

# Regulated and constitutive activation of specific signalling pathways by the human S1P<sub>5</sub> receptor

<sup>1</sup>Anke Niedernberg, <sup>2</sup>Andree Blaukat, <sup>3</sup>Torsten Schöneberg & <sup>\*,1,4</sup>Evi Kostenis

<sup>1</sup>Disease group Cardiovascular Diseases, Aventis Pharma, Building H825, 65926 Frankfurt, Germany; <sup>2</sup>Pharmakologisches Institut, Ruprecht-Karls-Universität Heidelberg, Im Neuenheimer Feld 366, 69120 Heidelberg, Germany and <sup>3</sup>Institut für Pharmakologie, Universitätsklinikum Benjamin Franklin, Freie Universität Berlin, Thielallee 69-73, 14195 Berlin, Germany

**1** We tested the hypothesis, whether G Protein-coupled receptors (GPCRs) may differentially regulate specific signalling pathways by constitutive and agonist-induced activation using the human sphingosine 1-phosphate receptor S1P<sub>5</sub> as a model.

**2** S1P<sub>5</sub> receptor-expressing HEK293 cells exhibited a high degree of basal activity for both inhibition of adenylyl cyclase and extracellular signal regulated kinase (ERK) when cultured in serum, which contains high levels of sphingosine 1-phosphate (S1P). However, basal activity was independent of the presence of S1P: (i) constitutive activity remained when cells were cultured in delipidated serum, (ii) addition of S1P to delipidated serum did not increase basal S1P<sub>5</sub> receptor signalling.

**3** Conversely, constitutive inhibition of forskolin-stimulated adenylyl cyclase was further enhanced by S1P in S1P<sub>5</sub>-HEK293 cells.

**4** Transfection of several mammalian cell lines (CHO-K1, HEK293, NIH-3T3, RH7777) with the S1P<sub>5</sub> receptor induced cell rounding, which was more pronounced in the presence of S1P-containing serum. Rounded cell morphology did not correlate with apoptotic cell death, but led to detachment of cells.

**5** Cell surface ELISA assays showed that a fraction of plasma membrane S1P<sub>5</sub> receptors were dose-dependently internalized with S1P.

**6** These data reveal that intrinsic inhibition of unstimulated adenylyl cyclase or ERK activity by the S1P<sub>5</sub> receptor is insensitive to ligand modulation. Conversely, effects on forskolin-stimulated adenylyl cyclase, cell morphology and internalization can be further augmented with S1P. Our results suggest that different signal transduction pathways are not equally activated through constitutively active GPCRs with promiscuous signalling characteristics.

*British Journal of Pharmacology* (2003) **138**, 481–493. doi:10.1038/sj.bjp.0705055

**Keywords:** Human S1P<sub>5</sub>; cell rounding; constitutive activity; sphingosine 1-phosphate; cAMP assay; internalization

**Abbreviations:** csFBS, charcoal-stripped foetal bovine serum; dhS1P, dihydro sphingosine 1-phosphate; ERK, extracellular signal regulated kinase; FBS, foetal bovine serum; G protein, guanine nucleotide-binding protein; GPCR, G protein-coupled receptor; LPA, lysophosphatidic acid; PTX, pertussis toxin; S1P, sphingosine 1-phosphate

## Introduction

G protein-coupled receptors (GPCRs) constitute the largest protein family of integral membrane receptors. Despite the wide variety of activating ligands, they share a common seven transmembrane topology and common activation mechanisms (for review see Bockaert & Pin, 1999; Meng & Bourne, 2001). Information provided by extracellular stimuli is transduced into intracellular second messengers by their coupling to heterotrimeric G proteins that subsequently regulate diverse effector systems within the cells (Iiri *et al.*, 1998; Marinissen & Gutkind, 2001).

