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ACCELERATED COMMUNICATION

Expression and Characterization of a 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-Eicosatetraenoic Acid Receptor Highly Expressed on Human Eosinophils and Neutrophils

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ABSTRACT

Using a bioinformatics approach, we have isolated a novel G-protein-coupled receptor (GPCR), R527, and have demonstrated that this receptor shows no significant homology to previously deorphanized GPCRs. Quantitative reverse transcription-polymerase chain reaction analysis of the expression of GPCR R527 indicated a very high level of mRNA expression in eosinophils, with high expression also detected in neutrophils and lung macrophages. Stable cell lines were generated expressing this receptor together with the G-protein α-subunit $G\alpha_{16}$. These cells were used to screen an agonist collection in a calcium mobilization assay and 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-oxo-ETE) was identified as a putative ligand. 5(*S*)-Hydroxyperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid was also shown to activate the receptor, whereas the leukotrienes LTB_4, LTC_4, LTD_4, and LTE_4 failed to elicit a response. In cAMP assays, pertussis toxin reversed the inhibitory effects of 5-oxo-ETE on cAMP production, indicating that the receptor is $G\alpha_i$-coupled. The GPCR R527 shows pharmacological properties similar to those of the previously described 5-oxo-ETE receptor expressed on eosinophils, neutrophils, and monocytes. These cell types show chemotactic responses to 5-oxo-ETE, and this eicosanoid has been proposed to play a key role in the inflammatory response. The molecular identification of a receptor binding 5-oxo-ETE will expand our understanding of the physiological role of this mediator and may provide new therapeutic opportunities.

The eicosanoid 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid (5-oxo-ETE) is a lipid on the 5-lipoxygenase pathway and was previously shown to be a potent stimulator of chemotaxis (Powell et al., 1995). It is formed from 5-hydroxy-6,8,11,14 (*E*,*Z*,*Z*,*Z*)-eicosatetraenoic acid by the action of a highly specific dehydrogenase found in neutrophils, eosinophils, monocytes, and lymphocytes (Powell et al., 1992, 1995; Zhang et al., 1996). Dendritic cells have also recently been reported to be a physiological source of 5-oxo-ETE (Zimpfer et al., 2000). This mediator has been shown to have effects on a number of eosinophil, neutrophil, and monocyte functions. The effects on eosinophils include chemotaxis, calcium mobilization, and degranulation, all of which are strongly induced (Powell et al., 1995; Schwenk and Schroder, 1995; O'Flaherty et al., 1996b; Czech et al., 1997). 5-oxo-ETE has also been described as a stimulator of neutrophil chemotaxis (Powell et al., 1993), calcium mobilization (O'Flaherty et al., 1998), Cd11b expression, and actin polymerization (Powell et al., 1997). In addition, monocyte chemotaxis has been shown to be stimulated by 5-oxo-ETE and this lipid was also shown to synergize with monocyte chemotactic proteins 1 and 3 (Sozzani et al., 1996). All of these reported effects suggest that 5-oxo-ETE plays an

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ABBREVIATIONS: 5-oxo-ETE, 5-oxo-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; GPCR, G-protein-coupled receptor; 5(*S*)-HETE, 5(*S*)-hydroxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; 5(*S*)-HPETE, 5(*S*)-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid; HBECs, human bronchial epithelial cells; PCR, polymerase chain reaction; HEK, human embryonic kidney; RT, reverse transcriptase/transcription; FLIPR, fluorometric imaging plate reader; PTX, pertussis toxin; bp, base pair(s); nt, nucleotide(s); CGP45715A, iralukast; CGS25019C, 4-[5-{4-(aminoiminomethyl)phenoxy}pentoxy]-3-methoxy-*N*,*N*-bis(1-methylethyl)(*Z*)-2-butenedioate (1:1); 5-LO, 5-lipoxygenase; LY255283, 1-[5-ethyl-2-hydroxy-4-[[6-methyl-6-(1H-tetrazol-5-yl)heptyl]oxy]phenyl]ethanone.

important role in the inflammatory response. The chemotactic responses induced by 5-oxo-ETE have been suggested to be mediated via a GPCR (O' Flaherty et al., 2000); however, the receptor mediating these effects was not previously known at the molecular level.

In an effort to search for additional members of the GPCR family, we have performed human genome database searches. These studies led to the identification of a novel orphan GPCR termed R527 with high homology to the orphan receptor HM74. Expression of the full-length cDNA for R527 allowed screening for potential agonists, and this subsequently led to the identification of 5-oxo-ETE as an agonist of this GPCR. We found this receptor to be highly expressed on human macrophages, eosinophils, and neutrophils, suggesting it may be the previously described chemotactic 5-oxo-ETE receptor.

Materials and Methods

Materials. 5(*S*)-HETE and 5(*S*)-hydroperoxy-6*E*,8*Z*,11*Z*,14*Z*-eicosatetraenoic acid [5(*S*)-HPETE] were purchased from Biomol (Plymouth Meeting, PA). 5-oxo-ETE was purchased from Biomol or Caymen Biochemicals (Lausen, Switzerland).

