Double Mutation at the Putative Protein Kinase C Phosphorylation Sites Thr$^{151}$ Plus Thr$^{323}$ in the Mouse LeukotrieneD$_4$ Receptor Eliminates Homologous Desensitization

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Abstract

Background/Aims: Signalling via CysLT1 is involved in activation of volume sensitive K$^+$ channels and homologous desensitization of the LTD$_4$ receptor impairs regulatory volume decrease (RVD). The aim is to illustrate the effect of mutation of putative PKC consensus phosphorylation sites in the CysLT1R on desensitization and RVD. Methods: mCysLT1 contains 4 putative PKC consensus phosphorylation sites, and four mutants were created: Thr151Gly, Thr323Gly, Thr151Gly plus Thr323Gly, and Thr236Gly plus Ser243Gly. Functional mCysLT1 receptor activity after injection of in vitro transcribed cRNA into Xenopus laevis oocytes was visualized as a LTD$_4$-evoked, Ca$^{2+}$-activated Cl$^-$ currents recorded by two-electrode voltage clamp. Results: Repetitive LTD$_4$ administration (100 nM) desensitized the LTD$_4$-evoked currents in oocytes expressing wild type CysLT1. Single mutations as well as the double mutation Thr236Gly plus Ser243Gly had no or a slight effect on the LTD$_4$ induced desensitization. However, double mutation Thr323Gly plus Thr151Gly prevented the desensitization. As a functional consequence we find that inhibition of PKC accelerates RVD and prevents the inhibitory effect of LTD$_4$-pretreatment on RVD in Ehrlich ascites tumour cells. Conclusion: These data indicate that simultaneous PKC-mediated phosphorylation at the 2nd inner loop (Thr$^{151}$) and at the C-terminal domain (Thr$^{323}$) leads to mCysLT1 receptor desensitization and abrogates the RVD response following osmotic cell swelling.
Introduction

Cysteinyl leukotrienes (LTC₄, LTD₄ and LTE₄) are inflammatory mediators that exert their effects through G-protein coupled receptors (GPCRs) [1-3]. The high affinity receptor for LTD₄, CysLT1 (CysLT1R), has been implicated in various pathological conditions such as asthma [4] and antagonists of the CysLT1R are currently being used in asthma therapy [5, 6]. CysLT1R seems to be involved in colon cancer [7-9], and increased expression of the CysLT1R correlates with poorer prognosis in colon cancer patients [8, 10]. Moreover, it has been demonstrated that LTD₄ via the CysLT1R, mediates cell proliferation, migration and survival of intestinal epithelial cells [11-13]. Down-regulation and inhibition of the CysLT1R have been shown to abolish cell proliferation and induce cell death in non-transformed intestinal epithelial cells [14]. Considering these data, it is evident that further elucidation of the regulation of the CysLT1R and the related signalling pathways could potentially provide novel therapeutic targets in the treatment of asthma and colon cancer.

A change in cell volume is an important signal in regulation of cell proliferation and programmed cell death in mammalian cells [15, 16] and lipogenase products are found to be important messengers in the swelling-induced activation of e.g. TRPV4 channels [17, 18], K⁺ (Task-2) channels and transporters for organic osmolytes [19-21]. We have in a series of papers demonstrated that 5-lipoxygenase product LTD₄ plays a central role in cell volume control in Ehrlich ascites tumour cells (EATC) [15, 16, 22-25]. Stimulation of EATC with a low LTD₄ concentration (3-5 nM) activates the volume sensitive, Ca²⁺ independent K⁺ efflux, the volume sensitive K⁺ channel (EC₅₀ = 2 nM [19, 20]) and the volume sensitive transporter for organic osmolytes [21] and accelerates the volume regulatory response following osmotic cell swelling [24]. On the other hand, stimulation of EATC with a higher LTD₄ concentration results in a PLC-dependent increase in the intracellular Ca²⁺ concentration and in activation of a pertussis toxin sensitive, Ca²⁺ dependent K⁺ efflux (EC₅₀, approximately 15 nM LTD₄) [19]. Accordingly, characterization of the LTD₄ receptor in EATC revealed 2 binding affinities (Kd ≈ 1 nM / 43 nM) [26]. Thus, two different signalling pathways activated by LTD₄ seem to exist in EATC, one responsible for activation of the volume-sensitive K⁺ channels and one responsible for Ca²⁺ mobilization (see [27]). CysLT1R is desensitised and we have previously shown that homologous desensitization of the LTD₄ receptor impairs the volume regulatory response following osmotic cell swelling as well as LTD₄-induced Ca²⁺ mobilization in EATC [24].

