Regulation of Cysteinyl Leukotriene Type 1 Receptor Internalization and Signaling

Cysteinyl leukotrienes activate the cysteinyl leukotriene type 1 receptor (CysLT1R) to regulate numerous cell functions important in inflammatory processes and diseases such as asthma. Despite its physiologic importance, no studies to date have examined the regulation of CysLT1R signaling or trafficking. We have established model systems for analyzing recombinant human CysLT1R and found regulation of internalization and signaling of the CysLT1R to be unique among G protein-coupled receptors. Rapid and profound LTD4-stimulated internalization was observed for the wild type (WT) CysLT1R, whereas a C-terminal truncation mutant exhibited impaired internalization yet signaled robustly, suggesting a region within amino acids 310–321 as critical to internalization. Although overexpression of WT arrestins significantly increased WT CysLT1R internalization, expression of dominant-negative arrestins had minimal effects, and WT CysLT1R internalized in murine embryonic fibroblasts lacking both arrestin-2 and arrestin-3, suggesting that arrestins are not the primary physiologic regulators of CysLT1Rs. Instead, pharmacologic inhibition of protein kinase C (PKC) was shown to profoundly inhibit CysLT1R internalization while greatly increasing both phosphoinositide (PI) production and calcium mobilization stimulated by LTD4 yet had almost no effect on H1 histamine receptor internalization or signaling. Moreover, mutation of putative PKC phosphorylation sites within the CysLT1R C-tail (CysLT1R/S313–S316) reduced receptor internalization, increased PI production and calcium mobilization by LTD4, and significantly attenuated the effects of PKC inhibition. These findings characterized the CysLT1R as the first G protein-coupled receptor identified to date in which PKC is the principal regulator of both rapid agonist-dependent internalization and rapid agonist-dependent desensitization.

The cysteinyl (Cys) leukotriene (LT) LTC4, and its conversion products LTD4 and LTE4, regulate numerous cell and organ system functions (1, 2). Most notably, CysLTs have been identified as important mediators of asthmatic attacks and asthma pathogenesis; they are potent bronchoconstrictors (3), and also seem important in modulating airway inflammation and remodeling (4). Despite the detection of specific LTD4 binding to guinea pig lung membranes in 1993 by Metters et al. (5), the cloning of a high affinity CysLT receptor was frustrated for years, until the ultimate reporting of the human CysLT type 1 receptor (CysLT1R) by Lynch et al. (6) and Saura et al. (7) in 1999. The CysLT1R is expressed in spleen, peripheral blood leukocytes, and airway smooth muscle, has nanomolar affinity for LTD4, and couples to the heterotrimeric G protein Gq to promote calcium flux. LTC4 is also a full agonist of the CysLT1R but is 10 times less potent. A second CysLT receptor subtype, CysLT type 2 receptor, has recently been cloned (8, 9). CysLT type 2 receptor is expressed in leukocytes, heart, and brain, and binds LTD4 and LTC4 with equal affinity. Although no specific CysLT type 2 receptor antagonists currently exist, CysLT1R antagonists have been established as effective antiasthma drugs (10, 11).

Since the initial characterization of the CysLT1R only a handful of studies, focused primarily on pharmacologic properties, have been published examining this receptor. This lack of studies is due primarily to difficulties in expressing recombinant CysLT1R in mammalian cells (6, 12). Although expression of recombinant CysLT1R in HEK 293T cells appears useful for high-throughput screening of potential CysLT1R ligands using an automated assay of intracellular calcium mobilization (7, 9), features of the CysLT1R beyond basic pharmacologic receptor-ligand interactions remain uncharacterized. Understanding the mechanisms by which the responsiveness of a given G protein-coupled receptor (GPCR) is regulated not only can provide insight into the functional impact of the receptor in physiologic and disease states but also can identify regulatory molecules as potential therapeutic targets (13–15). For example, the responsiveness of the β2-adrenergic receptor (β2AR) is tightly regulated by phosphorylation of the receptor by GPCR kinases (GRKs) and the subsequent binding of arrestins.