Cloning of Human R527 cDNA. The complete coding region of R527 was PCR-amplified from human neutrophil cDNA using the primers R527F 5′-ATG GAA CTT CAT AAC CTG AGC TCT C-3′ and R527R 5′-TCA GCC CTG GGA GGA GCC TTC C-3′. The resulting PCR product was cloned into pCR2.1-TOPO (Invitrogen, Paisley, UK), and the entire insert was sequenced using an automated DNA sequencer (Solvias AG, Basel, Switzerland). The insert was then PCR-amplified to add a *c-myc* tag at the 3′ end of R527 using primers R527KF 5′-CAC CAT GGA ACT TCA TAA CCT GAG C-3′ and R527c-myc 5′-TCA CAG ATC TTC TTC AGA AAT AAG TTT TTG TTC GCC CTG GGA GGA GCC TTC C-3′. The resulting PCR fragment was directionally cloned into the mammalian expression vector pcDNA3.1 D/V5-His-TOPO (Invitrogen). The final PCR product was fully sequenced on both strands as described above.

Expression of R527. The human cDNA R527 cloned into pcDNA3.1 D/V5-His-TOPO was transfected into HEK293 cells stably expressing the G-protein α-subunit $G\alpha_{16}$ (Offermanns and Simon, 1995; Wu et al., 1995) using Transfast (Promega, Southampton, UK) according to the manufacturer's instructions. A clonal stable cell line expressing R527 was generated by antibiotic selection in the presence of G-418 (400 μg/ml). Receptor expression was confirmed by RT-PCR using primers CEJ536 5′-TCT TCA TCT TCT GCA TCC ACA CG-3′ and CEJ537 5′-AGT GGC AGG AAG AAC TCC AGC AG-3′ with an annealing temperature of 62°C. This primer pair combination gave a PCR product of 453 bp, the identity of which was confirmed by sequencing. Control RT-PCR reactions were performed with primers specific to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase with primers purchased from Stratagene (Amsterdam, The Netherlands) and using conditions suggested by the manufacturer.

Calcium Mobilization Assays. R527/HEK$G\alpha_{16}$ cells were seeded into 96-well Biocoat plates (BD Biosciences, Ermbodegen, Belgium) at 50,000 cells/well and maintained for approximately 24 h at 37°C in the presence of 5% CO_2. The cells were then incubated with Hanks' balanced salt solution containing 0.1% (w/v) bovine serum albumin, 20 mM HEPES, 2 μM Fluo-3-AM (Molecular Probes, Lieden, The Netherlands), 100 μM brilliant black, and 2.5 mM probenecid for 30 min at 37°C in the presence 5% CO_2. Plates were then transferred to a FLIPR (Molecular Devices Ltd, Wokingham, UK). Basal fluorescence was determined for 10 s, then 50 μl of agonist solution added per well and the fluorescence change monitored for 10 min. The data are presented as normalized fluorescence, calculated as (maximum fluorescence − minimum fluorescence)/minimum fluorescence and shown as mean ± S.E.M. of four independent experiments.

Cyclic AMP Assays. For measurements of adenylyl cyclase activity, cells were seeded in 24-well plates at a density of 100,000 cells per well and maintained in culture for 2 to 3 days. On the day of the assay, cells were washed and incubated for 4 h in serum-free medium containing [^{3}H]adenine (2 μCi/ml; Amersham Biosciences, Little Chalfont, Buckinghamshire, UK), to label the intracellular ATP pool. Where required, 100 ng/ml pertussis toxin (PTX; Sigma, Gillingham, UK) was included during this labeling period. Thereafter, plates were washed and cells were incubated in 500 μl of HEPES-buffered saline (130 mM NaCl, 0.8 mM $MgSO_4$, 5.4 mM KCl, 0.9 mM NaH_2PO_4, 1.8 mM CaCl2, 25 mM glucose, 20 mM HEPES, pH 7.4) supplemented with 1 μM isobutylmethylxanthine (Sigma) at 37°C. Cells were then stimulated with 3 μM of the diterpene forskolin (Sigma), and receptor agonists in appropriate concentrations. After 15 min of incubation, the reactions were stopped by aspiration of the buffer and cell extraction with 1 ml of ice-cold trichloroacetic acid (5%). Pools of [^{3}H]ATP and [^{3}H]cAMP were separated by sequential chromatography on Dowex and alumina columns. Data are shown as disintegrations per minute measured in the cAMP pool by liquid scintillation counting.

Cell Culture, RNA Extraction, and cDNA Synthesis. Primary human bronchial epithelial cells (HBECs), human umbilical vein endothelial cells, human airway smooth muscle cells, and human lung fibroblasts were purchased from BioWhittaker Europe (Verviers, Belgium) and grown according to the protocol provided by the supplier. Differentiated HBECs were grown as described in Danahay et al. (2002), and human neutrophils and eosinophils were isolated as described previously (Yousefi et al., 2001). Total RNA was isolated from cells using the RNeasy mini RNA isolation kit (QIAGEN, Crawley, UK) according to the manufacturer's instructions. First strand cDNA was prepared from total RNA using the reagents and protocol provided in the first strand cDNA synthesis kit (Roche Molecular Biochemicals, Lewes, UK). Genomic contamination of RNA samples was checked by performing control RT-PCR reactions without reverse transcriptase.

