$CysLT_1$ receptor is a target for extracellular nucleotide-induced heterologous desensitization: a possible feedback mechanism in inflammation

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Summary

Both cysteinyl-leukotrienes and extracellular nucleotides mediate inflammatory responses via specific G-protein-coupled receptors, the CysLT and the P2Y receptors, respectively. Since these mediators accumulate at sites of inflammation, and inflammatory cells express both classes of receptors, their responses are likely to be crossregulated. We investigated the molecular basis of desensitization and trafficking of the $CysLT_1$ receptor constitutively and transiently expressed in the human monocyte/macrophage-like U937 or COS-7 cells in response to LTD_4 or nucleotides. Exposure to agonist induced a rapid homologous desensitization of the $CysLT_1$ receptor [as measured by the reduction in the maximal agonist-induced intracellular cytosolic Ca^{2+} ($[Ca^{2+}]_i$) transient], followed by receptor internalization (as assessed by equilibrium binding and confocal microscopy). Activation of P2Y receptors with ATP or UDP induced heterologous desensitization of the $CysLT_1$ receptor. Conversely, LTD_4-induced $CysLT_1$ receptor activation had no effect on P2Y receptor responses, which suggests that the latter have a hierarchy in producing desensitizing signals. Furthermore, ATP/UDP-induced $CysLT_1$ receptor desensitization was unable to cause receptor internalization, induced a faster recovery of $CysLT_1$ functionality and was dependent upon protein kinase C. By contrast, homologous desensitization, which is probably dependent upon G-protein-receptor kinase 2 activation, induced a fast receptor downregulation and, accordingly, a slower recovery of $CysLT_1$ functionality. Hence, $CysLT_1$ receptor desensitization and trafficking are differentially regulated by the $CysLT_1$ cognate ligand or by extracellular nucleotides. This crosstalk may have a profound physiological implication in the regulation of responses at sites of inflammation, and may represent just an example of a feedback mechanism used by cells to fine-tune their responses.

Key words: $CysLT_1$ Receptor, Heterologous desensitization, Extracellular nucleotides, Inflammation, U937 cells

Introduction

Cysteinyl-leukotrienes (Cys-LTs) (i.e. LTC_4, LTD_4 and LTE_4) are lipid mediators derived from arachidonic acid through the involvement of the 5-lipoxygenase pathway (Samuelsson, 1983). They are known to be potent inflammatory mediators and to play an important role in asthma (Drazen, 2003; Nicosia et al., 2001), and have been implicated in a number of cardiovascular diseases, from coronary artery disease to atherosclerosis (Lotzer et al., 2003; Porreca et al., 1996) and stroke (Ciceri et al., 2001) or in cardiovascular complications of inflammatory processes (Folco et al., 2000).

Until now, two receptor subtypes for Cys-LTs have been cloned, namely $CysLT_1$ and $CysLT_2$ (Brink et al., 2003; Capra, 2004), both belonging to the G-protein-coupled receptor (GPCR) superfamily. $CysLT_1$ and $CysLT_2$ receptors can couple to distinct types of G proteins. In particular, when $CysLT_1$ receptor is expressed in recombinant systems it shows a preferential coupling to $G_{q/11}$, whereas when constitutively expressed it has been reported to activate both pertussis toxin (PTX)-sensitive and -insensitive G proteins (Brink et al., 2003). In fact, we have previously demonstrated that dimethylsulphoxyde-differentiated monocyte/macrophage-like U937 (dU937) cells (Frey et al., 1993) respond to LTD_4 with a strong $[Ca^{2+}]_i$ increase that is only partially sensitive to PTX (Capra et al., 2003), and with the activation of the Ras-MAPK cascade being totally dependent upon $G_{i/o}$ (Capra et al., 2004a). These signaling pathways were totally inhibited by different specific $CysLT_1$-receptor antagonists, and no $CysLT_2$ receptor mRNA was detected, which indicates that in U937 cells LTD_4-induced responses can be totally ascribed to a $CysLT_1$ receptor. Further, $CysLT_1$ receptor is expressed on pregranulocytic cells, where it has been hypothesized to prime maturation and differentiation of $CD34^+$ precursor cells to eosinophils, monocytes and macrophages (Figueroa et al., 2001). All these findings suggest a role for Cys-LTs in cellular differentiation and/or proliferation, thus amplifying the tissue damage and expanding the population of inflammatory cells (Kanaoka and Boyce, 2004; Miranda et al., 2002).

Extracellular nucleotides have also been postulated to play a role in differentiation and maturation of blood cells (Sak et al., 2003), a process that is often associated with an inflammatory response. Furthermore, purines are known to induce U937 phenotypic differentiation (Cowen et al., 1991), and to be involved in recruitment of neutrophils and monocytes to the site of injury (Kunapuli and Daniel, 1998). Thus, in addition to their intracellular functions in energy metabolism and nucleic acid synthesis, nucleotides are now recognized to have important roles as extracellular signaling molecules. Adenine (ATP, ADP) and uracil (UTP, UDP) nucleotides and, as has been more recently recognized, sugar nucleotides such as UDP-glucose and UDP-galactose (Abbracchio et al., 2003) are emerging as physiological regulators of a variety of functions such as development, proliferation, immune response and release of hormones and cytokines (Abbracchio and Burnstock, 1998). These actions are mediated by activation of seven ligand-gated channels (the P2X receptors) and eight P2Y receptors (the $P2Y_{1,2,4,6,11,12,13,14}$ receptor subtypes) that also belong to the GPCR superfamily (Abbracchio and Burnstock, 1994). In particular, U937 cells constitutively express a number of P2Y receptors, such as $P2Y_2$ (which specifically responds to both ATP and UTP) and $P2Y_6$ (which is activated by UDP) (Jin et al., 1998).

GPCRs represent the largest group of receptors for neurotransmitters and hormones, with more than 800 putative members identified in the human genome (Fredriksson et al., 2003). Activation of a GPCR by its ligand induces conformational changes and coupling to the heterotrimeric G proteins, but also initiates the process of receptor desensitization, i.e. the fading of GPCR responsiveness to agonist with time (Ferguson, 2001). Mainly, two types of desensitization can occur: homologous and heterologous (Chuang et al., 1996). Homologous desensitization is strictly agonist dependent, and thus only activated receptors desensitize. By contrast, in heterologous desensitization the activation of one receptor can result in reduced responses to activation of other unrelated, or apparently unrelated, receptors that have not been exposed to agonist (Bunemann et al., 1999). This latter process is generally ascribed to different kinases, such as protein kinases A and C (PKA and PKC) or, more recently, also to other protein kinases (Ferguson, 2001).

PKA and PKC, besides participating in GPCR signaling, also participate in a feedback mechanism in which the second messenger activates a kinase that decreases the activity of the receptor (Bunemann et al., 1999; Chuang et al., 1996). Nonetheless, second-messenger-dependent protein-kinase-mediated mechanisms of receptor desensitization have received less attention than G-protein-receptor kinase (GRK)-mediated mechanisms.

Given that $CysLT_1$ and P2Y receptors are known to be coexpressed in a number of inflammatory cells, such as peripheral blood monocytes, leukocytes and eosinophils, it is possible to hypothesize that their responses are crossregulated, as has been demonstrated for the chemoattractant receptor system (Ali et al., 1999). Although much has been learned about cellular activation and regulation by single receptors, mechanisms of receptor crossregulation leading to priming or desensitization are only beginning to be unrevealed. Therefore, this study was aimed at the characterization of crossregulation between $CysLT_1$ and P2Y receptors in a constitutive and a recombinant expression system, investigating the molecular basis of desensitization and trafficking of the $CysLT_1$ receptor in response to LTD_4 and extracellular nucleotides. We demonstrate that $CysLT_1$ receptor is the target for both agonist-mediated homologous desensitization, and purine-mediated heterologous desensitization. We also show that the desensitization and trafficking of the $CysLT_1$ receptor is regulated differently by its cognate ligand LTD_4 or by ATP and UDP, and that receptor crossregulation is unidirectional, since activation of the $CysLT_1$ receptor by LTD_4 had no effect on P2Y receptor responses. Finally, we show here that, although the $CysLT_1$ receptor is promiscuously coupled to G_q and G_i in dU937 cells, extracellular nucleotide-induced heterologous desensitization seems to affect mostly the G_i coupling.

