Agonist-Induced Desensitization/Resensitization of Human G Protein-Coupled Receptor 17: A Functional Cross-Talk between Purinergic and Cysteinyl-Leukotriene Ligands

S. Daniele, M. L. Trincavelli, P. Gabelloni, D. Lecca, P. Rosa, M. P. Abbracchio, and C. Martini

Department of Psychiatry, Neurobiology, Pharmacology and Biotechnology, University of Pisa, Pisa, Italy (S.D., M.L.T., P.G., C.M.); and Laboratory of Molecular and Cellular Pharmacology of Purinergic Transmission, Department of Pharmacological Sciences (D.L., M.P.A.), and Consiglio Nazionale delle Ricerche Institute of Neuroscience, Department of Medical Pharmacology, University of Milan, Milan, Italy (P.R.)

Received December 30, 2010; accepted April 28, 2011

ABSTRACT

G protein-coupled receptor (GPR) 17 is a P2Y-like receptor that responds to both uracil nucleotides (as UDP-glucose) and cysteinyl-leukotrienes (cysLTs, as LTD4). By bioinformatic analysis, two distinct binding sites have been hypothesized to be present on GPR17, but little is known on their putative cross-regulation and on GPR17 desensitization/resensitization upon agonist exposure. In this study, we investigated in GPR17-expressing 1321N1 cells the cross-regulation between purinergic- and cysLT-mediated responses and analyzed GPR17 regulation after prolonged agonist exposure. Because GPR17 receptors couple to G proteins and adenylyl cyclase inhibition, both guanosine 5'-O-(3-thio)triphosphate ([35S]GTP[S]) binding and the cAMP assay have been used to investigate receptor functional activity. UDP-glucose was found to enhance LTD4 potency in mediating activation of G proteins and vice versa, possibly through an allosteric mechanism. Both UDP-glucose and LTD4 induced a time- and concentration-dependent GPR17 loss of response (homologous desensitization) with similar kinetics. GPR17 homologous desensitization was accompanied by internalization of receptors inside cells, which occurred in a time-dependent manner with similar kinetics for both agonists. Upon agonist removal, receptor resensitization occurred with the typical kinetics of G protein-coupled receptors. Finally, activation of GPR17 by UDP-glucose (but not vice versa) induced a partial heterologous desensitization of LTD4-mediated responses, suggesting that nucleotides have a hierarchy in producing desensitizing signals. These findings suggest a functional cross-talk between purinergic and cysLT ligands at GPR17. Because of the recently suggested key role of GPR17 in brain oligodendrogligenesis and myelination, this cross-talk may have profound implications in fine-tuning cell responses to demyelinating and inflammatory conditions when these ligands accumulate at lesion sites.

Introduction

The P2Y-like, G protein-coupled receptor (GPR) GPR17 is structurally and phylogenetically related to previously identified P2Y receptors (P2YRs) for extracellular nucleotides and to the cysteiny1-leukotriene 1 and 2 receptors (CysLT1 and CysLT2, respectively), which respond to arachidonic acid-derived cysteiny1-leukotrienes (cysLTs).

Previously, it has been demonstrated that on both the native (Daniele et al., 2010; Ceruti et al., 2011; Fumagalli et al., 2011) and the recombinant receptors (Ciana et al., 2006; Lecca et al., 2008; Pugliese et al., 2009; Temporini et al., 2009; Calleri et al., 2010), GPR17 acts as a classic ligand-activated GPR that responds to both uracil nucleotides (UDP, UDP-glucose, and UDP-galactose; Jacobson and Boeynaems, 2009) and the cAMP assay have been used to investigate receptor functional activity. UDP-glucose was found to enhance LTD4 potency in mediating activation of G proteins and vice versa, possibly through an allosteric mechanism. Both UDP-glucose and LTD4 induced a time- and concentration-dependent GPR17 loss of response (homologous desensitization) with similar kinetics. GPR17 homologous desensitization was accompanied by internalization of receptors inside cells, which occurred in a time-dependent manner with similar kinetics for both agonists. Upon agonist removal, receptor resensitization occurred with the typical kinetics of G protein-coupled receptors. Finally, activation of GPR17 by UDP-glucose (but not vice versa) induced a partial heterologous desensitization of LTD4-mediated responses, suggesting that nucleotides have a hierarchy in producing desensitizing signals. These findings suggest a functional cross-talk between purinergic and cysLT ligands at GPR17. Because of the recently suggested key role of GPR17 in brain oligodendrogligenesis and myelination, this cross-talk may have profound implications in fine-tuning cell responses to demyelinating and inflammatory conditions when these ligands accumulate at lesion sites.

ABBREVIATIONS: GPR, G protein-coupled receptor; cysLT, cysteinyl-leukotriene; CysLT, cysteiny1-leukotriene receptor; DTT, dithiothreitol; FK, forskolin; hGPR17, human GPR17; OPC, oligodendrocyte precursor cell; P2YR, P2Y receptor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RT, reverse transcription; bp, base pair.
cysLT (Parravincini et al., 2008, 2010); however, it is still unclear if GPR17 predominantly behaves as a purinergic receptor that may be able to modulate responses to cysLTs through a protein-protein interaction mechanism.

