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ACCELERATED COMMUNICATION

Molecular Cloning and Characterization of a Second Human Cysteinyl Leukotriene Receptor: Discovery of a Subtype Selective Agonist

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ABSTRACT

The cysteinyl leukotrienes (CysLTs) are potent biological mediators in the pathophysiology of inflammatory diseases, in particular of airway obstruction in asthma. Pharmacological studies have suggested the existence of at least two types of CysLT receptors, designated $CysLT_1$ and $CysLT_2$. The $CysLT_1$ receptor has been cloned recently. Here we report the molecular cloning, expression, localization, and functional characterization of a human G protein-coupled receptor that has the expected characteristics of a $CysLT_2$ receptor. This new receptor is selectively activated by nanomolar concentrations of CysLTs with a rank order potency of $LTC_4 = LTD_4 \gg LTE_4$. The leukotriene analog BAY u9773, reported to be a dual $CysLT_1$/$CysLT_2$ antagonist, was found to be an antagonist at $CysLT_1$ sites but acted as a partial agonist at this new receptor. The structurally different $CysLT_1$ receptor-selective antagonists zafirlukast, montelukast, and MK-571 did not inhibit the agonist-mediated calcium mobilization of $CysLT_2$ receptors at physiological concentrations. Localization studies indicate highest expression of $CysLT_2$ receptors in adrenal glands, heart, and placenta; moderate levels in spleen, peripheral blood leukocytes, and lymph nodes; and low levels in the central nervous system and pituitary. The human $CysLT_2$ receptor gene is located on chromosome 13q14.12–21.1. The new receptor exhibits all characteristics of the thus far poorly defined $CysLT_2$ receptor. Moreover, we have identified BAY u9773 as a $CysLT_2$ selective agonist, which could prove to be of immediate use in understanding the functional roles of the $CysLT_2$ receptor.

Cysteinyl leukotrienes (CysLTs) are the products of the 5-lipoxygenase pathway in arachidonic acid metabolism. They are predominantly produced by myeloid cells associated with the inflammatory responses (Samuelsson et al., 1987) and are potent constrictors of pulmonary smooth muscles (Dahlén et al., 1980), trachea, and parenchyma in human airways, where they induce microvascular permeability (Dahlén et al., 1981) and mucus secretion (Marom et al., 1982). Leukotrienes have been implicated in a number of pathological inflammatory diseases including asthma, allergic rhinitis, inflammatory bowel disease, and psoriasis (Busse and Gaddy, 1991). The effects of CysLTs are mediated via specific plasma membrane receptors belonging to the superfamily of G protein-coupled receptors. There is evidence for the existence of two CysLT receptor subtypes (Fleisch et al., 1982; Labat et al., 1992; Coleman et al., 1995; Metters, 1995): $CysLT_1$ and the $CysLT_2$ receptors, the latter of which encompasses all receptors that cannot be inhibited by $CysLT_1$-specific antagonists (Coleman et al., 1995). The $CysLT_1$ receptor has been studied intensively because of the availability of $CysLT_1$-specific antagonists and of the existence of cell lines expressing it endogenously (Saussy et al., 1989). Recently, a cDNA encoding a $CysLT_1$ receptor was cloned (Lynch et al., 1999; Sarau et al., 1999). The pharmacological profile of the cloned $CysLT_1$ showed a rank order potency of $LTD_4 > LTC_4 > LTE_4$ and was potently inhibited by the $CysLT_1$ antagonists pranlukast, montelukast, zafirlukast, and pobilukast. The $CysLT_2$ receptor, on the other hand, is pharmacologically less defined, mainly be-

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ABBREVIATIONS: CysLT, cysteinyl leukotrienes; PCR, polymerase chain reaction; GPCR, G protein-coupled receptor; HEK 293T, human embryonic kidney cells stably expressing the simian virus 40 large T antigen.

cause of the lack of selective agonists and antagonists. In man, $CysLT_2$ receptors have been indirectly shown to be responsible for contracting pulmonary veins, contractions that were resistant to a number of $CysLT_1$-selective antagonists (Labat et al., 1992).

