0026-895X/00/020407-06$3.00/0
MOLECULAR PHARMACOLOGY

MOL 58:407–412, 2000 /13126/842491

Edg2 Receptor Functionality: Giα1 Coexpression and Fusion Protein Studies

GEORGE MCALLISTER, JOSEPHINE A. STANTON, KAMRAN SALIM, EMMA J. HANDFORD, and MARGARET S. BEER

Department of Biochemistry, Merck, Sharp & Dohme Research Laboratories, Neuroscience Research Centre, Terlings Park, Harlow, Essex, United Kingdom

Received November 29, 1999; accepted May 12, 2000

This paper is available online at http://www.molpharm.org

ABSTRACT

Recombinant receptor cell lines are widely used in G-protein-coupled receptor selectivity studies. To unequivocally interpret the results of such studies, it is essential that the host cell line does not endogenously express the receptor of interest and in addition is unresponsive to the receptor's natural ligand. Here we describe an approach to overcome such difficulties associated with orphan receptors or, as in the present case, receptors whose endogenous ligand ubiquitously affects mammalian cells. The functional heterologous assay system described is for the hEdg2 receptor, which uses lysophosphatidic acid as its endogenous ligand. Once activated, this receptor mediates its effects via multiple secondary messenger pathways, including a Gi-coupled pathway. We have transiently expressed a pertussis toxin-insensitive hEdg2 receptor-ratGiα1 fusion protein into human embryonic kidney cells and have monitored the ability of compounds to stimulate [^{35}S]GTPγS binding in membranes prepared from these cells after pretreatment with toxin. Because the assay conditions used favor Gi-mediated responses and because endogenous Giα subunits are rendered inactive, the response measured is, by definition, fusion protein-mediated. Consequently, we have developed an assay that monitors definitively Edg2 receptor-mediated responses in a mammalian cell line. A limited structure activity relationship study suggests that the lysophospholipid carbon chain has a role in receptor activation and in addition indicates that certain modifications to the phosphate group are tolerated.

The use of recombinant receptor cells lines is an established approach in the search for selective compounds for G-protein-coupled receptors (GPCRs). Indeed, since the advent of molecular cloning and the consequent discovery of an unexpected degree of receptor multiplicity within GPCR superfamilies (e.g., 5-hydroxytryptamine [5-HT]) combined with subtype colocalization within tissue types it is hard to imagine how selective compounds could be identified without such techniques. Great care, however, must be taken in the choice of host cell lines for such heterologous receptor expression systems. For example, it is essential that the receptor subtype under investigation is not endogenously expressed by the host cell line. To measure the assay endpoint with confidence, it is necessary to ensure that the host cell is completely unresponsive to the transfected receptor's endogenous ligand or closely related analogs. Generally this might, at worst, severely restrict the availability of suitable host cell types requiring perhaps the use of a less than ideal cell line in terms of, for example, growth characteristics. In the case of orphan receptors, i.e., receptors with no known endogenous ligand, or, as is the case in the present study, receptors whose endogenous ligand ubiquitously affects mammalian cells, such considerations become more problematic. Here we describe an approach to develop a functional assay for the human Edg2 (hEdg2) receptor that uses lysophosphatidic acid (LPA) as its endogenous ligand. Evidence is emerging to suggest that Edg2 receptors play a crucial role in nerve cell myelination and might offer a potential target in the treatment of demyelinating diseases such as multiple sclerosis. For example not only are Edg2 receptors expressed exclusively in adult oligodendrocytes and Schwann cells (Weiner et al., 1998) but also LPA protects against apoptosis in cultured Schwann cells (Weiner and Chun, 1999).

The Edg2 receptor belongs to a GPCR family so called because the first family member to be cloned was encoded by an endothelial differentiation gene (Hla and Maciag, 1990). Eight family members have been identified sharing closest resemblance to the cannabinoid receptor family. They play an important role in cell growth, development, and maintenance and in modulating cytoskeletal-dependent changes. These effects are achieved by activating multiple secondary messenger pathways involving coupling to numerous tri-

ABBREVIATIONS: GPCR, G-protein-coupled receptor; 5-HT, 5-hydroxytryptamine; hEdg2, human endothelial differentiation gene-2; LPA, oleoyl 1-acyl-2-hydroxy-s*n*-glycero-3-phosphate lysophosphatidic acid; PTX, pertussis toxin; GTPγS, guanosine 5′[γ-thio]triphosphate; S-1-P, sphingosine-1-phosphate; LPC, oleoyl 1-acyl-2-hydroxy-s*n*-glycero-3-phosphocholine choline phosphatidic acid; LPE, oleoyl and palmitoyl 1-acyl-2-hydroxy-*sn*-glycero-3-phosphoethanolaminephosphatidic acid; LPG, oleoyl 1-acyl-2-hydroxy-s*n*-glycero-3-[phospho-*rac*-(1-glycerol)]glycerol-phosphatidic acid.

meric guanine nucleotide-binding proteins (Gi, Gq/11, G12/13) and/or the small G-protein Rho (Goetzl and An, 1998).

LPA, the endogenous ligand for the Edg2 receptor, is the simplest of all glycerophospholipids and one of a growing list of lipid extracellular messengers. It is found in 0.5 μM concentrations in serum being formed either by de novo synthesis from glucose-derived dihydroxyacetone phosphate and a fatty acid-coenzyme A but more probably by enzymatic conversion of precursor glycerophospholipids (Goetzl and An, 1998). It is largely associated with, and released from, activated platelets and mediates its effects, at least in part, by Edg2, Edg4, and Edg7 receptor stimulation.