Previously, it was demonstrated that the presence of the GPR17 transcript and protein is restricted to very early stages of differentiation in oligodendrocyte precursor cells (OPCs) and is segregated completely from mature myelin proteins to the point that mature myelinating oligodendrocytes no longer express GPR17 (Lecca et al., 2008; Ceruti et al., 2011; Fumagalli et al., 2011). GPR17 endogenous ligands have also been found to promote OPC maturation (Lecca et al., 2008; Ceruti et al., 2011). Therefore, these ligands first induce early precursor cells to undergo their differentiation by binding to GPR17; then, at a certain stage of OPC differentiation, they also trigger receptor desensitization with subsequent intracellular internalization and degradation, contributing to its removal from the cell membrane. A similar process has been associated with the specification of other cell lineages (e.g., erythrocytes), in which the down-regulation of membrane receptors for key trophic or differentiating factors has been proposed as necessary to allow cells to proceed toward terminal maturation (Walrafen et al., 2005).

Desensitization is a common process that regulates the functional responsiveness of both P2YRs (Otero et al., 2000; Santiago-Pérez et al., 2001) and CysLTs (Winkler et al., 1988). Heterologous regulation between P2YRs and CysLTs has been demonstrated in human monocyte/macrophage-like cells, in which the activation of P2YRs with ATP or UDP induced the desensitization of the CysLT1. In contrast, LTD4-induced CysLT1 activation had no effect on the responses of P2YRs (Capra et al., 2005).

Although much has been learned about the cellular activation and regulation of each class of GPR17 ligand, studies still are being performed on the mechanisms of GPR17 cross-regulation that lead to priming or desensitization. Therefore, the present study was undertaken to assess the possible interactions between the two putative GPR17 purinergic and cysLT binding sites in a recombinant GPR17 expression system and to characterize GPR17 homologous/heterologous desensitization/resensitization by cysLT and purinergic ligands.

The two classes of GPR17 ligands were demonstrated to interact with each other through allosteric modulation. Furthermore, both UDP-glucose and LTD4 induced time-dependent receptor desensitization and internalization with kinetic rates comparable for the two classes of ligands. After induction of the desensitization, receptor responsiveness was recovered with kinetic values that were typical of GPRs. Finally, the activation of the GPR17 purinergic site by UDP-glucose (but not vice versa) induced the heterologous desensitization of LTD4-mediated responses, with resensitization kinetics comparable to those of the purinergic site.

These results suggest the existence of a functional cross-talk between cysLT and purinergic sites on GPR17 and that the latter have a hierarchy in producing desensitizing signals.

Materials and Methods

Chemicals and Reagents. Dulbecco's modified Eagle's medium and all of the reagents for cell culture were purchased from Lonza Milano, S.r.l. (Caravaggio, Italy). [35S]GTPγS and [3H]cAMP were purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). UDP-glucose was purchased from Sigma-Aldrich (St. Louis, MO), and LTD4 was purchased from Cayman Europe (Tallinn, Estonia). Cangrelor was provided by The Medicines Company (Parsippany, NJ). Other chemicals were supplied by other commercial sources.

Cell Culture and Stable Transfection. Astrocytoma cells (1321N1) were seeded onto 75-cm² flasks (10⁶ cells) in Dulbecco's modified Eagle's medium and then transfected with pcDNA 3.1-human GPR17 (hGPR17) or with the plasmid alone using the calcium phosphate precipitation method, as described previously (Pugliese et al., 2009).

RNA Isolation and Reverse Transcription Polymerase Chain Reaction. Total RNA (1 μg) was reverse-transcribed using a Quant-it Transcription kit (Qiagen, Valencia, CA) in a total volume of 20 μl. Two microliters of the reverse transcription mixture was used for polymerase chain reaction (PCR). The PCR mixture contained a total volume of 25 μl consisting of 200 μM of each deoxynucleotide triphosphate, 300 nM forward and reverse primers (Table 1), 1.5 mM MgCl2, and 1 μl of AmpliTaq Gold (Applied Biosystems, Foster City, CA) in an equipped buffer. Thermal cycling was performed with an initial denaturation of 5 min at 94°C and followed by 35 cycles of 1 min at 95°C, 30 s at annealing temperatures, and 1 min at 72°C, with a terminal extension of 7 min at 72°C. PCR products were separated by agarose gel electrophoresis on 2.5% UltraPure agarose-Tris-borate EDTA gels (Invitrogen, Carlsbad, CA), which were visualized by ethidium bromide staining under UV light.

[35S]GTPγS Binding Assay. Control and transfected cells were homogenized in 5 mM Tris-HCl and 2 mM EDTA (pH 7.4) and then centrifuged at 4,000 g for 15 min at 4°C. The resulting pellets (plasma membranes) were washed in 50 mM Tris-HCl and 10 mM MgCl2 (pH 7.4) and then stored at −80°C until needed. Nucleotide-cysLT-stimulated [35S]GTPγS binding assays in the membranes of cells expressing hGPR17 or pcDNA 3.1 were performed as described previously (Ciana et al., 2006; Pugliese et al., 2009; Calleri et al., 2010) in the absence or presence of nucleotides or cysLTs.

To determine the possible interaction between nucleotides or cysLTs, membranes were preincubated with LTD4 (5 or 50 nM) or UDP-glucose (10 or 100 μM) and then stimulated with different UDP-glucose or LTD4 concentrations, respectively.

