-RVS (5′-GCTGCCCGGCCTGCCGGC-3′) replaced the original sequence, which substituted amino acids Thr²⁰⁸ and Ser³⁰³, respectively, for proline and alanine. The introduction of silent Smal and StuI restriction sites allowed us to use this construction to create the single mutations T308P, T308A, and S310A, the sequence encoding the cytoplasmic tail of BLT1 was first transferred into the pUC31 vector. New unique restriction sites were available, and a cassette generated by the annealing product of the two primers T308P-S310A-RVS (5′-GG-GCTGGAGGCCCCTCCTGGGA GGCGGGCCCTCCCAGCACGC-3′) and T308P-S310A-FWD (5′-CTGGAGGC CGGCGGCGGCAGCG-3′) was inserted into the pJ3M-WT BLT1, generating the full-length receptor containing the specific mutation. cDNAs of mutant receptors were then sequenced (University of Calgary, Alberta, Canada) to confirm proper incorporation of stop codon or amino acid substitution and integrity of the receptor sequence. cDNAs corresponding to WT and truncated mutant BLT1 receptors were then cloned into the pcDNA3 vector (Invitrogen, Carsbad, CA). GRK2, GRK3, GRK5 (37, 38), and GRK6 (39, 40) cDNAs were also cloned into pcDNA3. The human Gα₁₅ and murine Gα₁₆ and PLCβ₅ cDNAs were also contained in the pcCIS and pM12 under the cytomegalovirus promoter, respectively (42–44).

Cell Culture and Transfection—COS-7 cells were grown in Dulbecco’s modified Eagle’s medium with high glucose, supplemented with 5% fetal bovine serum and gentamicin sulfate (40 μg/ml). For transfection, cells were plated in 30-mm dishes (2.0 × 10⁵ cells/dish) and transiently transfected, the following day, with constructions encoding WT or mutant BLT1 in combination with cDNA of Gα₁₅, Gα₁₆, or PLCβ₅. In some experiments, cDNA encoding GRK2, -3, -5, or -6 were also added, as indicated in the figure legends. Using 4 μl of Lipofectamine2000 or 2 μl of FuGENE-6 per dish, 0.2 μg of each cDNA was used for IP studies and experiments. 1 μg of cDNA for flow cytometry analysis. In binding assays, 2 μg of cDNA were used to transfect 1.2 × 10⁵ cells plated in Petri dishes. Experiments were performed 48 h after transfection. Total transfected cDNA quantities were adjusted, for each experiment, with the pcDNA3 vector DNA.

Radioligand Binding Assay—COS-7 cells expressing WT or mutant BLT1 receptors were harvested and washed twice in phosphate-buffered saline (PBS), twice in Hepes (pH 7.4) and twice in Hepes-5% bovine serum albumin (50% v/v), (0.15 μl) bovine serum albumin (45) in which cells were also resuspended for the assay. Competition binding curves were carried out on 2 × 10⁵ cells with 0.25 nm [³²P]LTB4 and increasing concentrations of nonradioactive LTB₄ for 2 h at 4 °C. Free radioactivity was separated from cells by centrifugation and a double wash with 1 ml of Hepes-Tyrode’s buffer. Radioactivity contained in the cell pellet was counted in a scintillation
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\(\beta\) counter. Nonspecific binding represented less than 10% of total binding with 500 nM nonradioactive LT\(_B4\).

**Inositol Phosphate Determination**—Transiently transfected COS-7 cells were labeled and stimulated with LT\(_B4\), IP was extracted, and radioactivity was counted as described previously (13). In some experiments, cells were pretreated with PKC activators (mezerein, phorbol esters (\(4\beta\)-PMA or the inactive form \(4\alpha\)-PMA)) or inhibitors (staurosporine, GF109203X) at the indicated concentrations and times or with CK1 or CK2 inhibitors (N-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide, 50 \(\mu\)M, 30 min; 5,6-dichloro-1-(\(\beta\)-ribofuranosyl)benzimidazole, 10 \(\mu\)M, 30 min).