Materials and Methods

Materials

Phosphate-buffered saline (PBS), RPMI 1640, fetal bovine serum (FBS), bovine serum albumin (BSA), EGTA, penicillin, streptomycin, L-glutamine, dimethylsulphoxyde, probenecid, penicillamine, forskolin, PMA (phorbol 12-myristate 13-acetate), ionomycin, ATP, UDP, serine-borate complex, cysteine, glycine, protein G-Sepharose 4B Fast Flow Recombinant Protein G, deoxycholate, phenylmethylsulfonyl fluoride, NaF, Na_3VO_4, Triton X-100, Tween 20 and HEPES were from Sigma. All salts for saline and Tris solution and digitonin were from Merck. LTD_4 and rabbit polyclonal antibody against $CysLT_1$ receptor were purchased from Cayman Chemical Co.; [^{3}H]LTD_4, Filter count and Ultima Gold were from Perkin Elmer. GF109203X, H89, PTX and GRK2 inhibitor (Methyl 5-[2-(5-nitro-2-furyl)vinyl]-2-furoate) were from Calbiochem. YM-254890 was a kind gift from J. Takasaki (Astellas Pharma, Ibaraki, Japan). The protease inhibitor complex Complete™ was from Roche Applied Sciences; DMEM and Lipofectamine 2000 were from Invitrogen-Life Technologies. Monoclonal antibody HA.11 was from Covance. Fluo3/AM, Fura 2/AM, pluronic F-127 and Alexa-488 goat anti-rabbit antibody were purchased from Molecular Probes. Disposable culture flasks, Petri dishes, and filters were from Corning Glassworks. Reagents and films for chemoluminescence were from Amersham Bioscience. All reagents and supplies for electrophoresis and DC™Protein assay were from Bio-Rad Laboratories.

Cell culture

U937 cells (ATCC) were routinely cultured into flasks in RPMI 1640 medium supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin at 37C°C (5% CO_2) and differentiated for 96 hours with 1.3% dimethylsulphoxyde, as previously described (Capra et al., 2003). For the U937 D5 clone (a generous gift from C. Shayo, Instituto de Biologia y Medicina Experimental, Buenos Aires, Argentina), the culture medium was supplemented with 0.8 mg/ml G-418 to maintain selection. COS-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS, 2 mM glutamine, 50 U/mL penicillin, 100 μg/ml streptomycin and 20 mM HEPES buffer pH 7.4, at 37°C in a humidified atmosphere of 95% O_2 and 5% CO_2.

Construction of expression plasmid and transfection

An epitope-tagged $CysLT_1$ receptor (pCEFL-HA-$CysLT_1$) was generated by inserting the coding region of $CysLT_1$ receptor into the pCEFL-HA expression vector (kindly provided by S. J. Gutkind,

HIDCR, NIH, Bethesda, MD). For transient transfections, COS-7 were grown to 80-90% confluence and transfected with 7.5 μg of DNA in 10 cm tissue culture dishes (binding studies), 2.5 μg of DNA in 60 mm tissue culture dishes (immunoprecipitation) or 0.15 μg in 12 mm diameter glass coverslips ($[Ca^{2+}]_i$ measurement, confocal microscopy), using Lipofectamine 2000 according to the manufacturer's instructions. Cells were incubated with the DNA/Lipofectamine mixture (1:2 ratio) for 24 hours and the media replaced immediately before the challenge.

Determination of cytosolic free Ca^{2+} levels

Non adherent cells

Determination of free $[Ca^{2+}]_i$ has been performed as previously described (Capra et al., 2003). Briefly, dU937 cells were incubated for 30 minutes at 30°C in the dark with 2 μM Fluo3/AM. After loading, Fluo3/AM was removed and cells were further incubated for 30 minutes at 30°C to complete the hydrolysis of Fluo3/AM. Then, cells were centrifuged, resuspended, diluted to the concentration of 10^6 cells/ml, and transferred to the spectrofluorimeter (Perkin Elmer LS50), where fluorescence was monitored at 37°C (506 nm excitation, 530 nm emission). Calibration was performed by adding 2 μM ionomycin and 100 μM digitonin (F_{max}, maximal fluorescence of the system) and by adding 5 mM EGTA and 60 mM Tris-base (F_{min} minimal fluorescence of the system). $[Ca^{2+}]_i$ elevation has been expressed as the ratio of stimulated over basal (s/b).

Adherent cells

In the recovery experiments dU937 cells were seeded on sterile coverslips coated with poly-D-lysine for 30 minutes at 33°C in saline solution containing 2 μM Fura 2/AM. By contrast, adherent COS-7 cells were incubated for 45 minutes at 37°C in the dark with 5 μM Fura 2/AM. Determination of free $[Ca^{2+}]_i$ has been performed as previously described (Capra et al., 2004b). Briefly, after loading, cells were washed twice with a saline solution, and transferred to the spectrofluorimeter, where fluorescence was monitored at 37°C (505 nm emission, 340 and 380 nm excitation). To measure the concentration of $[Ca^{2+}]_i$, from the fluorescence recording, the system was calibrated as follows: F_{max} was obtained by adding 2 μM ionomycin and 100 μM digitonin, and F_{min} was obtained by adding 5 mM EGTA and 60 mM Tris base.

Equilibrium binding studies and acid wash

Equilibrium binding studies in intact dU937 cells were performed as previously described (Capra et al., 2003). Briefly, cells were incubated at 25°C for 60 minutes using 0.3 nM [^{3}H]LTD$_4$ and unlabeled LTD$_4$ at the indicated concentrations. dU937 cells (5-10×10^6 cells/sample), saline solution pH 7.4, 20 mM $CaCl_2$ and 20 mM penicillamine were added to the incubation mixture to achieve a final volume of 500 μl. Unbound ligand was separated by centrifugation, the pellet was washed once with 1 ml of saline solution at 4°C and dissolved in 1 N NaOH, and radioactivity was measured in a liquid scintillation counter (Packard instruments). For the acid-wash procedure, pellets obtained after binding at 37°C or 0°C for 30 minutes on dU937 cells were resuspended in 800 μl of ice-cold acidic buffer [0.2 M acetic acid/0.5 M NaCl/0.2% (w/v) BSA, pH 2.5] for 10 minutes and centrifuged at 16,000 ***g*** for 10 minutes. The radioactivity was determined both in the resulting pellets and in the supernatants. COS-7 crude membranes were prepared from cells as previously described with minor variations (Ravasi et al., 2002). Briefly, cells were minced and homogenized at 4°C in 10 mM HEPES-KOH pH 7.4, centrifuged at 1000 ***g*** for 10 minutes and the supernatant centrifuged at 27,000 ***g*** for 30 minutes. The pellet was resuspended and centrifuged twice under the same conditions. Prior to use, enzymatic inhibitors were added to the membrane suspension to avoid Cys-LT metabolism. Equilibrium binding studies were performed at 25°C for 60 minutes using a mixed type protocol with 0.03-0.5 nM [^{3}H]LTD$_4$ and unlabeled LTD$_4$ at the indicated concentrations (Capra et al., 2003; Rovati, 1998). COS-7 membranes (150 μg/sample), 10 mM HEPES-KOH pH 7.4, 10 mM $CaCl_2$ and 10 mM $MgCl_2$ were added to the incubation mixture to achieve a final volume of 250 μl. Unbound ligand was separated by rapid vacuum filtration (Brandel Cell Harvester) onto glass-fiber GF/C filters soaked in 2.5% polyvinylalchool and the filters were washed twice with 4 ml of HEPES buffer at 4°C. Radioactivity was measured in a liquid scintillation counter. Nonspecific binding was calculated, using the LIGAND computer program (Munson and Rodbard, 1980), as one of the unknown parameters of the model (25-30%).

Confocal microscopy of intact dU937 and COS-7 cells

dU937 were serum-starved for 1 hour in saline solution, let adhere on sterile coverslips coated with poly-D-lysine for 15 minutes at 37°C, and treated with appropriate stimuli for 5 minutes. Confocal microscopy analysis of dU937 and COS-7 cells was performed as previously described (Citro et al., 2005). Briefly, dU937 and COS-7 cells were washed with PBS, fixed with 3.7% paraformaldehyde and permeabilized with 0.1% Triton X-100. Cells adherent to coverslips were incubated with 2% BSA as blocking solution and with antibody against the C-terminus of CysLT$_1$ receptor (1:250 v/v) for 1 hour at 37°C, and then were washed and incubated with Alexa 488 goat anti-rabbit antibody (1:250 v/v) for 45 minutes. Confocal laser scanning microscopy was performed using a Bio-Rad confocal microscope (Radiance 2100 equipped with a ×100/×60 lens).