Quantitative PCR (TaqMan Analysis). Messenger RNA levels in total RNA samples were measured by TaqMan RT-PCR. Primers and probes were designed using Primer Express Software (Applied Biosystems, Foster City, CA). The probe used for R527 was 6-FAM labeled 5′-TCC ACC AAC CGC ACG GCC A-3′ and primers R527 RTF 5′-GCT GCC TGC AAA GTC AAC CT-3′ and R527 RTR 5′-CGA TGG CTG TGA GGA AGA CA-3′. Quantitative RT-PCR reactions were performed in triplicate in 25 μl final volumes and contained final concentrations 1× TaqMan Universal PCR master mix with 10 ng of the target cDNA preparation in each reaction. The optimized primer concentrations were 900 nM for the forward primer, 300 nM for the reverse primer, and 225 nM for the labeled probe. Experiments were performed using an ABI PRISM 7700 sequence detector (Applied Biosystems) and analyzed using ABI PRISM 7700 Sequence Detection System software. Amplification conditions were as follows: 50°C for 2 min and 95°C for 10 min followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The data were quantitated by extrapolation from the standard curve, normalized to glyceraldehyde-3-phosphate dehydrogenase, and the mean ± S.E.M. was plotted.

Northern Blot Analysis. Northern blot analysis was performed using 12-lane multiple tissue Northern blots (BD Clontech, Oxford, UK) according to the manufacturer's instructions. Briefly, a 453-bp R527 PCR fragment was generated as described above and was labeled with fresh [α-^{32}P]dCTP using the Rediprime labeling kit (Amersham Biosciences) according to the manufacturer's instructions. Prehybridization was carried out for 30 min in ExpressHyb solution (BD Clontech) containing herring sperm DNA (0.1 mg/ml). Hybridization was performed at 68°C for approximately 2 h with the denatured probe followed by four washes with 2× standard saline citrate, 0.05% SDS at RT for 10 min then two washes in 0.1× standard saline citrate, 0.1% SDS for 20 min at

50°C. Blots were exposed to a phosphorimager screen (Amersham Biosciences, Little Chalfont, UK) overnight and images processed using a Storm PhosphorImager machine (Amersham Biosciences) and with ImageQuant 5.0 software. Blots were stripped by boiling for 10 min in 0.5% SDS and rehybridized with a β-actin (BD Clontech) ^{32}P-labeled probe.

Results

Cloning and Sequence Analysis of the R527 G-Protein-Coupled Receptor. We have identified R527 as a part of a human genome-mining program to identify novel orphan GPCRs. The strategy for mining GPCRs involved searching the nonredundant amino acid database, followed by searching genomic databases such as the public High Throughput Genomic database. The datamining strategy used an iterative approach as described previously (Mickanin et al., 2001). R527 is encoded on the complementary strand of human genomic sequence from chromosome 2, GenBank accession number AC013396, from 98405 to 97251 bp. The identified region encodes a 1155-bp putative seven transmembrane receptor. RT-PCR was used to clone the coding region of this putative GPCR from human neutrophil cDNA, and the re-

```
   1  ATGGAACTTCATAACCTGAGCTCTCCATCTCCCTCTCTCTCCTCCTCTGTTCTCCCTCCC   60
      M  E  L  H  N  L  S  S  P  S  P  S  L  S  S  S  V  L  P  P

  61  TCCTTCTCTCCCTCACCCTCCTCTGCTCCCTCTGCCTTTACCACTGTGGGGGGGTCCTCT  120
      S  F  S  P  S  P  S  P  S  S  A  P  S  A  F  T  T  V  G  G  S  S

 121  GGAGGGCCCTGCCACCCCACCTCTTCCTCGCTGGTGTCTGCCTTCCTGGCACCAATCCTG  180
      G  G  P  C  H  P  T  S  S  S  L  V  S  A  F  L  A  P  I  L

                                                  TMI
 181  GCCCTGGAGTTTGTCCTGGGCCTGGTGGGGAACAGTTTGGCCCTCTTCATCTTCTGCATC  240
      A  L  E  F  V  L  G  L  V  G  N  S  L  A  L  F  I  F  C  I

                                                 TMII
 241  CACACGCGGCCCTGGACCTCCAACACGGTGTTCCTGGTCAGCCTGGTGGCCGCTGACTTC  300
      H  T  R  P  W  T  S  N  T  V  F  L  V  S  L  V  A  A  D  F

 301  CTCCTGATCAGCAACCTGCCCCTCCGCGTGGACTACTACCTCCTCCATGAGACCTGGCGC  360
      L  L  I  S  N  L  P  L  R  V  D  Y  Y  L  L  H  E  T  W  R

                                                 TMIII
 361  TTTGGGGCTGCTGCCTGCAAAGTCAACCTCTTCATGCTGTCCACCAACCGCACGGCCAGC  420
      F  G  A  A  A  C  K  V  N  L  F  M  L  S  T  N  R  T  A  S

 421  GTTGTCTTCCTCACAGCCATCGCACTCAACCGCTACCTGAAGGTGGTGCAGCCCCACCAC  480
      V  V  F  L  T  A  I  A  L  N  R  Y  L  K  V  V  Q  P  H  H

 481  GTGCTGAGCCGTGCTTCCGTGGGGGCAGCTGCCCGGGTGGCCGGGGGACTCTGGGTGGGC  540
      V  L  S  R  A  S  V  G  A  A  A  R  V  A  G  G  L  W  V  G

            TMIV
 541  ATCCTGCTCCTCAACGGGCACCTGCTCCTGAGCACCTTCTCCGGCCCCTCCTGCCTCAGC  600
      I  L  L  L  N  G  H  L  L  L  S  T  F  S  G  P  S  C  L  S

 601  TACAGGGTGGGCACGAAGCCCTCGGCCTCGCTCCGCTGGCACCAGGCACTGTACCTGCTG  660
      Y  R  V  G  T  K  P  S  A  S  L  R  W  H  Q  A  L  Y  L  L

                  TMV
 661  GAGTTCTTCCTGCCACTGGCACTCATCCTCTTTGCTATTGTGAGCATTGGGCTCACCATC  720
      E  F  F  L  P  L  A  L  I  L  F  A  I  V  S  I  G  L  T  I

 721  CGGAACCGTGGTCTGGGCGGGCAGGCAGGCCCGCAGAGGGCCATGCGTGTGCTGGCCATG  780
      R  N  R  G  L  G  G  Q  A  G  P  Q  R  A  M  R  V  L  A  M

                                                  TMVI
 781  GTGGTGGCCGTCTACACCATCTGCTTCTTGCCCAGCATCATCTTTGGCATGGCTTCCATG  840
      V  V  A  V  Y  T  I  C  F  L  P  S  I  I  F  G  M  A  S  M

 841  GTGGCTTTCTGGCTGTCCGCCTGCCGCTCCCTGGACCTCTGCACACAGCTCTTCCATGGC  900
      V  A  F  W  L  S  A  C  R  S  L  D  L  C  T  Q  L  F  H  G

      TMVII
 901  TCCCTGGCCTTCACCTACCTCAACAGTGTCCTGGACCCCGTGCTCTACTGCTTCTCTAGC  960
      S  L  A  F  T  Y  L  N  S  V  L  D  P  V  L  Y  C  F  S  S

 961  CCCAACTTCCTCCACCAGAGCCGGGCCTTGCTGGGCCTCACGCGGGGCCGGCAGGGCCCA  1020
      P  N  F  L  H  Q  S  R  A  L  L  G  L  T  R  G  R  Q  G  P

1021  GTGAGCGACGAGAGCTCCTACCAACCCTCCAGGCAGTGGCGCTACCGGGAGGCCTCTAGG  1080
      V  S  D  E  S  S  Y  Q  P  S  R  Q  W  R  Y  R  E  A  S  R

1081  AAGGCGGAGGCCATAGGGAAGGTGAAAGTGCAGGGCGAGGTCTCTCTGGAAAAGGAAGGC  1140
      K  A  E  A  I  G  K  V  K  V  Q  G  E  V  S  L  E  K  E  G

1141  TCCTCCCAGGGCTGA  1155
      S  S  Q  G  *
```