**Phosphorylation**—Forty-eight hours after transfection, cells were washed twice with Tris-buffered saline and then radiolabeled with \([32\text{P}]\text{orthophosphate (50 g/ml)}\) for 2 h at 37 \({}^\circ\text{C}\), in phosphate-free modified Eagle’s medium, pH 7.4. Agonist was applied as indicated in the figure legends in the presence of the phosphatase inhibitor calyculin A (10 nM). Treatment was stopped by transferring the plates onto ice, removing the medium, and washing twice with ice-cold Tris-buffered saline. Cells were harvested with 1 ml of Tris-buffered saline, followed by a brief centrifugation. The supernatant was then discarded, and cells were immediately used for immunoprecipitation.

**Immunoprecipitation**—Cells were lysed in 500 \(\mu\)l of radioimmunoprecipitation assay buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% SDS, 5 mM EDTA, 1 \(\mu\)g/ml leupeptin, 5 \(\mu\)g/ml aprotinin, 10 \(\mu\)g/ml soybean trypsin inhibitor, 100 \(\mu\)g/ml 4-(2-aminoethyl)-5-chloro-2-methyl-4-imidazolecarboxamide-7-amino-2-heptanone, 1 mM Na\(_3\)VO\(_4\), 10 mM calyculin A). Samples were precleared for 45 min at 4 \({}^\circ\text{C}\) using Protein A-Sepharose. Supernatants were collected and incubated for 1 h with anti-Myc antibody (9E10 hybridoma; American Tissue Culture Collection, Manassas, VA). The mixture was then incubated for 2 h at 4 \({}^\circ\text{C}\) with 50 \(\mu\)l of Protein A-Sepharose with gentle mixing. Protein A-Sepharose was pelleted by brief centrifugation, washed three times in radioimmunoprecipitation assay buffer, and resuspended in SDS-PAGE sample loading buffer (2% SDS, 50 mM Tris-HCl, pH 6.8, 10% glycerol, 5% \(\beta\)-mercaptoethanol, and 0.1% bromophenol blue). Phosphorylated proteins were separated by SDS-PAGE and visualized by autoradiography, and receptor phosphorylation was analyzed using NIH Image software (obtained from the National Institutes of Health Web site: rsb.info.nih.gov/nih-image). Gel image was scanned and imported into NIH Image from Adobe Photo-deluxe (Adobe Systems, San Jose, CA).

**Flow Cytometry Studies**—COS-7 cells transiently transfected with the Myc-tagged WT or mutant BLT1 receptors were subjected to flow cytometry analysis. 2.5 \(\times\) 10\(^6\) cells were labeled, as previously described (13), with anti-Myc, followed by incubation with fluorescein isothiocyanate-conjugated goat anti-mouse IgG antibody (Bio/Can Scientific, Mississauga, Ontario, Canada). All measures were performed on a FACScan flow cytometer (Becton-Dickinson).

**Statistical Analysis**—Data were analyzed for statistical significance using Student’s paired \(t\) test or analysis of variance, as appropriate. Differences were considered significant at \(p < 0.05\).

**RESULTS**

In the present study, we used a cotransfection system in COS-7 cells to define the signaling pathways involved in heterologous and homologous desensitization of BLT1. In addition, we defined a structural determinant of BLT1, which is targeted by a specific GRK.