Immunoprecipitation of CysLT$_1$ receptor in COS-7 cells

24 hours after transfection, COS-7 cells were washed twice with ice-cold PBS and lysed in 250 μl ice-cold lysis buffer (50 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride, the protease inhibitor complex Complete^{TM}, 1 mM NaF, 1 mM Na_3VO_4 and 0.1% Triton X-100). Cell suspensions were incubated at 4°C for 60 minutes, freeze/thawed and homogenized with Teflon/glass homogenizer. The particulate fraction was removed by centrifugation at 16,000 ***g*** for 10 minutes, and 300 μg of supernatant protein was incubated in the presence of anti-HA-specific monoclonal antibody (16B12, Babco 1:500) and 100 μl of 20% slurry protein G Sepharose beads in immunoprecipitation buffer (300 nM NaCl, 2% deoxycholate and 2% Triton X-100) at 4°C for 12-18 hours. The beads were washed three times with immunoprecipitation buffer and solubilized in 2× SDS sample buffer and the immunoprecipitates subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. CysLT$_1$ receptor was detected by immunoblotting at 4°C for 12-18 hours with a polyclonal anti-CysLT$_1$ receptor antibody diluted 1:7500 in TBS-T (150 mM NaCl, 10 mM Tris-HCl, pH 7.0, and 0.05% Tween 20) wash buffer containing 3% dried skimmed milk. After three 10 minute washes with TBS-T, membranes were incubated for 1 hour with horseradish peroxidase-conjugated goat anti-rabbit IgG. The blots were developed using ECL chemiluminescent substrate.

Statistical analysis

Ligand-binding studies were analyzed using the LIGAND computer program (Munson and Rodbard, 1980). Parameter errors are all expressed as percentage coefficient of variation (%CV) and calculated by simultaneous analysis of at least two different independent experiments performed in duplicate or triplicate. Nonspecific binding was determined by computer as one of the unknown parameters of the model and was always <30% of total binding. Statistical comparison of multiple groups were analyzed using one way ANOVA followed by the Dunnett post hoc test. Data are expressed as means±s.e.m. All curves are computer generated.

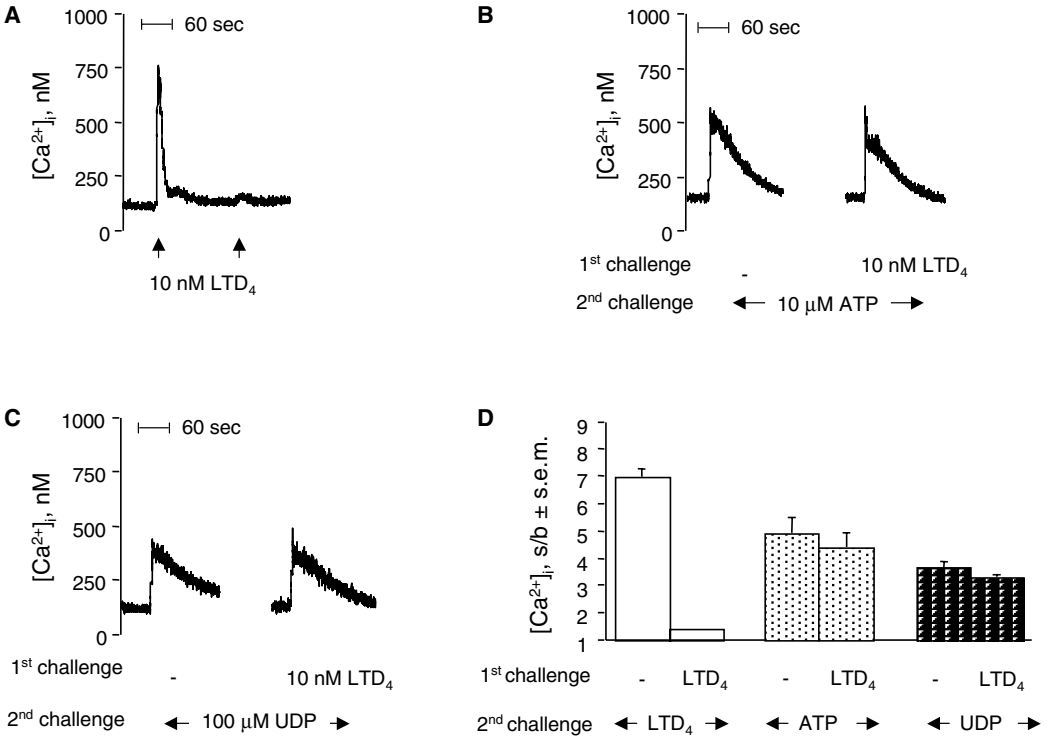

Fig. 1. Calcium responses to LTD_4, ATP and UDP following LTD_4-induced $CysLT_1$ receptor activation. (A) Representative traces of the $[Ca^{2+}]_i$ transient induced by 10 nM LTD_4 before and after an initial challenge with the same concentration of LTD_4. (B,C) Representative traces of the $[Ca^{2+}]_i$ transient induced by 10 μM ATP (B) and 100 μM UDP (C) (2nd challenge) before and after an initial challenge with 10 nM LTD_4. (D) Values shown representative means of $[Ca^{2+}]_i$ stimulation over basal (s/b) ± s.e.m. of at least three independent experiments.

Results

Effect of $CysLT_1$ receptor activation on the LTD_4-induced $[Ca^{2+}]_i$ transient (homologous desensitization)

To verify the extent of agonist-induced $CysLT_1$ desensitization, we measured the LTD_4-induced $[Ca^{2+}]_i$ transient following an initial challenge with LTD_4 in dU937 cells. Fig. 1A shows that 10 nM LTD_4 (submaximal concentration) (Capra et al., 2003) was able to elicit only a tiny response after an initial challenge with the same concentration of LTD_4, resulting in almost complete inhibition (>90%) of $CysLT_1$ activation (Fig. 1D).

Effect of $CysLT_1$ receptor activation on the ATP- and UDP-induced $[Ca^{2+}]_i$ transient (heterologous desensitization)

To verify a possible influence of $CysLT_1$ activation on P2Y receptor responses, we measured the ATP- and UDP-induced $[Ca^{2+}]_i$ transient following an initial challenge with LTD_4. Fig. 1B,C show that ATP- and UDP-induced responses (submaximal concentrations, data not shown) were unaffected by a prior challenge with 10 nM LTD_4, demonstrating that $CysLT_1$ activation is not able to influence P2Y receptor functionality in dU937 cells (Fig. 1D).

Effect of P2Y receptor activation on LTD_4-induced $[Ca^{2+}]_i$ transient (heterologous desensitization)

To verify the extent of a possible influence of P2Y receptor activation on the $CysLT_1$ response, we measured the agonist-induced $[Ca^{2+}]_i$ transient following an initial challenge with graded concentrations of ATP or UDP. Fig. 2A,B show the concentration-dependent effects of an initial challenge with extracellular nucleotides on the response elicited by 10 nM LTD_4 in dU937 cells. It is clear that ATP and UDP were able to provoke a reduction in agonist-induced Ca^{2+} response in a concentration-dependent manner, reaching a maximal $CysLT_1$ desensitization of 78% and 66%, respectively (Fig. 2C).

Effect of second-messenger-activated protein kinases on $CysLT_1$ homologous and heterologous desensitization

Following the previous results, we investigated the participation of the second-messenger-activated protein kinases in nucleotide-induced $CysLT_1$ heterologous desensitization. We measured the effect of a 5 minute pretreatment of increasing concentrations of the potent PKC inhibitor GF109203X (GFX) on the response elicited by 10 nM LTD_4, following an initial challenge with 10 μM ATP or 100 μM UDP (Fig. 3A,B). It is clear that 10 μM GFX was able to dose-dependently revert ATP- and UDP-induced $CysLT_1$ desensitization. However, this effect was only partial, with the Ca^{2+} response in the presence of 10 μM GFX reaching 54% and 69% of LTD_4 signal for ATP and UDP, respectively (Fig. 3C). By contrast, the same concentration of GFX had no effect on agonist-induced homologous desensitization (Fig. 3C). Accordingly, pretreatment with 10 μM GFX was also able to almost completely revert $CysLT_1$ desensitization induced by an initial challenge with 500 nM phorbol 12-myristate 13-acetate (PMA), a known activator of PKC (Fig. 4A,B), with an effect similar to that obtained on nucleotide-induced desensitization. Taken together, these data suggest that P2Y-induced $CysLT_1$ heterologous desensitization is dependent upon PKC activity.