The ubiquitous responsiveness of mammalian cells lines to LPA has led not only to a limited choice of suitable host cell lines for recombinant receptor cell line studies but also to difficulties in identifying it as the Edg2 receptor endogenous ligand. Erickson et al. (1998) sought to overcome this problem by transfecting the hEdg2 receptor in *Saccharomyces cerevisiae* and monitoring the activation of the pheromone-inducible mitogen-activated protein kinase cascade. The main disadvantage of this approach is the very weak potency of compounds tested, presumably due to inefficient coupling of the mammalian receptor with the yeast G-protein, limiting potential structure activity relationship studies.

An alternative approach described in this study and based on the Edg2 receptor/Gi signaling pathway has been to construct a fusion protein in which the N terminus of the rGiα1 subunit is linked, in-frame, to the C terminus of the Edg2 receptor (Bertin et al., 1994; Stables et al., 1997; Wise et al., 1997a,b). In addition, the rGiα1 subunit has been rendered pertussis toxin (PTX)-insensitive by the mutation of cysteine 351 to glycine (Milligan, 1988). An agonist-induced [^{35}S]guanosine 5′[γ-thio] triphosphate ([^{35}S]GTPγS) binding assay with membranes from HEK cells transiently expressing the hEdg2 receptor-rGiα1 fusion protein has then been developed (Stanton and Beer, 1997). Pretreating the cells with PTX ensures that the agonist-induced [^{35}S]GTPγS binding is, by definition, mediated by the transfected receptor. This fusion protein approach has enabled us to confirm that LPA activates hEdg2 receptors in mammalian cells. This assay gives a measure of potency and efficacy and is amenable to high throughput application. Identification of compounds with a range of intrinsic efficacies should be invaluable in exploring the physiological/pathophysiological role of the Edg2 receptor.

Experimental Procedures

Materials. [^{35}S]GTPγS (>1000 Ci/mmol) was obtained from Amersham Pharmacia Biotech (UK). All tissue culture reagents were from Sigma (St. Louis, MO) or Life Technologies Inc. (Rockville, MD). PTX and sphingosine-1-phosphate (S-1-P) were purchased from Sigma and oleoyl 1-acyl-2-hydroxy-*sn*-glycero-3-phosphate, sodium salt (LPA), oleoyl (and palmitoyl) 1-acyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (LPE), oleoyl 1-acyl-2-hydroxy-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)], sodium salt (LPG), and oleoyl 1-acyl-2-hydroxy-*sn*-glycero-3-phosphocholine (LPC) were from Avanti Polar Lipids Inc. (Alabaster, AL). Primers were purchased from Life Technologies Inc. and restriction enzymes from New England BioLabs Inc. (Beverly, MA).

Cloning of hEdg2 Receptor. The cDNA encoding the hEdg2 receptor was cloned with polymerase chain reaction (PCR) techniques with a *klenTaq* polymerase mix (Advantage cDNA PCR kit from Clontech, Palo Alto, CA). PCR reactions were performed on human substantia nigra cDNA (Clontech) with the primers 5′-GTCATGGCTGCCATCTCTACT-3′ (sense) and 5′-GTTCTAAACCACAGAGTGGTC-3′ (antisense). The PCR-amplified fragment was subcloned into pCR-Blunt II-TOPO (Invitrogen, Carlsbad, CA) and confirmed as that encoding for the hEdg2 receptor by sequencing. For use in the cotransfection studies the hEdg2 receptor cDNA was excised with the restriction enzymes *Xho*I and *Hin*dIII and ligated into the mammalian expression vector pcDNA3.1(−) (Invitrogen). For the hEdg2 receptor-Cys^{351} → Gly rGiα1 fusion studies the open reading frame (ORF) of the hEdg2 receptor underwent further PCR with the following primers containing *Hin*dIII and *Nco*I restriction sites, respectively (underlined), 5′-AAGCTTATGGCTGCCATCTCTACTTCCATC-3′ (sense) and 5′-CCATGGCAACCACAGAGTGGTCATTGCTGT-3′ (antisense). The PCR-amplified fragment was then subcloned into pCRII-TOPO (Invitrogen).

Construction of Cys^{351} → Gly rGiα1. Site-directed mutagenesis on the rGiα1 subunit [with a QuikChange kit (Stratagene, La Jolla, CA)], was carried out to remove a *Nco*I site (Ala^{87}, GCC→GCG) within the ORF. This allowed a *Nco*I site at the beginning of the ORF to be used as the site of fusion of the hEdg2 receptor to the rGiα1 subunit. To render the rGiα1 subunit PTX-insensitive, the cDNA underwent PCR with the Advantage cDNA PCR kit and the primers 5′-GAATTCCCATGGGCTGCACACTGAGCGCTGAGGACA-3′ (sense) and 5′-GCGGCCGCTCTAGATTAGAAGAGACC**ACC**GTCTTTTAGGTTATT-3′ (antisense) (Cys^{351} → Gly in bold; restriction sites for *Eco*RI, Nco 1, *Not*I, and *Xba*I underlined) resulting in a Cys^{351} → Gly mutation in the 3′ end of the rGiα1 subunit and hence to PTX insensitivity. This PCR product was then reinserted into the *Eco*RI/*Xba*I site of pBluescript SK(−), and the mutation was confirmed by sequence analysis.