Fig. 1. Nucleotide and predicted protein sequence of R527. Putative transmembrane regions are underlined and numbered I to VII. The transmembrane regions were predicted as described in Krogh et al. (2001). This sequence has been deposited in GenBank with accession number AY158687.

sulting cDNA clone was named R527 (Fig. 1). The sequence was found to have three base changes in the coding region of R527 compared with Genbank AC013396 at nt 681 (G to A), nt 867 (A to C) and nt 1102 (C to G). The open reading frame encodes a 384-amino acid protein, and multiple sequence alignment of R527, with its closest homologs, is shown in Fig. 2. R527 shows closest sequence identity to the putative chemokine GPCR HM74 with 39.6% identity over 283 amino acids and also with orphan receptors of unknown function GPR81 and GPR31, with 40 and 34% sequence identity over 281 and 288 amino acids, respectively. During the preparation of this article, Hosoi et al. (2002) reported a sequence of a receptor TG1019 with 99.7% sequence identity to R527. R527 differs from TG1019 by a truncation 39 amino acids at the N terminus of R527 and a single amino acid change (Leu to Val) at amino acid 368.

Generation of Stable Cell Lines Expressing R527. To identify the ligand for R527, a stable cell line was generated for the receptor in HEK293 cells stably expressing the G-protein α-subunit $G\alpha_{16}$. An expression construct was prepared with a *c-myc* tag engineered at the C terminus of R527 and was used for transfection and generation of stable clonal cell lines isolated by antibiotic selection. Expression of R527 mRNA was confirmed by RT-PCR and at the protein level by immunofluorescent detection with a fluorescein isothiocyanate-labeled anti–c-Myc antibody (data not shown).

Identification of Ligands for R527. Many G protein-coupled receptors can be functionally linked to the phosphoinositide signaling pathway via $G\alpha_{16}$ (Offermanns and Simon, 1995). We used this approach to search for potential ligands of R527. Changes in intracellular calcium in response to agonist stimulation of a selected R527/HEK$G\alpha_{16}$ cell line

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R527          ---------------------------------------MELHNLSSPSPSLSSSVLPPS
TG1019        MLCHRGGQLIVPIIPLCPEHSCRGRRLQNLLSGPWPKQPMELHNLSSPSPSLSSSVLPPS
GPR31         ---------------------------------------MPFPNCSAP------------
HM74          --------------------------MNRHHLQDHFLEIDKKNCCVFR-----------
GPR81         ---------------------------------------MYNGSCCRIE-----------
                                                      :   . .