In some experiments, the effects of GPR17 antagonists on agonist-mediated effects were evaluated. In particular, the adenosine-based P2Y12–13 antagonist cangrelor (formerly AR-C69931MX; Marteau et al., 2003; Fumagalli et al., 2004; Srinivasan et al., 2009) and the CysLT1 antagonist 2-[2-[(3R)-2-[(3R)-2-(7-chloroquinolin-2-yl)ethyl]phenyl]-3-sulfanylpropyl]phenylpropan-2-ol (montelukast; Lynch et al., 1999; Heise et al., 2000), which have been characterized previously and demonstrated to have an high affinity for GPR17 (Ciana et al., 2006; Pugliese et al., 2009), were used. The antagonists were added for 5 min before addition of UDP-glucose and LTD4 to determine the inhibition of the agonist-mediated G protein activation.

Measurement of cAMP Levels. Intracellular cAMP levels were measured using a competitive protein binding method, as reported previously (Colotta et al., 2008; Trincavelli et al., 2008; Fumagalli et al., 2009).

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primer sequences and annealing temperature for GPR17, P2YR14, CysLT receptors, and β-actin</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>Primer Sequences</th>
<th>Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P2Y14</td>
<td>Forward: 5'-CGG AAC ATG TTA AAC ATC AGT TGG T-3'</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-GCT GTA AGG ACC TCG GGT CGT AC-3'</td>
<td>51</td>
</tr>
<tr>
<td>GPR17</td>
<td>Forward: 5'-CCC TGG CTC TCT TGT GCG TTC T-3'</td>
<td>51</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-TCT CTG TGT TTT CCC CTT CG-3'</td>
<td>56</td>
</tr>
<tr>
<td>CysLT</td>
<td>Forward: 5'-ATGAGCGACATGACCTTTTTTC-3'</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CATCTTTCTGATGCCGTACCA-3'</td>
<td>56</td>
</tr>
<tr>
<td>CysLT</td>
<td>Forward: 5'-CGCCGATTTGAATCTCCCTGTT-3'</td>
<td>57</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CGCGCGCCGCTTCGCGAGCGG-3'</td>
<td>60</td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward: 5'-CGG CTC TCC CAC GAG CTC CAC AGC ACC-3'</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Reverse: 5'-CGG CCG CCA ATG CAC ACC AGC-3'</td>
<td>50</td>
</tr>
</tbody>
</table>
2011). In brief, 1321N1 cells were seeded on 24-well plates (5.0 × 10^4 cells/well) in 0.5 ml of medium. After 24 h, the entire medium was removed, and the cells were incubated at 37°C for 15 min with 0.4 ml of Dulbecco’s modified Eagle’s medium in the presence of the phosphodiesterase inhibitor 4-[[(3-butoxy-4-methoxyphenyl)-methyl]-2-imidazo-lidonine (Ro20-1724) (20 μM).

The functional responses of purinergic and cysLT ligands on hGPR17 were evaluated by assessing their abilities to inhibit cAMP accumulation stimulated by 10 μM forskolin (PK). Cells were stirred for 15 min with 10 μM UDP-glucose or 10 nM LTD4. The reaction was terminated by the removal of the medium and the addition of 200 μl of 0.4 N HCl. After 30 min, lysates were neutralized with 50 μl of 4 N KOH, and the suspension was centrifuged at 800g for 5 min. For the determination of cAMP production, the cAMP binding protein, isolated from bovine adrenal glands, was incubated with [3H]cAMP (2 nM), and 50 μl of cell lysate or cAMP standard (0–16 fmol) at 0°C for 150 min in a total volume of 300 μl. The bound radioactivity was separated by rapid filtration through GF/C glass fiber filters (PerkinElmer Life and Analytical Sciences) and washed twice with 4 ml of 50 mM Tris-HCl (pH 7.4). The radioactivity was measured by liquid scintillation spectrometry.

For homologous and heterologous desensitization experiments, the cells were treated with UDP-glucose (100 nM to 100 μM) or LTD4 (0.5–100 nM) at different times (5–90 min). Then, cells were washed and stimulated with UDP-glucose (10 μM) or LTD4 (10 nM) for 15 min, and CAMP levels were quantified. In desensitization experiments, the cells were treated with UDP-glucose (100 μM) or LTD4 (100 nM) for 90 min to induce GPR17 desensitization, and then the cells were washed in the absence of agonists for 30 to 120 min.