Construction of the hEdg2 Receptor-Cys^{351} → Gly rGiα1 Fusion Protein. The hEdg2 cDNA in pCRII-TOPO was digested with *Hin*dIII and *Nco*I and ligated into the Cys^{351} → Gly rGiα1 subunit containing pBluescript SK(−), itself having been digested with *Hin*dIII and *Nco*I. Introduction of the *Nco*I site at the 3′ end of the ORF of the receptor resulted in the removal of the stop codon and insertion of an extra alanine between the receptor and rGiα1 subunit. The full fusion construct was then excised from pBluescript SK(−) with *Hin*dIII and *Not*I, ligated into the mammalian expression vector pcDNA3.1(+) and confirmed by sequence analysis.

Construction of the myc and Hemaglutinin (HA) Epitope-Tagged rGiα1 Subunits. The myc (EQKLISEED) and HA (DVPDYA) epitope tags were engineered into the rGiα1 subunit after the sixth amino acid residue from its N terminus. The 5′-oligonucleotides containing the myc and HA tags (underlined) and an *Eco*RI (GAA TTC) restriction site were ATC GAA TTC ATG GGC TGC ACA CTG AGC GCT GAG CAG AAG CTG ATC TCC GAG GAG GAC CTG GAG GAC AAG GCG GCC GTG GAG and ATC GAA TTC ATG GGC TGC ACA CTG AGC GCT GAC GTC CCC GAC TAC GCG GAG GAC AAG GCG GCC GTG GAG, respectively. The 3′-oligonucleotide containing the stop codon and an *Xba*I restriction site (ATC TAG) was AT ATC TAG ACT AGC AGA GCT TAG AAG AGA CCA CAG TCT. Introduction of the epitope tags was achieved with standard PCR methodologies. The PCR products were then restriction enzyme-digested and subcloned into pcDNA3.1(+). The sequence of these constructs was confirmed by an ABI 375 automated fluorescence sequencer.

Transient Transfection of Human Embryonic Kidney (HEK) 293 Cells. HEK cells were plated out at a density of 3×10^5 cells/ml in cell culture dishes containing 10 ml of Dulbecco's modified Eagle's medium (with 8% fetal calf serum) and incubated overnight. The following day 0.5 ml of 0.25 M $CaCl_2$, 0.5 ml 2× BBS buffer (50 mM *N*,*N*-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid, 280 mM NaCl, 1.5 mM Na_2HPO_4), pH 6.95, and DNA (7.5 μg of hEdg2 receptor plus 7.5 μg of rGiα1 subunit [tagged, wild type, or Cys^{351} → Gly-mutated] for cotransfection studies and 15 μg for the hEdg2 receptor-rGiα1 fusion protein) was made up, left at room temperature (RT) for 15 min, and added to each cell culture dish. The HEK

cells were incubated at 37°C in 3% CO_2 for 48 to 60 h before being harvested in phosphate-buffered saline and stored at −70°C. For the PTX studies 100 ng/ml PTX was added to the cell medium 20 h before harvesting.

Agonist-Induced [^{35}S]GTPγS Binding. Untransfected HEK 293, CHO-K1, Ltk$^-$, HeLa, COS-7, PC12, or HEK 293 cells transiently expressing the hEdg2 receptor either alone or cotransfected with the rGiα1 subunit (HA or myc epitope tagged at the N terminus of the subunit, wild type, or Cys351→Gly-mutated) were homogenized in ice-cold 20 mM HEPES buffer containing 10 mM EDTA (pH 7.4 at RT) with a Kinematica polytron (setting 5, 10 s) and centrifuged at 48,000*g*, 4°C for 15 min. The pellet was resuspended in ice-cold 20 mM HEPES buffer containing 0.1 mM EDTA (pH 7.4 at RT) and recentrifuged at 48,000*g*, 4°C for 15 min. The final pellet was resuspended in 20 mM HEPES buffer containing 100 mM NaCl and 10 mM $MgCl_2$, pH 7.4, at RT.

Membranes (2.5 mg wet weight) were incubated with GDP (1 μM) and diluted with test drug in assay buffer containing 1% fatty acid free BSA (final assay concentration of 0.1% BSA) for 20 min at 30°C before being placed on ice for 15 min. [^{35}S]GTPγS (100 pM) was then added to a final volume of 1 ml, and the tubes were incubated for a further 30 min at 30°C. The incubation was terminated by filtering over GF/B filters with a cell harvester (Brandel Research and Development Laboratories, Inc., Gaithersburg, MD), and the filters were washed once with 5 ml of water. Radioactivity was counted by liquid scintillation spectrometry at an efficiency of >90%.

Data Analysis. Background filter counts, i.e., residual radioactivity bound to the filter in the absence of membrane, were subtracted from each sample count. Test results are represented as either percentage increase in binding above basal (that seen in the absence of test compound) or stimulated − basal dpm. Dose-response curves were plotted and analyzed by nonlinear, least-squares regression analysis with an iterative curve-fitting routine (Marquardt-Levenberg method) provided by the data manipulation software RS/1 (Software Products Corp., Cambridge, MA). Potency values are expressed as pEC_{50} values ($-\log_{10}$ concentration of agonist required to give 50% of its own maximal stimulation). Efficacy values are given as a percentage of the maximal response seen with LPA. Data are expressed as arithmetic mean ± S.E. from ≥3 experiments.

