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$CysLT_1$ signal transduction in differentiated U937 cells involves the activation of the small GTP-binding protein Ras

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Abstract

We investigated the signal transduction pathway(s) of leukotriene D_4 (LTD_4) in the human promonocytic U937 cells, a cell line known to constitutively express $CysLT_1$ receptors. Herein, we demonstrate that LTD_4 specifically acts on a $CysLT_1$ receptor to dose-dependently increase (three to five-fold over basal) RasGTP through a $G_{i/o}$ protein. In fact, while cytosolic Ca^{2+} ($[Ca^{2+}]_i$) increase was only partially sensitive to pertussis toxin (PTx), Ras activation was almost completely inhibited by the same toxin. Furthermore, the phospholipase C (PLC) inhibitor U73122 completely inhibited both $[Ca^{2+}]_i$ and RasGTP increase, suggesting that in these cells PLC is the point of convergence for both PTx insensitive and sensitive pathways leading to $[Ca^{2+}]_i$ release and Ras activation. Indeed, chelating intracellular Ca^{2+} strongly (>70%) prevented LTD_4-induced Ras activation, indicating that this ion plays an essential role for $CysLT_1$-induced downstream signaling in differentiated U937 (dU937) cells. In addition, while Src did not appear to be substantially involved in $CysLT_1$-induced signaling, genistein was able to partially inhibit LTD_4-induced $[Ca^{2+}]_i$ transient (∼34%) and almost completely prevented Ras activation (>90%), suggesting a potential role for other Ca^{2+}-dependent tyrosine kinases in LTD_4-induced signaling. Finally, agonist-induced $CysLT_1$ stimulation was followed by a specific extracellular regulated kinase (ERK) 1/2 phosphorylation, an event with a pharmacological profile similar to that of Ras activation, partially (∼40%) sensitive to *Clostridium sordellii lethal toxin* and totally blocked by PTx. In conclusion, LTD_4-induced $CysLT_1$ receptor activation in dU937 cells leads to Ras activation and ERK phosphorylation mostly through a PTx-sensitive $G_{i/o}$ protein, PLC, and Ca^{2+}-dependent tyrosine kinase(s).

Keywords: Cysteinyl-leukotriene; $CysLT_1$ receptor; U937; Ras; MAPK; Signal transduction

1. Introduction

Cysteine-containing leukotrienes (cys-LTs) are pivotal inflammatory mediators formed through the 5-lipoxygenase pathway of arachidonic acid in a number of inflammatory cells including eosinophils, basophils, monocytes, and macrophages. It is now widely recognized that cys-LTs might play an important role in a number of inflammatory diseases, such as asthma, participating both to the early bronchoconstriction but also to the late chronic inflammatory component of the disease [1]. In addition, cys-LTs have been implicated in a number of cardiovascular diseases, from coronary artery disease to atherosclerosis [2,3] or in cardiovascular complications of inflammatory processes [4].

Until now, two receptor subtypes have been cloned, namely $CysLT_1$ [5,6] and $CysLT_2$ [7–9], both belonging to the seven transmembrane domains superfamily of receptors, largely confirming previous pharmacological characterization based on sensitivity to classical antagonists. The distribution of each subtype has been also clarified, and in particular, the $CysLT_1$ receptor has been localized, besides in human lung smooth muscle cells and macrophages, also in most peripheral blood cells, including eosinophils and in subsets of monocytes and B lymphocytes and $CD34^+$ progenitor cells [10,11]. In turn, $CysLT_2$ is more

Abbreviations: cys-LTs, cysteine-containing leukotrienes; PTx, pertussis toxin; GPCR, G protein-coupled receptors; LTD_4, leukotriene D_4; $[Ca^{2+}]_i$, cytosolic Ca^{2+} concentration; dU937, differentiated U937; PLC, phospholipase C; MAPK, mitogen-activated protein kinase; RBD, Ras binding domain; Fluo3/AM, Fluo3 acetoxymethyl ester; PTK, protein tyrosine kinase; ERK, extracellular regulated kinase; IP_3, inositol 1,4,5-triphosphate.

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abundant in heart, peripheral blood leukocytes and brain [7]. Therefore, $CysLT_1$ distribution is quite consistent with the anti-bronchoconstrictive and anti-inflammatory actions of $CysLT_1$ receptor antagonists [1].

While in recombinant cell systems $CysLT_1$ was shown to be preferentially coupled to $G_{q/11}$ [5,6], soon it became clear that heterogeneity of pathways has to be expected among endogenous tissues and cells. Indeed, naturally expressed $CysLT_1$ receptor has been reported to activate both pertussis toxin (PTx)-sensitive and -insensitive G-proteins [12,13]. This by no means a surprise, as it has been recognized that the use of recombinant systems, especially when dealing with signal transduction pathway(s) of G protein-coupled receptors (GPCRs) [14], might produce results whose general validity is sometimes questionable and cannot be easily extrapolated to endogenous cells.

It is widely recognized that the result of the interaction between leukotriene D_4 (LTD_4) and its receptor is the rise in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$). However, differences exist between cell types with respect to both the role of Ca^{2+} and the mechanisms of its elevation [15]. For example, we have recently demonstrated that in dimethylsulphoxyde differentiated U937 cells LTD_4-induced $[Ca^{2+}]_i$ elevation not only was partially PTx sensitive, but also regulated by isoprenylated proteins, possibly the β subunits of heterotrimeric G proteins and/or a member of the small GTPase Ras family [16]. Indeed, it has been suggested that LTD_4 is able to activate Ras in mesangial cells, but this activation was somehow attributed to the activation of a $CysLT_2$ receptor [17].

The aim of this work was, therefore, to elucidate the signal transduction pathways of LTD_4 in dU937 cells. The promonocytic leukemia cell U937, an immortalized cell line known to constitutively express a high density of $CysLT_1$ receptors upon differentiation to monocytes/macrophages [6,18], was selected because closely related to the inflammatory cells responsible of many cys-LT biological actions. Indeed, monocyte/macrophages activation leads to the release of a wide spectrum of cytokines and chemokines that have key roles in all inflammatory diseases, such as chronic asthma or in the formation and rupture of the atherosclerotic plaques. Herein, we demonstrate for the first time that $CysLT_1$ activates Ras via a PTx sensitive G protein and through the essential recruitment of a Ca^{2+}-dependent factor, possibly a Ca^{2+}-dependent kinase. Furthermore, we have also shown that this signaling pathway depends on PLC activation, and that Ras activation is able to induce ERK1/2 phosphorylation.