The abbreviations used are: Cys, cysteinyl; LT, leukotriene; CysLT1R, CysLT type 1 receptor; GPCR, G protein-coupled receptor; β2AR, β2-adrenergic receptor; GRK, GPCR kinase; PI, phosphoinositide; PRC, protein kinase C; PKA, protein kinase A; MEF, murine embryonic fibroblast; H1 HR, H1 histamine receptor; DMEM, Dulbecco’s modified Eagle’s medium; PMA, phorbol 12-myristate 13-acetate; Bis, bisindolylmaleimide; TP, thromboxane A2; GFP, green fluorescent protein; ELISA, enzyme-linked immunosorbent assay.

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**Recipient of a Career Investigator Award from the American Lung Association. To whom correspondence should be addressed: Wake Forest University Health Sciences Center, Center for Human Genomics, Medical Center Blvd., Winston-Salem, NC 27157. Tel.: 336-713-7541; Fax: 336-713-7566; E-mail: rpen@wfubmc.edu.

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tin proteins. GRK-mediated phosphorylation partially uncouples the β2AR from Gα and promotes the binding of arrestins to the receptor, which in turn sterically inhibits β2AR-Gi interaction while promoting receptor internalization into endocytic vesicles. Sensitivity of the β2AR to this mode of regulation may explain a differential efficacy of β-agonists among airway cells that influences airway function and response to therapy (16) and may contribute to the pathology of chronic heart failure, in which β2AR hyporesponsiveness is associated with elevated GRK levels in cardiac myocytes (17). Importantly, cardiac expression of a GRK2 “minigene” that effectively inhibits GRK2 activity can reverse β2AR hyporesponsiveness and the pathologic phenotype in animal models of heart failure (18–20), thereby establishing the utility of targeting GRK2, and possibly other GPCR regulatory molecules, in disease therapy. Moreover, differential sensitivity to GRK/arrestin-mediated regulation seems to explain, in part, differences in the signaling capacity and functional effects among GPCRs in a given cell type. The prostaglandin E2 EP2 receptor, which is resistant to GRK-mediated phosphorylation and arrestin binding, is much more efficacious than the β2AR in stimulating cAMP production in analyses of both recombinant and endogenous receptors (21). The enhanced signaling capacity of EP2 receptors in human airway smooth muscle likely contributes to the significantly greater effect of prostaglandin E2 (relative to β-agonists) in modulating growth, migration, and contraction of human airway smooth muscle (22–24).

In the current study, we have provided mechanistic insight into the regulatory features of the signaling and trafficking of the human CysLT1R. Results demonstrated that the CysLT1R undergoes rapid agonist-dependent internalization, yet, unlike most GPCRs characterized to date, this effect appeared largely GRK- and arrestin-independent. Instead, internalization and desensitization were most dramatically affected by PKC activity, characterizing the CysLT1R as the only GPCR examined to date in which both agonist-dependent internalization and desensitization are primarily PKC-dependent phenomena.

**EXPERIMENTAL PROCEDURES**

**Materials—**FuGENE 6 transfection reagent and all reagents used for cloning were purchased from Roche Applied Science. Anti-CysLT1R antibody and LTD4 were purchased from Cayman Chemical Company (Ann Arbor, MI). [3H]LTD4 (183 Ci/mmol) and myo-[2-3H]-inositol (10–25 Ci/mmol) were purchased from PerkinElmer Life Sciences. All biosynthesis and transfection reagents were purchased from Calbiochem. Anti-FLAG M1 and M2 antibodies were purchased from Sigma. Anti-PKCα antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-GFP antibody was from Covance (Princeton, NJ). A construct encoding PKCβ-GFP was purchased from Clontech. The HG55 cDNA (6) encoding human CysLT1R was provided by Jilly Evans of Merck (West Point, PA). Murine embryonic fibroblast (MEF) cultures, derived from transgenic mice in which arrestin-2 and arrestin-3 expression were ablated (arr2−/− arr3−/−) and from paired nontransgenic controls, were provided by Robert Lefkowitz (Duke University, Durham, NC). Adenovirus shuttle plasmids and corresponding viral vectors pAdEGI and pAdVgRXR (25) were provided by David Johns (Johns Hopkins University, Baltimore, MD).