R527          FSPSPSSAPSAFTTVGGSSGGPCHPTSSSLVSAFLAPILALEFVLGLVGNSLALFIFCIH
TG1019        FSPSPSSAPSAFTTVGGSSGGPCHPTSSSLVSAFLAPILALEFVLGLVGNSLALFIFCIH
GPR31         --------------------------STVVATAVGVLLGLECGLGLLGNAVALWTFLFR
HM74          --------------------------DDFIAKVLPPVLGLEFIFGLLGNGLALWIFCFH
GPR81         --------------------------GDTISQVMPPLLIVAFVLGALGNGVALCGFCFH
                                       .  ::  :  :* :   :* :**.:**  * ::

R527          TRPWTSNTVFLVSLVAADFLLISNLPLRVDYYLLHETWRFGAAACKVNLFMLSTNRTASV
TG1019        TRPWTSNTVFLVSLVAADFLLISNLPLRVDYYLLHETWRFGAAACKVNLFMLSTNRTASV
GPR31         VRVWKPYAVYLLNLALADLLLAACLPFLAAFYLSLQAWHLGRVGCWALRFLLDLSRSVGM
HM74          LKSWKSSRIFLFNLAVADFLLIICLPFVMDYYVRRSDWNFGDIPCRLVLFMFAMNRQGSI
GPR81         MKTWKPSTVYLFNLAVADFLLMICLPFRTDYYLRRRHWAFGDIPCRVGLFTLAMNRAGSI
               : *..  ::*..*. **:**   **:   :*:    * :*   *    * :  .*  .:

R527          VFLTAIALNRYLKVVQPHHVLSRASVGAAARVAGGLWVGILLLNGHLLLSTFSGP---SC
TG1019        VFLTAIALNRYLKVVQPHHVLSRASVGAAARVAGGLWVGILLLNGHLLLSTFSGP---SC
GPR31         AFLAAVALDRYLRVVHPRLKVNLLSPQAALGVSGLVWLLMVALTCPGLLISEAAQNSTRC
HM74          IFLTVVAVDRYFRVVHPHHALNKISNWTAAIISCLLWGITVGLTVHLLKKKLLIQN--GP
GPR81         VFLTVVAADRYFKVVHPHHAVNTISTRVAAGIVCTLWALVILGTVYLLLENHLCVQ--ET
               **:.:* :**::**:*:  :.  *  .*  :   :*   :  .   *  .

R527          LSYRVGTKPSASLRWHQALYLLEFFLPLALILFAIVSIG--LTIRNRGLGGQAGPQRAMR
TG1019        LSYRVGTKPSASLRWHQALYLLEFFLPLALILFAIVSIG--LTIRNRGLGGQAGPQRAMR
GPR31         HSFYSRADGSFSIIWQEALSCLQFVLPFGLIVFCNAGIIRALQKRLREPEKQPKLQRAQA
HM74          ANVCISFSICHTFRWHEAMFLLEFLLPLGIILFCSARIIWSLRQRQ--MDRHAKIKRAIT
GPR81         AVSCESFIMESANGWHDIMFQLEFFMPLGIILFCSFKIVWSLRRRQQ-LARQARMKKATR
                        :  *:: :  *:*.:*:.:*:*.   *   *  *      :.  ::*

R527          VLAMVVAVYTICFLPSIIFGMASMVAFWLS---ACRSLDLCTQLFHGSLAFTYLNSVLDP
TG1019        VLAMVVAVYTICFLPSIIFGMASMVAFWLS---ACRSLDLCTQLFHGSLAFTYLNSVLDP
GPR31         LVTLVVVLFALCFLPCFLARVLMHIFQNLG---SCRALCAVAHTSDVTGSLTYLHSVVNP
HM74          FIMVVAIVFVICFLPSVVVRIRIFWLLHTSGTQNCEVYRSVDLAFFITLSFTYMNSMLDP
GPR81         FIMVVAIVFITCYLPSVSARLYFLWTVPSS---ACDP--SVHGALHITLSFTYMNSMLDP
              .: :*. ::  *:**..   :        .    *            : ::**::*:::*

R527          VLYCFSSPNFLHQSRALLGLTRGRQGPVSD-------------------ESSYQPSRQWR
TG1019        VLYCFSSPNFLHQSRALLGLTRGRQGPVSD-------------------ESSYQPSRQWR
GPR31         VVYCFSSPTFRSSYRRVFHTLRGK-GQAAE-------------------PPDFNPRDSYS
HM74          VVYYFSSPSFPNFFSTLINRCLQRKMTGEPDNNRSTSVELTGDPNKTRGAPEALMANSGE
GPR81         LVYYFSSPSFPKFYNKLKICSLKPKQPG---------------HSKTQRPEEMPISNLGR
              ::* ****.*      :

R527          YREASRKAEAIGKVKVQGEVSLEKEGSSQG------
TG1019        YREASRKAEAIGKLKVQGEVSLEKEGSSQG------
GPR31         -------------------------------------
HM74          PWSPSYLGPTSNNHSKKGHCHQEPASLEKQLGCCIE
GPR81         ---RSCIS-VANSFQSQSDGQWDPHIVEWH-----
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Fig. 2. Multiple sequence alignment of R527 and related sequences. R527 was aligned with HM74 (GenBank D10923), GPR81 (GenBank AF41110), GPR31 (GenBank HSU65402), and TG1019 (GenBank AB083055) using the multiple sequence alignment program ClustalW. *, conserved residues; ·, semiconservative changes; :, conservative changes.