2. Materials and methods

2.1. Materials

Phosphate buffer saline, RPMI 1640, fetal calf serum, bovine serum albumin, EGTA, phenylmethylsulphonylfluoride, aprotinin, penicillin, streptomycin, L-glutamine, dimethylsulphoxyde, probenecid, penicillamine, and Hepes, were from Sigma Chem. Co. All salts for saline and Tris solution were from Merck. U73122, PP1, PP2 and pertussis toxin were from Calbiochem. *Clostridium sordellii lethal toxin* was a generous gift from Dr. Popoff (Institut Pasteur, Unité des Toxines Microbiennes, Paris, France). LTD_4 was purchased from Cayman Chemical Co.; zafirlukast and pranlukast were a kind gift from Merck. "TRIZOL® Reagent" and Taq Platinum Polymerase and PCR-Buffer are from Invitrogen-Life Technologies; the ReTRO script Kit is from Ambion; Anti-p-ERK1/2 (threonine 202 and tyrosine 204) monoclonal antibody are from Signaling Technology; Rabbit polyclonal antibody against $CysLT_1$ receptor is from Cayman. Ras activation assay kit containing a GST fusion protein corresponding to the human RBD of Raf-1 and a pan-Ras mouse monoclonal antibody (clone RAS10) was purchased from Upstate biotechnology. Horseradish peroxidase-conjugated goat anti-mouse/rabbit IgG and ECL chemiluminescent substrate were from Amersham Pharmacia Biotech. Fluo3/AM and pluronic F-127 were purchased from Molecular Probes. Disposable culture flasks, petri dishes, and filters were from Corning Glassworks.

2.2. Cell culture

U937 cells (ATCC) were routinely cultured into flasks in RPMI 1640 medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37 °C (5% CO_2) and differentiated for 96 h with 1.3% dimethylsulphoxyde.

RT-PCR of $CysLT_1$ receptor. Total human dU937 cells RNA were extracted from cells using "TRIZOL Reagent," according to the manufacturer's instructions (GIBCO BRL—Invitrogen). After denaturation (75 °C, 3 min), 1–2 μg of total purified RNA was retro-transcribed in the presence of MMLV-RTase (5 U/μl) under optimized reaction conditions (RT-buffer: 50 mM Tris–HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 5 mM DTT, 2 mM dNTPs, 0.5 U/μl RNase inhibitors 42–44 °C, 1 h). Specific amino- and carboxyl-terminal primers for $CysLT_1$ receptor (N-terminal, 5′-GGCGGATCCATGGATGAAACAGGAAATCTG-3′ and C-terminal, 5′-CGGCTCGAGCTATACTTTACATATTTCTTC-3′) were selected on the basis of the sequences previously published [5]. The PCR-mediated amplification of cDNA was performed using Taq Platinum Polymerase (0.03 U/μl) under optimized conditions (PCR-Buffer: 20 mM Tris–HCl, pH 8.4, 75 mM KCl, 0.2 mM dNTPs, 2 mM $MgCl_2$, 0.2 μM forward and reverse primers) using a Bio-Rad/I-Cycler PCR system. A specific cDNA fragment of 1013 bp was amplified (30 cycles: denaturation: 95 °C, 30 s; annealing: 60 °C, 20 s; extension: 68 °C, 45 s) and visualized after electrophoresis in 1% agarose gels by UV irradiation.

2.3. Determination of cytosolic free Ca^{2+} levels

Determination of $[Ca^{2+}]_i$ has been performed as previously described [16]. Briefly, dU937 cells were incubated for 30 min at 30 °C in the dark with 2 μM Fluo3/AM. After loading, Fluo3/AM was removed and cells were further incubated for 30 min at 30 °C to complete the hydrolysis of Fluo3/AM. Then, cells were centrifuged, resuspended, diluted to the concentration of 10^6 cells/ml, transferred to the spectrofluorimeter (Perkin Elmer LS50) and fluorescence monitored at 37 °C (506 nm excitation, 530 nm emission). Calibration was performed by adding 2 μM ionomycin and 100 μM digitonin (F_{max}) and by adding 5 mM EGTA and 60 mM Tris-base (F_{min}). $[Ca^{2+}]_i$ elevation has been expressed as fold increase over basal.

2.4. Subcellular fractionation, electrophoresis, and immunoblot analysis

dU937 cells were serum deprived overnight, treated with appropriate stimuli and then washed with ice-cold phosphate buffer saline and lysed by addition of 0.5 ml "Hypotonic Buffer" (5 mM Tris–HCl, pH 7.5, 1 mM $MgCl_2$, 1 mM EGTA, 0.1 mM EDTA, with 0.0067 IU/ml aprotinin and 0.2 mM PMSF as protease inhibitors). Cells suspensions were incubated at 4 °C for 30 min, freeze/thawed and homogenized with Teflon/glass homogenizer. Following centrifugation at 600 × *g* for 10 min at 4 °C to remove unbroken cells and the nuclear pellets, the samples were centrifuged for 1 h at 100,000 × *g* at 4 °C in a Beckman TL100 centrifuge. The pellets (particulate fraction, P), the cytosolic supernatants (soluble fraction, S) and the total lysates (total fraction, T), containing the same amounts of proteins (20–30 ng/ml), were boiled for 5 min at 95 °C in Laemmli buffer (200 mM Tris pH 6.5, 6% SDS, 15% 2-β-mercaptoethanol, 24% glycerol, 3% bromophenol blue), separated on SDS-polyacrylamide gel (11% for Ras, 15% for $CysLT_1$ and ERK). After semi-dry transfer to nitrocellulose, membranes were incubates overnight at 4 °C in TBS-T (20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.2% Tween 20) containing 5% powdered skim milk. After three 10 min washes with TBS-T, membranes were incubated for 1 h with primary antibodies diluted in TBS-T/3% milk (Ras: 1 μg/ml; ERK: 0.5 μg/ml; $CysLT_1$: 2 ng/ml) and for 1 h with horseradish peroxidase-conjugated goat anti-mouse/rabbit IgG. The blots were developed using ECL chemiluminescent substrate.

2.5. Ras activation assay

Ras activation assay was performed following the affinity precipitation protocol provided by the manufacturer (Ras pull-down assay kit, Upstate Biotechnology), which relies on the use of the GST fusion-protein of the human Ras Binding Domain (RBD) of Raf-1 to specifically bind and precipitate Ras GTP from cell lysates. Briefly, cells were serum-starved overnight, treated with appropriate stimuli, and then lysed as previously described (see above). Lysates (1 mg/ml of total cell proteins in each sample) were incubated with 10 μg of Raf-1 RBD for 45 min at 4 °C and then centrifuged for 15 sec at 14,000 × *g* to pellet the agarose beads. After discarding the supernatant, agarose beads were washed with 1 ml PBS and then the pellets were resuspended in 2× Laemmli sample buffer containing DTT, boiled for 5 min, and finally centrifuged for 15 s at 14,000 × *g*. The supernatant was collected and cellular proteins resolved by SDS-PAGE using 11% (w/v) acrylamide and analyzed by western blotting (see above).