**Biotinylation Experiments.** GPR17-transfected cells (1321N1) were seeded onto 100-mm2 Petri dishes to reach 80% confluence. When required, cells were incubated subsequently in noncompleting medium in the absence or presence of 100 μM UDP-glucose or 10 nM LTD4 for 15 or 30 min. After being washed with ice-cold PBS containing 0.1 mM CaCl2 and 1 mM MgCl2 for 30 min at 4°C, the labeled cells were washed twice for 10 min with 50 mM glycine in Tris-buffered saline (25 mM Tris, 85 mM NaCl, 5 mM KCl, 1 mM CaCl2, and 1 mM MgCl2) to quench the free biotin. When required, cells were incubated subsequently in noncompleting medium in the absence or presence of 100 μM UDP-glucose or 100 nM LTD4 for 15 or 30 min. After being washed at 4°C with ice-cold PBS containing 0.1 mM CaCl2 and 1 mM MgCl2, the remaining biotin label on the surface was removed by incubating cells twice at 4°C with 50 mM dithiothreitol (DTT) in PBS with 0.1 mM CaCl2 and 1 mM MgCl2 for 15 min each time. DTT was neutralized with iodoacetamide (10 mM) in PBS containing 0.1 mM CaCl2 and 1 mM MgCl2, and cells were then lysed in buffer A, which contained 1% Triton X-100 and a protease inhibitor cocktail. After centrifugation (20,000g for 20 min at 4°C), supernatants containing equal amount of protein were incubated with streptavidin beads to immunoprecipitate the biotinylated proteins (Martin and Henley, 2004; Winterstein et al., 2008). After extensive washes in extraction buffer, proteins were eluted from the streptavidin beads by heating at 65°C in Laemmli sample buffer and then were analyzed by SDS-polyacrylamide gel electrophoresis followed by Western blot analysis using a homemade antibody against GPR17 (1:1000) (Lecca et al., 2008; Fumagalli et al., 2010). The primary antibody was detected using anti-rabbit IgG light chains conjugated to peroxidase (diluted 1:50,000). The peroxidase was detected using a chemiluminescent substrate (PerkinElmer Life and Analytical Sciences).

**Data Analysis.** A nonlinear multipurpose curve-fitting program, Prism (GraphPad Software Inc., San Diego, CA), was used for the binding data analysis of intracellular cAMP and [35S]GTPγS; in addition, the EC_{50} values were derived. Data are reported as the means ± S.E.M. of three or four different experiments (performed in duplicate). Statistical analyses of binding data were performed using a one-way analysis of variance study followed by the Bonferroni test for repeated measurements. Differences were considered statistically significant when P < 0.05. The densitometric analysis of immunoreactive bands was performed using the ImageJ program (National Institutes of Health, Bethesda, MD).

**Results**

**Expression Profile of 1321N1 Cells.** Because the GPR17 ligands UDP-glucose and LTD4 also activate P2Y$_{14}$R (Das et al., 2010) and CysLT1Rs (Lynch et al., 1999; Takasaki et al., 2000), the expression profile and stability of these receptors were evaluated in 1321N1 cells transfected with hGPR17. Reverse transcription (RT)-PCR analysis, performed using specifically designed primers for various cloned receptors, revealed that fragments sizes corresponded to the expected amplified products for GPR17 [891 base pair (bp)] and P2Y$_{14}$ (102 bp) even if to a lesser extent. No amplification products were observed using specific primers for both CysLT1 (479 bp) and CysLT2 (370 bp) (Fig. 1).

**Interaction between Purinergic and cysLT GPR17 Binding Sites.** To detect interactions between nucleotides and cysLTs in GPR17-expressing cells, an analysis of [35S]GTPγS binding was performed. This assay was based on the fact that GPR stimulation by agonists results in increased binding of GTP to G proteins, which can be quantified by measuring [35S]GTPγS binding to purified membranes (Martearu et al., 2003). This assay has been used widely for the characterization of GPR17 responses by testing the increase of [35S]GTPγS binding by exogenously added agonists in transfected cells (Ciana et al., 2006; Lecca et al., 2008; Pugliese et al., 2009; Temporini et al., 2009; Calleri et al., 2010). Optimal [35S]GTPγS binding conditions were determined in preliminary experiments. To determine purinergic/cysLT interactions, crude membranes were preincubated with LTD4 (5 or 50 nM) or UDP-glucose (10 or 100 μM) and then stimulated with different UDP-glucose or LTD4 concentrations, respectively. Absolute values of bound [35S]GTPγS corresponded to 46.78 ± 2.77 fmol/mg protein under basal, unstimulated conditions; in the presence of 10 and 100 μM UDP-glucose alone, these values were increased to 65.24 ± 4.72 and 69.49 ± 3.80 fmol/mg protein, respectively; in the presence of 5 and 50 nM LTD4 alone, basal levels were increased to 55.83 ± 1.58 and 68.89 ± 3.16 fmol/mg, respectively. The

**Fig. 1. RT-PCR analysis of P2Y$_{14}$R, GPR17, and CysLT1 and CysLT2 receptors in human 1321N1 cells expressing GPR17.** As expected, the RT-PCR analysis with specific primers under Materials and Methods revealed the presence of amplified products corresponding to GPR17 (lane 2) and low levels of P2Y$_{14}$R (lane 1). β-Actin also was amplified as an internal control (lane 5). No amplification products were obtained for either CysLT1 or CysLT2 (lanes 3 and 4).
results from the interaction experiments demonstrate that LTD₄, at two concentrations (one corresponding to its EC₅₀ value and the other one 10-fold over it), was able to induce a leftward shift of the UDP-glucose dose-response curve, causing a significant decrease in the UDP-glucose EC₅₀ value from 8.8 ± 0.5 μM to 176.8 ± 11.2 nM (P < 0.001) and 180.9 ± 14.0 nM (P < 0.001), respectively (Fig. 2A; Supplemental Fig. 1A). These results demonstrate that LTD₄ increased the potency of UDP-glucose toward GPR17. In a similar manner, UDP-glucose, at two concentrations (one corresponding to its EC₅₀ value and the other one 10-fold over it), was able to enhance the LTD₄-induced response, causing a leftward shift of the LTD₄ dose-response curve and an increase in its potency (Fig. 2B; Supplemental Fig. 1B). In particular, the LTD₄ EC₅₀ value significantly decreased from 9.06 ± 0.75 to 0.33 ± 0.02 nM (P < 0.001) and 0.32 ± 0.01 nM (P < 0.001), respectively. In this case, an increase of the maximal effect also was observed (Fig. 2B). The activation of GPR17 by either purinergic or cysLT ligands was speculated to induce a change in the receptor conformational state, enhancing the functional responsiveness of either class of endogenous ligands. Both agonists did not induce any significant stimulation of [³⁵S]GTPγS binding in pcDNA 3.1-transfected cells, demonstrating that their effects were mediated specifically by GPR17 activation (data not shown).