Most G protein coupled receptors (GPCR) are subjected to homologous desensitization [28] in a process involving Ser/Thr-targeted phosphorylation of cytoplasmic residues on the receptor on the 3rd intracellular loop or the C-terminal domain of the receptor by GPCR kinases (GRKs) and/or by protein kinase C (PKC) and A (PKA) [28, 29]. Intracellular signalling from activated CysLT1R has been found to involve inositol 1, 4, 5-triphosphate- (IP₃) and diacylglycerol (DAG) synthesis and hence activation of PKC [3, 30]. Homologous [31] and heterologous [32] desensitization of LTD₄ signaling has previously been shown to involve PKC and it has been suggested that desensitization of the CysLT1 receptor is unique as PKC is the principal regulator of both agonist-dependent internalization and desensitization [31, 33]. Accordingly, Capra and co-workers demonstrated that ATP/UDP-induced heterologous desensitization involved GRKs whereas homologous desensitization involved PKC [34]. It has been demonstrated that desensitization of CysLT1 involves internalization of the receptor [10, 31] in a PKC dependent process [31]. Deshpande and co-workers demonstrated that relief of PKC and PKC-mediated desensitization augments LTD₄ induced Ca²⁺ mobilization and airway smooth muscle contraction [33].

The human cysteinyl leukotriene receptor (hCysLT1) was cloned by two groups in 1999 [35, 36] and the murine leukotriene receptor (mCysLT1) in 2001 [37, 38]. The mCysLT1 receptor has 87% sequence identity with the hCysLT1 receptor but differs from the hCysLT1 receptor with respect to PTX sensitivity, receptor-mediated Ca²⁺ influx, and antagonist sensitivity [38]. Expressing the mCysLT1 in Xenopus oocytes revealed that activation by
LTD\textsubscript{4} leads to an initial Ca\textsuperscript{2+} release from intracellular stores, subsequently activating Ca\textsuperscript{2+}-activated or store-operated Ca\textsuperscript{2+} channels leading to influx of extracellular Ca\textsuperscript{2+} [38].

The CysLT1R in EATC contain four potential phosphorylation sites for PKC located to the 2\textsuperscript{nd} (Thr\textsuperscript{151}) and the 3\textsuperscript{rd} (Thr\textsuperscript{236}, Ser\textsuperscript{243}) intracellular loop plus the C-terminal domain (Thr\textsuperscript{323}) [38]. Truncation of the human CysLT1R C-terminal domain (amino acids 310-321), i.e., close to Thr\textsuperscript{309} which is equivalent to the murine Thr\textsuperscript{323} (see [38]), has previously been shown to reduce LTD\textsubscript{4} induced receptor internalisation, whereas mutation at putative PKC sites in CysLT1R C-terminal [amino acids 313-316] prevents desensitization in the human CysLT1R [31].

To identify specific residues involved in the desensitization process we have used the murine CysLT1 receptor and expressed the murine wild type receptor and receptor clones mutated in four PKC sites (Thr\textsuperscript{236} plus Ser\textsuperscript{243}; Thr\textsuperscript{151}; Thr\textsuperscript{236} plus Thr\textsuperscript{323}) in the Xenopus laevis expression system. We have previously demonstrated that non-injected oocytes do not respond to LTD\textsubscript{4}, whereas oocytes expressing the wild type mCysLT1 respond to LTD\textsubscript{4} with high affinity [38]. Double mutation of Thr\textsuperscript{236}Gly plus Ser\textsuperscript{243}Gly in the 3\textsuperscript{rd} loop did not prevent LTD\textsubscript{4} induced desensitization. Single mutation of Thr\textsuperscript{323}Gly in the C-terminal domain had no effect on the LTD\textsubscript{4} induced desensitization, whereas single mutation of Thr\textsuperscript{151}Gly in the 2\textsuperscript{nd} inner loop had a slight inhibitory effect on the desensitization. Double mutation in Thr\textsuperscript{323}Gly plus Thr\textsuperscript{151}Gly prevented LTD\textsubscript{4} induced desensitization. A functional consequence of prevention of CysLT1 desensitization through PKC inhibition on volume regulation is also demonstrated.