2.6. Statistical analysis

Statistical comparison of two groups was performed using and independent *t* test; multiple groups were analyzed using one-way ANOVA followed by Dunnett post hoc test. Data are expressed as means ± S.E.M. All gels were analyzed using NIH Image computer software. Each experiment was performed at least three times. Where possible triplicates have been performed.

3. Results

3.1. Characterization of $CysLT_1$ receptors

In order to confirm that dU937 cells express a receptor for LTD_4, we routinely performed RT-PCR reaction on total RNA from U937 and dU937 cells to amplify a $CysLT_1$ specific DNA sequence. A standard PCR reaction was run with a set of amino- and carboxyl-terminal primers to obtain

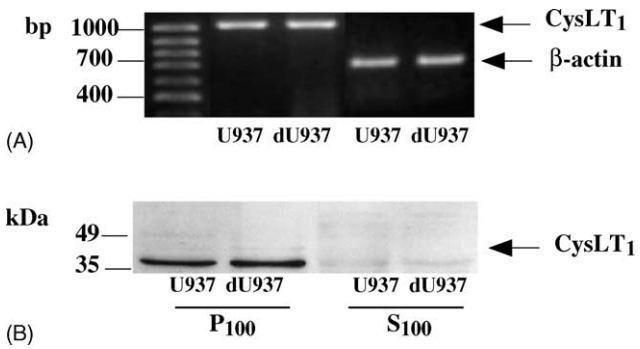

Fig. 1. RT-PCR and western blot of $CysLT_1$ receptor in dU937 cells. Analysis of $CysLT_1$ receptor expression was performed on U937 and dU937 cells cultured as described in Section 2. (A) Agarose gel electrophoresis of RT-PCR products derived from U937 and dU937 first DNA template: $CysLT_1$ 1013-bp fragment product was obtained using specific amino- and carboxyl-terminal primers: N-terminal, 5′-GGCGGATCCATGGATGAAACAGGAAATCTG-3′ and C-terminal, 5′-CGGCTCGAGCTATACTTTACATATTTCTTC-3′. Amplification product of β-actin is also shown. (B) Western immunoblotting analysis of particulate (P100) and soluble (S100) fractions from U937 and dU937 cells performed with a rabbit polyclonal antibody raised against $CysLT_1$ receptor. A single protein band with similar intensity was detected in both particulate fractions with a molecular mass close to the expected molecular weight of the $CysLT_1$ receptor (38 kDa).

the expected product of 1013 bp (Fig. 1A). Furthermore, we also performed western blot analysis with a polyclonal antibody specific for $CysLT_1$ receptor to demonstrate expression of the protein at the membrane level. Fig. 1B shows that no signal was present in the soluble fraction, whereas only a single immunoreactive band was detected in the particulate fraction migrating between 35 and 49 kDa.

3.2. Effect of LTD_4 on the amount of activated Ras (RasGTP)

To demonstrate a specific activation of Ras by LTD_4, dU937 cells were treated with the agonist and then the amount of RasGTP present in the homogenates was evaluated. Fig. 2 demonstrates that 10 nM LTD_4 was able to rapidly (1–2 min) produce a ∼five-fold increase in RasGTP over basal condition (Fig. 2A), and that Ras activation is dose-dependent with maximal efficacy at 10 nM (Fig. 2B). As expected, 100 ng/ml *Clostridium sordellii lethal toxin*, a specific inhibitor of the small GTPases Ras, Rap and Rac [19], was able to completely prevent RasGTP formation (data not shown).

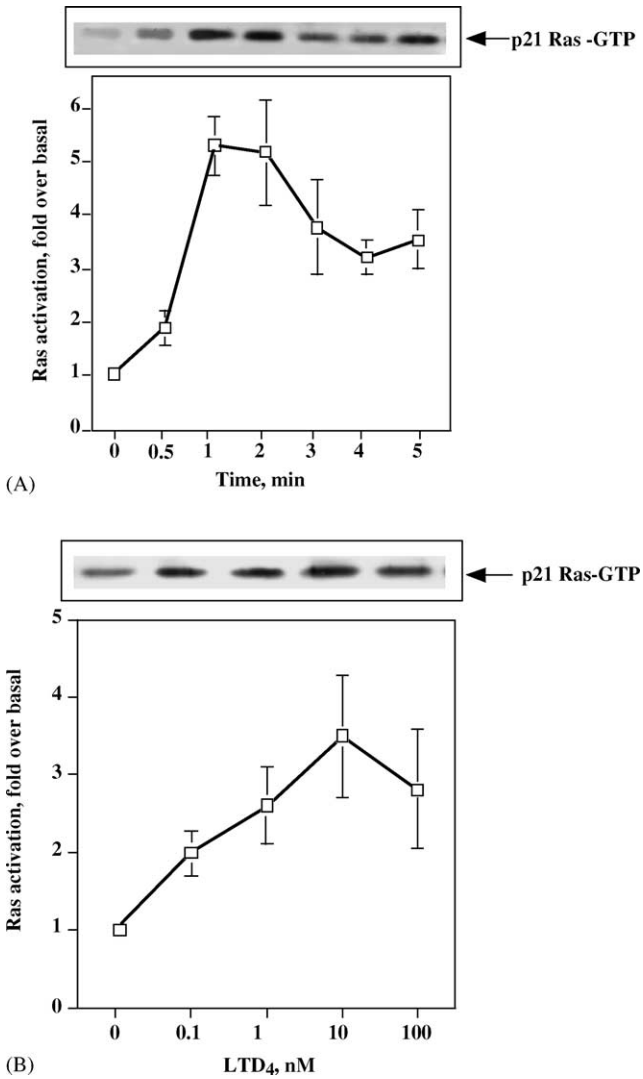

Fig. 2. Time course and concentration–response curve of LTD_4-induced Ras activation. Activated Ras (p21 RasGTP) was co-immunoprecipitated with Raf-1 RBD from cell lysates and detected by immunoblotting the same amount of proteins for each sample with a pan-Ras antibody. (A) Time-course of Ras activation induced by 10 nM LTD_4; (B) concentration–response curve of Ras activation induced by 2 min stimulation with LTD_4 at the indicated concentration. (Upper panel) Typical gel is shown. (Lower panel) Values shown represent means ± S.E.M. of densitometric analysis of at least three independent experiments.

3.3. Pharmacological profile of LTD_4-induced Ras activation

Zafirlukast and pranlukast, two selective $CysLT_1$ receptor antagonists, were used to characterize the receptor involved in LTD_4-induced Ras activation. Pretreatment for 30 min with 1 μM of each antagonist completely prevented LTD_4-induced response (Fig. 3).