Results

All assays were carried out in the presence of 1 μM GDP. Addition of GDP favors binding to Giα subunits, which have a relatively high spontaneous rate of GDP dissociation. As expected, LPA dose dependently increased [^{35}S]GTPγS binding in all the nontransfected cell lines tested with varying degrees of efficacy (Fig. 1), and as anticipated this response was abolished after pretreatment of membranes (HEK cells) with PTX (100 ng/ml) due to ADP-ribosylation of endogenous Gi/o-proteins (data not shown). The following rank order of responsiveness was seen: Ltk$^-$ = CHO ≫ PC12 > HeLa = HEK = COS-7. HEK cells were used for all subsequent transient expression studies, because they displayed a relatively low endogenous LPA response and were known to have good transfection efficiency.

The LPA-mediated [^{35}S]GTPγS binding in the hEdg2 receptor-expressing cell line, either alone or together with the various Gi constructs or as a PTX-insensitive hEdg2 receptor-rGiα1 fusion protein, is shown in Fig. 2. The maximal response to LPA after transient transfection of the hEdg2 receptor alone was only modestly improved compared with that seen in the mock or untransfected cells (10%). The response was improved by cotransfection of the receptor with the wild-type rat Giα1 (rGiα1) subunit (25 ± 1%), was further enhanced when the wild-type rGiα1 subunit was replaced with HA (27 ± 2%) or myc (30 ± 5%) N-terminal-tagged rGiα1 subunits and even more so when replaced with the mutant rGiα1 subunit (48 ± 3%). The transient expression of the PTX-insensitive hEdg2 receptor-rGiα1 fusion protein yielded the most efficacious response (69 ± 10%). The potency values of LPA, under the various assay conditions, are presented in Table 1 as pEC_{50} values.

In a second series of experiments, the effects of PTX pretreatment (100 ng/ml) on the hEdg2 receptor-expressing cells cotransfected with the various Gi constructs or as a PTX-insensitive hEdg2 receptor-rGiα1 fusion protein were investigated (Fig. 3). Compared with the equivalent untreated cells, PTX treatment led to a dramatic reduction in the measurable LPA-mediated response seen in the rGiα1HA(N) subunit and the rGiα1 myc(N) subunit cotransfected cell lines (77 ± 8% and 73 ± 5%, respectively) and abolished the response in the wild-type rGiα1 subunit-cotransfected cell line. In contrast, PTX pretreatment of the cells transiently transfected with the hEdg2 receptor-rGiα1 fusion protein

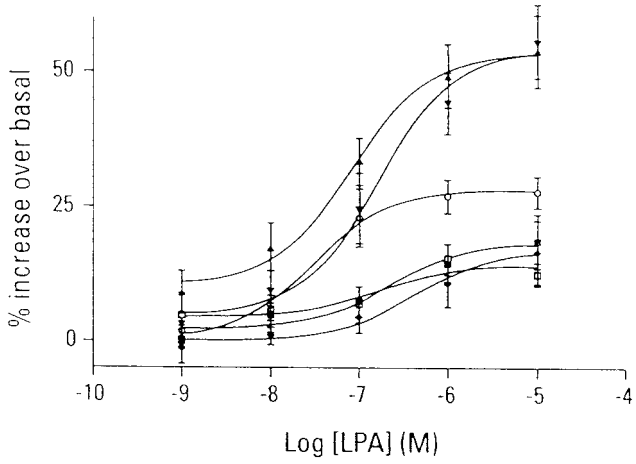

Fig. 1. LPA-induced [^{35}S]GTPγS binding, in the presence of 1 μM GDP, mediated by endogenous receptors in HEK 293 (■), CHO-K1 (▲), Ltk$^-$ (▼), HeLa (♦), COS-7 (□), and PC12 (○) cells. Data are expressed as percentage increase in binding above basal. Curves are a composite of three or more separate experiments, mean ± S.E.

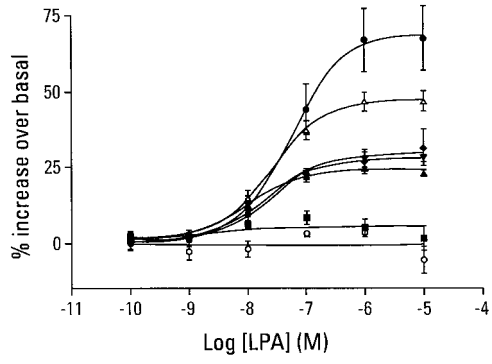

Fig. 2. LPA-induced [^{35}S]GTPγS binding, in the presence of 1 μM GDP, mediated by mock (○), hEdg2 receptor (■), hEdg2 receptor + wild-type rGiα1 subunit (▲), hEdg2 receptor + rGiα1HA(N) subunit (▼), hEdg2 receptor + rGiα1 myc(N) subunit (♦), hEdg2 receptor + mutant rGiα1 subunit (△), and the hEdg2 receptor-rGiα1 fusion protein (●) transiently transfected HEK cells. For potency (pEC_{50}) and efficacy (E_{max}) values see Table 1. Data are expressed as percentage increase in binding above basal. Each curve is a composite of four experiments, mean ± S.E.

was without effect on either the efficacy or potency values generated.