Conversely, neither pretreatment with 10 μM H89, a PKA inhibitor (Fig. 4C), nor with 10 μM KN93, a Ca^{2+} calmodulin-dependent kinase II (CaMKII) inhibitor (data not shown),

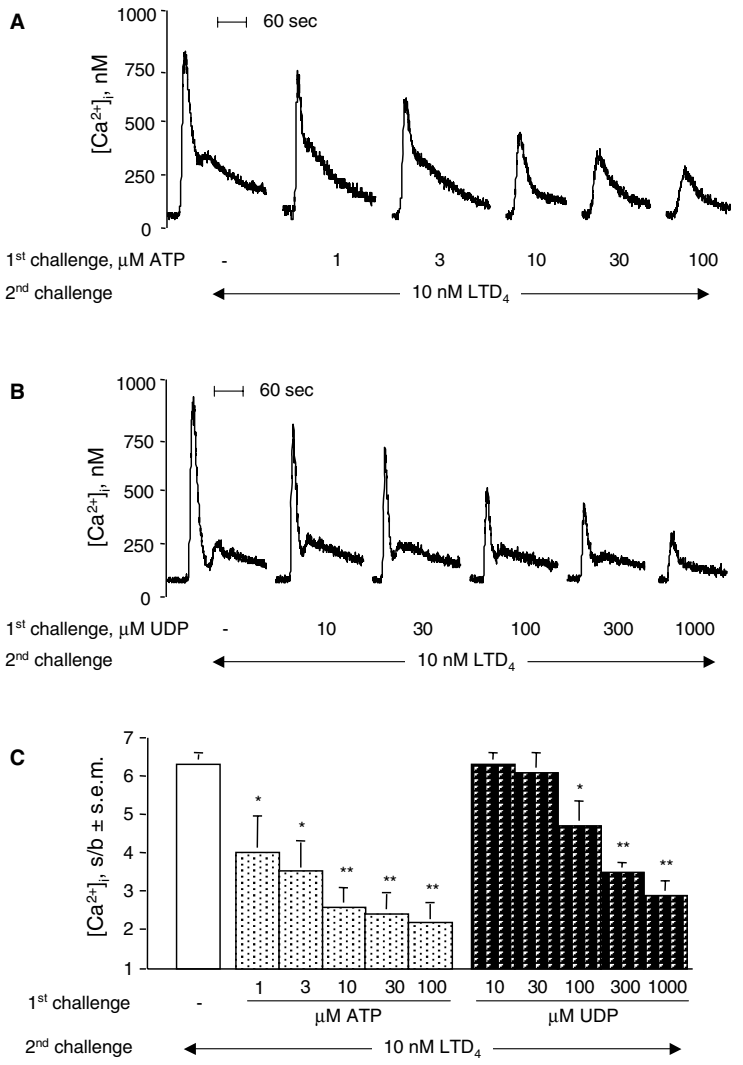

Fig. 2. Calcium responses to LTD_4 following ATP- and UDP-induced P2Y receptor activation. Representative traces of the $[Ca^{2+}]_i$ transient induced by 10 nM LTD_4 (2nd challenge) after an initial challenge with increasing concentrations of ATP (A) and UDP (B). (C) Values shown represent means of $[Ca^{2+}]_i$ stimulation over basal (s/b) ± s.e.m. of at least three independent experiments. Statistical comparison of multiple groups were analyzed using one way ANOVA followed by Dunnett post hoc test (*P<0.05; **P<0.01, vs control).

was able to affect agonist- or nucleotide-induced $CysLT_1$ desensitization.

Rate of recovery of $CysLT_1$ functionality after homologous or heterologous desensitization in dU937 cells and in D5 clones

To better characterize the different mechanisms of homologous and heterologous desensitization, we performed a time-course of the rate of recovery of $CysLT_1$ functionality after LTD_4- or ATP-induced desensitization. Fig. 5A shows the $[Ca^{2+}]_i$ transient elicited by 10 nM LTD_4 (2nd challenge) after the indicated periods of times in dU937 cells. It is clear that whereas $CysLT_1$ receptor is completely re-sensitized at 30 minutes after ATP-induced heterologous desensitization, it is only partially re-sensitized after an initial challenge with LTD_4.

Furthermore, Fig. 5B shows that $CysLT_1$ receptor functionality is recovered faster in a stable U937 clone (D5 clone) with reduced GRK2 expression (Fernandez et al., 2002) than in control dU937 cells after agonist-induced homologous desensitization. In addition, a 30 minute pre-treatment with 1 μM of the GRK2 inhibitor (Iino et al., 2002) decreased $CysLT_1$ homologous desensitization to 78% (data not shown) compared with control cells (>90%, see Fig. 1D).

Effect of PTX and of YM-254890 on extracellular nucleotide-induced heterologous desensitization

We have previously reported that the LTD_4-elicited $[Ca^{2+}]_i$ increase is partially inhibited by PTX pre-treatment in dU937 cells, demonstrating a promiscuous coupling for $CysLT_1$ receptor in these cells with G_q and G_i (Capra et al., 2003). Therefore, to assess whether or not the observed extracellular-nucleotide-induced desensitization was able to affect both signaling pathways, we treated the dU937 cells with 300 ng/ml PTX overnight or with 60 nM of the $G\alpha_q$ inhibitor, YM-254890 (Takasaki et al., 2004), to selectively inhibit only one signaling pathway at a time. Particularly UDP- but also ATP-induced $CysLT_1$ desensitization were reduced (20% and 45%, respectively) when G_i signaling was inhibited and G_q coupling preserved and enhanced (>80% for UDP) when $G\alpha_q$ signaling was inhibited (Fig. 5C,D). As a control, the same concentration of YM-254890 did not affect fMLP receptor signaling, a known pure G_i-coupled receptor, while completely inhibiting PAF signaling, a classically G_q-coupled receptor (data not shown).

Effect of LTD_4, ATP and UDP on $CysLT_1$ internalization

Equilibrium binding studies and acid wash

To examine the possibility that ATP and UDP interact with the orthosteric site labeled by $[^3H]LTD_4$, we performed equilibrium binding studies in intact dU937 cells. Fig. 6A shows that, in contrast to unlabeled LTD_4, neither ATP nor UDP was able to compete with $[^3H]LTD_4$. We also considered whether $CysLT_1$ expression on the membrane surface would be affected by different agonist pretreatment, and found that while a 30 minute pretreatment with 0.1 μM LTD_4 almost completely abolished the amount of $[^3H]LTD_4$ surface-specific binding, pretreatment with 100 μM ATP or 1 mM UDP failed to affect it (Fig. 6B). To further confirm that the observed reduction of $[^3H]LTD_4$-specific binding was due to loss of $CysLT_1$ receptor from the cell surface, acid wash procedure was used to remove the radioactivity associated with the cell surface (supernatant), separating it from the one present inside the cell (pellet) (Giovanazzi et al., 1997). Fig. 6C shows that a 30 minute incubation with 0.3 nM $[^3H]LTD_4$ at 0°C (a temperature at which receptor internalization is inhibited) equally distributed the radioactivity between pellet (P) and supernatant (S). Conversely, incubation at 37°C

(a temperature permissive for receptor internalization) significantly increased the amount of radioactivity present inside the cell, concomitantly reducing the one present on the cell surface. These data suggest that the reduction of surface-specific binding following LTD_4 pretreatment might be due to receptor internalization.

Immunocytochemistry of $CysLT_1$ receptor in dU937 cells

To further confirm that the decrease in $[^3H]LTD_4$-binding sites induced by LTD_4 pretreatment was due to receptor internalization, we visualized by confocal microscopy the expression of $CysLT_1$ receptors using immunocytochemical staining with a specific antibody raised against the C-terminal of the receptor. Fig. 7 shows that in dU937 cells fluorescence was predominantly associated with the surface, which suggests a plasma membrane localization of the $CysLT_1$ receptor at the steady state. Treatment with 10 nM LTD_4 for 5 minutes induced a rapid loss of the receptor from the cell surface and the trafficking of the receptors inside the cells. By contrast, neither 100 μM ATP and 1 mM UDP, nor 500 nM PMA was able to induce any modification of the $CysLT_1$ receptor localization. In addition, a 30 minute pretreatment with 10 μM GFX was unable to prevent agonist-induced $CysLT_1$ receptor internalization, thus confirming independence of receptor trafficking from PKC activity.