3.4. Effect of pertussis toxin on LTD_4-induced $[Ca^{2+}]_i$ elevation and Ras activation

To identify which class of G protein is involved in Ras activation, dU937 cells were treated for 20 h with 300 ng/ml (maximal efficacious concentration, data not shown) of PTx and then LTD_4-induced $[Ca^{2+}]_i$ elevation was recorded. Fig. 4A shows that only half (∼51%) of the $CysLT_1$-mediated signal was PTx-sensitive, and can thus be ascribed to a G protein of the $G_{i/o}$ family, while the remaining part has to be ascribed to a PTx-insensitive mechanism. At variance, 1 μM *N*-formyl-methionyl-leucyl-phenylalanine (fMLP)-induced $[Ca^{2+}]_i$ elevation was completely abolished by PTx treatment. Furthermore, to

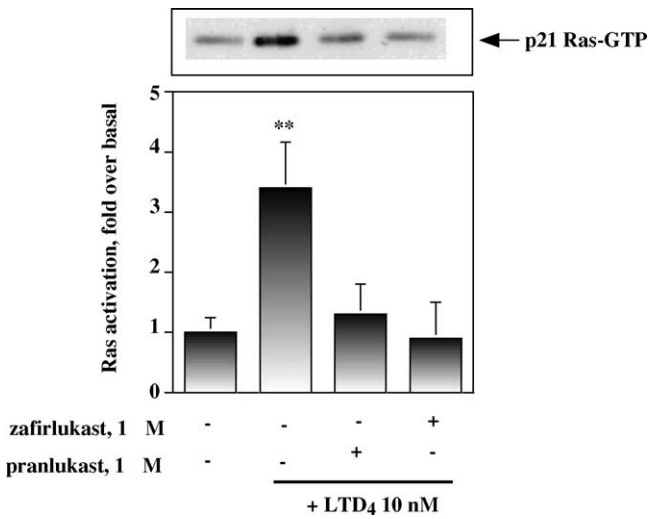

Fig. 3. Pharmacological specificity of LTD_4-induced Ras activation. dU937 cells were processed as described in the legend to Fig. 2 to evaluate Ras activation. Effect of 30 min pretreatment with 1 μM $CysLT_1$ receptor antagonists zafirlukast and pranlukast, before 10 nM LTD_4 challenge (2 min). (Upper panel) Typical gel is shown. (Lower panel) Values shown represent means ± S.E.M. of densitometric analysis of four independent experiments. $^{**}P < 0.01$, ANOVA followed by Dunnett post hoc test vs. control.

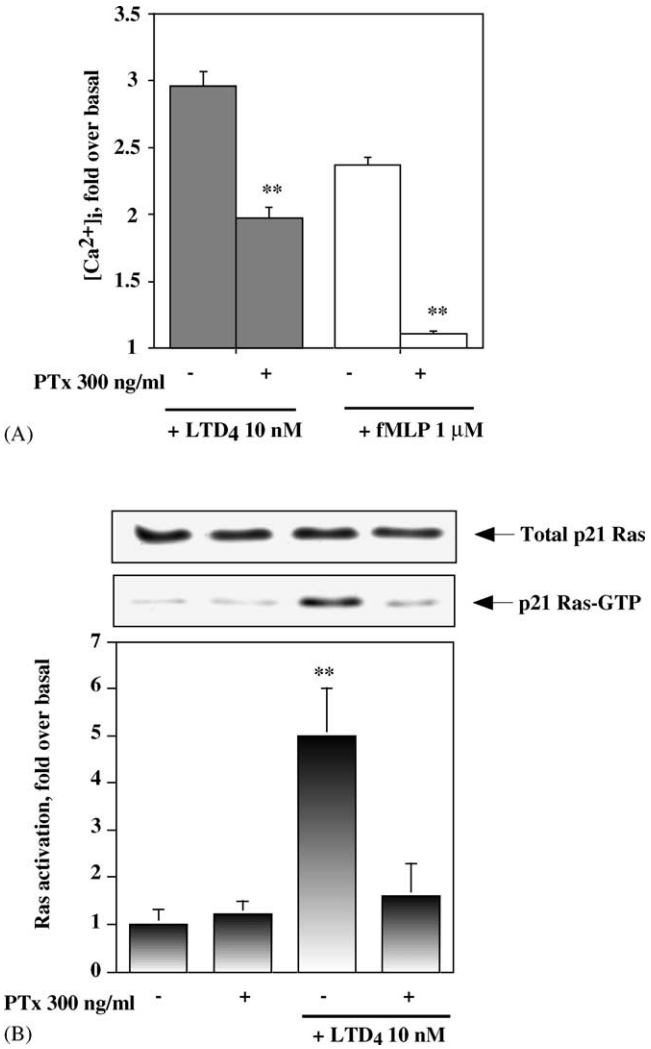

Fig. 4. Effect of pertussis toxin on LTD_4-induced $[Ca^{2+}]_i$ elevation and Ras activation. (A) dU937 cells were treated for 20 h with 300 ng/ml PTx and then challenged with 10 nM LTD_4 or 1 μM *N*-formyl-methionyl-leucyl-phenylalanine (fMLP). Data are presented as fold increase over basal ± S.E.M. of three independent experiments. $^{**}P < 0.01$, independent *t* test. (B) Cells were treated as in panel (A), challenged with 10 nM LTD_4 (2 min) and subjected to the Ras pull-down assay performed as described in the legend to Fig. 2. (Upper panel) Typical gel is shown. (Lower panel) Values shown represent means ± S.E.M. of densitometric analysis of three independent experiments. $^{**}P < 0.01$, ANOVA followed by Dunnett post hoc test vs. control.