The potency and efficacy values (with respect to LPA) for several lysophospholipids in the PTX-pretreated hEdg2 receptor-rGiα1 fusion protein-expressing HEK cells are given in Table 2. LPA was, by over an order of magnitude, the most potent compound tested. LPG was marginally less efficacious. LPE (oleoyl) and S-1-P were able to induce hEdg2 receptor-mediated [^{35}S]GTPγS binding albeit with much reduced potency and efficacy. LPC (oleoyl) and LPE (palmitoyl) were inactive at up to 100 and 30 μM concentrations, respectively.

Discussion

Edg receptors are classified into two subfamilies, depending on their amino acid sequence similarity (within a subgroup, 44–52%; between subgroups, 27–34%) and their preferred endogenous ligand. Edg1, -3, -5, and -8 receptors are activated by S-1-P and Edg2, -4, and -7 receptors by LPA. The orphan Edg6 receptor displays a 30 to 40% amino acid identity with both subgroups. These receptors lack the commonly seen disulfide bridge between extracellular loops 1 and 2, which might be relevant in providing a suitable binding pocket for the bulky ligand.

Members of this family activate multiple secondary messenger pathways (Gi, Gq/11, G12/13, and/or Rho coupled). The effector system(s) activated could conceivably depend on the receptor subtype, the ligand concentration, and, therefore, the level of receptor activation as well as the internal milieu of the host cell type in both native tissue and recombinant receptor cell lines.

Edg receptors are expressed in most mammalian cells, with each subtype having a distinct distribution pattern, raising the possibility of tissue-specific biological roles that could be explored in drug discovery programs. Several subtypes are often expressed within the same tissue and possibly on differing cells types. Expression on the same cell may allow subtle control mechanisms that increase the repertoire of endogenous ligands, perhaps involving synergism between phospholipids.

Exploration of the physiological/pathophysiological roles of these receptors requires the identification of selective compounds, preferably with a range of intrinsic efficacies. Here we describe an assay that allows efficient profiling of compounds at the hEdg2 receptor.

The complex intracellular pathways activated by Edg receptors offer several potential biochemical markers of receptor activation. However, monitoring an early event in the signaling cascade should increase the probability of developing an assay design, which could be applied to all Edg receptors, rather than an assay based on a more downstream signaling event, when divergence between receptor subtypes is more likely to have occurred. Agonist-induced [^{35}S]GTPγS binding, employing recombinant receptor cell lines, is an established in vitro functional assay for GPCRs and fulfills

TABLE 1

Basal constitutive activity and LPA-induced [^{35}S]GTPγS binding following transient transfections

Levels of basal activity, i.e., in the absence of agonist, seen in membranes prepared from HEK cells transiently transfected with the hEdg2 receptor (alone or together with the various Gi constructs) or the hEdg2 receptor-rGiα1 fusion protein or mock-transfected HEK cells are presented as dpm bound. Potency values for LPA-induced [^{35}S]GTPγS binding carried out in membranes prepared from each condition are given as pEC_{50} values, and efficacy values (E_{max}) are the maximal stimulation achieved. Results are arithmetic means ± S.E. of four experiments.

	Basal ± S.E.	pEC_{50} ± S.E.	E_{max} ± S.E.
	dpm		*%*
hEdg2	20,099 ± 1,931	8.3[a]	10[a]
hEdg2 + wild-type rGiα1	25,037 ± 3,984	8.0 ± 0.2	25 ± 1
hEdg2 + rGiα1HA(N)	23,279 ± 2,085	7.7 ± 0.1	27 ± 2
hEdg2 + rGiα1myc(N)	30,557 ± 4,352	7.6 ± 0.1	30 ± 5
hEdg2 + mutant rGiα	19,986 ± 1,851	7.6 ± 0.1	48 ± 3
hEdg2-rGiα1 fusion	22,693 ± 2,506	7.3 ± 0.1	69 ± 10
No DNA	12,472 ± 720	NA	NA

[a] Only two determinations were made.