In our quest to identify the natural ligands of orphan

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GTCTGTTTCTTTTTAACCCAGCATGGAGAGAAAATTTATGTCCTTGCAACCATCCATCTCCGTATCAGAA    70
                      M  E  R  K  F  M  S  L  Q  P  S  I  S  V  S  E
ATGGAACCAAATGGCACCTTCAGCAATAACAACAGCAGGAACTGCACAATTGAAAACTTCAAGAGAGAAT   140
 M  E  P  N  G  T  F  S  N  N  N  S  R  N  C  T  I  E  N  F  K  R  E
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CCTGCAGCCTTATAAGAAGTCCACATCTGTGAACGTTTTCATGCTAAATCTGGCCATTTCAGATCTCCTG   280
  L  Q  P  Y  K  K  S  T  S  V  N  V  F  M  L  N  L  A  I  S  D  L  L
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 F  I  S  T  L  P  F  R  A  D  Y  Y  L  R  G  S  N  W  I  F  G  D  L
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A  C  R  I  M  S  Y  S  L  Y  V  N  M  Y  S  S  I  Y  F  L  T  V  L  S
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  V  V  R  F  L  A  M  V  H  P  F  R  L  L  H  V  T  S  I  R  S  A  W
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 I  L  C  G  I  I  W  I  L  I  M  A  S  S  I  M  L  L  D  S  G  S  E
AGAACGGCAGTGTCACATCATGCTTAGAGCTGAATCTCTATAAAATTGCTAAGCTGCAGACCATGAACTA   630
Q  N  G  S  V  T  S  C  L  E  L  N  L  Y  K  I  A  K  L  Q  T  M  N  Y
TATTGCCTTGGTGGTGGGCTGCCTGCTGCCATTTTTCACACTCAGCATCTGTTATCTGCTGATCATTCGG   700
  I  A  L  V  V  G  C  L  L  P  F  F  T  L  S  I  C  Y  L  L  I  I  R
GTTCTGTTAAAAGTGGAGGTCCCAGAATCGGGGCTGCGGGTTTCTCACAGGAAGGCACTGACCACCATCA   770
 V  L  L  K  V  E  V  P  E  S  G  L  R  V  S  H  R  K  A  L  T  T  I
TCATCACCTTGATCATCTTCTTCTTGTGTTTCCTGCCCTATCACACACTGAGGACCGTCCACTTGACGAC   840
I  I  T  L  I  I  F  F  L  C  F  L  P  Y  H  T  L  R  T  V  H  L  T  T
ATGGAAAGTGGGTTTATGCAAAGACAGACTGCATAAAGCTTTGGTTATCACACTGGCCTTGGCAGCAGCC   910
  W  K  V  G  L  C  K  D  R  L  H  K  A  L  V  I  T  L  A  L  A  A  A
AATGCCTGCTTCAATCCTCTGCTCTATTACTTTGCTGGGGAGAATTTTAAGGACAGACTAAAGTCTGCAC   980
 N  A  C  F  N  P  L  L  Y  Y  F  A  G  E  N  F  K  D  R  L  K  S  A
TCAGAAAAGGCCATCCACAGAAGGCAAAGACAAAGTGTGTTTTCCCTGTTAGTGTGTGGTTGAGAAAGGA  1050
L  R  K  G  H  P  Q  K  A  K  T  K  C  V  F  P  V  S  V  W  L  R  K  E
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  T  R  V  *
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AGATGCTGCCTTCCCTTTTGAGATAGTGTAGAAAAACACTAGATAGTGTGAGAGGTTCCTTTCTGTCCAC  2100
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TAGTAAGAGTTTTAAAAATCTCTGTGCAGAAGTGTTGGCTGGGTGCTCTCCCCACCACTACCCTTGTAAA  2380
CTTCCAGGAAGATTGGTTGAAAGTCTGAATAAAAGCTGTCCTTTCCTACC                       2430
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Fig. 1. Nucleotide- and deduced amino acid sequence of the human HPN321($CysLT_2$) receptor as derived from clone RP11–108P5 (AL137118). Putative poly-adenylation signals are underlined. Position 2123 to 2430 corresponds to an expressed sequence tag (AW235714) found in the dBEST database from a subtracted kidney library. The sequence of HPN321 has been deposited in GenBank (accession no. AF279611).

GPCRs, we cloned in silico a receptor, HPN321, that exhibited moderate similarity to the $CysLT_1$ receptor. We describe here the pharmacological characterization of this novel receptor and conclude that we cloned a receptor exhibiting the expected characteristics of the $CysLT_2$ receptor.

Experimental Procedures

Materials. LTB_4, LTC_4, LTD_4, and LTE_4 were from Cayman Chemical (Ann Arbor, MI). BAY u9773 and MK-571 were from BIOMOL Research Laboratories (Plymouth Meeting, PA). LY-17883 was from Sigma (St. Louis, MO). Zafirlukast (ICI 204,219; Accolate) and montelukast (MK-476; Singulair) were purchased from the local pharmacy. All other standard chemicals used were either from Fisher or Sigma.

Identification, Cloning, and Sequencing of HPN321 and $CysLT_1$ receptors. A human chromosomal clone (AL137118) was identified during our systematic computational queries of novel DNA sequences coding for GPCR-related proteins. The corresponding open reading frame was identified using TBLAST algorithm and known GPCR protein sequences as queries. The DNA sequence was used to design two sets of nested primers (first set 5′-GATAGTATTGCTCCCTGTTTCATT-3′ [−112 to −89] and 5′-GAAGATGGACACAAGGATACAAGA-3′[1064 to 1087] and second set 5′-ATGTAATCAGTAAGCAAGAAGGA-3′[−75 to −53] and 5′-ACAGGTCTCATCTAAG AGCTCCTT-3′ [1063 to 1040]). The first polymerase chain reaction (PCR) was carried out with the ExpandLong Template PCR system (Hoffman La Roche, Nutley, NJ) on 0.1 μg of human genomic DNA (CLONTECH, Palo Alto, CA) according to the manufacturer's recommendation. Second PCR was performed with 2 μl of first-round PCR product and 1.0 U of *Taq* polymerase (Promega, Madison, WI) in 50 μl of the buffer supplied by the manufacturer containing 2.5 mM $MgCl_2$ and was carried out for 30 cycles at 94°C for 30 s, 50°C for 30 s, and 72°C for 1.5 min. The resulting product was analyzed by agarose gel electrophoresis and subcloned into pCDNA3.1 (Invitrogen, San Diego, CA). The accuracy of the sequence was confirmed by DNA sequencing using a dideoxy termination kit from Amersham Pharmacia Biotech (Piscataway, NJ). $CysLT_1$ was cloned from an expressed sequence tag available through the I.M.A.G.E. consortium (clone 1357580). The insert that contained the whole open reading frame, was excised by*Eco*RI/*Not*I digestion and directionally cloned into pCDNA3.1 (Invitrogen).

Expression in HEK 293T Cells. HPN321/$CysLT_1$ receptor plasmids were transiently expressed in human embryonic kidney cells stably expressing the simian virus 40 large T antigen (HEK 293T) cells using LipofectAMINE PLUS (Life Technologies, Gaithersburg, MD) according to the manufacturer's protocol. Transfections were done in 100-mm tissue culture dishes and seeded after 24 h into microtiter plates for subsequent assays. Assays (see below) were performed 48 h after cell transfections. Cell lines stably expressing HPN321 were established by selecting hygromycin-resistant clones.