were measured in a FLIPR assay. Screening of a collection of approximately 2000 known and putative GPCR agonists identified two lipids that activated the receptor, 5-oxo-ETE and 5(*S*)-HPETE. Concentration dependent dose-response curves for these eicosanoids in a FLIPR assay are shown in Fig. 3. The EC_{50} for 5-oxo-ETE was determined to be 114 ± 15 nM (n = 4). 5(*S*)-HPETE and was found to be a much poorer agonist with potency approximately 100-fold less than those observed for 5-oxo-ETE. Another structurally related lipid 5(*S*)-HETE had only a very weak effect in the assay. No activity was detected for any of the ligands against vector-only transfected cells up to a concentration of 3 μM (Fig. 3). Leukotrienes LTB_4, LTC_4, LTD_4, and LTE_4 were tested for their activity against the stable R527/HEKGα_{16} cell line but no calcium mobilization activity was observed. The cysLT1R antagonist CGP45715A (Bray et al., 1991) and the LTB_4 receptor antagonist CGS25019C (Raychaudhuri et al., 1995) were analyzed for their effects on 5-oxo-ETE induced increases in intracellular calcium. No inhibition of the 5-oxo-ETE signal was observed for antagonist concentrations of up to 10 μM (data not shown).

Cyclic AMP Assays. The effects of 5-oxo-ETE on forskolin-stimulated cAMP release were examined using the R527/HEKGα_{16} stable cell line in a radiochemical assay. Increasing concentrations of 5-oxo-ETE were found to inhibit forskolin-induced cAMP formation, with maximal effects seen at 100 nM 5-oxo-ETE (Fig. 4). The EC_{50} for 5-oxo-ETE in this assay was 0.33 ± 0.1 nM (n = 6). The 5-oxo-ETE-induced inhibition of adenylyl cyclase could be reversed by preincubation of the cells with pertussis toxin, suggesting that R527 is Gα_i coupled.

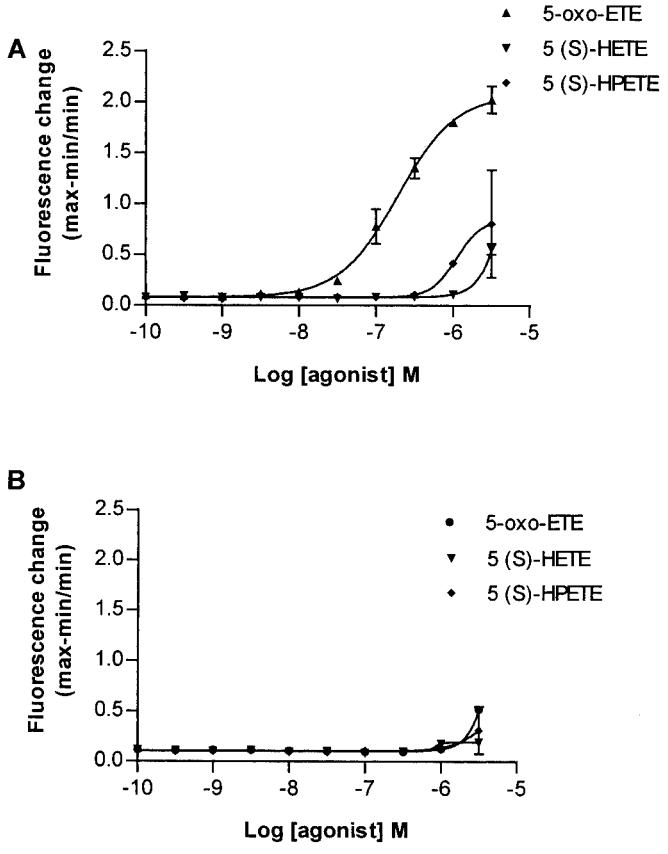

Fig. 3. Effects of 5-oxo-ETE and other eicosanoids on calcium mobilization. HEKGα_{16} cells stably transfected with R527 (A) or vector control (B) were incubated with increasing concentrations of 5-oxo-ETE, 5(*S*)-HETE, or 5(*S*)-HPETE, and changes in intracellular calcium were measured by FLIPR.

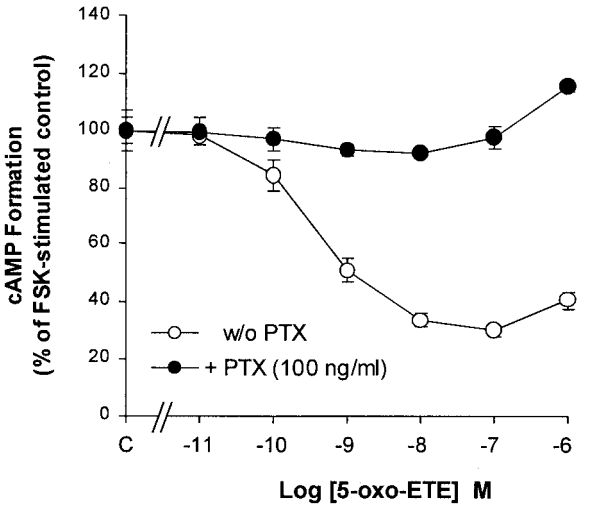

Fig. 4. Effects of 5-oxo-ETE on cAMP release and PTX sensitivity. HEKGα_{16} cells stably transfected with R527 were untreated or treated with 100 ng/ml pertussis toxin for 4 h. The effects of various concentrations of 5-oxo-ETE on forskolin induced cAMP production are shown (○). The effects of PTX on cAMP production are also shown (●).