Sources of other reagents either are identified below or are from previously identified sources (21). **Generation of Receptor Constructs—**More detailed descriptions of construct generation are provided in supplemental material. A signal sequence (26) was inserted upstream of the 3-FLAG cassette in pcDNA3–3FLAG, and this plasmid was used to generate all WT and mutant CysLT1R clones. The open reading frame encoding the human CysLT1R in HG55 was amplified by PCR and inserted in-frame immediately downstream of the 3-FLAG cassette. C-terminal truncation mutants CysLT1R321stop, CysLT1R309stop, and CysLT1R300stop, as well as CysLT1R321stop, CysLT1R309stop, and CysLT1R300stop, as well as CysLT1R(313–316)A, in which serines 313, 315, and 316 were mutated to alanines, were similarly generated by PCR cloning with specific antisense primers. Generation of pcDNA3 plasmid encoding FLAG-tagged human H1 histamine receptor (H1 HR) in pcDNA3 was described previously (27).

The sequence encoding the CysLT1R open reading frame, the signal sequence motif, and the 3-FLAG epitope were PCR-amplified and cloned into the shuttle vector pAdEGI to make pAdEGI-CysLT1R. Recombinant adenovirus was generated by co-transfection of pAdEGI-CysLT1R DNA with φ5 viral DNA as described previously (25). The resulting virus, AdEGI-CysLT1R, was plaque-purified, expanded, and purified by two rounds of cesium chloride density centrifugation followed by exhaustive dialysis against 10 mM Tris, pH 8.0, 140 mM NaCl, 1 mM MgCl2, and 5% w/v sucrose. Viral particle numbers were determined by dilution in 0.1% SDS followed by measuring absorbance at 260 nm, and viral titers were determined by plaque assay on 911 cells. The particle to infective/particle ratio was between 25 and 45 for all of the viruses used. Sequence fidelity of all of the clones was verified by deoxyribonucleotide sequencing.

**Cell Culture, Transfection, Infection—**HEK 293 cells and COS-1 cells were maintained in Dulbecco’s modified Eagles medium (DMEM) supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. Cells were plated into 60-mm dishes 1 day prior to transfection so that they were 65–75% confluent immediately prior to transfection. All transfections were carried out using 10 μl of FuGENE 6 transfection reagent and 3 μg of total DNA. Eighteen h after transfection, cells were harvested and plated on 6- or 24-well plates for subsequent immunoblot analysis, receptor sequestration ELISA, analysis of cAMP production, or quantitation of anti-a7 mAb binding for immunochemical analysis of receptor subcellular distribution. For transfection of MEFs with pcDNA3–FLAGβ2AR, 20 μl of Lipofectamine 2000 (Invitrogen) in 500 μl of OptiMEM (Invitrogen) were mixed with 8 μg of pcDNA3–FLAGβ2AR, and the mixture was added dropwise to MEFs seeded in 60-mm dishes; the cells were passaged 24 h later into 24-well plates. For infection of MEFs using AdEGI-CysLT1R, MEFs were harvested with tryple/EDTA, were seeded in phosphate-buffered saline, and resuspended in DMEM/10% fetal bovine serum medium lacking antibiotics. 1 × 105 particles of CysLT1R adenovirus plus 5 × 105 particles of receptor plasmid VgKXR adenovirus, or 5 × 105 particles of VgKXR alone, were combined with 500,000 cells and were plated into 60-mm dishes. Fifteen h later, cells were again harvested, washed, and plated into 24-well plates in the presence of 10% serum medium lacking antibiotics to induce expression. Twenty-four h later, receptor internalization was assessed by ELISA as described below.

**Receptor Internalization—**Agonist-induced changes in cell surface receptor distribution were measured by ELISA. Forty-eight h after transfection, cells in 24-well plates were washed once and then incubated at 37 °C for 30 min in plain DMEM with various inhibitors or vehicle (typically 0.03% Me2SO). After pretreatment, cells were stimulated with LTD4, histamine, isoproterenol, or vehicle (typically 0.05% ETOH) for 0–30 min. The medium was aspirated, and cells were fixed with 3.7% formaldehyde for 10 min at room temperature, washed three times with Tris-buffered saline, and then blocked for 45 min with 1% bovine serum albumin in Tris-buffered saline. Cells were then incubated with a 1/1000 dilution of an anti-Flag M2 antibody conjugated to alkaline phosphatase, washed three times with Tris-buffered saline, and then incubated with alkaline phosphatase substrate at 37 °C until adequate color development was visible. Reactions were stopped by adding 0.1 ml from each sample well into 0.1 ml of 0.4 M NaOH, and the sample absorbances were read at 405 nm using a Bio-Rad microplate reader and Microplate Manager software.