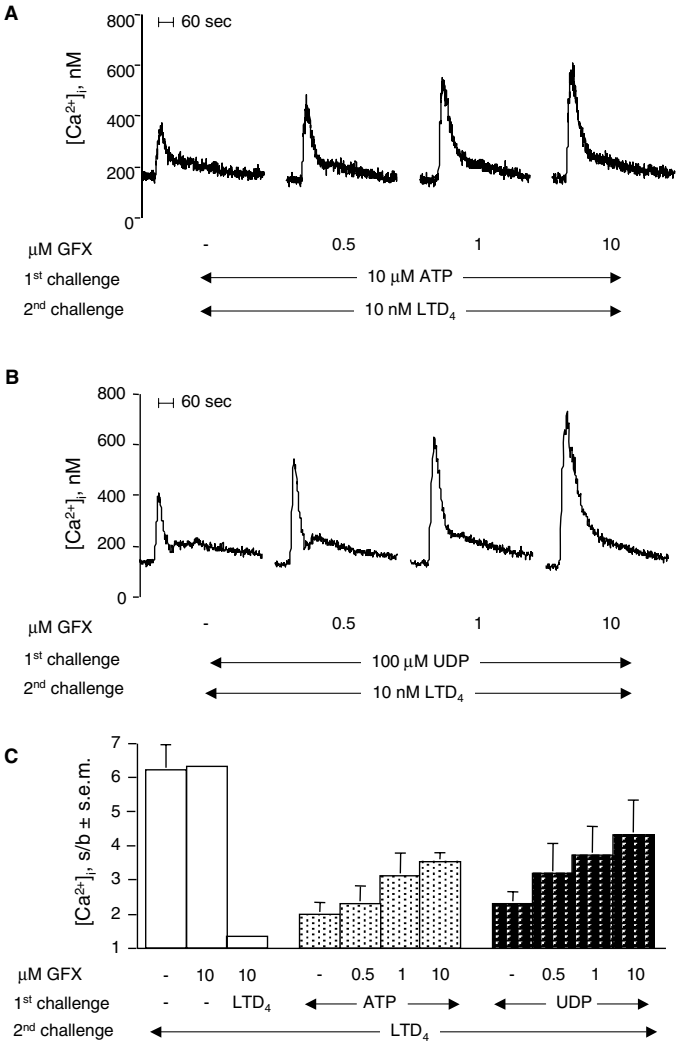

Fig. 3. Effect of GFX on $CysLT_1$ receptor activation after LTD_4, ATP and UDP challenge. Representative traces of the effect of increasing concentrations of GFX pretreatment (5 minutes) on $[Ca^{2+}]_i$ transient induced by 10 nM LTD_4 (2nd challenge) after an initial challenge with 10 μM ATP (A) and 100 μM UDP (B). (C) Values shown represent means of $[Ca^{2+}]_i$ stimulation over basal (s/b) ± s.e.m. of at least three independent experiments.

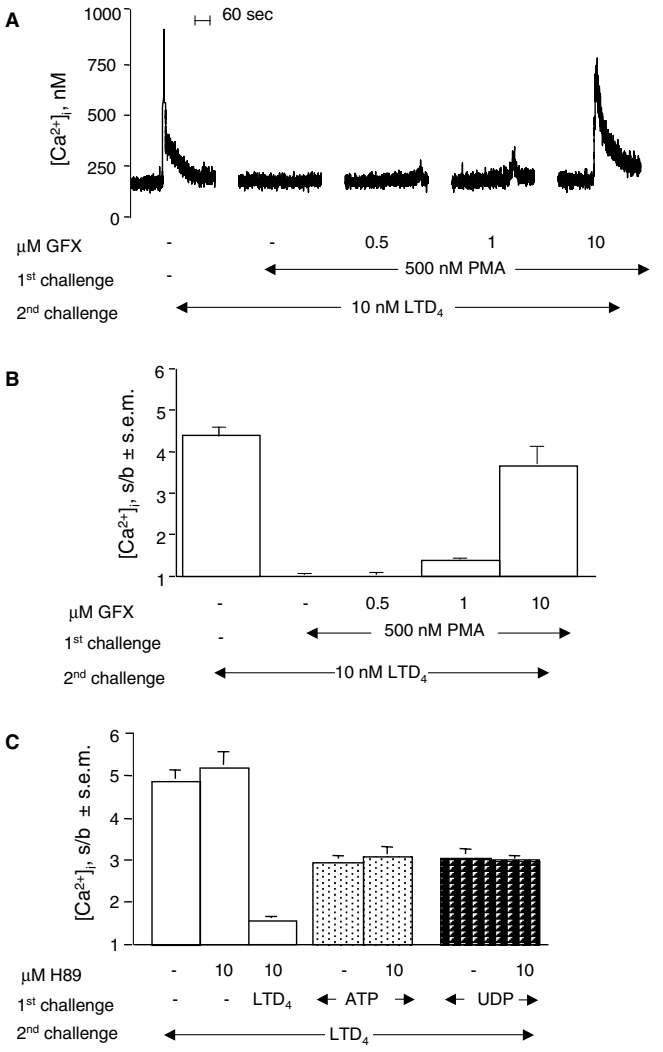

Fig. 4. Effect of GFX on $CysLT_1$ receptor activation after PMA challenge, and effect of H89 on ATP- and UDP-induced $CysLT_1$ desensitization. (A) Representative trace of the effect of increasing concentrations of GFX pretreatment (5 minutes) on the $[Ca^{2+}]_i$ transient induced by 10 nM LTD_4 (2nd challenge) after an initial challenge with 500 nM PMA (5 minutes). (B) Values shown represent means of $[Ca^{2+}]_i$ stimulation over basal (s/b) ± s.e.m. of at least five independent experiments. (C) Effect of 10 μM H89 pretreatment (5 minutes) on the $[Ca^{2+}]_i$ transient induced by 10 nM LTD_4 (2nd challenge) after an initial challenge with 10 nM LTD_4, 10 μM ATP or 100 μM UDP.

CysLT$_1$ receptor expression and regulation in COS-7 cells

CysLT$_1$ receptor expression

To verify that CysLT$_1$ homologous and heterologous desensitization were not limited to dU937 cells, we also studied receptor regulation in a recombinant system, using COS-7 cells transiently expressing the CysLT$_1$ receptor but, conversely, constitutively expressing the ATP- and UTP-responsive P2Y$_2$ receptors (Herold et al., 1997). Fig. 8A shows the immunoblot of COS-7 cells transiently transfected with a construct encoding an HA-tagged CysLT$_1$ receptor immunoprecipitated with a monoclonal antibody raised against the N-terminal HA epitope. A protein band of ~45 kDa is correctly recognized by the CysLT$_1$ polyclonal antibody. Furthermore, this protein possesses the binding characteristics of a CysLT$_1$ receptor (Fig. 8B), with K_{d1} = 0.062 nM ± 24%CV and K_{d2} = 6.25 nM ± 27%CV, as previously reported (Capra et al., 1998a; Capra et al., 1998b; Ravasi et al., 2000) expressed at a level of B_{max1} of 0.15 ± 21%CV and B_{max2} of 568 ± 21%CV fmol per mg of membrane protein. In accordance with results obtained in dU937 cells, the extracellular nucleotides ATP and UDP were unable to compete with the site labeled by [^{3}H]LTD$_4$ (Fig. 8B, inset)

CysLT$_1$ receptor homologous and heterologous desensitization

As expected, in COS-7 cells LTD$_4$-induced CysLT$_1$ receptor Ca^{2+} response undergoes agonist-induced desensitization (data not shown). Furthermore, similarly to what we have observed in dU937 cells, the response to 1 μM LTD$_4$, following an initial challenge with 10 μM ATP, was almost completely abolished (Fig. 8D). Conversely, the ATP-induced $[Ca^{2+}]_i$ transient was unaffected by a prior challenge with 10 μM LTD$_4$ (Fig. 8C), confirming also in COS-7 cells an unidirectional regulation of CysLT$_1$ receptor.

Confocal microscopy analysis of CysLT$_1$ receptor trafficking in COS-7 cells indicates that the basal receptor localization at the plasma membrane was greatly affected by a 5 minute treatment with cognate agonist, whereas it was basically unchanged by treatment with 30 μM ATP (Fig. 8E).