Materials and Methods

Media and chemicals

ND96 contained (in mM): Na\textsuperscript{+} 96, Cl\textsuperscript{−} 101, K\textsuperscript{+} 2, Ca\textsuperscript{2+} 1.8, Mg\textsuperscript{2+} 1, and HEPES 5. ND96ps was identical to ND96 but supplemented with sodium pyruvate (2.5 mM), streptomycin (0.01 mg/ml), and penicillin (0.01 mg/ml). Ca\textsuperscript{2+}-free ND96 contained (in mM): Na\textsuperscript{+} 96, Cl\textsuperscript{−} 99, K\textsuperscript{+} 2, Mg\textsuperscript{2+} 1, and HEPES 5. All media were adjusted to pH 7.65. All chemicals were of analytical grade (Sigma Chemical) on less otherwise stated. Collagenase IA (50 mg/ml), tricine [N-tris-(hydroxymethyl)methyl-3-aminoethane sulfonic acid, 0.15% (w/v)] and sodium pyruvate (100 mM) were all dissolved in dH\textsubscript{2}O. Stock solutions of LTD\textsubscript{4} (Cayman Chemical Company) were all prepared in ethanol (201 µM). For K\textsuperscript{+} flux release experiments we used isotonic NaCl Ringer (300 mOsm) containing in mM 143 NaCl, 5 KCl, 1 MgSO\textsubscript{4}, 1 NaHPO\textsubscript{4}, 1 CaCl\textsubscript{2}, 3.3 MOPS 3-(N-morpholino)propane-sulfonic acid, 3.3 TES (N-tris(hydroxymethyl)methyl-3-aminoethane sulfonic acid) and 5 HEPES (N-2-hydroxyethyl)piperazine-N\textsuperscript{′}-2-ethane sulfonic acid)(pH 7.4) and hypotonic NaCl Ringer (150 mOsm) obtained from the isotonic solution by dilution with buffered water containing 3.3 mM MOPS, 3.3 mM TES and 5 mM HEPES (pH 7.4). For cell volume estimations we used K\textsuperscript{+}/Nar equilibrium Ringer which contained 50 mM K\textsuperscript{+} and was prepared by substitution of the appropriate amount of KCl for NaCl [39], isotonic Ringer with low ion strength, i.e., hypotonic NaCl Ringer supplemented with sucrose to isotonicity (0.85 mmol sucrose per mmol NaCl), and hypotonic ND96Cl Ringer where N-methyl-D-glucammonium\textsuperscript{+} was substituted for Na\textsuperscript{+}.

In vitro transcription and construction of cRNA

mCysLT1 cDNA clone was obtained from J Mollerup [38]. cRNA for oocyte injection were obtained by linearizing pBS-mCysLT1 with XhoI and performing in vitro transcription and capping (mCAP mRNA capping kit, Stratagene, La Jolla, USA) according to the manufacturer’s instructions. cRNA quality was monitored by linearizing pBS-mCysLT1 with XhoI and performing in vitro transcription and capping (mCAP mRNA capping kit, Stratagene, La Jolla, USA) according to the manufacturer’s instructions. cRNA quality was monitored by 1% agarose gel analysis, and the cRNA concentration was estimated by UV spectrophotometry. Aliquots of cRNA in water (0.50 ng/µl) were kept at −80°C.

Site-directed Mutagenese

Mutant mCysLT1’s were generated by extension-overlap PCR using high Fidelity Platinum Polymerase (Invitrogen), with primers incorporating the desired nucleotide substitutions. Thr\textsuperscript{151}Gly (Thr\textsuperscript{151} to glycine), 2xdeltap (Thr\textsuperscript{236} and Ser\textsuperscript{243} to glycine) and Thr\textsuperscript{323}Gly (Thr\textsuperscript{323} to glycine). PCR fragments encoded mutated mCysLT1’s were subsequently purified, digested with enzymes and inserted into the pBS expression
Vector, resulting in pBS-mCysLT1. In order to construct the double mutant Thr151Gly/Thr323Gly, mutant Thr152Gly- and Thr323Gly- mCysLT1 were excised with NSPI, purified and ligated. Constructs were fully sequenced (MWG-AG biotech, Ebersberg, Germany) to confirm mutations and receptor sequence. cRNA for oocyte injection were obtained by linearizing pBS-mCysLT1 with Xhol and performing in vitro transcription and capping as indicated above.

Preparation of oocytes and injection of cRNA

_Xenopus laevis_ frogs were anaesthetized form 20 min in 0.15% (w/v) tricaine before removal 5 to 7.5 ml oocytes. During surgery, the frog was kept on ice. Surgical removal of oocytes from Xenopus laevis was approved by “Dyreforsøgstilsynet” 2007/561-1313. The oocytes were washed in Ca²⁺-free ND96, and de-folliculated by collagenase IA (2 mg/ml) in Ca²⁺-free ND96 (1h, room temperature). The oocytes were washed 7 times in Ca²⁺-free ND96, subsequently 3 times in Ca²⁺ containing ND96, and finally kept in ND96ps. Healthy stage V or VI oocytes were selected and kept in 80-mm petri dishes until the following day. cRNA (total 90 nl / 45 ng) was injected into each oocyte at the white, vegetative site of the oocyte using a Nanoliter Injector (World Precision Instruments, USA) and borosilicate glass micropipettes (K120-3) pulled on a Narishigi-puller (PP-830, Narishigi, Japan). After injection, the oocytes were maintained individually in 24-well dishes (NuncIon, Nunc) in ND96ps at 17°C. The medium was changed on the third day. Electrophysiological recordings (two-electrode voltage clamp) were performed on days 3−5 after cRNA injection. The number of successful sets of oocytes recordings from one frog are in average 1 to 4.