Treatment of cells with 10 μM cysteine throughout the course of cell treatment with LTD4, in experiments assessing either receptor internalization or PI production, had no effect on results, suggesting that the metabolism of LTD4 in culture media was not of consequence. Experimental results were also qualitatively similar whether cells were maintained in serum-containing or serum-free medium for 18 h prior to acute pretreatment and treatment. Whenever possible, the effect of experimental variables (e.g. receptor mutations, pharmacologic inhibition) was tested in assays performed under optimally matched conditions (e.g. simultaneously performed using same passage or transfected cells, same prepared reagents) to minimize intra- and inter-experimental variability.

**Radioligand Binding—**Binding of [3H]LTD4 (183 Ci/mmol) to membrane preparations prepared from COS-1 cells expressing WT CysLT1R was performed by incubating membranes in 10 mM HEPES, pH 7.4, containing 20 mM CaCl2, protease inhibitor mixture (Sigma), 20 mM l-penicillamine, and 0–1.5 nM [3H]LTD4, for 60 min at room temperature, followed by filtration on Whatman GF/C filters using a Brandel Cell Harvester and five 5-ml washes with 10 mM HEPES, 0.01% bovine serum albumin. Non-specific binding was determined using 10 μM MK-571.
RESULTS AND DISCUSSION

Initial attempts to generate a useful recombinant CysLT1R produced constructs that either expressed poorly or, when expressed, resulted in proteins that migrated at too small a size.
(-35 kDa), suggesting either folding problems or difficulties in processing through the endoplasmic reticulum and the Golgi (data not shown). Ultimately a construct encoding an N-terminal 3-FLAG epitope and an upstream signal sequence to facilitate endoplasmic reticulum processing (26) was generated that expressed well in both COS-1 and HEK 293 cells and migrated at the predicted size (Fig. 1A). Multiple bands ranging from 45 to 52 kDa in size were detected (as well as a possible dimer at 90 kDa), suggesting the existence of multiple glycosylation sites. Importantly, antibodies against the N-terminal FLAG epitope and the C terminus of the human CysLT1R identified the same size bands (see below). Radioligand binding analysis of this construct expressed in COS-1 revealed saturable binding of [3H]LTD4 ($B_{\text{max}} = 450$ fmol/mg protein) at levels 10-fold higher than that reported for the original HG55 clone (6), yet with a similar $K_d$ value (0.4 nM) (Fig. 1B). In unstimulated cells, CysLT1R was visualized primarily at the plasma membrane, although some puncta were frequently observed, suggesting that the tendency of receptors to internalize in the absence of agonist.

**Co-expression of CysLT1R with either arrestin-2 or arrestin-3 increased LTD4-stimulated internalization (Fig. 2C), suggesting a role for arrestins in mediating agonist-dependent internalization similar to that demonstrated for the β2AR and numerous other GPCRs (30). Co-expression of arrestin mutants (ARR2(R169E) or ARR2(R170E)), of which the binding to GPCRs is largely independent of receptor phosphorylation (21, 31), was no more effective in increasing CysLT1R internalization than was the corresponding wild type arrestin construct.
Co-expression of arrestin-2 or arrestin-3 also reduced LTD4-stimulated PI production, suggesting the capacity of arrestins to promote uncoupling/desensitization as well as internalization of the CysLT1R (Fig. 2D).

In an attempt to establish structural regions of the CysLT1R important in LTD4-stimulated internalization and desensitization, we generated CysLT1R mutants in which the receptor C terminus was progressively truncated (Fig. 3). Although lacking canonical GRK phosphorylation sites (32), the CysLT1R C-tail contains multiple serine/threonine residues that might serve as phosphorylation sites. Constructs encoding CysLT1R, truncated after amino acid 321 or 309, were expressed in COS-1 cells (Fig. 3B), and agonist-stimulated internalization was assessed. Truncation at amino acid 321 (CysLT1R321stop) had no effect on LTD4-stimulated internalization (Fig. 3C). Truncation at amino acid 309 (CysLT1R309stop) resulted in a receptor that internalized −20% compared with 44% for wt CysLT1R. However, LTD4-stimulated PI production mediated by CysLT1R309stop was slightly greater than that observed for wt CysLT1R; PI production by CysLT1R321stop was slightly lower (data not shown). These data implicate the region spanning amino acids 310 to 321 as important in mediating internalization of the CysLT1R.