Discussion

Almost every GPCR studied undergoes desensitization and, despite their diversity, all cells use universal mechanisms for desensitizing GPCRs, a process that has been strongly linked

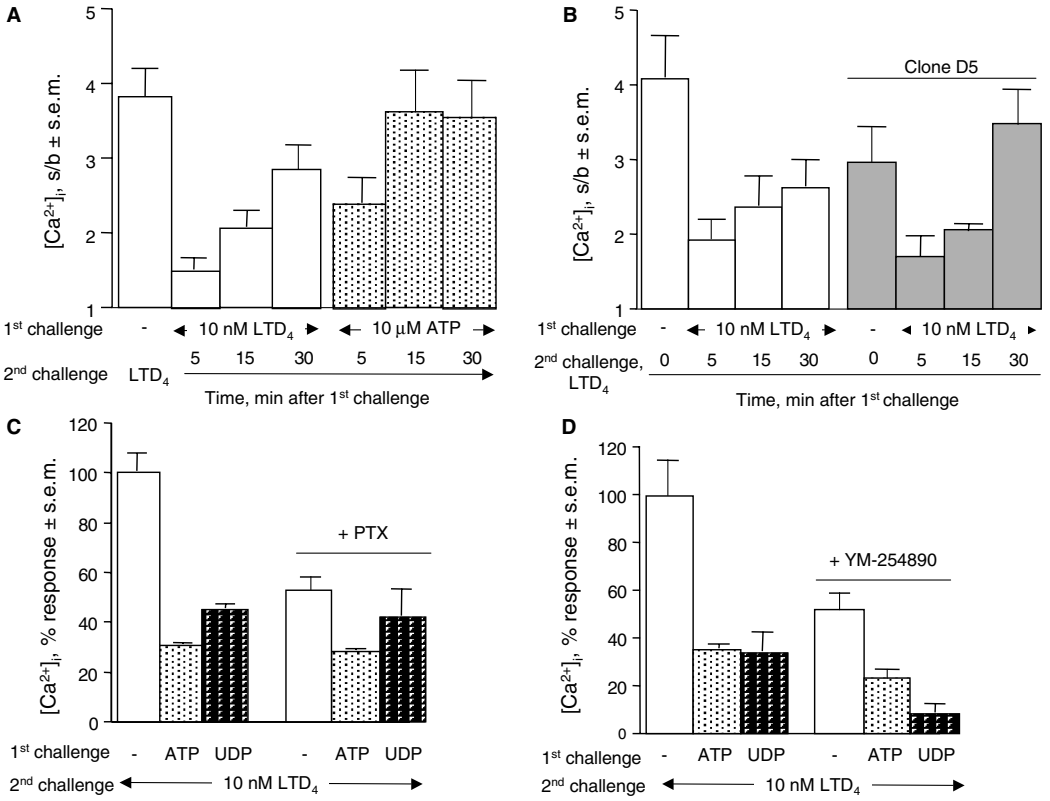

Fig. 5. Rate of recovery of CysLT$_1$ functionality after homologous or heterologous desensitization in dU937 cells and in the D5 clone, and effect of PTX or YM-254890 on extracellular nucleotide-induced heterologous desensitization. (A) Time course of recovery of CysLT$_1$ functionality (10 nM LTD$_4$, 2nd challenge) following homologous and heterologous desensitization in dU937 cells. Values shown represent means of $[Ca^{2+}]_i$ stimulation over basal (s/b) ± s.e.m. of five independent experiments. (B) Time course of recovery of CysLT$_1$ functionality (10 nM LTD$_4$, 2nd challenge) following homologous desensitization in control dU937 cells and D5 clone. Values shown represent means of $[Ca^{2+}]_i$ stimulation over basal (s/b) ± s.e.m. of at least three independent experiments. (C) Effect of 300 ng/ml PTX pretreatment (overnight) on the $[Ca^{2+}]_i$ transient induced by 10 nM LTD$_4$ (2nd challenge) after an initial challenge with 10 μM ATP or 100 μM UDP. Values shown represent the percentage response ± s.e.m. of three independent experiments. (D) Effect of 60 nM YM-254890 pretreatment (5 minutes) on the $[Ca^{2+}]_i$ transient induced by 10 nM LTD$_4$ (2nd challenge) after an initial challenge with 10 μM ATP or 100 μM UDP. Values shown represent the percentage response ± s.e.m. of three independent experiments.

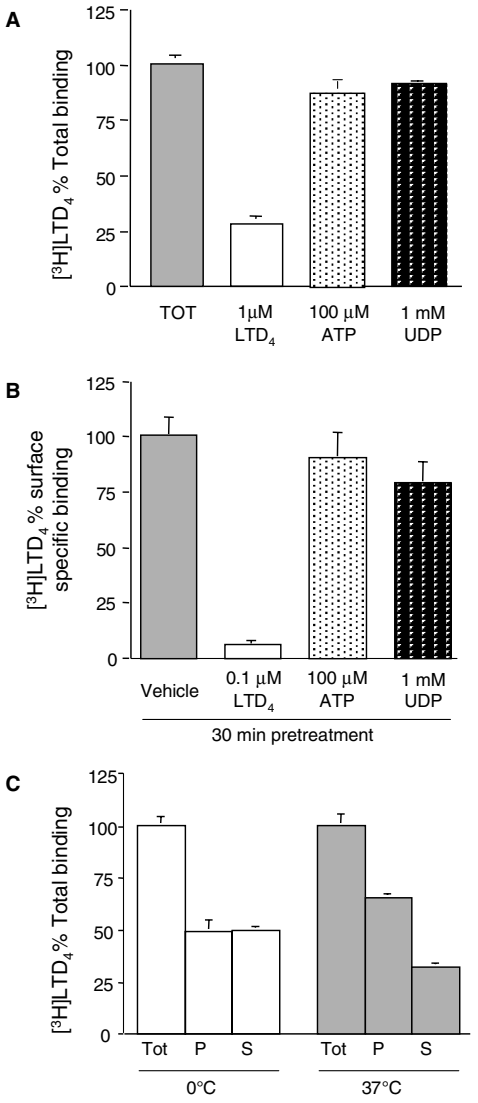

Fig. 6. Equilibrium binding and acid wash of [^{3}H]LTD$_4$ in intact dU937 cells. (A) Percentage of total [^{3}H]LTD$_4$ binding and displacement by unlabeled LTD$_4$, ATP or UDP. (B) Effect of a 30 minute pretreatment with 0.1 μM LTD$_4$, 100 μM ATP or 1 mM UDP on surface-specific [^{3}H]LTD$_4$ binding. (C) Acid wash: effect of the incubation at 0°C or 37°C (30 minutes) on the distribution of total [^{3}H]LTD$_4$ radioactivity (Tot); P, pellet; S, supernatant. Values shown represent means±s.e.m. of at least two independent experiments.

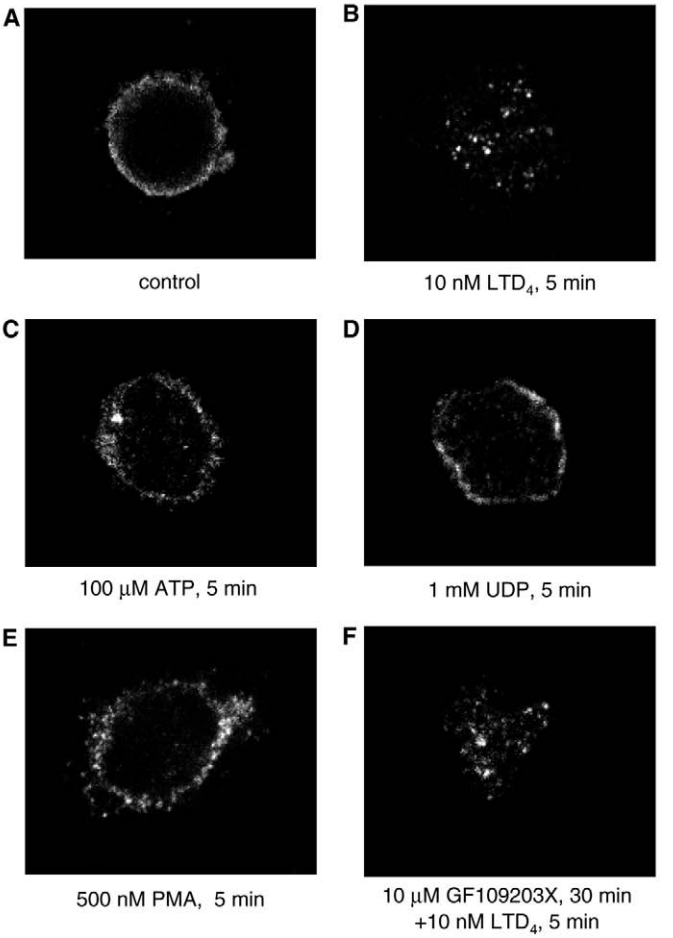

Fig. 7. Confocal microscopy of the CysLT$_1$ receptor trafficking in intact dU937 cells. Confocal laser scanning microscopy of the subcellular distribution of the CysLT$_1$ receptors was performed using a specific antibody raised against the C-terminal tail. Cells were fixed with paraformaldehyde and permeabilized with Triton X-100 in basal conditions (control), and after 5 minutes were challenged with the indicated stimuli. Immunocytochemical staining of five representative dU937 cells after stimulation with vehicle (A), 10 nM LTD$_4$ (B), 100 μM ATP (C), 1 mM UDP (D), 500 nM PMA (E) and 10 nM LTD$_4$ after a 30 minute pretreatment with 10 μM GFX (F). The experiment shown is representative of at least two others performed.