Measurement of Agonist-Induced Calcium Release. Calcium mobilization assays were carried out using transiently transfected HEK 293T-HPN321 or $CysLT_1$ cells loaded with Fluo-4 AM fluorescent indicator dye (Molecular Probes, Eugene, OR) in the fluorescent imaging plate reader system (Molecular Devices, Sunnyvale, CA). Briefly, cells were seeded in poly-D-lysine-treated black microtiter plates at 8×10^4 cells/well and grown to confluence. The cells were loaded with 2 μM Fluo-4 in growth medium (Dulbecco's modified Eagle's medium, 10% fetal bovine serum) supplemented with 2.5 mM probenecid for 1 h at 37°C, 5% CO_2. The cells were washed thrice with Hanks' balanced salt solution containing 20 mM HEPES and 2.5 mM probenecid. Calcium transient curves were generated by reading baseline fluorescence values for 10 s, followed by addition of test compounds. For the first minute, fluorescence values were collected in 1-s intervals; for the next 2 min, data were collected in 3-s intervals. For calculation of dose-response curves, the peak fluorescence values for each agonist concentration were determined and analyzed by nonlinear regression using PRISM software (GraphPad, San Diego, CA). Antagonist studies were performed under the same conditions but test compounds were added 2 to 5 min before addition of agonist (at EC_{50} value). EC_{50} is defined as the agonist concentration generating 50% of the peak fluorescence values. IC_{50} is the concentration required to inhibit 50% of the peak fluorescence.

Radioligand Binding Studies. Transiently transfected HEK 293T cells were grown and harvested, and crude membranes were prepared as described (Nothacker et al., 1999). For competition binding studies, membranes (200 μg of total membrane protein) were incubated with 1.5 nM [^{3}H]LT D_4 (NEN Life Science, Boston, MA) and variable concentrations of competitors in 250 μl of 50 mM Tris-HCl pH 7.4, 20 mM $CaCl_2$, 25 mM $MgCl_2$, 10 mM glycine, and 10 mM cysteine for 1 h at 22°C. The membranes were filtered over Whatman GF/C filters using a Brandel cell harvester, washed thrice with ice-cold binding buffer, and counted in the presence of 5-ml CytoScint (ICN, Irvine, CA) in a Beckman LS 3801 liquid scintillation counter. Nonspecific binding was determined in the presence of 2 μM unlabeled LTD_4. Under these conditions, specific binding accounted for ~60% of total binding. Data were analyzed by nonlinear regression analysis using PRISM software (GraphPad).

RNA-Array and Northern Blot Analysis. Human multiple tissue arrays (CLONTECH) were analyzed by hybridization to an

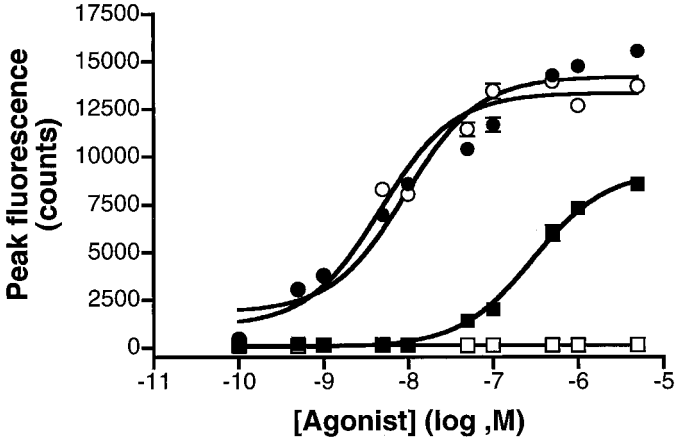

Fig. 2. Concentration-response curve of HPN321 ($CysLT_2$) expressing HEK 293T cells incubated with various leukotrienes. Cells transiently expressing HPN321 responded specifically to CysLT treatment with calcium mobilization responses. Average values ± S.E.M. of a typical experiment with each point determined in triplicate are shown. ●, LTC_4; ○, LTD_4; ■, LTE_4, □, LTB_4.

TABLE 1

Comparison of efficacies of agonists and antagonists at $CysLT_1$ and HPN321 ($CysLT_2$) receptors

Calcium mobilization assays were done as described under *Experimental Procedures*. Data are expressed as the mean ± S.E.M. (n = 3).

	$CysLT_1$	HPN321
Agonist	EC_{50} (nM)	EC_{50} (nM)
LTC_4	43 ± 4	8.9 ± 1.4
LTD_4	0.9 ± 0.1	4.4 ± 0.2
LTE_4	104 ± 6	293 ± 37
BAY u9773	>10,000	92 ± 15
Antagonist	IC_{50} (nM)[a]	IC_{50} (nM)[a]
BAY u9773	440 ± 182	300 ± 92
Zafirlukast	0.26[b]	>1000
Montelukast	2.3[b]	>5000
MK-571	10.4[c]	>1000
LY-17883	ND	>5000

[a] IC_{50} values were determined relative to a 10 nM LTD_4 stimulation.
[b] Sarau et al., 1999
[c] Lynch et al., 1999
ND, not determined.

[α-^{32}P]dCTP-labeled, 600-bp *Bam*HI-fragment of HPN321. Hybridization was conducted according to the manufacturer's recommendation and with final washes of 0.1× standard saline citrate, 0.5% SDS at 55°C for 30 min before exposition to X-OMAT film (Kodak, Rochester, NY) at −80°C. Lymphoma-derived U-937 cell lines and leukemia-derived HL-60 cell lines were differentiated with 1.3% dimethyl sulfoxide in RPMI 1640 (Life Technologies) supplemented with 10% fetal bovine serum for three days at 37°C, 5% CO_2. The cells were harvested and total RNA prepared using TRIZOL reagent (Life Technologies). Northern blots were prepared from 5 or 15 μg of total RNA according to standard molecular biology techniques (Sambrook et al., 1989) and hybridized to the probe described above. Control hybridization was done with a $CysLT_1$ specific probe composed of a 3′-*Hin*cII/*Not*I fragment of I.M.A.G.E. clone 1357580.