To verify if two distinct binding sites (one for nucleotides and the other for cysLTs) were present on GPR17 (Parravicini et al., 2008, 2010), the effects of the CysLT antagonist (montelukast) and the purinergic antagonist (cangrelor) were tested on the LTD₄- and UDP-glucose-induced responses, respectively. Montelukast inhibited LTD₄-mediated responses with an IC₅₀ value of 23.0 ± 1.4 nM that was comparable with that from previous studies (Ciana et al., 2006). This CysLT antagonist also was able to partially impair UDP-glucose-mediated responses with a potency in the micromolar range (IC₅₀ = 1.10 ± 0.65 μM), although the purinergic agonist effects were not abolished completely (Fig. 3A). Cangrelor antagonized LTD₄- and UDP-glucose-mediated responses with a potency in the nanomolar and sub-nanomolar ranges (IC₅₀ = 104.0 ± 7.5 and 0.81 ± 0.05 nM), respectively (Fig. 3B). As demonstrated for montelukast, the antagonistic effect of cangrelor toward LTD₄ was not complete. These data suggest that montelukast and cangrelor act as allosteric modulators for the purinergic and cysLT sites, respectively.

**Homologous Desensitization/Resensitization of GPR17.**

To verify the kinetics and the extent of agonist-induced GPR17 desensitization, the cAMP assay was used. Previous data on both recombinant (Ciana et al., 2006) and native GPR17 (Fumagalli et al., 2011) have shown that, as expected for a G₁₅-coupled receptor, GPR17 inhibits adenylyl cyclase activity and cAMP formation. Therefore, the activation of GPR17 can be studied by assessing the abilities of its agonists to inhibit the cAMP formation stimulated by the direct adenylyl cyclase activator FK (Fumagalli et al., 2011). As a first step, 1321N1 cells were treated at different time points (5–90 min) with 10 nM LTD₄ or 10 μM UDP-glucose. Cells then were washed with 400 μl of saline buffer, and the cAMP assay was performed, as described above. Figure 4 shows that, at submaximal concentrations, both UDP-glucose (Fig. 4A and LTD₄ (Fig. 4B) decreased GPR17 responses after an initial challenge with the same concentration of either UDP-glucose or LTD₄. These effects occurred in a time-dependent manner, resulting in an almost complete inhibition (>85%) of GPR17 activation after 90 min of preincubation with either agonist. The two agonists induced homologous desensitization of GPR17 with a similar kinetics. In a different set of experiments, the maximal percentage reduction of the UDP-glucose-mediated responses after an initial challenge with 10 nM LTD₄ or 10 μM UDP-glucose was determined. These effects occurred in a time-dependent manner, resulting in an almost complete inhibition (>85%) of GPR17 activation after 90 min of preincubation with either agonist. The two agonists induced homologous desensitization of GPR17 with a similar kinetics. In a different set of experiments, the maximal percentage reduction of the UDP-glucose-mediated responses after an initial challenge with 10 nM LTD₄ or 10 μM UDP-glucose was determined.
glucose and LTD₄ responses after homologous desensitization ranged from 77 to 83% and from 78 to 89%, respectively.

Cells then were pretreated with graded UDP-glucose or LTD₄ concentrations for 90 min (the time at which maximal desensitization had been observed with the experiments described in Fig. 4), and after the agonist was removed and the cells were washed, GPR17 responsiveness was measured by assessing the FK-stimulated cAMP response in the presence of either 10 μM UDP-glucose or 10 nM LTD₄, respectively. The results demonstrated that responses to both UDP-glucose (Fig. 5A) and LTD₄ (Fig. 5B) were markedly reduced after a 90-min initial exposure to either agonist. The loss of GPR17 responsiveness was clearly dependent on the agonist concentration used for the initial challenge, with a minor loss of response at low agonist concentrations and an almost total loss of responsiveness in cells that had been preincubated with the highest UDP-glucose (100 μM) or LTD₄ (100 nM) concentration.

To assess whether the agonist-induced loss of GPR17 responsiveness could be reverted by placing cells in agonist-free medium, a time course recovery rate analysis of the receptor functionality was performed after LTD₄- or UDP-glucose-induced desensitization. Thus, for both UDP-glucose and LTD₄, an exposure time and agonist concentration were chosen at the maximal values observed for the desensitization, based on data reported in Figs. 5 and 6. Thus, cells were incubated for 90 min with UDP-glucose (100 μM) and then washed and maintained in agonist-free medium for various times periods (30–120 min). After each wash-out period, GPR17 responsiveness on cAMP assays was assessed by incubating cells with FK and 10 μM UDP-glucose. Results showed that, after UDP-glucose-induced desensitization, the GPR17 response to the uracil nucleotides was restored completely after 90 min. The recovery of responsiveness was gradual and time-dependent, with a kinetics trend typical of that of a GPR (Fig. 6A).