Expression of R527 in Primary Human Cells. The expression of R527 in primary human cells was examined by TaqMan quantitative RT-PCR. A receptor binding 5-oxo-ETE has been previously described on neutrophils (Powell et al., 1993), eosinophils (Powell et al., 1995), and monocytes (Sozzani et al., 1996), so expression profiling was initially performed on several inflammatory cell types. The expression of R527 was most abundant in eosinophils with expression levels more than 200-fold greater than those observed in human lung macrophages (Table 1). High levels of expression were also observed in neutrophils and lung macrophages, although much lower than the levels detected in eosinophils. Very low levels of expression of R527 were found in HBECs, differentiated HBECs, fibroblasts, and T cells, with no expression observed in airway smooth muscle cells.

Tissue Expression Profile of R527. The tissue expression profile of R527 was obtained by Northern blot analysis. Hybridization of a radiolabeled R527 probe to a blot containing poly(A^+) RNA from 12 human tissues gave the results shown in Fig. 5. R527 is highly expressed in kidney and liver,

TABLE 1

TaqMan quantitative PCR analysis of R527 levels in human cells

Data are normalized to glyceraldehyde-3-phosphate dehydrogenase and are shown relative to the R527 expression level in macrophages.

Sample	Relative Expression
	-fold
Eosinophil	210 ± 42
Neutrophil	5.8 ± 0.57
Lung bronchoalveolar lavage macrophage	1 ± 0.087
T cell	0.06 ± 0.01
Differentiated HBEC	0.012 ± 0.001
HBEC	<0.001
Fibroblast	<0.001
Human umbilical vein endothelial cells	<0.001
Smooth muscle cells	0

with weak expression in placenta, and gave a single transcript of approximately 7.5 kilobase pairs. Weak expression could be detected in PBLs, which gave a smaller transcript of 1.6 kilobase pairs. No expression could be detected in any of the other tissues examined.

Discussion

The eicosanoid 5-oxo-ETE was previously suggested to play a role in the inflammatory response. A receptor for this mediator has been described on human eosinophils, neutrophils, and monocytes (Powell et al., 1993, 1995; Sozzani et al., 1996), and many of the responses have been shown to be sensitive to pertussis toxin, suggesting that the effects are mediated via a $G\alpha_i$-coupled GPCR (Sozzani et al., 1996; Powell et al., 1997). A role for 5-oxo-ETE in the inflammatory response has also been suggested in vivo, and this eicosanoid has been proposed to be a physiological mediator of inflammation in asthma (Stamatiou et al., 1998). Indeed, in a Brown Norway rat model, 5-oxo-ETE administered to the airways by tracheal insufflation induced a dramatic increase in the number of eosinophils around the walls of the airways (Stamatiou et al., 1998). The reported increase in eosinophil numbers was not inhibited by antagonists of LTB_4 or platelet-activating factor receptors, suggesting that 5-oxo-ETE acts via a different receptor. 5-oxo-ETE has also been proposed to play a role in dermal inflammatory diseases via its involvement in the migration of neutrophils from the basal layer to the upper compartments of the epidermis, contributing to the histopathological features of this disease (Zimpfer et al., 1998). We have demonstrated that receptor R527 is highly expressed in the kidney, a feature also observed by Hosoi et al. (2002). Previous reports have suggested a role of metabolites of the 5-lipoxygenase (5-LO) pathway and in particular the leukotrienes, in renal hemodynamics (Imig, 2000). Studies using 5-LO–deficient mice have also suggested a role for 5-LO metabolites in allograft rejection (Goulet et al., 2001); however, the precise role of 5-oxo-ETE and its receptor is not clear.

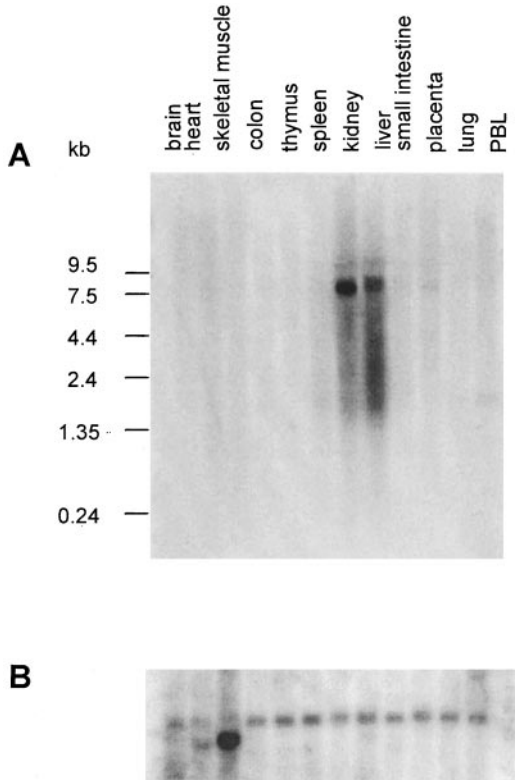

Fig. 5. Northern blot analysis of the tissue distribution of R527. A human multiple tissue Northern blot was probed with a ^{32}P-labeled probe to analyze the tissue expression of R527 (A). The blot was stripped and re-hybridized with the housekeeping gene control β-actin (B)