**Binding Characteristics**—COS-7 cells were transiently transfected with cDNAs for WT BLT1 or constructions of BLT1 in which either the receptor was truncated or specific residues were substituted (Fig. 1). Cell surface expression of WT and mutant receptors was confirmed by flow cytometry using an antibody directed against the Myc tag at the N terminus of each receptor. No specific labeling or \(^{3}H\)-LT\(_B4\) binding could be detected in whole cell extracts transfected with pcDNA3 (data not shown). On the other hand, whole cells expressing WT and mutant forms of BLT1 showed specific \(^{3}H\)-LT\(_B4\) binding, which was displaced in a concentration-dependent manner by unlabeled LT\(_B4\) (0–500 nM). The nonlinear regression analysis of these competition binding curves revealed the presence of one class of binding sites exhibiting high affinity for LT\(_B4\) (dissociation constant \(K_d\) = 1.01 \(\pm\) 0.13 nM). Moreover, the addition of the Myc epitope did not alter the affinity for LT\(_B4\) as compared with the reported \(K_d\) (6, 46, 47). Compared with the WT receptor, all mutant receptors displayed similar affinity for the LT\(_B4\). Similar \(B_{\text{max}}\) values were also observed, ranging from 1.5 \(\times\) 10\(^5\) to 2.8 \(\times\) 10\(^5\) sites, with the exception of the mutant G291stop, which was expressed at higher levels. Binding characteristics are shown in Table 1.

**Ligand-induced IP Production**—As previously shown (13), LT\(_B4\) induces a significant increase in IP accumulation in COS-7 cells cotransfected with BLT1 and Go\(_{\alpha}\). Since BLT1 contains six potential phosphorylation sites for PKC and two potential sites for CK2, we first confirmed previous studies on the involvement of a second messenger-activated kinase in the heterologous desensitization of BLT1 signaling.

Pretreatment of these cells with the nonphorbol PKC activator mezerein caused a time- and concentration-dependent inhibition of ligand-induced IP accumulation (Fig. 2, A and B). It reached 30–40% of inhibition at 60 min at 1 \(\mu\)M mezerein and was prevented by concomitant treatment with the PKC inhibitor, staurosporine (Fig. 2C). Similar findings with \(4\beta\)-PMA (Table II) indicated that the effect was mediated by PKC but was not dependent on the phorbol moiety. These results suggested a partial role for PKC in regulating BLT1 signal transduction and suggested that other kinases would participate to a greater extent. Protein kinase CK1\(\alpha\) was shown to be involved in the phosphorylation and regulation of the m\(_3\)-muscarinic receptor (27, 28). We therefore assessed the involvement of CK in BLT1 signal transduction; treatment of cells with the CK2 inhibitor 5,6-dichloro-1-(\(\beta\)-d-ribofuranosyl)benzimidazole or the CK1 inhibitor N-(2-aminoethyl)-5-chloroisoquinoline-8-sulfonamide before stimulation with LT\(_B4\) failed to modify the IP response (data not shown).

As part of the general regulation of BLT1 responsiveness, homologous desensitization of BLT1 signaling has been demonstrated in various systems and models (32–34). We further investigated the mechanism involved in this homologous inactivation of BLT1 by targeting specific GRKs. The expression of the GRK2, -3, -5, and -6 was assessed by Western blot analysis of a total cell lysate from transfected COS-7 cells (Fig. 3A), indicating that equal amounts of transfected cDNA resulted in similar expression levels of GRKs. When COS-7 cells were
Table I

| Receptors     | Binding parameters |
|---------------|--------------------|
|               | $K_d$ (nM) | $B_{\text{max}}$ (sites/cell) |
| WT            | 1.01 ± 0.13 | 20172 ± 5080 |
| G291stop      | 1.01 ± 0.15 | 222872 ± 36302 |
| G319stop      | 0.79 ± 0.04 | 10375 ± 4800 |
| T308P         | 0.86 ± 0.19 | 25514 ± 7234 |
| T308P/S310A   | 0.94 ± 0.36 | 28015 ± 15248 |
| S310A         | 0.79 ± 0.16 | 15506 ± 3502 |
| T308A         | 0.5 ± 0.02  | 17555 ± 7227 |

The candidate phosphorylation site for LTB4-triggered homologous desensitization of BLT1 was located at Thr308. The Thr308 site could also be used by GRKs.