to receptor phosphorylation (Bouvier et al., 1988; Ferguson, 2001). Desensitization is a complex process that plays an important role in turning off receptor-mediated signal transduction pathways, i.e. a physiological feedback mechanism that protects against acute and chronic receptor stimulation, and filters information from multiple receptor inputs into an integrated and meaningful biological signal (Bunemann et al., 1999). In particular, heterologous desensitization plays a critical role in regulating intracellular signaling by diverse members of the GPCRs superfamily and may unveil unexpected hierarchies in the cellular integration of multiple signals (Selbie and Hill, 1998).

We demonstrate that the CysLT$_1$ receptor is the target of agonist-mediated homologous desensitization, but also of ATP/UDP-induced heterologous desensitization. Whereas the first process is most likely due to a GRK, the latter is mediated by the activation of PKC. Moreover, using radiolabeled-binding analysis and confocal microscopy, we found that LTD$_4$-induced desensitization is followed by receptor internalization and downregulation, whereas the nucleotide-mediated desensitization was unable to affect CysLT$_1$ receptor localization and trafficking and induced a faster recovery of its functionality. In addition, extracellular nucleotide-induced heterologous desensitization seems to affect G_i coupling to a greater extent.

The human promonocytic leukemia U937 cell line is known to constitutively express a high density of CysLT$_1$ receptors. These cells also express at least two P2Y receptors, namely

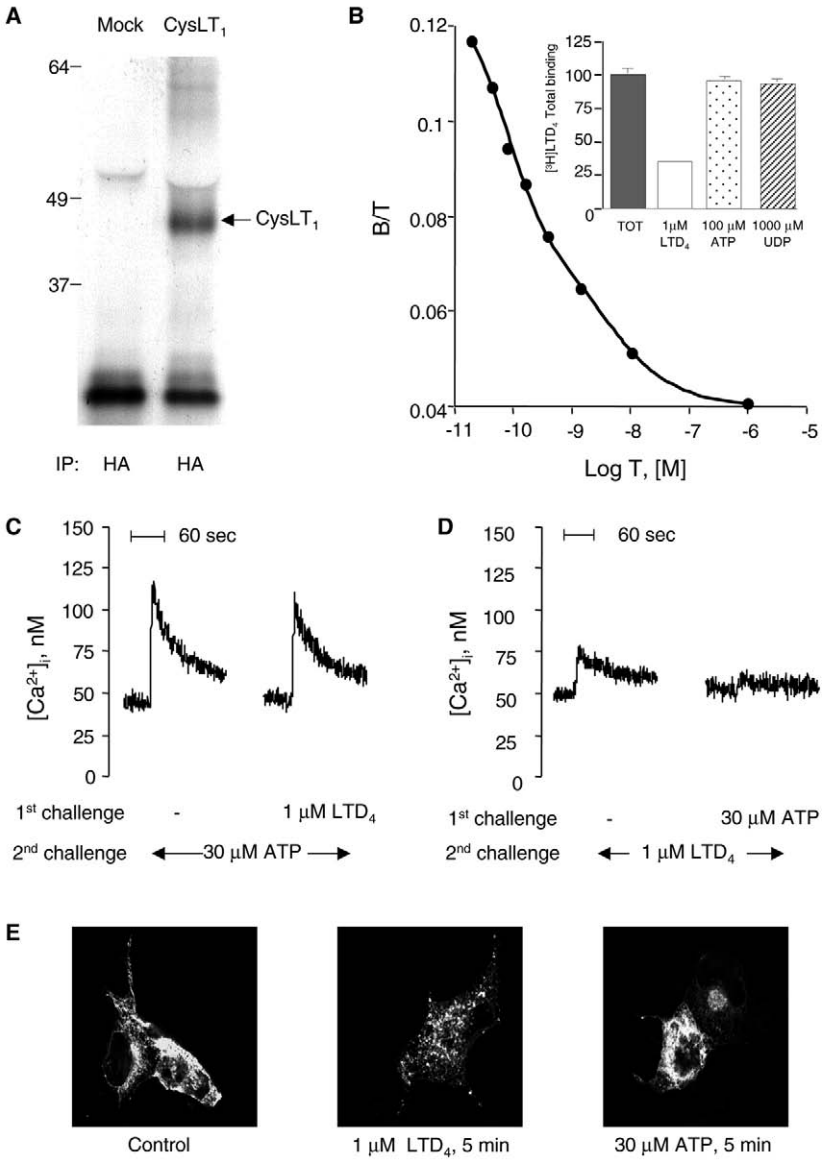

Fig. 8. CysLT$_1$ receptor expression and heterologous desensitization in COS-7 cells. Confocal microscopy of the CysLT$_1$ receptor trafficking in intact COS-7 cells. (A) COS-7 cells expressing HA-tagged CysLT$_1$ receptor were immunoprecipitated with HA monoclonal antibody and visualized with an CysLT$_1$ polyclonal antibody. (B) Equilibrium mixed-type binding curve of [^{3}H]LTD$_4$ in membranes from COS-7 cells transiently transfected with HA-tagged CysLT$_1$ receptor. Binding of [^{3}H]LTD$_4$ is expressed as the ratio of bound ligand concentration to total ligand concentration (B/T, dimensionless) versus the logarithm of total ligand concentration (Log T). Two independent [^{3}H]LTD$_4$ mixed-type experiments were performed, each with triplicate determinations, and analyzed simultaneously. For the sake of clarity, only one representative curve is shown; the curve is computer generated. (Inset) The percentage of total [^{3}H]LTD$_4$ binding and displacement by unlabeled LTD$_4$, ATP or UDP. (C) Representative traces of the $[Ca^{2+}]_i$ transient induced by 30 μM ATP (2nd challenge) before and after an initial challenge with 1 μM LTD$_4$. (D) Representative traces of the $[Ca^{2+}]_i$ transient induced by 1 μM LTD$_4$ (2nd challenge) before and after an initial challenge with 30 μM of ATP. (E) Confocal laser scanning microscopy of the subcellular distribution of the CysLT$_1$ receptors performed using a specific antibody raised against the C-terminal tail. Cells were fixed with paraformaldehyde and permeabilized with Triton X-100 in basal conditions (control) and after a 5 minute challenge with the indicated stimuli. Immunocytochemical staining of COS-7 cells after stimulation with vehicle (left), 1 μM LTD$_4$ (middle), 30 μM ATP (right). The experiment shown is representative of two others performed.

P2Y$_2$ and P2Y$_6$ (Jin et al., 1998), coupled to G$_q$, and thus to intracellular Ca^{2+} homeostasis. On this basis, we used ATP and UDP, known to activate P2Y$_2$ and P2Y$_6$ receptors, respectively (Communi et al., 2000). The pharmacodynamic parameters of these compounds in our cells are consistent with those from previous reports (data not shown), and will not be discussed further.

As expected, CysLT$_1$ receptors undergo agonist-induced homologous desensitization (Winkler et al., 1988) followed by a reduction of CysLT$_1$ receptor present on the plasma membrane, associated with a simultaneous increase of the receptor protein inside the cells, as demonstrated by binding studies with labeled LTD$_4$. Accordingly, confocal images of the CysLT$_1$ receptor demonstrate a very rapid disappearance of the receptor from the cell surface and its trafficking inside the cells, and an apparent reduction of the fluorescent signal associated with the receptor protein. The punctate appearance of the CysLT$_1$ receptor has already been reported in peripheral blood cells, and has been suggested to reflect a possible oligomerization and/or membrane compartmentalization (Figueroa et al., 2001). LTD$_4$ was unable to affect the ATP/UDP-induced $[Ca^{2+}]_i$ transient, indicating that the CysLT$_1$ receptor is not able to desensitize P2Y receptors present in dU937 cells. On the contrary, both ATP and UDP have been found to desensitize the LTD$_4$-induced $[Ca^{2+}]_i$ response in a concentration-dependent manner. This phenomenon of unidirectional desensitization has already been observed for a number of different chemoattractant receptors (Ali et al., 1999). Thus, it is possible to hypothesize that P2Y receptors have developed a hierarchy in producing desensitizing signals that profoundly impact on CysLT$_1$ receptor function.