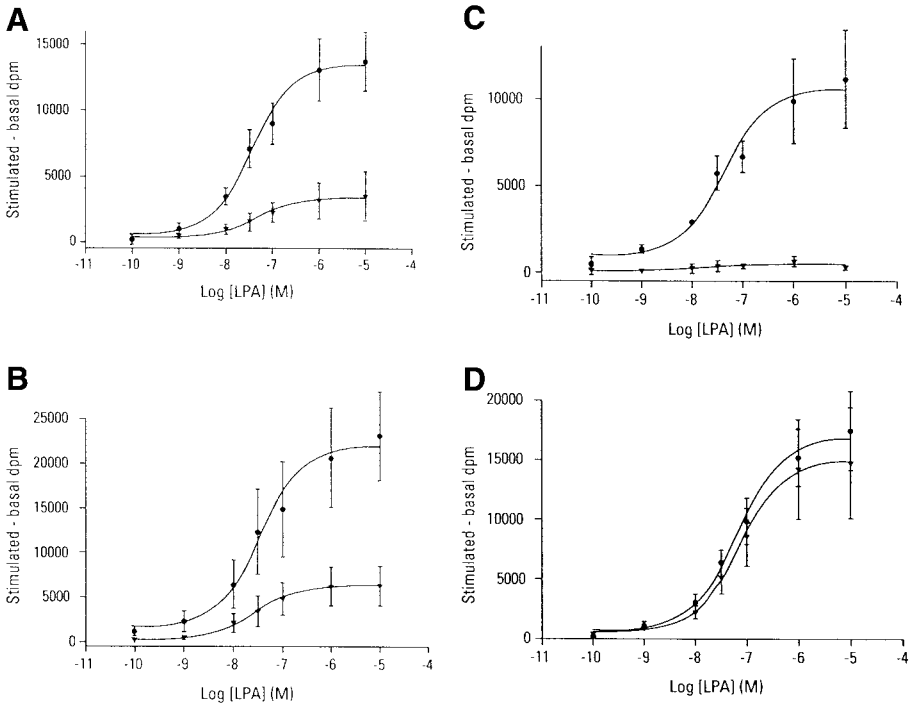

Fig. 3. LPA-induced [^{35}S]GTPγS binding mediated, in the presence of 1 μM GDP, by (A) hEdg2 receptor + rGiα1HA(N) subunit, (B) hEdg2 receptor + rGiα1 myc(N) subunit, (C) hEdg2 receptor + wild-type rGiα1 subunit, and (D) hEdg2 receptor-rGiα1 fusion protein transiently transfected HEK cells, in the absence (●) or presence (▼) of 100 ng/ml PTX. Data are expressed as stimulated − basal dpm. Each curve is a composite of three or more experiments, mean ± S.E. PTX treatment completely abolished the measurable LPA response in the wild-type rGiα1 cotransfected cell line and reduced the response seen in the rGiα1 HA(N) and the rGiα1 myc(N) cotransfected cell lines when compared with the equivalent untreated cells (77% and 73%, respectively). In contrast, PTX pretreatment of hEdg2 receptor-rGiα1 fusion protein-expressing cells was without effect on either the efficacy or potency values generated.

this condition. The assay measures the accumulation of the nonhydrolyzable GTP analog, [^{35}S]GTPγS, which displaces α-subunit-bound GDP after receptor activation.

The ubiquitous responsiveness of mammalian cells to LPA restricts the availability of suitable host cell lines, because responses might be due to endogenous receptor activation. We therefore took the approach of developing an assay with a PTX-insensitive hEdg2 receptor-rGiα1 fusion protein-expressing cell line (Bertin et al., 1994; Wise et al., 1997b).

Transient transfection of the hEdg2 receptor into HEK cells led to a modest increase in the maximal LPA-mediated response compared with mock-transfected cells. Not unexpectedly, an improved stimulation was seen after coexpression with the wild-type rGiα1 subunit. This response was further enhanced by replacing wild-type rGiα1 subunits with either HA or myc N-epitope tagged rGiα1 subunits. These tags were inserted near the N terminus to minimize possible interference with the secondary signaling cascade and in particular with the conformational changes required for GTPγS acceptance in the α-subunit binding pocket. The simplest explanation for this enhanced response is a more efficient subunit expression. However, because a similar effect was also seen with the poorly coupled h5-HT$_{5A}$ receptor but not the efficiently coupled hEdg4 receptor (unpublished data), it is probably due to improved receptor-subunit coupling.

After ligand activation, GPCRs interact with G-proteins to promote an α-subunit "open" conformation. The Giα1 subunit N-terminal region is involved in contact sites with the receptor, the effector binding surface, and the α-$\beta\gamma$ subunit interface (Wall et al., 1995; Lambright et al., 1996) in addition to cell membrane anchorage (Wise and Milligan, 1997). Hence, enhanced efficacy seen with the N-terminal-tagged subunits might be due to either a direct effect at contact sites involved in the conformational changes (Kisselev et al., 1995a,b; Taylor et al., 1996) or to improved maneuverability of the subunit at its point of attachment to the cell membrane, i.e., opposite to fusion protein compartmentalization effects (see below). Similar effects on compound efficacy were reported after α_{2A}-adrenoceptor-Cys^{351} $\rightarrow$ Gly Giα1 fusion protein β1γ2 coexpression studies and argued to be due to enhanced $\beta\gamma$-subunit interactions allowing more effective transduction (Wise et al., 1997a; Wise and Milligan, 1997).

The most efficacious LPA responses were seen after mutant rGiα1 receptor cotransfection and hEdg2 receptor-rGiα1 fusion protein transfection, although the fusion protein gave a greater response. The direct in-frame fusion of the N terminus of the α-subunit to the C terminus of the receptor was achieved by substituting the stop codon of the receptor with alanine and maintaining the α-subunit initiator methionine (Bertin et al., 1994; Wise et al., 1997a) and the PTX insensitivity by mutation of the α-subunit cysteine 351 to glycine (Hunt et al., 1994; Wise et al., 1997b). These results suggest that the enhanced response seen with the fusion protein is, at least in part, due to the cysteine 351 to glycine mutation.

The LPA response seen after transfection of the fusion protein was unaffected by pretreatment of the cells with PTX (Fig. 3D). In contrast, PTX pretreatment in coexpression studies resulted in a dramatic reduction in the maximal stimulation (Fig. 3, A–C). The residual activity is possibly due to interference of the mutation in the efficiency of ADP-ribosylation.