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We used site-directed mutagenesis to disrupt the CK2 site and examine its potential implication in GRK6-mediated BLT1 desensitization. Threonine 308 and serine 310 were changed to alanine.
proline and alanine, respectively. In addition to an increased IP production compared with the WT receptor (data reported in the legend to Fig. 7), the T308P mutant receptor was totally resistant to GRK6-mediated desensitization of IP accumulation (93.8 ± 5.2% of control without GRK6), in contrast to WT BLT1 (33.2 ± 3.1%) in response to 100 nM LTB₄ (Fig. 7). The double mutation, T308P/S310A, was equally resistant to desensitization by GRK6 (88.5 ± 8.4%) (Fig. 7). On the other hand, the single S308A mutation had no significant effect (47.1 ± 5.8%), showing an inhibition similar to that of the WT BLT1. Since changing Thr³⁰⁸ to proline may have affected the general conformation of the C tail, we also substituted it for alanine. The T308A mutant receptor, when coexpressed with GRK6, was also resistant to desensitization (80.6 ± 2.5%), albeit to a lesser degree than T308P (Fig. 7).

**GRK6 Phosphorylates BLT1**—It is well accepted that agonist-dependent desensitization by GRKs is mediated by their phosphorylation of the occupied receptor (18). To this end, we

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**TABLE II**

Effects of PMA treatment on LTB₄-induced IP accumulation

COS-7 cells transiently expressing the WT or truncated mutant BLT1 in presence of Goαₖ were pretreated with 4×-PMA (100 nM) or its inactive form 4β-PMA (as a control) and then stimulated with 100 nM LTB₄ for the indicated time. Total IP were extracted as described under “Materials and Methods.” Data represent IP accumulation over basal (nonstimulated) levels and are relative to those obtained in cells pretreated with 4×-PMA (defined as 100%). The results are the means ± S.E. of at least three independent experiments, each done in duplicate except for the G319stop, which was done once in triplicate. ***, p < 0.001. ND, not determined.

| Pretreatment (100 nM) | Duration of LTB₄ stimulation | Inositol phosphate accumulation |
|-----------------------|-----------------------------|--------------------------------|
|                       | min                         | WT | G291stop | G319stop |
| 4α-PMA                | 30                          | 100 ± 0 | 100 ± 0 | 100 ± 0 |
| 4β-PMA                | 30                          | 62 ± 5.8*** | 95 ± 4.8 | 103 ± 12.9 |
| 4α-PMA                | 60                          | 100 ± 0 | 100 ± 0 | ND |
| 4β-PMA                | 60                          | 70 ± 3.8*** | 95 ± 2.5 | ND |

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**Fig. 3.** Effects of GRK overexpression on LTB₄-induced IP accumulation in COS-7 cells. A, representative Western blot of GRKs overexpression in COS-7 cells. B, cells transiently coexpressing WT BLT1 together with Goαₖ and either GRK2, -3, -5, -6, or pcDNA3 (as control) were tested for IP production in response to 100 nM LTB₄ for 30 min. C, IP accumulation measured in COS-7 cells coexpressing WT BLT1 and expressing Goαₖ with either GRK6 or pcDNA3 in a time course study. D, a concentration-response study using graded concentrations of LTB₄. Values are means ± S.E. of 3–6 independent experiments, each done in duplicate. They are expressed as percentage of IP accumulation in WT BLT1-expressing cells stimulated with 1 μM LTB₄, defined as 100%. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus pcDNA3-transfected cells.
sought to determine whether GRK6-mediated inhibition of BLT1 IP response involved phosphorylation of BLT1, since Thr308 is essential for BLT1 desensitization. COS-7 cells expressing BLT1 were metabolically labeled with [32P]orthophosphate and exposed to 100 nM LTB4. BLT1 was then immunoprecipitated, and the phosphorylation of BLT1 was visualized by autoradiography (Fig. 8). Only a slight increase in BLT1 phosphorylation was observed upon agonist stimulation when cells were transfected with BLT1 cDNA alone (Fig. 8, lanes 5 and 6). Phosphorylated BLT1 proteins were observed as a diffuse band between 48 and 98 kDa, which represents differentially N-glycosylated forms of BLT1.2 The coexpression of GRK6 led to an increase in BLT1 phosphorylation (Fig. 8A, lanes 7 and 8). In contrast to its strong effect on desensitization by GRK6, substitution of Thr308 to alanine only marginally reduced GRK6-mediated phosphorylation of BLT1 (Fig. 8A, lanes 11 and 12). These findings suggest that GRK6-mediated, agonist-dependent, phosphorylation of BLT1 and desensitization of its response in terms of IP production may involve different structures.