Since cellular responses can be downregulated by receptor phosphorylation through second messengers-activated protein kinases (Bunemann et al., 1999; Zamah et al., 2002), we examined the role of PKA and PKC as possible mediators of P2Y-induced heterologous desensitization. The specific PKC inhibitor GFX reverted ATP/UDP-induced CysLT$_1$ desensitization in a concentration-dependent manner. Accordingly, pretreatment with PMA, a known nonspecific activator of various isoforms of PKC, inhibited LTD$_4$-

induced $[Ca^{2+}]_i$ transient, while pretreatment with GFX almost completely prevented this PMA-induced $CysLT_1$ desensitization. These data consistently support a role for PKC in the heterologous desensitization of the $CysLT_1$ receptor, in agreement with previously published observations (Vegesna et al., 1988) and with the presence of PKC consensus sequences in the C-terminal tail of the $CysLT_1$ receptor (Lynch et al., 1999).

Furthermore, we show here that, although the $CysLT_1$ receptor is promiscuously coupled to G_q and G_i in dU937 cells (Capra et al., 2003), extracellular nucleotide (particularly UDP)-induced heterologous desensitization was reduced when $G\alpha_i$ signaling was inhibited and G_q coupling preserved, indicating that PKC-induced-desensitization affects the G_i pathway more. Thus, extracellular nucleotides modulate the $CysLT_1$ receptor by inhibiting only the signaling events downstream of the PTX-sensitive G proteins that in dU937 cells are linked to the activation of the MAPK cascade (Capra et al., 2003; Capra et al., 2004a). This selective inhibition seems to suggest that heterologous desensitization might be a mechanism used by cells to obtain inhibition of specific functions exerted by a promiscuously coupled receptor.

In contrast to data recently published using a recombinant receptor system (Naik et al., 2005), we found that PKC was not involved in agonist-induced $CysLT_1$ receptor desensitization in dU937 cells. On the contrary, GRKs seem to be involved in this process, considering that in a stable U937 clone with reduced GRK2 expression (Fernandez et al., 2002) the rate of recovery of $CysLT_1$ functionality was faster than that in control U937 cells, and a GRK2 inhibitor partially reduced agonist-induced desensitization. However, a 30 minute pretreatment with GFX was able to increase LTD_4-induced $[Ca^{2+}]_i$ levels (data not shown), suggesting a 'tonic' desensitization of the $CysLT_1$ receptor by PKC. Furthermore, PKC inhibition also failed to affect agonist-induced $CysLT_1$ receptor trafficking, confirming the lack of a role of PKC in the homologous regulation of this receptor in a constitutive system. These data also indicate that PKC, a known downstream target of the $CysLT_1$ receptor in other cells (Accomazzo et al., 2001), does not exert a negative feedback mechanism on agonist-induced $CysLT_1$ activation, as is the case for other receptor systems either directly or indirectly (Bunemann et al., 1999; Chuang et al., 1996). We also examined the possibility that PKA or CaMKII is involved in $CysLT_1$ receptor desensitization and found that agonist-induced as well as ATP/UDP-induced desensitization were unaffected by inhibitors of PKA and CaMKII.

As discussed above, LTD_4-induced homologous desensitization is followed by rapid receptor internalization and trafficking. In striking contrast to this agonist-induced effect, neither ATP nor UDP was able to affect $CysLT_1$ receptor distribution. In fact, both nucleotides induced functional desensitization of the $CysLT_1$ receptor, but were totally unable to affect the specific binding of labeled LTD_4 at the cell surface, in contrast to the effect induced by the orthosteric agonist LTD_4. In addition, confocal microscopy images undoubtedly demonstrate the incapacity of ATP, UDP and PMA to induce receptor internalization and trafficking. Accordingly, ATP-dependent heterologous desensitization was associated with a faster recovery of $CysLT_1$ functionality compared with that in agonist-induced homologous desensitization.

Because it has been proposed that mouse $CysLT_1$ receptor might also be activated by UDP (Mellor et al., 2001), we performed equilibrium ligand binding studies with labeled LTD_4, and demonstrated that neither ATP nor UDP is able to compete with the binding sites labeled by LTD_4, thus excluding a competitive interaction of these nucleotides with the $CysLT_1$ receptor. Although an allosteric interaction is theoretically possible, it is unlikely to occur in our system, considering the lack of cross-desensitization, the different mechanism of agonist- or nucleotides-induced $CysLT_1$ desensitization, and the different trafficking induced by LTD_4 or by extracellular nucleotides.

Similar results were also obtained in COS-7 cells, where the $CysLT_1$ receptor has been transiently transfected and the P2Y receptors are, conversely, constitutively expressed. These data suggest, therefore, that this phenomenon is not limited to dU937 cells, but can be extended to other cell types known to coexpress these receptors, such as eosinophils, monocytes and macrophages.

In conclusion, we have demonstrated here that $CysLT_1$ receptor function and signaling is finely regulated at different levels, in view of the important pathophysiological roles accomplished by Cys-LTs. LTD_4-induced receptor activation not only induces homologous desensitization, but also rapid receptor internalization and trafficking. These data, therefore, suggest that the $CysLT_1$ receptor is a B class GPCR, as far as intracellular trafficking is concerned (i.e. a GPCR class characterized by slow recycling/degradation) (Luttrell and Lefkowitz, 2002). Moreover, $CysLT_1$ receptor is also heterologously desensitized by at least two P2Y receptors in an unidirectional way, suggesting a hierarchy in producing desensitizing signals. Interestingly, this second level of receptor regulation does not induce receptor sequestration nor internalization, and is associated with a more rapid functional recovery, suggesting that this mechanism may represent a more dynamic and promptly reversible means to regulate receptor activity. This is particularly interesting in view of recent data suggesting that, for other receptors (e.g. chemoattractant receptors), differential regulation by cognate ligands or by other GPCRs endows receptors with dramatically different function and/or biological activities (Ali et al., 1999; Luttrell and Lefkowitz, 2002). We may thus speculate that the functional consequences of $CysLT_1$ receptor desensitization in U937 cells are different depending upon the desensitizing signal, an issue that is currently under investigation in our laboratory. In this respect, it is intriguing that extracellular nucleotides seem to even exert a selective inhibition on one of the intracellular pathway activated by the $CysLT_1$ receptor [i.e. the G_i-dependent signaling cascade responsible for the airway smooth muscle cell proliferation (our unpublished observation)]. Finally, we also showed that heterologous desensitization is dependent upon the activation of the second messenger activated PKC in dU937 cells.

Given that cell differentiation and proliferation are often associated with the expansion of inflammation and that Cys-LTs and extracellular nucleotides have been postulated to have a role in differentiation and maturation of inflammatory blood cells known to coexpress their receptors, the crosstalk between $CysLT_1$ and P2Y receptors may have profound physiological importance as a mechanism to fine-tune the inflammatory response. In this particular case, it is possible that nucleotide-

induced desensitization of the CysLT$_1$ receptor is a feedback mechanism used by the cells to protect themselves from the vast increase of inflammatory mediators characteristic of several pathological processes. It is also possible that other inflammatory stimuli heterologously desensitize the CysLT$_1$ receptor in a similar way, as is the case for the chemoattractant receptor system, and this is an issue of ongoing investigation in our laboratory. Interestingly, CysLT, P2Y and some chemoattractant receptors, such as formyl peptide receptors or platelet-activating factor receptors, all belong to the same cluster of receptors (i.e. the purine receptor cluster, δ-group of the rhodopsin receptor family) (Fredriksson et al., 2003). To date, these receptors have no clear homologs in invertebrates, unlike the monoamine or neuropeptide receptors, suggesting a relatively recent evolutionary origin (Adams et al., 2000), and might have developed a peculiar mechanism of crossregulation. We think that this system may represent just an example of a more general mechanism to regulate receptor function in vivo and thus may lead to new drug combination therapies that modulate a variety of cell responses.

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