To evaluate the recovery of GPR17 functional responsiveness to cysLTs, cells were incubated for 90 min with 100 nM LTD₄, washed out, and maintained in agonist-free medium for the indicated times (30–120 min). After each wash-out period, GPR17 responsiveness on cAMP assay was assessed by incubating cells with FK and 10 nM LTD₄. Results indicated that, after LTD₄-induced desensitization, recovery of GPR17 responsiveness to this cysLT occurred with kinetics values that were basically similar to those observed after

---

**Fig. 4.** Time dependence of GPR17 homologous desensitization of GPR17. Cells were pretreated for the indicated time periods (5–90 min) with the medium or with a fixed concentration of the agonist [10 μM UDP-glucose (A) or 10 nM LTD₄ (B)]. Agonists then were removed, cells were washed, and the GPR17 responsiveness was assessed by adding 10 μM FK with either 10 μM UDP-glucose (A) or 10 nM LTD₄ (B), respectively. Intracellular levels of cAMP then were evaluated. Data are expressed as the percentage of basal cAMP levels set to 100% and represent the means ± S.E.M. of three separate experiments, each performed in duplicate. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus FK; #, P < 0.05; ##, P < 0.01; ###, P < 0.001 versus UDP-glucose or LTD₄.

**Fig. 5.** Concentration dependence of GPR17 homologous desensitization of GPR17. Cells were pretreated for a fixed time period (90 min) with only the medium or with the indicated concentrations of UDP-glucose (A) or LTD₄ (B). Agonists then were removed, cells were washed, and the GPR17 responsiveness was assessed in the presence of 10 μM FK and either 10 μM UDP-glucose or 10 nM LTD₄, respectively. Intracellular levels of cAMP then were evaluated. Data are expressed as the percentage of basal cAMP levels set to 100% and represent the means ± S.E.M. of three separate experiments, each performed in duplicate. *, P < 0.05; **, P < 0.01; ***, P < 0.001 versus FK; #, P < 0.05; ##, P < 0.01; ###, P < 0.001 versus UDP-glucose or LTD₄.
UDP-glucose, although the resensitization seemed complete within 60 min (Fig. 6B).

**Heterologous Desensitization/Resensitization of GPR17.**
To investigate the existence of the cross-desensitization between the two putative purinergic and cysLT sites on GPR17, the GPR17 responsiveness to UDP-glucose was measured after an initial challenge with LTD4 and vice versa.

Figure 7B shows that UDP-glucose-induced responses were totally unaffected by a prior 90-min challenge with different LTD4 concentrations, demonstrating that activation of the putative cysLT binding site on GPR17 was not able to influence the functionality of the GPR17 purinergic binding site, at least in 1321N1 cells.

In contrast, after a 90-min treatment with UDP-glucose, a modest but statistically significant decrease in the LTD4-mediated response was detected, especially at the highest UDP-glucose concentrations used (Fig. 7A). In a different set of experiments, the maximal percentage reduction of the LTD4 response after UDP-glucose cell treatments ranged from 52 to 74%.

It is clear from these data that the purinergic ligand was able to partially mediate a heterologous regulation of the putative cysLT binding site on GPR17, whereas the cysLT GPR17 ligand did not affect the functional responses mediated by the purinergic binding site.

To assess the recovery rate of GPR17 responsiveness to cysLT ligands, after heterologous desensitization, cells were incubated for 90 min with UDP-glucose (100 μM), washed with saline buffer, and then maintained in agonist-free medium for various time periods (30–120 min). After each washout period, GPR17 responsiveness on cAMP assays was assessed by incubating cells with FK in the presence of 10 nM LTD4. After heterologous desensitization, the kinetics of recovery for GPR17 functional responsiveness to LTD4 occurred within 90 to 120 min (Fig. 8A). In parallel, GPR17 functional response to UDP-glucose after LTD4 cell treat-
ment was evaluated during all of the time periods of the cell washout. Cells were incubated for 90 min with LTD₄ (100 nM), washed with saline buffer, and then maintained in agonist-free medium for various times (30–120 min). After each wash-out period, GPR17 responsiveness on cAMP assays was assessed by incubating cells with FK in the presence of 10 μM UDP-glucose. The results (Fig. 8B) demonstrate that GPR17 remained completely responsive to UDP-glucose after the LTD₄ cell challenge and subsequent washout, confirming that LTD₄ does not affect UDP-glucose-mediated responses.

**GPR17 Intracellular Trafficking after Agonist Administration.** To further investigate the mechanisms at the basis of GPR17 desensitization upon ligand binding and to specifically assess induction of receptor intracellular internalization, we decided to use biotinylation assays to follow receptor destiny after agonist exposure. To this purpose, receptors on the cell plasma membrane surface were labeled with a disulfide-containing biotinylating reagent under conditions that prevent receptor trafficking (incubation of the cells at 4°C). After being washed, cells were extracted either immediately or after biotin stripping of cell surface proteins using a disulfide-reducing reagent (DTT). Extracts then were blotted with an anti-GPR17 antibody (Fig. 9A). In the absence of DTT, a single band corresponding to an apparent molecular mass of approximately 55 kDa was detected. DTT treatment and biotin stripping from the plasma membrane resulted in the almost complete disappearance of this band (Fig. 9A), demonstrating that, under these conditions, all of the receptors are at the cell surface.