**DISCUSSION**

In the present study, we investigated the structural elements involved in the heterologous and homologous desensitization of the human high affinity LTB4 receptor. We have

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shown that GRK6 targets Thr308 within a putative CK2 site to alter BLT1 responsiveness in COS-7 cells.

When LTB4 binds to BLT1, it purportedly changes the conformation of the receptor leading to the activation of PI-specific PLC-β via pertussis toxin-sensitive (Gia1) and resistant (Gia6) G-proteins (6, 13). Hydrolysis of phosphatidylinositol 4,5-bisphosphate to IP and diacylglycerol with subsequent increase of intracellular Ca2+ concentrations is a major signaling pathway of BLT1 (6, 14–17). Ligand-induced desensitization of this signaling pathway as well as the associated responses (chemotaxis, secretory, and respiratory burst) has been extensively studied in inflammatory cells (32–34, 46, 48, 49). For instance, Goldman and Goetzl (46) showed that leukocyte deactivation by prior exposure to LTB4 led to a loss of high affinity binding sites associated with a decrease in the chemotactic response to LTB4. In addition, in vivo deactivation of BLT1 signaling was achieved in rabbits under prolonged infusion of LTB4, which completely prevented subsequent neutrophil migration induced by a bolus injection of LTB4 (49). Nevertheless, little is known regarding which regulators mediate the desensitization of BLT1 and which structural determinant(s) of the receptor are involved.

GPCR phosphorylation by serine and threonine kinases is commonly associated with desensitization, be it homologous, ligand-induced desensitization or heterologous desensitization resulting from cross-talk with other receptors or from PKC activation. Analysis of BLT1 amino acid sequence revealed numerous putative consensus sites for PKC ((S/T)XXR) suggesting that BLT1 might be subjected to regulation by PKC. As a result of PKC activation, partial but significant reduction of BLT1-mediated IP production was observed. Our data are in accordance with results obtained in neutrophils and eosinophils in which PKC activation potently inhibited agonist-mediated BLT1 responsiveness (30, 31, 33, 51). Furthermore, removing three of the six putative intracellular PKC sites, by truncation of the cytoplasmic tail, generated a mutant receptor (G291stop) totally resistant to PMA-induced inhibition of LTB4-triggered IP production. These results indicate that
ger-activated kinases: G-proteins (22, 25) and PLC (23, 24, 26). In fact, several components of the signaling ma-

may account for a major part of heterologous desensitization. Our initial results with truncated receptors showed, however, that desensitization of BLT1 was largely dependent on a proximal portion of the C-tail. Hence, we attempted to identify specific residues targeted by GRK6. CK1 and -2 phosphorylate Ser and Thr within an acidic environ-

ment (35, 50). Moreover, CK1 has been proposed to influence m3-muscarinic receptor signaling (28). In our system, however, selective inhibitors of CK1 and CK2 had no detectable effect on IP accumulation, suggesting that these kinases are not implicated in modulation of BLT1 signaling. The eventual importance of the CK2 sites in the C-tail and in the third intracellular loop of BLT1 remains to be determined.

BlT1 signaling via the PLCβ pathway does not require any of the structural determinants found in the C terminus tail of BLT1. On the other hand, structural elements important for regulation of this BLT1 signaling pathway are present in the C terminus.

As with the platelet-activating factor and chemokine recep-
tors expressed in rat basophil leukemia (RBL-1) cells, phosho-
rylation of one or more components of the signaling pathway may account for a major part of heterologous desensitization (23, 24, 26). In fact, several components of the signaling machinery have been shown to be modulated by second messen-
ger-activated kinases: G-proteins (22, 25) and PLCβ isoforms (23, 26). This multilevel regulation of signaling confers to the cross-desensitization of chemotransceptor receptors all of its complexity (reviewed in Ref. 24).