According to the two-state model, GPCRs can spontaneously isomerize between an inactive (R) and an active state (R\*) (Samama *et al.*, 1993; Weiss *et al.*, 1996). The active state R\* triggers exchange of GTP for GDP on the  $\alpha$  subunit of heterotrimeric G proteins, resulting in a measurable cellular or pharmacological response (Samama *et al.*, 1993). Several GPCRs show constitutive activity when overex-

pressed in recombinant systems (Samama *et al.*, 1993; van Sande *et al.*, 1995; Kenakin, 1996; Chen *et al.*, 2000) and this feature can be used in screenings of GPCRs. Ligands with affinity are applied to redistribute the receptor species: inverse agonist ligands preferentially stabilize the inactive conformation (R), agonist ligands preferentially stabilize the active conformation (R\*) (Milligan *et al.*, 1995), and neutral antagonists do not display any preference for a given receptor conformation. Interestingly, several established antagonist drugs targeting GPCRs have been recognized to be inverse agonists (McCune *et al.*, 2000; Stevens *et al.*, 2000; Miserey-Lenkei *et al.*, 2002). Besides constitutively active receptors (CARs), many constitutively active mutants (CAMs) have been generated that mimic the active state and signal in the absence of a ligand (e.g. Pei *et al.*, 1994; Jensen *et al.*, 2000; Stevens *et al.*, 2000; Scheer *et al.*, 2000; Ramsay *et al.*, 2001; Greasley *et al.*, 2001; Miserey-Lenkei *et al.*, 2002). Naturally occurring activating mutations are associated with human disease states like male-limited precocious puberty (luteinizing hormone receptor, Shenker *et al.*, 1993), hyperfunctioning thyroid adenoma (thyrotropin receptor, Parma *et al.*, 1993), retinitis pigmentosa (rhodopsin,

\*Author for correspondence: E. Kostenis; E-mail: ek@7tm.com

<sup>4</sup>Current address: Laboratory for Molecular Pharmacology, The Panum Institute, University of Copenhagen, Blegdamsvej 3, DK-2200 Copenhagen, Denmark

Rao *et al.*, 1994), and autosomal dominant hypocalcaemia ( $Ca^{2+}$  sensing receptor, Brown, 1999), for review see Coughlin, 1994). CAMs have been extensively used in recent years to provide insight into conformational changes which occur in GPCRs upon ligand-binding (Lefkowitz *et al.*, 1993; Scheer & Cotecchia, 1997; Leurs *et al.*, 1998; Pauwels & Wurch, 1998). In contrast, little information is available whether CAMs can differentially alter separate signalling cascades. A rare example for which this issue has been investigated is the  $\alpha_{1b}$ -adrenoceptor: a Cys128Phe mutant displays constitutive activity when phospholipase  $C\beta$  activation is monitored but not when phospholipase A2 activity is measured, whereas an Ala293Glu mutant of the same receptor shows constitutive activity in both pathways (Perez *et al.*, 1996). Likewise, it is not known so far whether CARs with the ability to signal through more than one pathway differentially alter separate signalling cascades.

Lipid receptors, in particular the family of sphingosine 1-phosphate (S1P) receptors  $SIP_{1-5}$  (formerly known as edge receptors; Chun *et al.*, 2002) have received increasing attention in recent years, and many reports show that expression of various S1P receptor subtypes in mammalian (primary) cells causes phenotypical changes such as morphological alterations, stress fibre formation, cytoskeletal reorganization and cell proliferation (Lee *et al.*, 1998; 1999; Moolenaar, 1999; van Brocklyn *et al.*, 1999; Robert *et al.*, 2001). In addition, it has been suggested that the plethora of effects mediated by S1P receptors is in part due to their ability to signal through more than one pathway. In all cases, however, phenotypical changes were dependent on the presence of S1P (Lee *et al.*, 1998; van Brocklyn *et al.*, 1999) and no reports are available about ligand-independent activities of S1P receptor subtypes.