Cell surface biotinylated cells then were incubated with or without agonists at 37°C (i.e., under conditions allowing receptor trafficking). After a 15- or 30-min incubation, cells were cooled, and the remaining surface biotin was stripped with DTT as described above. Exposure of cells to UDP-glucose activated the endocytosis of GPR17, as shown by the fact that stimulated cells had more biotinylated internalized GPR17 than nonstimulated cells (constitutive internalization, white column in Fig. 9C). Quantitative analysis of at least three independent experiments showed that, after cell treatment with UDP-glucose for 15 and 30 min, 17.35 ± 2.25% and 60.0 ± 4.49% of surface biotinylated receptors, respectively, internalized inside cells. When cells were probed with LTD₄, GPR17 underwent less efficient endocytosis: only 12.1 ± 1.7% and 34.5 ± 2.7% of the receptors were found internalized inside cells after agonist exposure for 15 and 30 min, respectively.

**Discussion**

Data from our group obtained from a number of different assays, such as [³⁵S]GTPγS binding assays (Ciana et al., 2006; Lecca et al., 2008; Calleri et al., 2010), adenylyl cyclase assays (Ciana et al., 2006; Fumagalli et al., 2011), frontal affinity chromatography-mass spectrometry (Temporini et al., 2009; Calleri et al., 2010), whole-cell patch-clamp recording (Pugliese et al., 2009), and extracellular signal-regulated kinase 1/2 and p38 phosphorylation (Daniele et al., 2010), have shown that heterologously expressed GPR17 is responsive to two classes of ligands: uracil nucleotides and cysLTs (Ciana et al., 2006; Pugliese et al., 2009). This pharmacological profile also has been confirmed recently in two cell systems that natively express GPR17 by using some of the assays mentioned above as well as survival and cell differentiation assays (Daniele et al., 2010; Fumagalli et al., 2011). However, the “dual” pharmacological profile of GPR17 remains to be confirmed because of conflicting data that have been reported. Recently, the purinergic component of GPR17 has been confirmed by another independent group (Benned-Jensen and Rosenkilde, 2010). Using both [³⁵S]GTPγS binding and cAMP response element-binding protein reporter assays, these authors have shown that UDP-glucose, UDP-galactose, and UDP activated human recombinant GPR17 in a concentration-dependent manner with EC₅₀ values in the micromolar range, which closely match those previously reported by us. These authors also have shown that GPR17 is not activated, internalized, or bound by cysLTs (Benned-Jensen and Rosenkilde, 2010).

In contrast, results from in vitro recombinant systems have suggested that GPR17 also may act as a direct negative regulator of the CysLT₁ receptor response to LTD₄ via the formation of receptor-receptor dimers, an action that apparently does not require GPR17 activation by endogenous li-
gands (Maekawa et al., 2009). It may be that GPR17 can act as either a ligand-dependent or a ligand-independent pathway, depending on the specific cellular system and specific pathophysiological conditions.

In this study, we confirmed by using both [35S]GTPγS binding and adenyl cyclase assays that GPR17 is activated by uracil nucleotides and cysLTs. To determine the functional interactions between purinergic and cysLT-mediated responses, [35S]GTPγS binding assays were used to investigate the reciprocal regulation between UDP-glucose and LTD₄ in mediating GPR17 coupling to G proteins in 1321N1 GPR17-transfected cells. Evidence was produced that demonstrates that UDP-glucose was able to enhance LTD₄-induced GPR17 G protein coupling and vice versa. Thus, the activation of GPR17 by either purinergic or cysLT ligands induced a change in receptor conformational state that enhanced the functional responsiveness of either class of endogenous ligands. Analysis of agonist dose-response curves suggested that the modulatory effect between the two classes of GPR17 agonists occurs by an allosteric mechanism that may involve 1) two distinct binding sites on the same proteins or 2) two different proteins interacting on the plasma membrane. By RT-PCR, 1321N1 cells were demonstrated to express P2Y₁₄,R at low levels and not to express CysLTRs. In pcDNA 3.1-transfected cells, no functional responses to either UDP-glucose or LTD₄ were observed. These data suggest that, in 1321N1 cells, 1) P2Y₁₄,R, although expressed at low levels, is not involved in the functional response to sugar nucleotides (Communi et al., 2001) and 2) LTD₄-evoked responses cannot be ascribed to the activation of any CysLTR subtypes in these cells.

This allosteric interaction between purinergic and cysLT ligands also was confirmed by data obtained using antagonists toward the two classes of GPR17 ligands. Montelukast and cangrelor, antagonists for the cysLT and purinergic sites, respectively, partially prevented the responses evoked by UDP-glucose and LTD₄, with an IC₅₀ value not comparable with the affinity of these ligands for the homologous site. These data suggest that montelukast and cangrelor act as allosteric antagonists for purinergic and leukotriene sites on GPR17. These data were in line with data previously reported in the literature. In 1321N1 cells heterologously expressing human P2Y₁₂,₄₉,Rs, CysLTR₁ antagonists inhibited both P2YR agonist-induced activation of phospholipase C and intracellular Ca²⁺ mobilization but in an insurmountable manner. These data demonstrated that CysLTR₁ antagonists can interact functionally with the signaling pathways of P2YRs via an allosteric mechanism that did not involve direct interaction of the two receptors at the membrane level (Mamedova et al., 2005). Despite the fact that nucleotides and cysLTs originated from totally independent metabolic pathways, several results suggest important functional interactions between the two families of signaling molecules and their receptors. P2Y₁₄,R has been found to be promiscuously activated by both nucleotides and LTE₄ (Nonaka et al., 2005), further underlying the close relationship between the two families.