A mutant lacking the entire cytosolic C-tail (CysLT1R300stop) was also generated and was shown to express at levels approximately one-half that of wt CysLT1R (estimated by immunoblot analysis and cell-surface fluorescence detected by ELISA). However, CysLT1R300stop did not internalize or stimulate PI production in response to LTD4 (data not shown), thus precluding any clear interpretation of data derived from this mutant.

Although the observed effects of co-expression of arrestin-2/3 suggest a role for GRKs and arrestins in CysLT1R regulation, the relevance of these findings is unclear given the supraphysiologic levels of arrestins expressed in this model. More definitive evidence of the role of arrestins in CysLT1R internalization and desensitization was therefore sought by examining the competitive effect of dominant-negative arrestin mutants on wt CysLT1R internalization in HEK 293 cells. The magnitude of
agonist-induced internalization of wt CysLT1R in HEK 293 cells was greater than that observed in COS-1 cells (−60% loss of cell surface receptors compared with −35–40% loss in COS-1 cells) and was also much higher than that observed for isoproterenol-stimulated β2ARs (Fig. 4A). Surprisingly, expression of LIE LD/F391A arrestin-2 (previously shown to bind GPCRs but not clathrin/AP-2, thus effectively inhibiting GPCR internalization mediated by endogenous arrestins) or R169E/LIE LD/F391A arrestin-2 (the same construct with high affinity for GPCRs independent of receptor phosphorylation state) (33) had only a small effect on wt CysLT1R internalization (−15% inhibition by each construct), whereas significantly inhibiting β2AR internalization. Co-expression of CysLT1R with the dynamin mutant DynK44A (34) only partially inhibited (50%) LTD4-stimulated internalization, whereas β2AR internalization was completely inhibited, suggesting that CysLT1R inter-
myoinositol, washed, and then pretreated for 15 min with vehicle (A293 (D–F) cells expressing either WT CysLT1R (1.6 nM) cells. Bis I pretreatment caused a slight reduction in EC50 values for LTD4-stimulated PI production by wt CysLT1R in both COS-1 (3.5–0.8 represent mean S.E. values from four to six experiments.

Fig. 7. Effect of PKC inhibition on WT CysLT1R-, CysLT1RS(313–316)A-, and H1 HR-mediated PI production. COS-1 (A–C) and HEK 293 (D–F) cells expressing either WT CysLT1R (A and D), CysLT1RS(313–316)A (B and E), or H1 HR (C and F) were loaded for 18 h with [3H]myoinositol, washed, and then pretreated for 15 min with vehicle (Veh) or 10 μM Bis I in DMEM containing 5 mM LiCl. Cells were then stimulated with various concentrations of LTD4 or histamine for 30 min, and PI were subsequently extracted and quantified as described under “Experimental Procedures.” The EC50 value for LTD4-stimulated PI production by CysLT1RS(313–316)A was lower than the corresponding value for wt CysLT1R in vehicle-pretreated COS-1 (1.8 nM for CysLT1RS(313–316)A versus 3.5 nM for wt CysLT1R) but not in HEK 293 (1.9 nM versus 1.6 nM) cells. Bis I pretreatment caused a slight reduction in EC50 values for LTD4-stimulated PI production by wt CysLT1R in both COS-1 (3.5–0.8 nM) and HEK 293 (5.0–1.6 nM) cells. Pretreatment with Bis I had no effect on basal PI production assessed in each group (data not shown). Data represent mean ± S.E. values from four to six experiments.

We therefore examined the effect of numerous protein kinase inhibitors on LTD4-stimulated internalization of wt CysLT1R expressed in both COS-1 and HEK 293 cells. Pretreatment with H89, wortmannin, U0126, and genistein all failed to affect CysLT1R internalization (Fig. 6D and 6E). Collectively, these data suggest that the actions of arres-2 and arrestin-3 expression was ablated (arr2/3−/−) (35). Although expression of wt CysLT1R via FuGENE-mediated transfection of pcDNA3-3FLAG CysLT1R proved unsuccessful in MEFs, conditions were established for expression of a human 3-FLAG CysLT1R using a recombinant adenovirus (see “Experimental Procedures”) that enabled analysis of receptor internalization by ELISA. When expressed in arr2/3−/− MEFs, the CysLT1R internalized in response to LTD4 at a level (18% loss of cell surface receptors) comparable with that observed when expressed in MEFs derived from matched nontransgenic controls (~20%, Fig. 4B). Conversely, agonist-stimulated β2AR internalization was minimal in arr2/3−/− MEFs. Collectively, results depicted in Fig. 4 suggest that the actions of arrestins are not required to effect CysLT1R internalization and led us to consider a role for other regulatory molecules.