Two-electrode voltage clamp

The two-electrode voltage clamp experiments were performed as previously described [40]. Briefly, we used 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. Microelectrodes were pulled from GC150F-10/GF160F-8 borosilicate glass on a patch electrode puller (PP-830, Narishigi, Japan) and filled with 3 M KCl. Data were sampled at 1000 Hz or 500 Hz and low-pass filtered at 10 Hz. The potential and the current microelectrodes had resistances between 0.6−0.7 M Ω. 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 −21 mV and −101 mV, respectively (calculated from concentrations given in [41]). In order to avoid saturation of the amplifier during Ca²⁺-activated Cl⁻ currents we used oocytes voltage clamped in the range +50 to −60 mV instead of −95 mV see [40].

$K^+$ efflux

$K^+$ release after osmotic cell swelling was estimated as the release of $^{86}$Rb from EATC as previously described [42]. Briefly, EATC were equilibrated with $^{86}$Rb⁺ (2x10⁴ Bq/ml, 30 min), was washed and at time zero resuspended at a cyt crit of 2% in hypotonic NaCl Ringer (150 mOsm) containing 0.01% bovine serum albumin, bumetanide (30 µM, added to avoid $^{86}$Rb⁺ reuptake via the Na,K,2Cl co-transporter, and $^3$H-inulin (added for estimation of extracellular trapped water space). $^{86}$Rb⁺ efflux from the cells was determined as the gain in the extracellular $^{86}$Rb⁺ activity (cpm per ml medium) within the initial 8 min by serially isolating cell free efflux medium by centrifugation (15000g, 30s) through a silicone oil phase (300 µl 5:1 albumin, bumetanide (30 µM, added to avoid) and KCl co-transporter, and $^3$H-inulin (added for estimation of extracellular trapped water space). $^{86}$Rb⁺ efflux from the cells was determined as the gain in the extracellular $^{86}$Rb⁺ activity (cpm per ml medium) within the initial 8 min by serially isolating cell free efflux medium by centrifugation (15000g, 30s) through a silicone oil phase (300 µl 5:1 albumin, bumetanide (30 µM, added to avoid). For each sample, a 200 µl efflux aliquot was added for estimation of extracellular trapped water space). $^{86}$Rb⁺ efflux from the cells was determined as the gain in the extracellular $^{86}$Rb⁺ activity (cpm ml medium⁻¹) and the cell dry weight (g ml medium⁻¹), was plotted versus time, and the rate constant for $^{86}$Rb⁺ efflux (min⁻¹) was determined by fitting the efflux curve between 1 and 8 min to an exponential rise to a maximum function. The $^{86}$Rb⁺ efflux (cpm ml cell water⁻¹ min⁻¹) was calculated as the product of the rate constant and the cellular concentration (cpm ml cell water⁻¹). The $K^+$ efflux (µmoles ml cell water⁻¹ •

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min⁻¹) was calculated from the ⁸⁶Rb⁺ efflux by division with the specific activity (cpm • ml cell water⁻¹ / µmol K⁺ • ml cell water⁻¹) and converted to µmol • g dry weight⁻¹ • min⁻¹ by multiplication with the water content (ml cell water • g cell dry wt⁻¹).

**Cell volume measurements**

Absolute cell volume was determined by electronic cell sizing on EATC cells using a Coulter Multisizer 3 (Coulter, Luton, UK, Orifice 100 µm) as previously described [42]. Briefly, EATC cells were washed and transferred to isotonic K⁺/Na⁺ equilibrium Ringer, incubated for 30 min before dilution (100 fold) in either isotonic Ringer with low ion strength or hypotonic NMDGCl Ringer (final cell density 50.000 cells per ml). Cell volume was calculated from the median of cell volume distribution curves after calibration with latex beads.

**Statistics**

All data are presented either as individual experiments or as mean values ± standard error of the mean (SEM). Statistical evaluation is based on paired Student’s t-test or Welch t-test when the variance was significantly different (indicated in legends). For statistical evaluation p<0.05 was taken to indicate significant different.