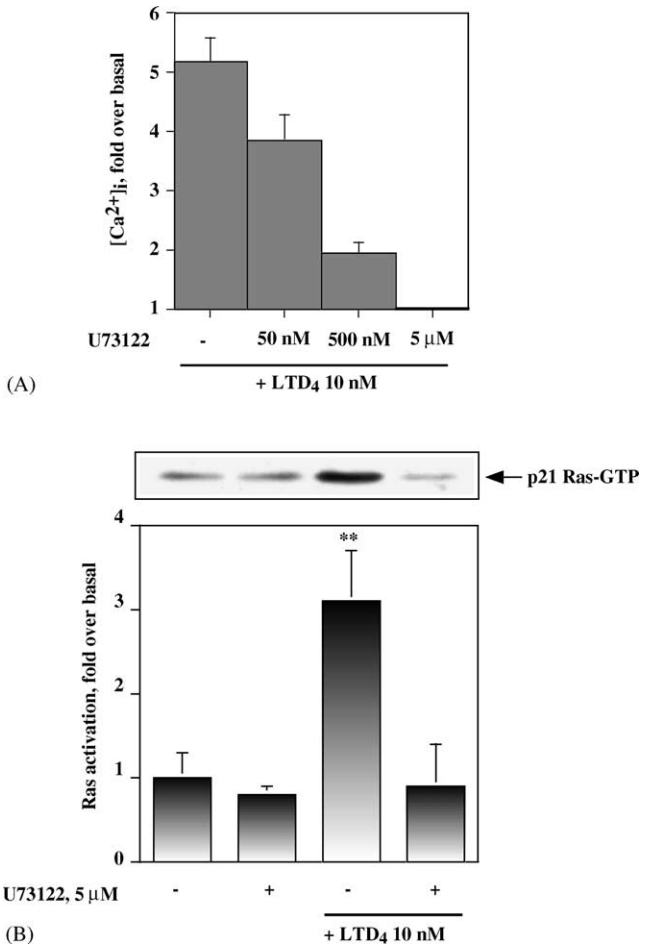

Fig. 5. Effect of U73122 on LTD_4-induced $[Ca^{2+}]_i$ elevation and Ras activation. (A) dU937 cells were treated for 5 min at the indicated concentrations of the PLC inhibitor U73122 and then challenged with 10 nM LTD_4. Data are presented as fold increase over basal ± S.E.M. of three independent experiments. (B) Cells were treated as in panel (A), challenged with 10 nM LTD_4 (2 min) and subjected to the Ras pull-down assay performed as described in the legend to Fig. 2. (Upper panel) Typical gel is shown. (Lower panel) Values shown represent means ± S.E.M. of densitometric analysis of three independent experiments. $^{**}P < 0.01$, ANOVA followed by Dunnett post hoc test vs. control.

investigate the involvement of PTx in LTD_4-induced Ras activation the amount of RasGTP was also measured. Fig. 4B indicates that PTx was able to inhibit almost completely (>85%) the activation of Ras.

3.5. Effect of U73122 and BAPTA on LTD_4-induced signaling

We have previously demonstrated that most of the $[Ca^{2+}]_i$ transient induced by LTD_4 in these cells is due to the release from intracellular stores [16]. Thus, we wanted to verify the effect of the PLC inhibitor U73122 in preventing $CysLT_1$-mediated downstream events in dU937 cells. It is clear from Fig. 5 that U73122 at the maximal concentration of 5 μM was able to completely abolish both LTD_4-induced $[Ca^{2+}]_i$ response (Fig. 5A) and the formation of RasGTP (Fig. 5B).

Furthermore, to verify the role of Ca^{2+} as a key second messenger, cells were loaded with BAPTA acetoxymethyl ester, a Ca^{2+}/Zn^{2+} chelator that blocks pore complex assembly. Similarly to U73122, 10 μM BAPTA strongly (>70%) prevented LTD_4-induced Ras activation (Fig. 6).

3.6. Effect of genistein and PP2 on LTD_4-induced $[Ca^{2+}]_i$ elevation and Ras activation

Activation of Ras by GPCR is usually associated with activation of a PTK, such as Src or a Src-like kinase, or other cytosolic kinases such as Pyk2, a proline-rich kinase activated by increase in $[Ca^{2+}]_i$. Fig. 7 shows the effect of

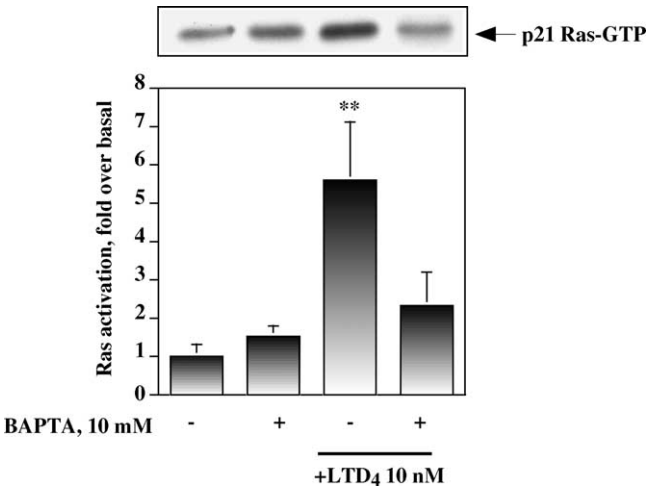

Fig. 6. Effect of BAPTA on LTD_4-induced Ras activation. dU937 cells were treated for 30 min with 10 μM BAPTA acetoxymethyl ester and then challenged with 10 nM LTD_4 (2 min). (Upper panel) Typical gel is shown. (Lower panel) Values shown represent means ± S.E.M. of densitometric analysis of three independent experiments. $^{**}P < 0.01$, ANOVA followed by Dunnett post hoc test vs. control.

the broad-spectrum tyrosine kinase inhibitor genistein and of the specific Src kinase inhibitor PP2. While genistein was able to partially inhibit LTD_4-induced $[Ca^{2+}]_i$ transient (∼34%) (Fig. 7A) and almost completely Ras activation (>90%, Fig. 7B), the specific Src kinase inhibitors PP1 (data not shown) and PP2 at a concentration of 10 μM were only able to marginally affect $[Ca^{2+}]_i$ signaling (Fig. 7A, ∼20%), without significantly affect Ras activation (Fig. 6B).

3.7. Extracellular regulated kinase 1/2 activation in dU937 cells

To test the hypothesis that the MAPKs ERK1/2 might be activated downstream of Ras, we measured the amount of phosphorylated form of ERK1/2. Time course and concentration–response curve were similar to that of LTD_4-induced Ras activation (data not shown). Fig. 8A shows a typical experiment in which 10 nM LTD_4 strongly activated ERK1/2 cascade (∼three-fold increase), while pranlukast and zafirlukast were able to completely prevent this activation. In addition, while PTx treatment (20 h with 300 ng/ml) completely inhibited ERK1/2 phosphorylation (Fig. 8B), 100 ng/ml *Clostridium sordellii lethal toxin* was able to inhibit LTD_4-induced ERK1/2 activation by only 40% (Fig. 8C).