Results

Identification and Molecular Characterization of the $CysLT_1$-Like Receptor, HPN321. Computational screenings of public expressed sequence tag and genomic databases were carried out to identify novel sequences displaying structural characteristics common to GPCRs. One genomic clone (GenBank accession no. AL137118) contained an intronless open reading frame of 347 amino acids (Fig. 1) exhibiting seven putative membrane-spanning helices characteristic of GPCR sequences and sharing 36% amino acid identity with the recently cloned $CysLT_1$ receptor (Lynch et al., 1999; Sarau et al., 1999). This sequence, named HPN321, was amplified by PCR from human genomic DNA, inserted into a mammalian expression vector under the control of a CMV promotor, and transiently expressed in HEK 293T cells. The transfected cells were challenged with either LTC_4 or LTD_4 and simultaneously monitored for changes of intracellular calcium. Both induced a strong calcium mobilization at low concentrations, whereas no changes could be observed in mock-transfected control cells.

Pharmacological Characterization of the HPN321 Receptor. To further characterize the pharmacological profile of the receptor, the responses to four different CysLTs were determined. The HPN321 receptor transfected HEK 293T cells responded to LTC_4, LTD_4, and LTE_4 in a dose-dependent manner, with respective EC_{50} values of 8.9, 4.4, and 293 nM (n = 3, Fig. 2). No activation could be observed by addition of the non-peptido-leukotriene LTB_4, even at concentrations up to 5 μM. The highest concentrations of LTC_4 and LTD_4 produced similar maximal responses, whereas LTE_4 behaved as a partial agonist, attaining only 60% of the maximal response of that elicited by LTC_4 and LTD_4. Consequently, the rank order of potency was LTC_4 = LTD_4 ≫ LTE_4, distinct from that of the $CysLT_1$ receptor (Table 1). We also tested several structurally different and selective $CysLT_1$ antagonists (LY-17883, MK-571, montelukast and zafirlukast) to assess their ability to block HPN321 activation. All of the $CysLT_1$ antagonists tested were practically inactive, showing only weak inhibition at concentrations of >1 μM (Table 1).

Pharmacological Properties of BAY u9773 at HPN321 and $CysLT_1$. To further characterize this novel receptor, we tested BAY u9773, a compound reported to behave as a dual $CysLT_1$/$CysLT_2$ antagonist. When applied simultaneously with the agonist LTD_4 (10 nM, in presence of 5 μM BAY u9773) BAY u9773 exhibited no inhibition. We then administered BAY u9773 alone and found that BAY u9773 was able to activate HPN321 with an EC_{50} of 100 nM, thus more potently than LTE_4, but less potently than LTC_4 or LTD_4. The response was concentration dependent and reached 67% of the maximal LTC_4 and LTD_4 responses (Fig. 3A), indicating that BAY u9773 acts as a partial agonist. We next tested whether BAY u9773 would also antagonize the effects of full agonists such as LTC_4/LTD_4. We therefore

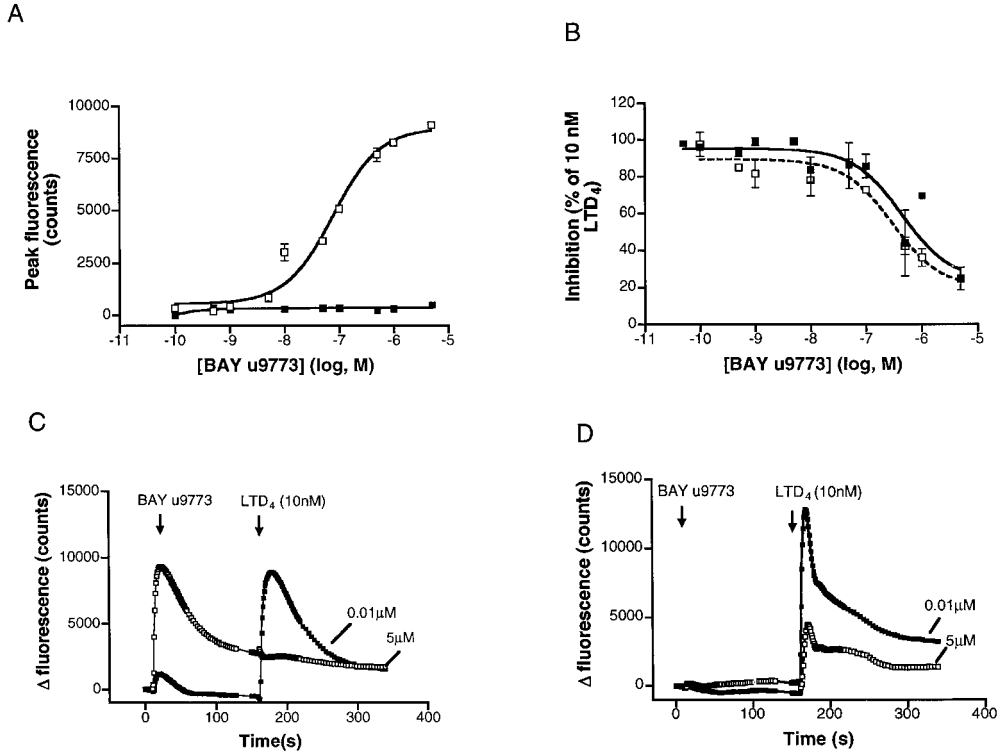

Fig. 3. Action of BAY u9773 at $CysLT_1$ and HPN321 ($CysLT_2$) receptors in transiently transfected HEK 293T cells. A, calcium mobilization responses to increasing concentrations of BAY u9773 in HEK 293T cells transfected with either HPN321 (□) or $CysLT_1$ (■). Values are expressed as optical counts and average values ± S.E.M. of triplicates from a typical experiment are shown. B, dose-dependent inhibition of LTD_4 induced calcium mobilization by BAY u9773 in HEK 293T cells transfected with either HPN321 (□) or $CysLT_1$ (■). C and D, time course of calcium mobilization responses to BAY u9773 in HPN321 (C) and $CysLT_1$ (D) expressing HEK 293T cells. In a first sequence, the indicated concentrations of BAY u9773 were applied to the cells and fluorescence changes because of calcium mobilization recorded. In a second sequence, the cells were challenged with 10 nM LTD_4 and fluorescence recordings resumed.