**Results**

**Desensitization of the LTD₄ response in mCysLT1 expressing oocytes**

Native Xenopus laevis oocytes express endogenous Ca²⁺-activated Cl⁻ channels which allows whole-cell Cl⁻ currents to be used in the detection of receptor activity, elicited through intracellular Ca²⁺ signalling [43]. Cysteinyl leukotrienes receptors has been cloned and previously characterized by this method [35, 38, 44]. We have used the two-electrode voltage-clamp technique to follow Ca²⁺ sensitive Cl⁻ current following repetitive stimulation with 100 nM LTD₄ in Xenopus oocytes expressing in vitro transcribed cRNA, encoding the wild type mCysLT1. In accordance with previous data using the same clone [38] it is seen from Fig. 1A that addition of LTD₄ leads to a fast, transient inward current, which returns to baseline approximately 1 min after removal of LTD₄. It should be noticed that the EC₅₀ value for LTD₄ induced Cl⁻ current is estimated at 10 nM [38]. A 2nd and 3rd stimulation with 100 nM LTD₄ at 5 minutes intervals did not result in any Cl⁻ current (Figs. 1A & 1B). The LTD₄ induced suppression of a second response lasted for less than 30 min (data not shown). Together these data are taken to indicate desensitization of the mCysLT1 by addition of 100 nM LTD₄. To exclude that the desensitization could be caused by a reduction in the Ca²⁺ sensitivity of the Cl⁻ channel we exposed the mCysLT1 expressing oocytes to the calcium ionophore A23187 (1µM) for 5 min in Ca²⁺ free medium. The oocytes were then exposed to Ca²⁺ (10 mM) twice for 20 sec with 1 min interval. The Ca²⁺ induced current was not reduced during the 2nd stimulation compared to the 1st stimulation, i.e., the peak current was -568 ± 116 nA and -417 ± 76 nA for the 1st and 2nd response, respectively.

**Double mCysLT1 mutation - Thr236Gly plus Ser243Gly – has no effect on LTD₄ induced desensitization of mCysLT1 expressed in oocytes**

The CysLT1R in EATC contain four potential phosphorylation sites for PKC located to the 2nd intracellular loop (Thr²³³), the 3rd intracellular loop (Thr²³⁶, Ser²⁴³) and the C-terminal domain (Thr¹⁵³) [38] (see Fig 2A). In order to investigate the importance of the PKC sites in the 3rd intracellular loop we substituted Thr²³⁶ as well as Ser²⁴³ with glycine in the CysLT1R, injected the clone in Xenopus oocytes, and followed the Cl⁻ current following three repetitive stimulations with 100 nM LTD₄. Figure 2B shows the 1st, 2nd and 3rd response (the maximum current amplitudes) for oocytes expressing the wild type CysLT1R and the double mutant Thr²³⁶Gly/Ser²⁴³Gly. It is seen that the 2nd and the 3rd response to LTD₄ are reduced in
Fig. 1. LTD₄ induced desensitization of mCysLT1. Activity of the mCysLT1 expressed in *Xenopus laevis* oocytes was recorded by two-electrode voltage clamp technique as the Ca²⁺-sensitive Cl⁻ current. Panel A: Oocytes were administered LTD₄ (100 nM) three times with 5 min intervals, i.e., for a few seconds (first stimulation) and up to 1 min (2nd and 3rd stimulation) as indicated by the bars. The figure shows a representative current trace. Panel B: Mean values for the maximal Cl⁻ currents ± SEM induced by LTD₄ at the 1st (n = 54), the 2nd (n = 44) and the 3rd (n = 42) exposure, where n is the number of oocytes from ≈ 10 frogs. * indicates significantly reduced compared to the current induced by LTD₄ at the 1st exposure.

Fig. 2. Double mCysLT1 mutation - Thr236Gly plus Ser243Gly – has no effect on LTD₄ induced desensitization of mCysLT1. Panel A: A schematic representation of mCysLT1 receptor based on data from [38]. Mutations of PKC sites (predicted by Procite) are indicated. Panel B: The Ca²⁺-sensitive Cl⁻ current following exposure to LTD₄ (100 nM) was recorded in *Xenopus laevis* oocytes expressing mCysLT1 as shown in Fig. 1. The figure shows mean values for the maximal Cl⁻ currents ± SEM induced by LTD₄ at the 1st, 2nd and 3rd response for the wild type (black bars) and the double mCysLT1 mutant (Thr236Gly plus Ser243Gly, grey bars). The number of experiments (oocytes) for the sequential responses was 14, 12 and 12 for wild type and 18, 16 and 16 for the double mutant. Oocytes are from 4 to 5 frogs. The LTD₄ induced Cl⁻ current at the 2nd and 3rd response was in wild type and double mutant significantly reduced compared to the 1st response. There was no significant difference between the LTD₄ induced Cl⁻ current in the wild type and the double mutant at the 2nd or 3rd LTD₄-induced response (Welch's approximate t-test).