4. Discussion

Here we report that $CysLT_1$ receptor activates Ras via a PTx-sensitive G protein and through the recruitment of a Ca^{2+}-dependent factor, possibly a Ca^{2+}-dependent tyrosine kinase. We have obtained evidence that while this

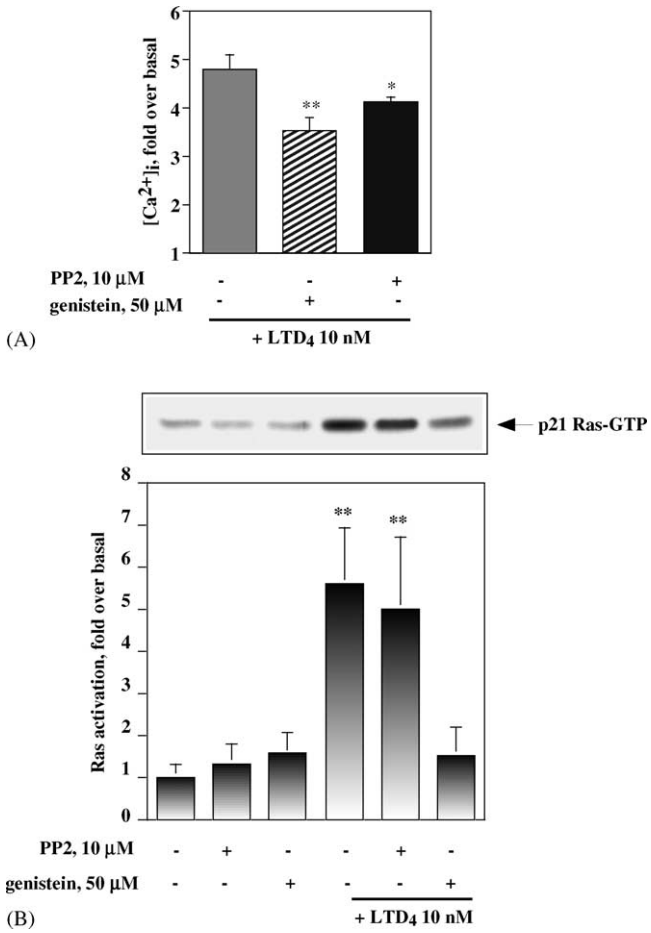

Fig. 7. Effect of genistein and PP2 on LTD_4-induced $[Ca^{2+}]_i$ elevation and Ras activation. dU937 cells were treated for 30 min with 50 μM genistein or with 10 μM PP2 and then challenged with 10 nM LTD_4. (A) Data are presented as fold increase over basal ± S.E.M. of three independent experiments. (B) Cells were treated as in panel (A), challenged with 10 nm LTD_4 (2 min) and subjected to the Ras pull-down assay performed as described in the legend to Fig. 2. (Upper panel) Typical gel is shown. (Lower panel) Values shown represent means ± S.E.M. of densitometric analysis of three independent experiments. $^{*}P < 0.05$ and $^{**}P < 0.01$, ANOVA followed by Dunnett post hoc test vs. control.

activation is mostly PTx-sensitive, $CysLT_1$ transduction pathways in dU937 cells involve both PTx-sensitive and -insensitive G proteins-mediated pathways converging at the level of PLC. We have also demonstrated that LTD_4-induced $CysLT_1$ activation stimulates ERK1/2 phosphorylation, and that MAPK activation is partially dependent from Ras (Fig. 9).

Classical view of Ras signaling cascade involves receptor tyrosine kinases as triggering elements, whereas GPCRs usually activate or inhibit second messenger regulating enzymes and/or ion channels. However, pathways for activation of Ras in response to GPCRs stimulation have now been clearly established [20] and involve an always increasing number of receptors.

We have recently reported that LTD_4-induced $[Ca^{2+}]_i$ elevation in dU937 cells is significantly inhibited by fluvastatin or by specific isoprenylation inhibitors, eventually

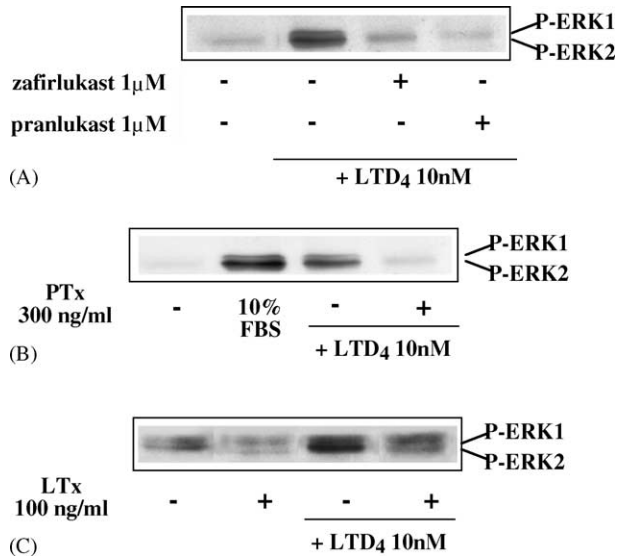

Fig. 8. ERK1/2 phosphorylation in dU937 cells. dU937 cells were cultured as described in Section 2. Western blot analysis was performed with an anti-p-ERK1/2 monoclonal antibody on the particulate fraction from dU937 cells containing the same amount of proteins. (A) ERK1/2 phosphorylation induced by 10 nM LTD_4 (2 min) and effect of 30 min pretreatment with 1 μM zafirlukast and pranlukast. (B and C) Cells were treated for 20 h with 300 ng/ml PTx (B) or 100 ng/ml *Clostridium sordellii lethal toxin* (C) and then challenged with 10 nM LTD_4. Control is represented by MEM additioned with 0.1% FBS. Gels were analyzed using NIH Image computer software. Data are representative of at least two other independent experiments.

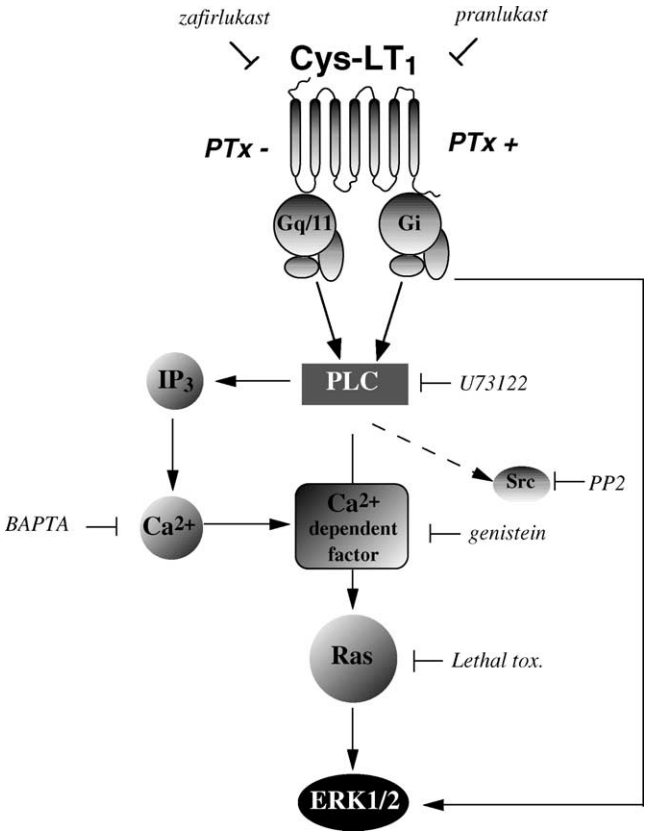

Fig. 9. Schematic representation of $CysLT_1$-induced signaling pathway in dU937 cells. See test for details.

suggesting a specific role for isoprenylated proteins, such as Gβγ or a small GTP-binding protein [16]. Furthermore, we have also demonstrated that *Clostridium sordellii lethal toxin* was able to greatly inhibit LTD_4-induced $[Ca^{2+}]_i$ elevation, suggesting that a Ras family member (Ras and/or Rap) might be involved in $CysLT_1$ signaling in these cells [16]. Here we demonstrate that LTD_4 is indeed able to induce Ras activation, and that this activation is not only rapid and concentration-dependent, but has the pharmacological profile of a $CysLT_1$ receptor, i.e. sensitive to the classical antagonists pranlukast and zafirlukast.