To test whether lipid receptors exhibit constitutive signalling characteristics and differentially alter various effector systems, signalling and cellular responses have to be determined in delipidated serum, that does not contain S1P. In this study, we have analysed several S1P receptors and chose the recently cloned human  $SIP_5$  receptor as an example to demonstrate that it constitutively activates some signalling pathways while others still remain sensitive to agonist stimulation.

## Methods

### *Molecular cloning of various human $SIP_5$ receptor constructs*

As the human  $SIP_5$  receptor (AC073749) sequence is intronless, we cloned the receptor from human genomic DNA *via* PCR. PCR conditions, established to amplify the  $SIP_5$  receptor sequence, were 94°C for 10 min followed by 35 cycles of 94°C, 1 min, 60°C, 1 min, 72°C, 2 min using the GC-melt kit. Primers designed to amplify the  $SIP_5$  receptor sequence contained a *Hind*III site in the forward, and an *Eco*RI-site in the reverse primer, respectively. The 1197-bp PCR product was cloned into the pcDNA3.1(+) mammalian expression vector and sequenced in both directions.

To clone the construct used for generation of stable cell lines the  $SIP_5$  receptor coding region was excised from pcDNA3.1(+) with *Hind*III(5') and *Not*I(3') and inserted into the peak 8 vector (Edge Biosystems) using  $T_4$ -DNA ligase.

To clone the  $SIP_5$ -GFP construct the stop codon was deleted by PCR. Primers were designed to introduce an *Eco*RI site upstream of the initiating methionine codon and a *Bam*HI site 5' of the stop codon. The PCR product was cloned into the *Eco*RI-*Bam*HI site of the pEGFP-N3 vector using  $T_4$ -DNA ligase.

To monitor cell surface expression of the  $SIP_5$  receptor, the HA epitop tag (YPYDVPDYA) was introduced immediately after the initiating methionine codon of the  $SIP_5$  receptor sequence into the pcDNA3.1(+) expression vector.

### *Cell culture and transfection*

HEK293 cells were grown in Dulbecco's modified Eagles Medium (DMEM) (with Glutamax-1, w/o sodiumpyruvate, with 4500 mg  $l^{-1}$  glucose, with pyridoxine) supplemented with 10% foetal bovine serum (FBS), penicillin-streptomycin (10,000 IU ml/10,000  $\mu$ g  $ml^{-1}$ ) and 25 mM HEPES pH 7.0. Peak stable cells were cultivated in DMEM (with Glutamax-1, w/o sodiumpyruvate, with 4500 mg  $l^{-1}$  glucose, with pyridoxine) supplemented with 10% FBS, penicillin-streptomycin (10,000 IU ml per 10,000  $\mu$ g  $ml^{-1}$ ), 25 mM HEPES pH 7.0 and G418 (400  $\mu$ g per ml). CHO-K1 cells were cultured in basal Iscove medium supplemented with 10% FBS, penicillin-streptomycin (10,000 IU ml per 10,000  $\mu$ g  $ml^{-1}$ ), 50  $\mu$ g  $ml^{-1}$  gentamicin, 2 mM L-Glutamin. All cells were cultivated at 37°C in a humidified 5%  $CO_2$  incubator.

For transient transfections  $4 \times 10^5$  HEK293 or  $2 \times 10^5$  CHO-K1 cells were seeded onto 35-mm dishes. About 24 h later cells were transiently transfected at 50–80% confluency with the indicated receptor (1  $\mu$ g of plasmid DNA) using the FuGENE 6 transfection reagent for HEK293 or the LipofectAMINE reagent for CHO cells according to the manufacturer's instructions. For visualization of transfected cells, receptors were cotransfected with the cDNA encoding the green fluorescent protein (GFP) at a 4:1 ratio. Transfection efficiencies typically amounted to 30–40% as determined by counting green, GFP-transfected cells.