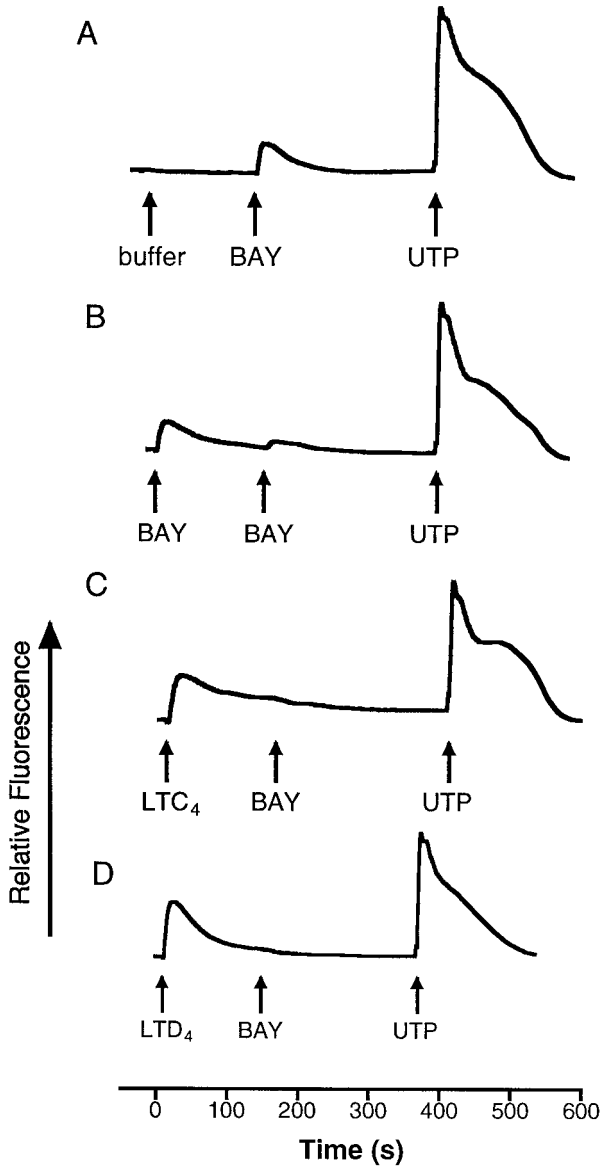

Fig. 4. Desensitization of calcium mobilization responses in HPN321($CysLT_2$) transfected HEK 293T cells. Cells were treated with 10 nM LTC_4, 10 nM LTD_4, or 100 nM BAY u9773. Agonists were added sequentially as indicated by arrows and changes in fluorescence intensity were recorded over the whole time course. Finally, the cells were challenged with 10 μM UTP to demonstrate the integrity of the signaling system. Results of a typical experiment are shown; each experiment was repeated at least three times.

monitored LTD_4 responses obtained 2 min after the addition of BAY u9773 at variable concentrations. BAY u9773 exhibited its intrinsic agonistic effect but in addition inhibited that elicited by LTD_4 in a dose-dependent manner (Fig. 3, B and C). When BAY u9773 was tested on $CysLT_1$ transfected cells, it had no intrinsic agonistic effect but elicited an antagonistic effect on a subsequent LTD_4 challenge (Fig. 3D). The IC_{50} values of BAY u9773 on LTD_4 stimulation were very similar for both receptors (Fig. 3B; Table 1) albeit the antagonistic mechanism at both receptors seemed to be different. The most probable explanation for the antagonistic properties of BAY u9773 at HPN321 is that the drug desensitizes the system in a manner similar to that seen by repeated challenge with LTC_4. To provide additional data demonstrating that BAY u9773 is a partial agonist at HPN321 receptor and that all of its effects in HEK 293T cells can be explained by desensitization of $[Ca^{2+}]_i$ mobilization responses, we carried out a series of mutual desensitization experiments (Fig. 4). Sequential addition of 100 nM BAY u9773 leads to a desensitization of the HPN321 specific response, leaving other calcium mobilization agonists unaltered as shown by the normal response to UTP (Fig. 4B). LTC_4 and LTD_4 applied at an equipotent concentration desensitized a secondary BAY u9773 challenge (Fig. 4, C and D). The observed homologous desensitization events strongly suggest a similar mode of action for the natural agonists as well for BAY u9773.

Displacement of [^{3}H]LTD_4. Competition binding experiments carried out on membranes of transiently transfected HEK 293T cells (Fig. 5) revealed the same rank order of potency for the competition of [^{3}H]LTD_4 binding sites as obtained in the functional assays. LTC_4 and LTD_4 showed high binding affinity, with IC_{50} values of 4 and 7 nM, respectively. In contrast, LTE_4 competed rather weakly, as reflected by its IC_{50} value of 0.7 μM. BAY u9773 fully inhibited [^{3}H]LTD_4 binding with an IC_{50} value of 0.4 μM, very similar to the values obtained for the calcium mobilization assay (Table 1).

Tissue Distribution of HPN321. Multiple human tissues were examined by Northern hybridization and dot blot analysis to determine sites of HPN321 expression (Fig. 6A). Using a 600-base pair 5′-probe that did not cross-hybridize with $CysLT_1$-DNA, we found the strongest expression in the adrenal gland, the heart, and the placenta. Cardiac expression could be detected throughout the entire heart, including ventricles, atrium, septum, and apex. Moderate expression could be detected in the immune system, in particular spleen, lymph nodes, and peripheral blood leukocytes. No signals were found in HL-60, a cell line known to express $CysLT_1$ receptors. We also investigated U-973 myeloid leukemia cells for HPN321 expression because this cell line has been used to develop $CysLT_1$ antagonists (Sarau et al., 1999). However, in neither undifferentiated nor differentiated U-973 cells could HPN321 expression be detected, whereas $CysLT_1$ mRNA was easily detected (Fig. 6B). HPN321-expression was present in lower levels throughout the central nervous system, with