Furthermore, at variance with $[Ca^{2+}]_i$ modulation, Ras activation has been found to be totally PTx-sensitive, suggesting that in dU937 cells the major transducer for $CysLT_1$-induced Ras activation is a PTx-sensitive G protein of the $G_{i/o}$ class. Thus, $CysLT_1$ receptors seem to be simultaneously coupled to a $G_{q/11}$ protein classically activating PLCβ to produce IP_3 and Ca^{2+} and to a $G_{i/o}$ protein that seems to be involved in both Ca^{2+} signaling (likely through βγ subunits) and Ras activation (Fig. 9). It is a fact that most GPCRs simultaneously activate multiple G proteins in natural as well as in recombinant systems and, particularly, G_i linked receptors are often coupled to G_q proteins as well [21].

Intriguingly, the PLC inhibitor U73122 completely abolished LTD_4-induced $[Ca^{2+}]_i$ increase as well as RasGTP accumulation, indicating that in these cells PLC (or different isoforms of PLC) is the point of convergence for both the PTx-sensitive and insensitive pathways leading to $[Ca^{2+}]_i$ modulation and Ras activation (Fig. 9). Furthermore, chelating intracellular Ca^{2+} with BAPTA strongly prevented the formation of RasGTP, demonstrating that the release of intracellular Ca^{2+} is essential for $CysLT_1$-induced Ras activation in these cells. These findings confirm our previous observations demonstrating that most of the LTD_4-induced $[Ca^{2+}]_i$ transient in dU937 cells is due to the release from intracellular stores induced by PLC-catalyzed IP_3 generation [16]. Thus, PLC and Ca^{2+} are essential components acting upstream of Ras activation (Fig. 9).

A number of possible pathways may link $CysLT_1$ receptor to Ras, some of which are PTx-sensitive and some not [20]. The former, generally involves the activation of Ras by βγ [22], via the Shc-Grb2-Sos complex [23], either directly or through the intervention of a PTK, such as Src [24], whereas the latter involves the activation of a Ca^{2+}-dependent kinase such as the proline-rich kinase Pyk2 [25]. In our system, the specific Src inhibitor PP2 was only marginally able to inhibit $[Ca^{2+}]_i$ signaling and substantially unable to affect Ras activation, at variance with genistein that significantly inhibited the formation of RasGTP. Thus, these data seem to suggest only a limited involvement of Src in LTD_4 signaling, but, in turn, a possible role for a Ca^{2+}-dependent kinase such as Pyk2,

that is expressed in hematopoietic besides neuronal cells [26]. Alternatively, or in cooperation with Pyk2, a Guanine nucleotide Exchange Factor (GEF) for Ras known to be regulated by Ca^{2+} (such as RasGRF or RasGRP [27]), might be involved in $CysLT_1$-induced Ras activation. This is an issue that worth further investigation.

We did not find a role for the $G\alpha_i$ subunit, as LTD_4 was not able to inhibit forskolin-induced adenylate cyclase stimulation in membranes from dU937 cells (data not shown). However, we cannot completely rule out the possibility that a $G\alpha_i$ subunit might be implicated in the direct activation of Ras either alone [28], or in cooperation with a $G\alpha_q$ subunit [29].

At this point, it was tempting to speculate the activation of the MAPK cascade downstream of Ras [30]. Indeed, LTD_4 was able to induce ERK1/2 phosphorylation with a pharmacological profile compatible with a $CysLT_1$ receptor activation, similarly to the results obtained with Ras. Furthermore, to confirm a link between $CysLT_1$ receptor-Ras and MAPKs, we demonstrated that ERK1/2 activation was, at least in part, inhibited by *Clostridium sordellii lethal toxin*. This, in turn suggest that other pathways might contribute to LTD_4-induced MAPK activation in dU937 cells. These data are in partial agreement with a report recently published showing that $CysLT_1$ receptor is indeed able to activate MAPK cascade in intestinal epithelial cells through a Ras-independent PKCε-dependent pathway [31].

In conclusion, we demonstrated that the involvement of Ras downstream of LTD_4-induced $CysLT_1$ receptor activation converges to PLC and Ca^{2+} for both PTx sensitive and insensitive pathways (Fig. 9). In addition to PLC, a Ca^{2+}-dependent PTK and/or a Ca^{2+}-dependent Ras GEF are essential components in $CysLT_1$ signaling to Ras and MAPKs. Indeed, the role of Ras and MAPKs in monocytes might be of crucial importance. The activation of the MAPK signaling cascade, that is known to regulate protein transcription and thus cellular proliferation or differentiation, suggests new and until now unexplored actions for these lipid mediators. Thus, the role of LTD_4 in both amplifying the immediate tissue damage and expanding the population of inflammatory cells seems deserving further investigation. Such a positive feedback mechanism may in part explain the clinically observed progressively refractory bronchospasm and inflammation characteristic of chronic asthma as well as the role of cys-LTs in the atherosclerotic and inflammatory processes of the vascular wall.