oocytes expressing the mutant to the same extent as in oocytes expressing the wild type, i.e., there is no significant difference between wild type mCysLT1R and the double mutated receptor. These data imply that double mutation at Thr²³⁶ plus Ser²⁴³ (Thr236Gly/Ser243Gly) does not affect the LTD₄ induced desensitization of the mCysLT1.
Double mCysLT1 mutation – Thr151Gly plus Thr323Gly – impairs LTD₄ induced desensitization of mCysLT1. The Ca²⁺-sensitive Cl⁻ current following exposure to LTD₄ (100 nM) was recorded in Xenopus laevis oocytes expressing mCysLT1 as shown in Fig. 1. Panel A: Maximal Cl⁻ currents ± SEM induced by LTD₄ at the first, second and third response for the wild type (black bars) and the single mCysLT1 mutant Thr151Gly. The number of experiments (oocytes) for the sequential responses was 17, 14 and 13 for wild type and 33, 31 and 29 for the Thr151Gly mutant. # significantly larger LTD₄-induced Cl⁻ current in the Thr151Gly mutant compared to the wild type. Panel B: Similar to Panel A but for the single mCysLT1 mutant Thr323Gly. The number of experiments (oocytes) for the sequential responses was 23, 18 and 17 for wild type and 26, 23 and 17 for the Thr323Gly mutant. # significantly reduced LTD₄-induced Cl⁻ current in the Thr323Gly mutant compared to the wild type. The 2nd and 3rd responses to LTD₄ in Thr323Gly mutant were not different from the wild type. It is noted that in two of the 3rd responses the current was much higher in Thr323Gly than the first response even though the second response was 0. These two values were regarded as outliers and are not included. Panel C: Representative Cl⁻ current trace in Xenopus laevis oocytes expressing the double mCysLT1 mutant Thr151Gly plus Thr323Gly during administration of LTD₄ (100 nM) three times with 5 min intervals as indicated by the bars. Panel D: Maximal Cl⁻ currents ± SEM induced by LTD₄ at the 2nd and 3rd response in oocytes expressing native mCysLT1 (black bars) and oocytes expressing the double mCysLT1 mutant Thr151Gly plus Thr323Gly (grey bars). Values are relative to the current following the 1st exposure to LTD₄ in the same oocyte (Wild type: 557 ± 163 nA, n=7; Double mutant: 436 ± 78 nA, n = 13). # indicates significantly larger LTD₄-induced Cl⁻ current in the double mutant compared to the wild type. There was no significant difference between the first, second and third response in the double mutant (paired t-test). Oocytes are from 4 to 8 frogs in all series of experiments.

Double mCysLT1 mutation – Thr151Gly plus Thr323Gly – impairs LTD₄ induced desensitization of mCysLT1 expressed in oocytes

To investigate the importance of the PKC sites in the 2nd intracellular loop we substituted threonine at position 151 to glycine, injected the receptor clone in Xenopus oocytes, and estimated the Cl⁻ current following three repetitive stimulations with 100 nM LTD₄. Figure 3A shows the 1st, 2nd and 3rd response for oocytes expressing the wild type receptor and the
Thr151Gly mutant. It is seen there is a significant difference between the wild type and Thr151Gly mutant in the size of the second response and the third response (Fig. 3A). It is noted that the 2nd and 3rd response to LTD4 in the Thr151Gly mutant was somehow oscillating.
(noisy) (data not shown). These data imply that phosphorylation of Thr\textsuperscript{151} might be involved in the LTD\textsubscript{4} induced desensitization of the mCysLT1 receptor. Substitution of threonine at 323 on the C-terminal domain end of the mCysLT1 receptor to glycine reduced the 1\textsuperscript{st} response to LTD\textsubscript{4} but the desensitization of mCysLT1 seemed not to be affected, i.e., the Cl current following the 2\textsuperscript{nd} and the 3\textsuperscript{rd} stimulation with 100 nM LTD\textsubscript{4} was not significantly different in oocytes expressing the mutant and the wild type mCysLT1 receptor (Fig. 3B). These data imply that phosphorylation of Thr\textsuperscript{323} alone has no significant effect on desensitization of the Cl\textsuperscript{−} current after repetitive LTD\textsubscript{4} addition. It is, however, noted that the 3\textsuperscript{rd} response in the Thr\textsuperscript{323} mutant is about 50% of the 1\textsuperscript{st} response, which was not significant different due to a large standard error on the recordings, thus some effect on desensitization of the receptor by phosphorylation of Thr\textsuperscript{323} cannot be ruled out.