Having demonstrated that the two classes of GPR17 ligands modulate each other’s responses, we then investigated the cross-talk between the two classes of ligands in inducing receptor desensitization. To do so, we took advantage of a cAMP assay that is typically used for this type of study. GPR17 homologous and heterologous desensitization induced by purinergic and cysLT ligands were characterized, and the recovery of receptor responsiveness was determined after agonist removal.

The results indicate that both purinergic and cysLT agonists (i.e., both UDP-glucose and LTD₄) induce time- and concentration-dependent homologous receptor desensitization with kinetics comparable for both classes of ligands. After induction of desensitization and cell washout, receptor responsiveness was recovered (resensitization) with comparable kinetics for UDP-glucose and LTD₄.

The heterologous desensitization between the purinergic and the cysLT GPR17 sites also was investigated. The results show that the activation of the GPR17 purinergic site by UDP-glucose induced a partial heterologous desensitization of LTD₄-mediated responses with a kinetic value comparable with that observed for the functional desensitization. In contrast, the activation of GPR17 with LTD₄ did not induce desensitization of UDP-glucose-mediated responses. These results were in line with data reported by Capra et al. (2005), who showed that activation of P2YRs with extracellular nucleotides in dU937 cells induced heterologous desensitization of CysLT₁ but not receptor trafficking or internalization. In contrast, LTD₄-induced CysLT₁ activation had no effect on P2YR responses, suggesting that the latter have a hierarchy in producing desensitizing signals (Capra et al., 2005).

After heterologous desensitization of cysLT responses, resensitization of signal occurred with kinetics slightly slower than that obtained in homologous desensitization studies and comparable with the resensitization kinetics typical of that of the purinergic site.

Because internalization of receptors also plays a role in receptor desensitization and leads to receptor endocytosis into vesicles and subsequent resensitization upon recycling of vesicles to the plasma membrane (Ferguson and Caron, 1998), we evaluated GPR17 internalization after agonist challenge. Results demonstrate that both agonists induce a time-dependent internalization of GPR17 receptors at the cell surface: the percentage of internalized receptors appeared to be higher after cell treatment with UDP-glucose with respect to that after cell treatment with LTD₄. These differences in agonist-mediated internalization may account for the subtle differences observed in the kinetics of receptor recycling. We speculate that LTD₄ induces a lower degree of receptor internalization that allows a more rapid recycling of receptors to the plasma membrane and a quicker functional recovery. Of course, mechanisms other than receptor internalization also could also contribute to GPR17 desensitization, but the present data represent the first molecular demonstration that GPR17 can undergo agonist-induced down-regulation by intracellular endocytosis as expected for a GPR.

The determination of the regulatory mechanisms controlling GPR17 responses over time was pivotal to clarify the role of GPR17 in the differentiation of OPCs, which natively express this receptor at very high levels (Lecca et al., 2008; Chen et al., 2009; Fumagalli et al., 2011). The pharmacological manipulation of GPR17 with its ligands has been shown to foster the progression of precursor cells toward mature myelinating cells, which no longer express the receptor. Loss of GPR17 at a relatively advanced differentiation state seems to be a prerequisite to allow the cells to proceed to terminal maturation, because the forced expression of GPR17 at later
stages of OPC differentiation in a transgenic mouse line prevented myelination and induced precocious death (Chen et al., 2009). On the basis of the present data, we speculate that, by binding to GPR17, receptor endogenous ligands induce receptor desensitization and removal from the cell membrane, with subsequent internalization and degradation, and that this event may contribute to the physiological loss of GPR17 that has been demonstrated to occur during OPC maturation.

Authorship Contributions

Participated in research design: Trincavelli, Rosa, Abbracchio, and Martini.

Conducted experiments: Daniele, Gabelloni, and Lecca.

Performed data analysis: Daniele and Trincavelli.

Wrote or contributed to the writing of the manuscript: Daniele, Trincavelli, and Abbracchio.

Other: Rosa contributed anti-GPR17 antibody.

References


Gla 95:363–378.


Ciampi O, Traini C, Pugliese AM, et al. (2008) Synthesis, ligand-receptor modeling studies and pharmacological evaluation of novel 4-modified-2-aryl-1,2,4-


Daniele, Gabelloni, and Lecca. 2010. Purinergic and cysLT Ligand-Mediated GPR17 Desensitization. 567

Supplemental Figure 1


Daniele S., Trincavelli M.L., Gabelloni P., Lecca D., Rosa P., Abbracchio M.P., Martini C.

JPET

A

B

Supplementary Fig. 1: Membrane aliquots (10 μg) obtained from 1321N1 cells transfected with human GPR17 were treated with A) the indicated concentrations of UDP-glucose, both in the absence or presence of 5 nM or 50 nM LTD₄; B) the indicated concentrations of LTD₄ in the absence or presence of 10 μM or 100 μM UDP-glucose. All data are expressed as percentage of basal [³⁵S]GTPγS binding (set to 100%), and represent the mean ± SEM of three different