We have described the isolation and identification of 5-oxo-ETE as an agonist of a novel GPCR, R527. The receptor seems to belong to a family of GPCRs comprising the three other orphan receptors, HM74, GPR81, and GPR31, all with significant sequence identity at the amino acid level. Analysis of the expression profile of R527 indicates that it is highly expressed in eosinophils, neutrophils, and lung macrophages, which parallels the activities of 5-oxo-ETE described on a previously unidentified GPCR on eosinophils, neutrophils, and monocytes (Powell et al., 1993, 1995; Sozzani et al., 1996). We have shown that in addition to 5-oxo-ETE, receptor R527 is also activated, but to a lesser extent, by the related lipid 5(*S*)-HPETE. 5-oxo-ETE and 5(*S*)-HPETE are both metabolites derived from arachidonic acid on the 5-LO pathway. A further structurally related lipid 5(*S*)-HETE has previously been shown to be approximately 350-fold less potent than 5-oxo-ETE in raising intracellular calcium levels in neutrophils (Powell et al., 1993), although there are no reports describing the activity of 5(*S*)-HPETE. Desensitization experiments have previously indicated that 5-oxo-ETE and 5-HETE probably act via the same receptor on neutrophils (Powell et al., 1993).

In FLIPR experiments, 5-oxo-ETE induced a concentration-dependent increase of intracellular calcium in a R527/HEK$G\alpha_{16}$ stable cell line; this effect was not inhibited by the cysLT1R and LTB4 receptor antagonists CGS25019C (Bray et al., 1991) or CGP45715A (Raychaudhuri et al., 1995), respectively. In addition, leukotrienes LTB_4, LTC_4, and LTD_4 did not induce calcium mobilization in the R527 stable cell line, suggesting that the effects of 5-oxo-ETE are independent of the LTB_4 or cysLT receptors. These observations are similar to those for 5-oxo-ETE on neutrophils and monocytes, where the LTB_4 receptor antagonist LY255283 was found to have no effect on 5-oxo-ETE–induced effects, whereas responses to LTB_4 were blocked by LY255283 (O'Flaherty et al., 1996a; Sozzani et al., 1996). Production of cAMP was inhibited by increasing concentrations of 5-oxo-ETE in the R527/HEK$G\alpha_{16}$ cell line, indicating that the receptor is $G\alpha_i$ coupled. This mode of G-protein coupling was confirmed by pretreating the cells with pertussis toxin, which inactivates G-proteins of the $G\alpha_i$ family (Milligan, 1988) and completely blocked the 5-oxo-ETE responses. The GPCR previously described on eosinophils, neutrophils, and monocytes was also shown to be sensitive to pertussis toxin and therefore described was as $G\alpha_i$-coupled (Sozzani et al., 1996; Powell et al., 1997).

The relative promiscuity of $G\alpha_{16}$ has been used to generate screening platforms for a broad range of different GPCRs (Stables et al., 1997), particularly where the physiological G-protein coupling of the receptor is unknown. Mammalian cells expressing $G\alpha_{16}$ have been shown to detect responses from $G\alpha_i$-, $G\alpha_s$-, or $G\alpha_q$-coupled receptors via activation of phospholipase C (Offermanns and Simon, 1995; Wu et al., 1995). The EC_{50} for 5-oxo-ETE via $G\alpha_{16}$ coupling in the

R527/HEKGα_{16} FLIPR assay was approximately 350-fold higher than the EC_{50} for the cAMP assay, where coupling is through Gα_i. Thus it seems that the coupling of the receptor through its natural G-protein partner is more efficient than activation through Gα_{16} in this case. Although there are many reports of coupling through Gα_{16} giving similar results to the natural G-protein coupling, there are also exceptions. For instance, the EC_{50} for the melatonin-induced activation of the ML_{1A} melatonin receptor was found to be approximately 100-fold higher through Gα_{16} than through Gα_i (Reppert et al., 1994; Stables et al., 1997).

R527 is almost identical to the TG1019 receptor published during the preparation of this article (Hosoi et al., 2002) and seems to be the same receptor, except for a difference in predicted start codon. The sequence described by Hosoi et al. (2002), TG1019, is longer at the N terminus by 39 amino acids. Analysis of the nucleotide sequence at the putative translation initiation sites for R527 and TG1019 reveals that TG1019 seems to have a stronger Kozak sequence. It contains an A at the −3 position, the most highly conserved position within the consensus sequence (Kozak, 1986) whereas R527 contains a C at this location. Although the putative coding region of R527 contains a G and TG1019 has a T at +4, the second most important determinant of start site strength, it is likely that the more upstream ATG of TG1019 is used in vivo consistent with the scanning model of translation initiation (Kozak, 1999). This may indicate that we have used an N-terminally truncated version of the native receptor in this study. Nevertheless, expression of the receptor has not been affected by this truncation, given that our findings concerning ligand binding compare well with those of Hosoi et al. (2002) and with earlier data on the 5-oxo-ETE receptor, suggesting that the N terminus of the receptor is not involved in these processes.

In summary, we report here the identification, cloning, and generation of a stable cell line of a novel GPCR, R527. 5-oxo-ETE was identified as the ligand for R527, and many of the pharmacological properties observed for this recombinant receptor were found to be similar to those of a 5-oxo-ETE receptor previously described on eosinophils, neutrophils, and monocytes. It remains possible that there are additional receptors that bind 5-oxo-ETE that have not yet been identified. The cell-type expression profile of receptor R527 and the reported chemotactic effects of 5-oxo-ETE suggest that it may play an important role in inflammatory diseases such as asthma and psoriasis. R527 may therefore represent an attractive therapeutic target for inflammatory diseases.

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