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References

[1] Drazen JM. Leukotrienes as mediators of airway obstruction. Am J Respir Crit Care Med 1998;158:S193–200.
[2] Spanbroek R, Grabner R, Lotzer K, Hildner M, Urbach A, Ruhling K, et al. Expanding expression of the 5-lipoxygenase pathway within the arterial wall during human atherogenesis. Proc Natl Acad Sci USA 2003;100:1238–43.
[3] Lotzer K, Spanbroek R, Hildner M, Urbach A, Heller R, Bretschneider E, et al. Differential leukotriene receptor expression and calcium responses in endothelial cells and macrophages indicate 5-lipoxygenase-dependent circuits of inflammation and atherogenesis. Arterioscler Thromb Vasc Biol 2003;23:E32–6.
[4] Folco G, Rossoni G, Buccellati C, Berti F, Sala A. Leukotrienes in cardiovascular diseases. Am J Respir Crit Care Med 2000;161: S112–6.
[5] Lynch KR, Gary P, O'neill GP, Qingyun Liu Q, Sawyer N, Metters KM, et al. Characterization of the human cysteinyl leukotriene $CysLT_1$ receptor. Nature 1999;399:789–93.
[6] Sarau HM, Ames RS, Chambers J, Ellis C, Elshourbagy N, Foley JJ, et al. Identification, molecular cloning, expression, and characterization of a cysteinyl leukotriene receptor. Mol Pharmacol 1999;56: 657–63.
[7] Takasaki J, Kamohara M, Matsumoto M, Saito T, Sugimoto T, Ohishi T, et al. The molecular characterization and tissue distribution of the human cysteinyl leukotriene CysLT(2) receptor. Biochem Biophys Res Commun 2000;274:316–22.
[8] Heise CE, O'Dowd BF, Figueroa DJ, Sawyer N, Nguyen T, Im D-S, et al. Characterization of the human cysteinyl leukotriene 2 receptor. J Biol Chem 2000;275:30531–6.
[9] Nothacker H-P, Wang Z, Zhu Y, Reinscheid RK, Lin SHS, Civelli O. Molecular cloning and characterization of a second human cysteinyl leukotriene receptor: discovery of a subtype selective agonist. Mol Pharmacol 2000;58:1601–8.
[10] Figueroa DJ, Breyer RM, Defoe SK, Kargman S, Daugherty BL, Waldburger K, et al. Expression of the cysteinyl leukotriene 1 receptor in normal human lung and peripheral blood leukocytes. Am J Respir Crit Care Med 2001;163:226–33.
[11] Bautz F, Denzlinger C, Kanz L, Mohle R. Chemotaxis and transendothelial migration of CD34(+) hematopoietic progenitor cells induced by the inflammatory mediator leukotriene D4 are mediated by the 7-transmembrane receptor CysLT1. Blood 2001; 97:3433–40.
[12] Crooke ST, Mattern M, Sarau HM, Winkler JD, Balcarek J, Wong A, et al. The signal transduction system of the leukotriene D_4 receptor. Trends Pharmacol Sci 1989;10:103–7.
[13] Pollock K, Creba J. Leukotriene D_4 induced calcium changes in U937 cells may utilize mechanisms additional to inositol phosphate production that are pertussis toxin insensitive but are blocked by phorbol myristate acetate. Cell Signal 1990;2:563–8.
[14] Kenakin T. Differences between natural and recombinant G protein-coupled receptor systems with varying receptor/G protein stoichiometry. Trends Pharmacol Sci 1997;18:456–64.
[15] Nicosia S, Capra V, Accomazzo MR, Galbiati E, Ragnini D, Saponara R, et al. Receptors and second messengers for Cys-Leukotrienes. In: Folco GC, Samuelsson B, Maclouf J, Velo GP, editors. Eicosanoids: from biotechnology to therapeutic applications. New York: Plenum Press; 1996. p. 127–36.

[16] Capra V, Accomazzo MR, Ravasi S, Parenti M, Macchia M, Nicosia S, et al. Involvement of prenylated proteins in calcium signaling induced by LTD_4 in differentiated U937 cells. Prostaglandins Other Lipid Mediat 2003;71:235–51.
[17] McMahon B, Mitchell D, Shattock R, Martin F, Brady HR, Godson C. Lipoxin, leukotriene, and PDGF receptors cross-talk to regulate mesangial cell proliferation. FASEB J 2002;16:1817–9.
[18] Frey EA, Nicholson DW, Metters KM. Characterization of the leukotriene D4 receptor in dimethylsulphoxide-differentiated U937 cells: comparison with the leukotriene D4 receptor in human lung and guinea-pig lung. Eur J Pharmacol 1993;244:239–50.
[19] Popoff MR, Chaves-Olarte E, Lemichez E, von Eichel-Streiber C, Thelestam M, Chardin P, et al. Ras, Rap, and Rac small GTP-binding proteins are targets for Clostridium sordellii lethal toxin glucosylation. J Biol Chem 1996;271:10217–24.
[20] Marinissen MJ, Gutkind JS. G-protein-coupled receptors and signaling networks: emerging paradigms. Trends Pharmacol Sci 2001;22: 368–76.
[21] Kenakin T. Agonist-receptor efficacy. I: Mechanisms of efficacy and receptor promiscuity. Trends Pharmacol Sci 1995;16:188–92.
[22] Crespo P, Xu N, Simonds WF, Gutkind JS. Ras-dependent activation of MAP kinase pathway mediated by G-protein beta gamma subunits. Nature 1994;369:418–20.
[23] van Biesen T, Hawes BE, Luttrell DK, Krueger KM, Touhara K, Porfiri E, et al. Receptor-tyrosine-kinase- and G beta gamma-mediated MAP kinase activation by a common signalling pathway. Nature 1995;376: 781–4.
[24] Luttrell LM, Hawes BE, van Biesen T, Luttrell DK, Lansing TJ, Lefkowitz RJ. Role of c-Src tyrosine kinase in G protein-coupled receptor- and Gbetagamma subunit-mediated activation of mitogen-activated protein kinases. J Biol Chem 1996;271:19443–50.
[25] Dikic I, Tokiwa G, Lev S, Courtneidge SA, Schlessinger J. A role for Pyk2 and Src in linking G-protein-coupled receptors with MAP kinase activation. Nature 1996;383:547–50.
[26] Avraham H, Park SY, Schinkmann K, Avraham S. RAFTK/Pyk2-mediated cellular signalling. Cell Signal 2000;12:123–33.
[27] Ebinu JO, Bottorff DA, Chan EY, Stang SL, Dunn RJ, Stone JC. RasGRPv, Ras guanyl nucleotide-releasing protein with calcium- and diacylglycerol-binding motifs. Science 1998;280:1082–6.
[28] Mochizuki N, Ohba Y, Kiyokawa E, Kurata T, Murakami T, Ozaki T, et al. Activation of the ERK/MAPK pathway by an isoform of rap1GAP associated with G alpha(i). Nature 1999;400:891–4.
[29] Blaukat A, Barac A, Cross MJ, Offermanns S, Dikic I. G protein-coupled receptor-mediated mitogen-activated protein kinase activation through cooperation of Galpha(q) and Galpha(i) signals. Mol Cell Biol 2000;20:6837–48.
[30] Gutkind JS. Regulation of mitogen-activated protein kinase signaling networks by G protein-coupled receptors. Sci STKE; 2000. Available at: http://stke.sciencemag.org/cgi/content/full/sigtrans;2000/40/re1.
[31] Paruchuri S, Hallberg B, Juhas M, Larsson C, Sjolander A. Leukotriene D(4) activates MAPK through a Ras-independent but PKCepsilon-dependent pathway in intestinal epithelial cells. J Cell Sci 2002;115:1883–93.