Published studies (e.g., Sautel and Milligan, 1998), with α_{2A}-adrenoceptors, indicate that fusion proteins result in compromised coupling efficiency possibly due to physical restrictions inhibiting protein conformational changes or effects on the compartmentalization of signaling elements within specific domains of the plasma membrane interfering with signal transduction (Huang et al., 1997; Sautel and Milligan, 1998). This study supports these findings, because the LPA potency with the fusion protein was 5- and 2-fold weaker than that seen with the wild-type and mutant rGiα1 cotransfection studies, respectively.

Finally we have investigated the ability of non-LPA phospholipids to activate the Edg2 receptor. All compounds tested were shown to have an LPA content of less than 0.2%. We have demonstrated with this fusion protein that attaching groups to the phosphate of LPA does not prevent hEdg2 receptor activation. Glycerol and ethanolamine, but not choline, are tolerated albeit with reduced potency and efficacy. Consistent with the findings of other groups (see below) the nature of the carbon chain seems to be critical, because 30 μM LPE (palmitoyl; C 16:0) in contrast to LPE (oleoyl; C18:1) was inactive. Additionally, the study indicates that two acyl side chains are also tolerated. S-1-P yielded an efficacy of 22% compared with LPA.

Erickson et al. (1998), with a reporter gene assay monitoring the pheromone response pathway in *Saccharomyces cerevisiae*, found LPA (18:1 oleoyl) to have an EC$_{50}$ of 20 to 30 μM, LPE to be weakly active, and S-1-P to be inactive. These weak activities probably reflect poor coupling of the mammalian receptor to the yeast G-protein and highlight the increased sensitivity of the approach taken in the present study. The yeast study also indicates the importance of ligand chain length. LPA analogs with increased chain length were more efficacious, and those with 6 and 10 carbon chains were inactive.

Consistent with the present study, Fukushima et al. (1998) report LPA (1 μM) induced [^{35}S]GTPγS binding after transient transfection of Edg2 receptors into Rh7777 rat hepatoma cells and stable transfection into B103 neuroblastoma cells. LPC, LPE, and LPG (1 μM) were inactive. These apparent discrepant findings might be explained by increased responsiveness and hence sensitivity of the fusion protein assay.

Finally, with hEdg2 receptors transiently expressed in Jurkat cells and a serum response element-driven luciferase

TABLE 2

Potency and efficacy values for LPA and analogs using agonist-induced [^{35}S]GTPγS binding mediated by the pertussis toxin-insensitive hEdg2 receptor-rGiα1 fusion protein

Potency (pEC$_{50}$) and efficacy values (E_{max}) for LPA and related phospholipids in the agonist-induced [^{35}S]GTPγS binding assay were carried out in membranes prepared from PTX treated (100 ng/ml for 20 h before harvesting) hEdg2 receptor-rGiα1 fusion protein transiently expressing HEK cells. Efficacy values are the maximal stimulation achieved and are presented as a percentage of the maximal LPA response. Results are arithmetic means ± S.E. of ≥3 experiments.

Compound	pEC$_{50}$ ± S.E.	Maximal LPA Response
		%
LPA (oleoyl C18:1)	6.7 ± 0.1	100
LPG (oleoyl C18:1)	5.0 ± 0.1	84 ± 3
LPE (oleoyl C18:1)	5.0 ± 0.2	29 ± 2
LPC (oleoyl C18:1)	Inactive	
LPE (palmitoyl C16:0)	Inactive	
S-1-P	5.7 ± 0.2	22 ± 1

expression reporter gene assay, An et al. (1998) report an EC_{50} value of 10 nM for LPA. This assay therefore appears to have increased sensitivity; however, the authors also report that the Jurkat cells might express an "as yet unidentified LPA receptor subtype," which would prove problematic when interpreting data from novel structurally diverse Edg receptor mimetics. In addition, S-1-P, LPC, and LPE were inactive, although compounds were tested only at concentrations up to 1 μM unlike the present study where compounds were tested up to 30 to 100 μM.

The present study, demonstrating dose-dependent LPA-induced [^{35}S]GTPγS binding in PTX-pretreated cells expressing the PTX-insensitive hEdg2 receptor-rGiα1 subunit fusion protein, clearly indicates that LPA activates this receptor. In addition to this certainty, the assay has good sensitivity, measures of compound potency, and efficacy and is amenable to high throughput application. The limited number of compounds tested indicates that structural modifications to LPA, in particular attachments to the phosphate group and the length and number of acyl side chains, are tolerated for Edg2 receptor activation.

GPCR fusion proteins have been used for several applications, e.g., to elucidate complex interactions between members of signaling pathways and the nature of agonist efficacy under conditions of fixed receptor/G-protein stoichiometry (Bertin et al., 1994; Wise et al., 1997a,b). Here we demonstrate a further application that, with the use of appropriate α-subunits (chimeric or otherwise), toxins, and assays, could be applied to other GPCRs that have a widespread endogenous expression in mammalian cells, e.g., thrombin, adenosine, UTP, and prostaglandin E receptors. Finally, the serendipitous finding that cotransfection of tagged/mutated rGiα1 subunits enhance the response compared with the wild-type α-subunit might be applicable to assay development for poorly coupled GPCRs.

Acknowledgment

We thank Karen Locker for carrying out HPLC analyses on all compounds tested.