From the time trace in Fig. 3C it is seen that simultaneous substitution of threonine at positions 323 and 151 in the receptor to glycine eliminates desensitization by repetitive stimulation with 100 nM LTD\textsubscript{4}. Figure 3D shows the 2\textsuperscript{nd} and the 3\textsuperscript{rd} response to LTD\textsubscript{4} exposure relative to the 1\textsuperscript{st} response in oocytes expressing wild type receptor (black bar) and the Thr323Gly/Thr151Gly mutant receptor (grey bar). In oocytes expressing the mutant there was no significant difference between the 1\textsuperscript{st} response and the 2\textsuperscript{nd} or 3\textsuperscript{rd} response, moreover the 2\textsuperscript{nd} and 3\textsuperscript{rd} response were significantly higher when compared to the 2\textsuperscript{nd} and 3\textsuperscript{rd} response in oocytes expressing the wild type receptor. Thus, simultaneous mutations of Thr\textsuperscript{151} and Thr\textsuperscript{323} at the second intracellular loop and the C-terminal domain of the receptor respectively prevent homologous desensitization of the LTD\textsubscript{4}-evoked currents in mCysLT1-expressing oocytes.

**Inhibition of PKC isoforms augments the swelling induced activation of K\textsuperscript{+} channel and impairs the inhibition of RVD seen after pre-treatment with LTD\textsubscript{4}**

As LTD\textsubscript{4} plays an essential role in the activation of swelling activated K\textsuperscript{+} efflux in EATC we have investigated whether an inhibition of CysLT1 receptor desensitization with a PKC inhibitor would augment the activation of the swelling induced K\textsuperscript{+} efflux. Figure 4A shows the release of Rb\textsuperscript{+}, used as a tracer for K\textsuperscript{+}, within the time period 0.8 min to 8 minutes following hypoosmotic exposure, i.e., the period where the K\textsuperscript{+} channel is maximally activated. From Fig. 4B it is seen that Gö6976 (inhibitor of classical PKC isoforms, PKCa, PKCB) and Gö6850 (an inhibitor of novel (PKC ε) plus classical PKC isoforms) both enhance the swelling activated efflux significantly. The simplest explanation for these observations is that pharmacological inhibition of PKC reduces the CysLT1 desensitization during RVD and thus increases the activity of the volume sensitive K\textsuperscript{+} channel. It has previously been shown that pre-treatment with LTD\textsubscript{4} impairs a subsequent RVD response probably due to desensitization of the CysLT1 receptor [24]. This is confirmed in Fig. 4D, which shows that the rate of the RVD response is reduced to 50% in cells exposed to 50 nM LTD\textsubscript{4} 6 min before the hypoosmotic challenge (compare Control white bar with Control black bar). Inhibition of PKC with Gö6850 prevents the reduction in the rate of RVD in LTD\textsubscript{4}-pre-treated cells (Fig. 4C and 4D), indicating absence of desensitization.