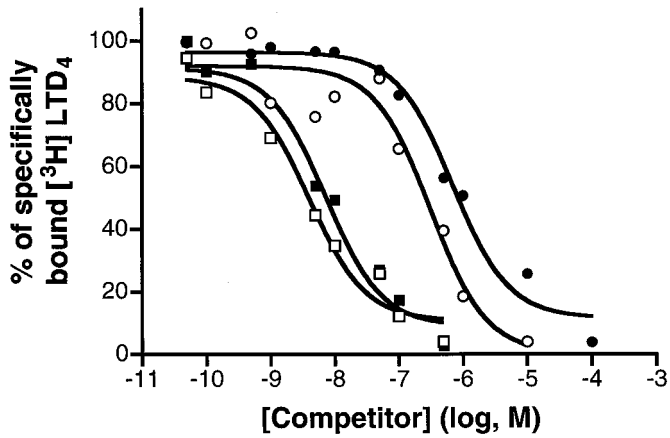

Fig. 5. Effects of CysLTs and BAY u9773 on competitive binding of [^{3}H]LTD_4 in membranes of HEK 293T cells transiently expressing the HPN321 receptor. Results of LTC_4 (□), LTD_4 (■), LTE_4 (●), and BAY u9773 (○) are presented as the mean of triplicate values from two independent experiments.

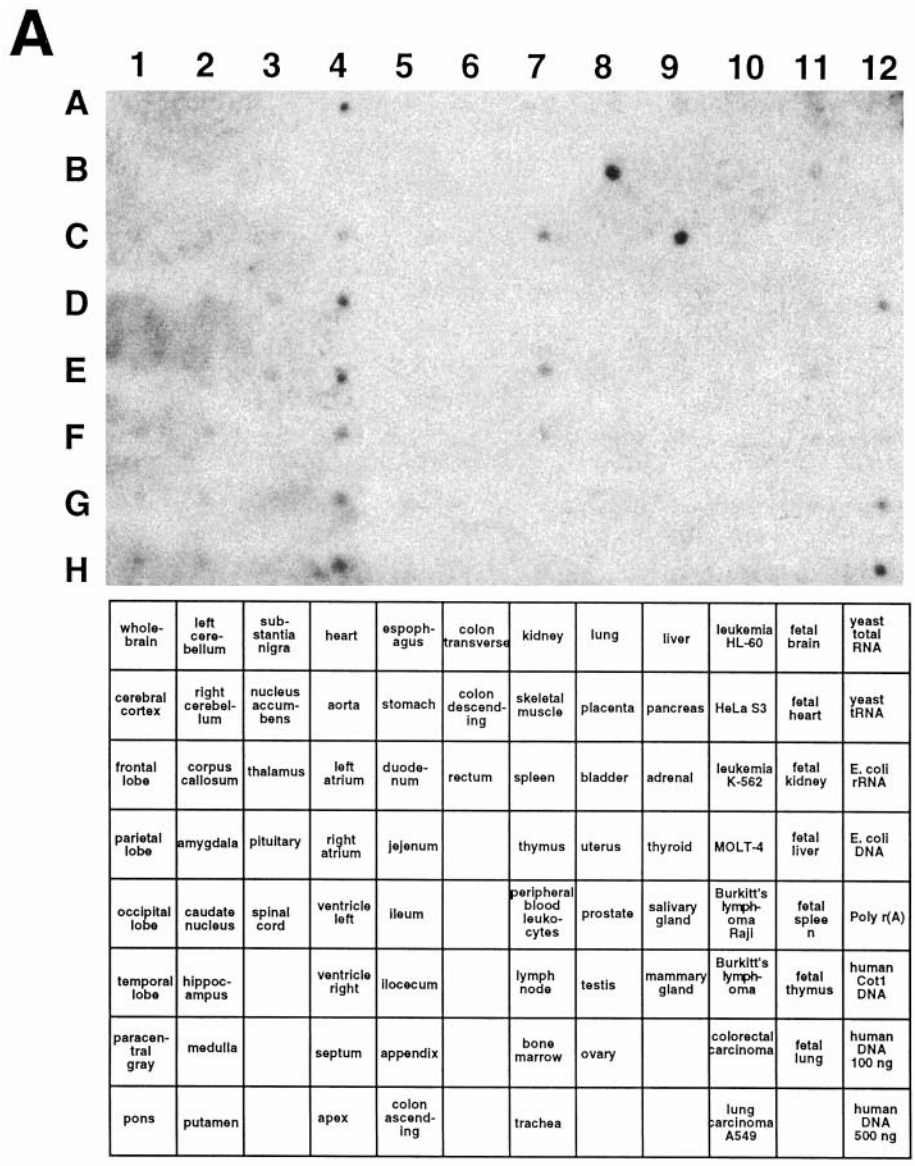

Fig. 6. Tissue distribution of HPN321 receptor mRNA. A, human normalized multiple tissue array. Top, array after hybridization to a 600-base-pair HPN321 specific probe. The diagram shows the type and position of poly(A^+) RNAs dotted on the membrane. B, Northern blot of total RNA isolated from undifferentiated (basal) and dimethyl sulfoxide-differentiated (differ.) HL-60 or U-937 cells. The identical membrane was either hybridized to a HPN321 (upper) or a $CysLT_1$ (middle) specific probe. Left and right panels in each column correspond to 5 and 15 μg of total RNA, respectively. HEK cells stably expressing HPN321 were used as a positive hybridization control.

highest levels in pituitary and spinal cord. This expression pattern is distinct from that of the $CysLT_1$ receptor (Lynch et al., 1999; Sarau et al., 1999) yet overlapping in some tissues.

Discussion

While searching in silico for novel GPCRs, we discovered a DNA sequence that could encode a putative GPCR sharing moderate sequence similarity (36%) with the recently described $CysLT_1$ receptor. When expressed in HEK 293T cells, this new receptor responded to low concentrations of CysLTs by inducing intracellular calcium mobilization. Furthermore, this receptor was not inhibited by antagonists specific to the $CysLT_1$receptor, fitting the postulated pharmacological profile of $CysLT_2$ receptor.