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Isoforms (see “Experimental Procedures”) that enabled analysis of receptor internalization by ELISA. When expressed in arr2/3−/− MEFs, the CysLT1R internalized in response to LTD4 at a level (18% loss of cell surface receptors) comparable with that observed when expressed in MEFs derived from matched nontransgenic controls (~20%, Fig. 4B). Conversely, agonist-stimulated β2AR internalization was minimal in arr2/3−/− MEFs. Collectively, results depicted in Fig. 4 suggest that the actions of arrestins are not required to effect CysLT1R internalization and led us to consider a role for other regulatory molecules.

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Comparable substrates for PKC include serine/threonine residues, preferably flanked by a lysine or arginine at positions −3, −2, or +2 with a hydrophobic residue at +1 (37), although this consensus is not an absolute requirement because protein secondary structure is also an important determinant. On the basis of our data demonstrating the resistance of the truncation mutant CysLT1R309stop to internalization, we generated a CysLT1R mutant (CysLT1RS(313–316)A) in which serines 313, 315, and 316 (representing putative PKC sites) were mutated to alanines. Comparison of this construct with wt CysLT1R revealed that the selected mutations caused an ~50% loss of maximal internalization in both COS-1 (Fig. 6, A and B) and HEK 293 (Fig. 6, C and D) cells. Interestingly, the EC50 was also slightly decreased for the CysLT1RS(313–316)A mutant in both cell types (see Fig. 6 legend). In addition, the effect of Bis I pretreatment on CysLT1RS(313–316)A was considerably less.
PKC inhibition also dramatically influenced LTD4-stimulated PI production. Pretreatment with Bis I (Fig. 7, A and D) or Bis IX, but not Bis V or other AGC kinase or tyrosine kinase inhibitors (data not shown), significantly increased maximal LTD4-stimulated PI generation by wt CysLT1R while decreasing the EC50 for LTD4 (see Fig. 7 legend). LTD4-stimulated PI production was 2–3-fold higher in CysLT1RS(313–316)A (Fig. 7, B and E) compared with that mediated by wt CysLT1R. Similar to our previous findings (27), only a very small effect of PKC inhibition was observed for histamine-stimulated PI production in cells expressing the H1 HR (Fig. 7, C and F), suggesting that PKC inhibition had minimal effect on either Gq or phospholipase C and that the receptor was the locus of regulation by PKC in LTD4-stimulated PI production. This interpretation was further supported by the observation that PI production in cells expressing CysLT1RS(313–316)A was minimally affected by PKC inhibition (Fig. 7, B and E).

Consistent with these data characterizing PKC-dependent regulation of PI production, pretreatment of COS-1 cells expressing wt CysLT1R with Bis I caused a large increase in LTD4-stimulated peak Ca2+ flux (Fig. 8). Conversely, Bis I pretreatment had only a small effect on peak Ca2+ flux in cells expressing CysLT1RS(313–316)A and no effect on cells expressing H1 HR. Moreover, peak Ca2+ flux mediated by CysLT1RS(313–316)A was significantly higher than that mediated by wt CysLT1R.

To explore the potential role of PKC in mediating heterogeneous (nonagonist-specific) CysLT1R regulation, CysLT1R internalization and signaling were examined in both COS-1 and HEK 293 cells. In COS-1 cells, treatment with 100 nM PMA for 30 min induced a small degree (10%) of internalization of both wt CysLT1R and CysLT1RS(313–316)A (Fig. 9), suggesting heterologous desensitization. The observed effects of PMA treatment on internalization and signaling were all partially reversed by Bis I pretreatment. Qualitatively similar results were obtained in HEK 293 cells (Fig. 9, B and D).

Because CysLT1RS(313–316)A retains the ability to internalize, we considered whether arrestins are important in this (residual) internalization. Co-expression of LIELD/F391A arrestin-2 or R169E/LIELD/F391A arrestin-2 had a slightly greater (25–40%) inhibition of LTD4-induced internalization (Fig. 10) than that observed for the wt CysLT1R (Fig. 2), suggesting that, under certain contexts of attenuated PKC effects, GRK/arrestin-dependent mechanisms of internalization may play a more substantive role.