Results obtained with the G291stop mutant receptor suggest that desensitization through activation of PKC might act di-
rectly at the receptor protein level, targeting structural deter-
minants of the cytoplasmic tail of BLT1. Interestingly, the putative PKC phosphorylation sites located upstream of the cytoplasmic tail, within the intracellular loops of BLT1, appear not to be implicated in this heterologous desensitization. As with many GPCRs, it would appear that GRKs are involved to a major degree in agonist-dependent BLT1 desensit-
zation. Our data demonstrate that BLT1 signaling can be inhibited by both second messenger-activated kinases and spe-
cific GRKs. Overexpression of GRKs in COS-7 cells, which express lower GRK levels than other cell lines (52), revealed BLT1 as a substrate for GRK2, GRK5, and especially GRK6.

We and others have shown that promyelocytic HL-60 cells differentiated toward the monocyte/macrophage or neutrophil lineages developed specific high affinity receptors for LTB4 (47, 53). This increased expression of BLT1 correlates with higher levels of GRK2 and GRK6 mRNA in myeloid cells (54). Conse-

quently, we further investigated structural elements involved in BLT1 regulation by GRK6. Compared with GRK2/3, GRK6 has been demonstrated to play a major role in attenuation of receptor signaling in only a small group of GPCRs, including the δ-opioid, calcitonin gene-related peptide, and follicle-stimu-
lating hormone receptors (55–58). Observations that LTB4 induced only low levels of BLT1 phosphorylation in COS-7 cells transiently expressing the receptor could also be attributed to the low level of endogenous GRKs (52). Overexpression of GRK6 further increased BLT1 phosphorylation, which was dependent on LTB4 stimulation. This suggested that GRK6 affected BLT1 both through inhibition of ligand-induced IP response and through ligand-activated phosphorylation of the receptor.

Whereas overexpression of GRK6 could readily inhibit LTB4-induced IP production by WT BLT1 as well as by the partially truncated G319stop mutant, it had no effect on G291stop BLT1 mutant. These findings indicated that a segment (residues 291–319) of the carboxyl terminus, which includes several Ser/Thr residues, contained the main site(s) of regulation involved in GRK6-mediated desensitization of BLT1.

Since several potential phosphorylation sites are distributed throughout the intracellular segments of BLT1, as seen with other GPCRs (18), the C-tail, and the third intracellular loop appeared as probable targets for kinases to mediate receptor desensitization. Our initial results with truncated receptors showed, however, that desensitization of BLT1 was largely dependent on a proximal portion of the C-tail. Hence, we attempted to identify specific residues targeted by GRK6. CK1 and -2 phosphorylate Ser and Thr within an acidic environ-

ment (35, 50). Moreover, CK1α has been proposed to influence m3-muscarinic receptor signaling (28). In our system, however, selective inhibitors of CK1 and CK2 had no detectable effect on IP accumulation, suggesting that these kinases are not implicated in modulation of BLT1 signaling. The eventual importance of the CK2 sites in the C-tail and in the third intracellular loop of BLT1 remains to be determined.

GRK2/3 have substrate specificities similar to CK (18, 50, 60), whereas GRK5 and GRK6 prefer Ser and Thr residues downstream of basic amino acids (18, 60). Moreover, Hall et al. (61) have mapped the phosphoacceptor site for GRK6 involved in constitutive phosphorylation of the nonreceptor protein NHERF, which totally correlates with previous phosphopeptide mapping studies (60, 61). GRK subtype preferences, however, may not be always restricted to such environmental specific-

ities, as observed by Oppermann and collaborators (62). They noticed that GRK3 could target phosphoacceptor residues not confined to the vicinity of acidic amino acids in the chemokine receptor CCR5 and, consequently, proposed that the general con-
formation of the receptor may be as important in substrate specific-
ificity as the environment of the phosphoacceptor residues. In this context, GRK6 could easily use the putative CK2 site in the 291–319 segment of BLT1. Cotransfection studies using point mutations of BLT1 within this segment indicated that Thr308 was crucial for GRK6-mediated desensitization.