Interestingly, this receptor could be partially activated by the leukotriene analog BAY u9773, originally described as a dual $CysLT_1$/$CysLT_2$ antagonist. In this report we demonstrate that BAY u9773 acts as a partial agonist at the new receptor and that this property may have led to its classification as a $CysLT_2$ antagonist. BAY u9773 has been a useful tool to define $CysLT_2$ receptors pharmacologically. It has been shown to inhibit physiological responses insensitive to $CysLT_1$ antagonists in several species including man (Labat et al., 1992; Tudhope et al., 1994; Bäck et al., 1996), thus allowing the detection of the $CysLT_2$ sites.

A functional model of $CysLT_1$ and $CysLT_2$ receptors in human lung has been developed (Gorenne et al., 1996). $CysLT_1$ receptors, mainly located in bronchial smooth muscle, mediate the contractions evoked by CysLTs. In contrast, $CysLT_2$ receptors located mainly in the vascular smooth muscles of pulmonary veins, induce contractions that cannot be blocked by $CysLT_1$ specific antagonists. In addition, the vascular endothelium contains both receptor subtypes, a $CysLT_1$ type associated with contractions and a $CysLT_2$ type associated with relaxation. We were able to demonstrate an important pharmacological difference in the action of BAY u9773 at $CysLT_1$ and $CysLT_2$ receptors, which enables us now to explain previous findings. Labat et al. had already reported that BAY u9773 elicits small contractile responses in human pulmonary veins and speculated that this effect was caused by a partial agonist activity (Labat et al., 1992). We show that BAY u9773 acts as a partial agonist at HPN321 receptor and thus suggest that it represents the target of BAY u9773 in the human pulmonary venous preparation. In addition, the same authors observed a BAY u9773-induced relaxation in human lung tissues. We can therefore speculate that the same receptor exists in human lung coupled to a relaxation effect, probably mediated through stimulation of endothelial nitric-oxide synthase. BAY u9773 was also reported to contract various tissue preparations from guinea pig (Tudhope et al., 1994; Wikström Jonsson et al., 1998). It must be emphasized, however, that the molecular targets of BAY u9773 in this species might not be identical to the CysLT receptors found in the human lung (Gorenne et al., 1996). One recent study in guinea pig lung parenchyma found that contractions evoked by BAY u9773 were antagonized by a $CysLT_1$ specific antagonist (Wikström Jonsson et al., 1998). This result suggests the existence of a $CysLT_1$-like molecular target for BAY u9773 in this guinea pig tissue. However, in our hands, the human $CysLT_1$ receptor (Lynch et al., 1999; Sarau et al., 1999) could not be activated by BAY u9773 and was sensitive to $CysLT_1$ antagonists. Our results thus support the existence of a pharmacological heterogeneity of CysLT receptors in different species.

Our finding that BAY u9773 acts as a subtype selective agonist offers a unique opportunity to study HPN321 receptor selective physiological activities, particularly in tissues in which HPN321 is dominantly expressed over $CysLT_1$, such as the adrenal gland and the heart. The antagonistic effects seen by pretreatment with BAY u9773 at $CysLT_2$ sites might be caused by receptor desensitization. Calcium mobilization responses mediated by HPN321 can be desensitized by repeated challenges of LTC_4, LTD_4, LTE_4, and BAYu9773 as well. Because BAY u9773 is structurally related to LTE_4, a partial agonist at both receptors, it probably competes with the full agonists LTC_4 and LTD_4 for the same binding site. We are currently studying the mechanism of BAY u9773 in greater detail, particularly the surprising agonistic selectivity toward HPN321.

The distribution data of HPN321 suggests major role(s) for this receptor in endocrine and cardiovascular systems. CysLTs are well known for their modulatory effects in cardiovascular functions, where they reduce myocardial contractility and coronary blood flow (Letts and Piper, 1982) and have vasoactive effects (Drazen et al., 1980). They are thus considered to be important players in cardiovascular diseases (for review, see Folco et al., 2000). The strong expression of HPN321 in adrenal gland points at a new tissue where to study the influence of CysLTs on endocrine circuits. Finally, leukotrienes have also been found to act on the pituitary to modulate the release of the pituitary hormones (Hulting et al., 1984; Saadi et al., 1990). Our discovery of the existence of the HPN321 message in pituitary adds a molecular credence to this concept. The HPN321 receptor may thus modulate a variety of different physiological functions, which can now be tested using BAY u9773.

During the preparation of this manuscript, two groups also reported the pharmacological characterization of a second human CysLT receptor (Heise et al., 2000; Takasaki et al., 2000). The sequences of the reported receptors (GenBank accession nos. AF254664 and AB038269) are identical with the one described herein. Relative potencies of the CysLTs are very similar, yet none report the selective activity of BAY u9773.

In summary, we present the molecular and pharmacological characterization of a novel human CysLT receptor subtype that we have named the $CysLT_2$ receptor and report the tissue distribution of its expression. The receptor shows a selective rank order of potency toward CysLTs and is the most preferred target for LTC_4. We also identified BAY u9773 as a subtype selective agonist for the $CysLT_2$ receptor and suggest the use of BAY u9773 as a selective tool in studies on the physiological roles of the $CysLT_2$ receptor in cardiac, neuronal, endocrine, and inflammatory circuits.

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References

Bäck M, Jonsson EW and Dahlén SE (1996) The cysteinyl-leukotriene receptor antagonist BAY u9773 is a competitive antagonist of leukotriene C4 in the guinea-pig ileum. *Eur J Pharmacol* **317:**107–113.

Busse WW and Gaddy JN (1991) The role of leukotriene antagonists and inhibitors in the treatment of airway disease. *Am Rev Respir Dis* **143:**S103–S107.

Coleman RA, Eglen RM, Jones RL, Narumiya S, Shimizu T, Smith WL, Dahlén SE, Drazen JM, Gardiner PJ, Jackson WT, Jones TR, Krell RD, and Nicosia S (1995) Prostanoid and leukotriene receptors: A progress report from the IUPHAR working parties on classification and nomenclature. *Adv Prostaglandin Thromboxane Leukot Res* **23:**283–285.