The role of second messenger-dependent kinases in GPCR regulation is frequently limited to the context of heterologous desensitization. Activation of second messenger kinases by...
other receptor-dependent or -independent pathways can promote the phosphorylation of a GPCR not occupied by its cognate ligand, and this phosphorylation can diminish subsequent agonist-stimulated receptor-G protein coupling. Many GPCRs exhibit this form of heterologous desensitization (38). For a handful of receptors including the CXCR4 (39), somatostatin 2A (40), and endothelial differentiation gene 1 (41) receptors, receptor phosphorylation by PKC seems sufficient to induce internalization of the receptor in the absence of agonist. For agonist-mediated internalization or desensitization, second messenger-dependent kinases typically play little if any role. For the overwhelming majority of GPCRs examined to date, GRK-mediated phosphorylation and arrestin binding are required for and are the principal determinants of agonist-promoted internalization and desensitization, as suggested by the effective inhibitory actions of dominant-negative GRKs and arrestins, and the lack of effect of various inhibitors of other kinases (42). For some GPCRs including the type 1 Angiotensin II (43), endothelial differentiation gene 1 (41), CXCR4 (44), chemokine receptor 5 (45), and somatostatin 2A (40, 46) receptors, PKC appears to play a role in agonist-stimulated receptor phosphorylation. However, the effect of pharmacologic inhibition of PKC is typically only a small to moderate reduction in receptor phosphorylation with little if any effect on agonist-induced receptor desensitization and internalization. These results suggest that PKC-mediated phosphorylation is an innocuous or redundant action that follows receptor stimulation and that GRKs and arrestins are the principal regulators of agonist-occupied GPCRs.

Receptors in which second messenger kinases may play a more significant role in agonist-induced receptor internalization or desensitization are the secretin receptor (a class II GPCR coupled to Gs), the metabotropic glutamate receptor 1a (mGlu1a; a seven-transmembrane domain receptor belonging to a unique class of receptors that bear little sequence or structural homology to class 1 or class 2 GPCRs) and mouse thromboxane A2 receptor (TP). In HEK 293 cells, PKA inhibitors do not inhibit secretin receptor desensitization but reduce secretin-stimulated receptor phosphorylation by 50% (47). Interestingly, dominant-negative arrestins do not inhibit secretin

**Fig. 9.** Effect of PMA treatment on signaling and internalization of wtCysLT1R, CysLT1RS(313–316)A, and H1 HR. A and B, COS-1 (A) and HEK 293 (B) cells expressing wtCysLT1R (wt), CysLT1RS(313–316)A (S(313–316)A), or H1 HR were pretreated for 30 min with vehicle (Veh) or 10 μM Bis I and then stimulated with 100 nM PMA for 30 min. Data represent mean ± S.E. values from four to six experiments. C and D, cells expressing the indicated receptors were loaded for 18 h with [3H]myoinositol, washed, and then pretreated for 15 min with vehicle (Veh) or 10 μM Bis I in DMEM containing 5 mM LiCl. Five min into this pretreatment, cells were then stimulated with 100 nM LTD4 or 10 μM histamine (HIST) for 30 min, and PI production was quantified as described under “Experimental Procedures.” Treatment with PMA alone had no affect on (basal) PI production in any of the groups (data not shown). Data represent mean ± S.E. values from three to five experiments.

**Fig. 10.** Effect of dominant-negative arrestins and dynamin on CysLT1RS(313–316)A internalization. CysLT1RS(313–316)A was co-expressed with dominant-negative arrestins, arrestin 2-LIELD/F391A or arrestin 2-R169E/LIELD/F391A, or with dynamin-K44A (DynK44A) in HEK 293 cells, and LTD4-stimulated internalization was assessed as described under “Experimental Procedures.” Data represent mean ± S.E. values from six experiments.
PKC inhibition has been shown to inhibit both agonist-induced internalization and agonist-induced desensitization of the metabotropic glutamate receptor 1a, although both of these effects have been shown to be GRK- and arrestin-dependent (49–51). Both PKC and GRKs/arrestins seem to play significant roles in agonist-induced desensitization of the Gαi-coupled mouse TP receptor and the human homologue thromboxane A2α receptor. The PKC inhibitor staurosporine partially reversed (~40%) homologous desensitization of the mouse TP receptor and also markedly attenuated agonist-induced receptor phosphorylation (52). Dominant-negative GRKs also inhibited agonist-induced phosphorylation and enhanced TP signaling (53), demonstrating a significant role for GRKs as well in homologous TP receptor desensitization. Agonist-induced phosphorylation of the human TP receptor is also significantly reversed by PKC inhibition (54). Interestingly, the TP-α receptor does not exhibit agonist-induced internalization, except when co-expressed with arrestin-2 or arrestin-3 (55).