Although CK inhibitors did not affect BLT1 desensitization, we cannot rule out the possibility that CK2 is involved in some form of regulation of BLT1 function or responsiveness. Little information is available on GPCR regulation by CKs in com-
parison with the numerous substrates identified for CK2 (reviewed in Ref. 35) and other CK subtypes. Tobin and collaborators (27) have shown that CK1α phosphorylates the m3-muscarinic receptor as well as rhodopsin in a stimulus-
dependent manner. Recently, another paper has highlighted a potential role for CK in regulation of GPCR signaling. Internalization of a fusion protein of GnRH and TRH receptors was based on the presence of CK2 sites located within its intracellular C-terminal domain promoting arrestin-2-dependent sequestration of the chimera (29).

Interestingly, mutating residue Thr308 to proline (T308P) completely prevented GRK6-mediated desensitization of BLT1 signaling. Proline residues are frequently found in transmembrane α-helix and can induce a kink in the helix backbone by 26° and thus impact the global structure of the protein. This finding further supports the proposition of Oppermann and collaborators (62), in that conformation strongly influences desensitization of GPCRs.

Several lines of evidence indicate that GRKs interact with regions of the receptor that may be distinct from their site of phosphorylation, as elegantly proposed by Pitcher et al. (18). Assessment of the phosphorylation of BLT1 suggested that substitution of Thr308 only partially prevented GRK6-mediated phosphorylation of the receptor as compared with the WT. If Thr308 was the only target of GRK6, mutating this site to alanine would be expected to produce a receptor resistant to GRK6-mediated desensitization and phosphorylation. However, GRK6 did phosphorylate the T308A mutant receptor, suggesting the presence of other phosphoacceptor residues, which may not be necessarily linked to desensitization. In another system, epinephrine-induced phosphorylation of the α1B-adrenergic receptor by GRK6 did not correlate with attenuation of receptor signaling in COS-7 cells (63). However, in several cases, clusters of serine and threonine residues have been mapped for their contribution to desensitization (64, 65). As with the β2-adrenergic receptor (65), BLT1 desensitization may not be dependent on a single residue. Our findings suggest that Thr308 is not the only site targeted by GRK6 phosphorylation, whereas it appears to be the major site for GRK6-mediated desensitization of BLT1 signaling.

Recently, it was shown that serine and threonine residues in the C-tail of the type 1 dopamine receptor (D1R) can be sorted according to their ability to participate in D1R desensitization or internalization (66). By extrapolation, phosphorylation of other cytoplasmic residues of BLT1 may be related to other functions, such as receptor internalization.

Direct evidence for a role of GRKs in LTB4-induced BLT1 phosphorylation and function in leukocytes may have to await the availability of selective GRK inhibitors. It must be noted, however, that GRK overexpression in COS-7 cells was not able to completely abrogate LTB4-induced BLT1-mediated signaling, suggesting that homologous desensitization could involve other events independent of GRK6-mediated phosphorylation.

In conclusion, our findings in the present study indicate that:
1) the cytoplasmic tail of BLT1 is not involved in ligand binding, G-protein coupling, or PLC activation but contains elements that regulate homologous and heterologous desensitization; 2) PKC activators inhibit LTB4-induced IP production; 3) consensus PKC sites in the C terminus tail of BLT1, but not those located within the intracellular loops, are required for heterologous desensitization; 4) GRK6-mediated BLT1 desensitization is dependent on the 291–319 segment of the C terminus tail; 5) amino acid substitution of phosphoacceptor Thr308 within the putative CK2 site practically abolishes GRK6-induced desensitization of BLT1; and 6) in contrast to GRK6-mediated desensitization, GRK6 phosphorylation of BLT1 is not restricted to residue Thr308.

We have defined a region within BLT1, involved in ligand-dependent, GRK6-mediated desensitization, and have identified Thr308 as the crucial targeted amino acid.
Thr308 Crucial for GRK6-mediated Desensitization of BLT1