Dahlén SE, Björk J, Hedqvist P, Arfors KE, Hammarström S, Lindgren JA and Samuelsson B (1981) Leukotrienes promote plasma leakage and leukocyte adhesion in postcapillary venules: In vivo effects with relevance to the acute inflammatory response. *Proc Natl Acad Sci USA* **78:**3887–3891.

Dahlén SE, Hedqvist P, Hammarström S and Samuelsson B (1980) Leukotrienes are potent constrictors of human bronchi. *Nature (Lond)* **288:**484–486.

Drazen JM, Austen KF, Lewis RA, Clark DA, Goto G, Marfat A and Corey EJ (1980) Comparative airway and vascular activities of leukotrienes C-1 and D in vivo and in vitro. *Proc Natl Acad Sci USA* **77:**4354–4358.

Fleisch JH, Rinkema LE and Baker SR (1982) Evidence for multiple leukotriene D4 receptors in smooth muscle. *Life Sci* **31:**577–581.

Folco G, Rossoni G, Buccellati C, Berti F, Maclouf J and Sala A (2000) Leukotrienes in cardiovascular diseases. *Am J Respir Crit Care Med* **161:**S112–S116.

Gorenne I, Norel X and Brink C (1996) Cysteinyl leukotriene receptors in the human lung: What's new? *Trends Pharmacol Sci* **17:**342–345.

Heise CE, O'Dowd BF, Figueroa DJ, Sawyer N, Nguyen T, Im DS, Stocco R, Bellefeuille JN, Abramovitz M, Cheng R, Williams DL Jr, Zeng Z, Liu Q, Ma L, Clements MK, Coulombe N, Liu Y, Austin CP, George SR, O'Neill GP, Metters KM, Lynch KR and Evans JF (2000) Characterization of the human cysteinyl leukotriene 2 receptor. J Biol Chem 2000 **275:**30531–30536.

Hulting AL, Lingren JA, Hökfelt T, Heidvall K, Eneroth P, Werner S, Patrono C and Samuelsson B (1984) Leukotriene C4 stimulates LH secretion from rat pituitary cells in vitro. *Eur J Pharmacol* **106:**459–460.

Labat C, Ortiz JL, Norel X, Gorenne I, Verley J, Abram TS, Cuthbert NJ, Tudhope SR, Norman P, Gardiner P and Brink C (1992) A second cysteinyl leukotriene receptor in human lung. *J Pharmacol Exp Ther* **263:**800–805.

Letts LG and Piper PJ (1982) The actions of leukotrienes C4 and D4 on guinea-pig isolated hearts. *Br J Pharmacol* **76:**169–176.

Lynch KR, O'Neill GP, Liu Q, Im DS, Sawyer N, Metters KM, Coulombe N, Abramovitz M, Figueroa, DJ Zeng Z, Connolly BM, Bai C, Austin CP, Chateauneuf A, Stocco R, Greig GM, Kargman S, Hooks SB, Hosfield E, Williams DL Jr, Ford-Hutchinson AW, Caskey CT and Evans JF (1999) Characterization of the human cysteinyl leukotriene CysLT1 receptor. *Nature (Lond)* **399:**789–793.

Marom Z, Shelhamer JH, Bach MK, Morton DR and Kaliner M (1982) Slow-reacting substances, leukotrienes C4 and D4, increase the release of mucus from human airways in vitro. *Am Rev Respir Dis* **126:**449–451.

Metters KM (1995) Leukotriene receptors. *J Lipid Mediat Cell Signal* **12:**413–427.

Nothacker HP, Wang ZH, McNeil AM, Saito Y, Merten S, O'Dowd B, Duckles SP and Civelli O (1999) Identification of the natural ligand of an orphan G-protein-coupled receptor involved in the regulation of vasoconstriction. *Nat Cell Biol* **1:**383–385.

Saadi M, Gerozissis K, Rougeot C, Minary P and Dray F (1990) Leukotriene C4-induced release of LHRH into the hypophyseal portal blood and of LH into the peripheral blood. *Life Sci* **46:**1857–1865.

Sambrook J, Maniatis T and Fritsch EF (1989). Molecular cloning: A laboratory manual. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

Samuelsson B, Dahlén SE, Lindgren JA, Rouzer CA and Serhan CN (1987) Leukotrienes and lipoxins: Structures, biosynthesis, and biological effects. *Science (Wash DC)* **237:**1171–1176.

Sarau, HM, Ames, RS, Chambers, J, Ellis, C, Elshourbagy, N, Foley, JJ, Schmidt, DB, Muccitelli, RM, Jenkins, O, Murdock, PR, Herrity, NC, Halsey, W, Sathe, G, Muir, AI, Nuthulaganti, P, Dytko, GM, Buckley, PT, Wilson, S, Bergsma, DJ and Hay, DW (1999) Identification, molecular cloning, expression, and characterization of a cysteinyl leukotriene receptor. *Mol Pharmacol* **56:**657–663.

Saussy DL, Sarau HM, Foley JJ, Mong S and Crooke ST (1989) Mechanism of leukotriene E4 partional agonist activity at leukotriene D4 receptors in differentiated U-937 cells. *J Biol Chem* **264:**19845–19855.

Takasaki J, Kamohara M, Matsumoto M, Saito T, Sugimoto T, Ohishi T, Ishii H, Ota T, Nishikawa T, Kawai Y, Masuho Y, Isogai T, Suzuki Y, Sugano S and Furuichi K (2000) The molecular characterization and tissue distribution of the human cysteinyl leukotriene $CysLT_2$ receptor. *Biochem Biophys Res Commun* **274:**316–322.

Tudhope SR, Cuthbert NJ, Abram TS, Jennings MA, Maxey RJ, Thompson AM, Norman P and Gardiner PJ (1994) BAY u9773, a novel antagonist of cysteinyl-leukotrienes with activity against two receptor subtypes. *Eur J Pharmacol* **264:**317–323.

Wikström Jonsson E, Rosenqvist U and Dahlén SE (1998) Agonist and antagonist activities of the leukotriene analogue BAY u9773 in guinea pig lung parenchyma. *Eur J Pharmacol* **357:**203–211

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