Unlike other class 1 GPCRs examined to date, the CysLT1R exhibits rapid agonist-induced internalization that is primarily dependent on PKC. Because the effects of dominant-negative arrestin overexpression and arrestin2/3 gene ablation are minimal on agonist-induced CysLT1R internalization, the mechanism by which PKC acts is dissociated from that involving GRKs/arrestins and therefore is novel among all GPCRs.

Moreover, PKC also appears to profoundly influence homologous desensitization, identifying CysLT1R as the only GPCR examined to date in which both agonist-induced internalization and agonist-induced desensitization are primarily PKC-dependent and arrestin-independent. LT4-stimulated PI production and calcium mobilization are greatly increased in the presence of Bis I in cells expressing wt CysLT1R but not in cells expressing either the CysLT1R(S313–316)A, which lacks putative PKC phosphorylation sites, or the H1 HR, which similarly couples to (apparently desensitization-resistant in COS-1 and HEK 293) Gαi and phospholipase C. Although the disparate effects of PKC inhibition on the regulation of wt CysLT1R versus CysLT1R(S313–316)A suggest that PKC-mediated phosphorylation of CysLT1R on residues within 313–316 mediates the effects of PKC on CysLT1R desensitization and internalization, it is important to note that this remains to be demonstrated directly. Our attempts to date to establish PKC-dependent phosphorylation of the CysLT1R have been frustrated by an inability to express the receptor at levels sufficient to permit detection of 32P incorporation in receptors immunoprecipitated from orthophosphate-loaded cells. Our experience is that to detect agonist-dependent phosphorylation of GPCRs, expression levels of at least 1 pmol/mg protein must be achieved. To date, we know of no group that has been able to achieve expression of the CysLT1R, in either cell lines or transgenic mice designed for overexpression (56), that approaches this level. Possible explanations for the failure to achieve such expression is either that the cellular synthetic machinery is constrained by inherent structural features of the receptor or that a high level of expression of CysLT1R may be toxic to the cell.

Of interest is the slight difference in magnitude of effect of PKC inhibition on CysLT1R internalization in COS-1 versus HEK 293 cells. In COS-1 cells, the effect of Bis I pretreatment on wt CysLT1R internalization is profound (>80% inhibition), whereas in HEK 293 cells the effect is less dramatic (~50%) but still prominent. In both cell types, maximal internalization of the CysLT1R(S313–316)A mutant is low (~50% of that of the wild type) and is only minimally affected by Bis I. Why Bis I has a greater effect in COS-1 cells is unclear. One possible explanation is that the greater levels of arrestins (57) and GRK2 (58) in HEK 293 cells may favor a slightly greater role for PKC/arrestin-mediated regulation, consistent with the results of Fig. 2 demonstrating that increased arrestin-2 or arrestin-3 expression can augment CysLT1R internalization. Moreover, in both COS-1 and HEK 293 cells, CysLT1R(S313–316)A retains some ability to internalize that is not inhibited by Bis I (Fig. 6) yet is slightly inhibited by dominant-negative arrestin 2 (Fig. 9), suggesting that a non-PKC (arrestin?)-dependent mechanism may acquire significance under certain conditions. Collectively, these findings suggest the intriguing possibility that PKC and GRKs/arrestins may be competitive with respect to CysLT1R regulation, and their relative effects may depend on relative expression levels and subcellular distribution, the latter influenced by competition among intracellular substrates for kinase activity. Future studies examining CysLT1R regulation employing strategies similar to those utilized by Kohout et al. (35) to assess arrestin selectivity among GPCRs, as well as analyses of primary cell types relating endogenous kinase/arrestin:receptor stoichiometry and CysLT1R signaling, will help clarify the physiologic roles of PKC, GRKs, and arrestins in CysLT1R regulation.

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