References

An S, Bleu T, Hallmark OG and Goetzl EJ (1998) Characterization of a novel subtype of human G protein-coupled receptor for lysophosphatidic acid. *J Biol Chem* **273:**7906–7910.

Bertin B, Freissmuth M, Jockers R, Strosberg AD and Marullo S (1994) Cellular signaling by an agonist-activated receptor/Gsα fusion protein. *Proc Natl Acad Sci USA* **91:**8827–8831.

Erickson JR, Wu JJ, Goddard JG, Tigyi G, Kawanishi K, Tomei LD and Kiefer MC (1998) Edg-2/Vzg-1 couples to the yeast pheromone response pathway selectively in response to lysophosphatidic acid. *J Biol Chem* **16:**1506–1510.

Fukushima N, Kimura Y and Chun J (1998) A single receptor encoded by *vzg-1/lp*$_{A1}$/*edg-2* couples to G proteins and mediates multiple cellular responses to lysophosphatidic acid. *Proc Natl Acad Sci USA* **95:**6151–6156.

Goetzl EJ and An S (1998) Diversity of cellular receptors and functions for the lysophospholipid growth factors lysophosphatidic acid and sphingosine 1-phosphate. *FASEB J* **12:**1589–1598.

Hla T and Maciag T (1990) An abundant transcript induced in differentiating human endothelial cells encodes a polypeptide with structural similarities to G-protein-coupled receptors. *J Biol Chem* **265:**9308–9313.

Huang C, Hepler JR, Chen LT, Gilman AG, Anderson RGW and Mumby SM (1997) Organization of G proteins and adenylyl cyclase at the plasma membrane. *Mol Biol Cell* **8:**2365–2378.

Hunt TW, Reed CC and Peralta EG (1994) Heterotrimeric G proteins containing $G_{\alpha i3}$ regulate multiple effector enzymes in the same cell. *J Biol Chem* **269:**29565–29570.

Kisselev O, Ermolaeva M and Gautam N (1995a) Efficient interaction with a receptor requires a specific type of prenyl group on the G protein γ subunit. *J Biol Chem* **270:**25356–25358.

Kisselev O, Pronin A, Ermolaeva M and Gautam N (1995b) Receptor-G protein coupling is established by a potential conformational switch in the beta gamma complex. *Proc Natl Acad Sci USA* **92:**9102–9106.

Lambright DG, Sondek J, Bohm A, Skiba NP, Hamm HE and Sigler PB (1996) The 2.0 A crystal structure of a heterotrimeric G protein. *Nature (Lond)* **379:**311–319.

Milligan G (1988) Techniques used in the identification and analysis of function of pertussis toxin-sensitive guanine nucleotide binding proteins. *Biochem J* **255:**1–13.

Sautel M and Milligan G (1998) Loss of activation of Gs but not Gi following expression of an α_{2A}-adrenoceptor-Gi1α fusion protein. *FEBS Lett* **436:**46–50.

Stables J, Green A, Marshall F, Fraser N, Knight E, Sautel M, Milligan G, Lee M and Rees S (1997) A bioluminescent assay for agonist activity at potentially any G-protein-coupled receptor. *Anal Biochem* **252:**115–126.

Stanton JA and Beer MS (1997) Characterisation of a cloned human 5-HT_{1A} receptor cell line using [^{35}S]GTPγS binding. *Eur J Pharmacol* **320:**267–275.

Taylor JM, Jacob-Mosier GG, Lawton RG, VanDort M and Neubig RR (1996) Receptor and membrane interaction sites on Gβ. *J Biol Chem* **271:**3336–3339.

Wall MA, Coleman DE, Lee E, Iniguez-Lluhi JA, Posner BA, Gilman AG and Sprang SR (1995) The structure of the G protein heterotrimer $G_{i\alpha1}\beta_1\gamma_2$. *Cell* **83:**1047–1058.

Weiner JA and Chun J (1999) Schwann cell survival mediated by the signaling phospholipid lysophosphatidic acid. *Proc Natl Acad Sci USA* **96:**5233–5238.

Weiner JA, Hecht JH and Chun J (1998) Lysophosphatidic acid receptor gene *vzg-1/lpA1/edg-2* is expressed by mature oligodendrocytes during myelination in the postnatal murine brain. *J Comp Neurol* **398:**587–598.

Wise A, Carr C and Milligan G (1997a) Measurement of agonist-induced guanine nucleotide turnover by the G-protein $G_{i1}\alpha$ when constrained within an α_{2a}-adrenoceptor-$G_{i1}\alpha$ fusion protein *Biochem J* **325:**17–21.

Wise A and Milligan G (1997) Rescue of functional interactions between the α_{2A}-adrenoreceptor and acylation-resistant forms of $G_{i1}\alpha$ by expressing the proteins from chimeric open reading frames. *J Biol Chem* **39:**24673–24678.

Wise A, Watson-Koken MA, Rees S, Lee M and Milligan G (1997b) Interactions of the α_{2a}-adrenoceptor with multiple G_i-family G-proteins: Studies with pertussis toxin-resistant G-protein mutants. *Biochem J* **321:**721–728.

Send reprint requests to: George McAllister, Department of Biochemistry, Merck, Sharp & Dohme Research Laboratories Neuroscience Research Centre, Terlings Park, Eastwick Rd., Harlow, Essex, CM20 2QR UK. E-mail: George_Mcallister@merck.com