**Discussion**

We have expressed a functional murine Cys LTR1 receptor in xenopus oocytes and demonstrated that stimulation with 100 nM LTD\textsubscript{4} leads to a reduced sensitivity towards the agonist which is rapid and lasts for less than 30 min. The fast onset and short duration of the response points to desensitization rather than internalisation of the receptor. The experiments with the Ca\textsuperscript{2+} stimulation in the presence of a Ca\textsuperscript{2+} ionophore excluded that it is the Cl channel which is desensitised by the repetitive Ca\textsuperscript{2+} stimulation. CysLTR1 belongs to the GPCR family and a classical model for GPCR’s desensitization involves phosphorylation of serine / threonine residues on the 3\textsuperscript{rd} intracellular loop and the C-terminal domain of the receptor [28]. It has turned out that CysLTR1 is rather special as it is the only GPCR...
where agonist-induced desensitization and internalisation is primarily dependent on PKC and independent of arrestin [31, 33]. Four potential PKC target sites have been identified in the murine CysLTR1, i.e., Thr\(^{236}\) (2\(^{\text{nd}}\) intracellular loop), Thr\(^{323}\) (3\(^{\text{rd}}\) intracellular loop) and Thr\(^{323}\) (at the C-terminal domain) (Fig. 2A, [38]). We have generated mutant of all these PKC sites in which threonine or serine (representing the PKC site) were mutated to glycine (Fig. 2A). The first response after expression of wild type and mutant receptors is identical in all mutant with the exception of the Thr\(^{323}\)Gly and we have consequently concluded that the receptors for wild type, Thr\(^{236}\)Gly plus Ser\(^{243}\)Gly (Fig. 2B), Thr\(^{151}\)Gly (Fig. 3A) and Thr\(^{151}\)Gly plus Thr\(^{323}\)Gly (see legend to Fig. 3C) are all expressed to a similar degree in the plasma membrane. Comparison of the second and third response with the first response in oocytes expressing wild type and mutant CysLTR1 revealed that a double mutation in the 3\(^{\text{rd}}\) intracellular loop, which is normally assumed to affect desensitization of members of the GPCR family [28], has no effect. On the other hand double mutation involving Thr\(^{151}\) in the 2\(^{\text{nd}}\) intracellular loop and Thr\(^{323}\) in the C-terminal domain resulted in complete loss of the ability to desensitize. Mutation of Thr\(^{151}\) alone gave a minor but significant reduction in the ability to desensitize and resulted in a somehow oscillating (noisy) 2\(^{\text{nd}}\) and 3\(^{\text{rd}}\) response. Whether this is a result of Ca\(^{2+}\) oscillation resulting from relative weak receptor stimulation has not been investigated. Whether mutation of Thr\(^{323}\) in the C-terminal domain alone has some effect on desensitization cannot be excluded (Fig. 3B). It is noted that for the human CysLTR1 it has been demonstrated that simultaneous mutation of Ser\(^{320}\), Ser\(^{330}\) and Ser\(^{331}\) (i.e., at sites equivalent to the murine Ser\(^{320}\), Ser\(^{330}\) and Ser\(^{331}\) (see [38]), prevents internalisation of the receptor by 50% [31, 45]. Moreover, the chemo attractant receptor C5a-R, which has some sequence identity to mCysLT1, has also been shown to be desensitised by phosphorylation of its C-terminal domain by PKC [46]. CysLTR1 is described as a promiscuous receptor, as it couples the G\(_i\) as well as G\(_i\) in trimeric GTP binding proteins, and it has been indicated that heterologous desensitization of the receptor involves the G\(_i\) mediated signalling [32]. The homologous desensitization described in the present work is suggested to be the result of a concomitant PKC mediated phosphorylation in the 2\(^{\text{nd}}\) intracellular loop and the C-terminal region of the receptor and hence uncoupling of the G\(_i\) mediated signaling. In GPCRs the 2\(^{\text{nd}}\) intracellular loop is known to be important for selective binding and activation of G-proteins [47, 48]. It would be interesting to test the double mutations in the equivalent PKC sites in the human receptors, i.e., at Thr\(^{151}\) in the 2\(^{\text{nd}}\) intracellular loop plus Thr\(^{323}\) at the C-terminal region.

As homologous desensitization of the CysLT1 receptor by pretreatment with LTD\(_4\) inhibits RVD our result predict that the presence of a PKC inhibitor during the pretreatment should prevent the inhibition of the RVD. This is indeed the case as seen in Fig. 4. We also find that inhibitor of classical PKC isoforms with G\(^{6850}\) and classical/novel PKC isoforms with G\(^{6976}\) during the RVD process accelerates the concomitant, swelling induced K\(^+\) efflux, likely because inhibition of PKC prevents the desensitization of CysLT1 and hence prolongs the activation of the volume sensitive K\(^+\) efflux pathway. Thodeti and co-workers have shown that classical PKC isoforms (PKC\(_{\alpha}\)) and novel PKC isoforms (PKC\(_{\delta}\), PKC\(_{\epsilon}\)) translocate to the plasma membrane in human intestinal epithelial cells following LTD\(_4\) exposure [49]. Furthermore, PKC\(_{\alpha}\) is found to play a major role for desensitization of the CysLT1 receptor in rat basophilic leukemia (RBL-1) [50]. The PKC activity is increased in EATC during osmotic cell swelling [51], although the PKC isoform was not determined. EATC express classical PKCs (PKC\(_{\alpha}\), PKC\(_{\beta}\)), novel PKCs (PKC\(_{\delta}\)) as well as atypical PKCs (PKC\(_{\iota}\)) (micro-array, unpublished data). The effect of G\(^{6850}\)/G\(^{6976}\) points to a role of a classical PKC in the swelling induced desensitization of CysLT1 in the EATC. Stutzin and coworkers have demonstrated that PKC\(_{\alpha}\) plays a role in the swelling induced activation of the volume sensitive Cl\(^-\) channel in HeLa cells [52]. Taken together we suggest that PKC could play a dual role in the regulation of volume sensitive channels, i.e., cell swelling induces release of LTD\(_4\) [53], activation of CysLT1 and subsequent activation of the volume sensitive K\(^+\) channel (TASK-2) [20, 54]. Simultaneously
PKC, most likely a classical isoform, is activated and leads to stimulation of the Cl⁻ channel (VRAC) as well as phosphorylation of CysLT1 (Thr¹⁵¹ / Thr³²³ ), i.e., desensitization of CysLT1 which results in a reduced stimulation and closure of TASK2.

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References