### *Generation of a stable $SIP_5$ receptor HEK293 cell line*

$4 \times 10^5$  peak stable cells (PSC, HEK293 cells resistant to G418) were seeded onto 35-mm dishes. About 24 h later cells were transfected at 50% confluency with 1  $\mu$ g receptor ( $SIP_5$  receptor in peak 8) or peak 8 (empty vector) cDNA using the FuGENE 6 transfection reagent. Forty-eight hours later cell selection with puromycin was initiated until a final concentration of 1  $\mu$ g  $ml^{-1}$  puromycin was reached. The stable cell lines were grown in DMEM (with Glutamax-1, w/o sodiumpyruvate, with 4500 mg  $l^{-1}$  glucose, with pyridoxine) supplemented with 10% FBS, penicillin-streptomycin (10,000 IU ml per 10,000  $\mu$ g  $ml^{-1}$ ), 25 mM HEPES pH 7.0 and 1  $\mu$ g  $ml^{-1}$  puromycin at 37°C in a humidified 5%  $CO_2$  incubator.

### *Measurement of cyclic AMP (cAMP) accumulation*

$1 \times 10^4$  HEK293 cells stably expressing the indicated receptor or vector DNA were cultivated in medium supplemented with 10% FBS or charcoal-stripped FBS (csFBS) ( $\pm$  S1P) for 24 h prior to the assay. Accumulation of cAMP was determined in the presence of 500  $\mu$ M isobutylmethylxanthine (IBMX) with

the alpha-screen<sup>®</sup> cAMP Detection Kit (Packard BioScience) according to the manufacturer's instructions. Luminescence was measured with the fusion  $\alpha$ -luminometer (Canberra-Packard GmbH). Data are means  $\pm$  s.e.mean of 5–10 independent experiments, each performed in quintuplets.

#### Cell rounding assay

CHO-K1 cells were plated at a density of  $1 \times 10^5$  cells/well on 35-mm dishes and transfected after 24 h with the indicated receptor expression plasmids as described above at a 4:1 ratio with the pEGFP N1 plasmid, which encodes a green fluorescence protein (GFP). Sixteen hours after transfection cells were washed once with PBS and grown for 32 h in Iscove supplemented with 10% FBS or 10% csFBS. Nuclei were stained with 4',6-diamino-2-phenylindole (DAPI). Transfected cells were visualized by phase contrast and fluorescence microscopy; apoptotic cells were identified by the blue colour of their nuclei representing fragmented DNA.

To determine pertussis toxin (PTX) sensitivity, PTX ( $100 \text{ ng ml}^{-1}$ ) was present in the medium during the last 24 h before analysis.

#### Caspase activity assay

$8 \times 10^5$  CHO-K1 cells were seeded onto 80-cm<sup>2</sup> dishes and transfected about 24 h later with cDNAs encoding for the S1P<sub>5</sub> receptor, rS1P<sub>3</sub> receptor or empty pcDNA3.1(+) vector cDNA as a control. Transfection medium was replaced after 16 h with basal Iscove medium supplemented with 10% FBS and cells were cultivated for an additional 8 h.  $4 \times 10^4$  cells were then seeded into 96-well plates and grown for 16 h in Iscove medium supplemented with 10% csFBS. Cells were serum starved for an additional 6 h in Iscove prior to addition of S1P or staurosporin ( $5 \mu\text{M}$ ). Caspase activity was then measured using the fluorometric homogeneous caspase assay<sup>®</sup> as per manufacturer's instructions. Fluorescence was recorded with the FluoStar Galaxy (BMG LabTechnologies GmbH) using an excitation filter at 485 nm and an emission filter at 520 nm. Data represent means  $\pm$  s.e.mean of six independent experiments.

#### ERK in vitro kinase reactions

Cell culture and transfections: CHO-K1 cells were grown on 6-well plates in Ham's F12 medium containing 10% FBS to about 50–70% confluency. Transfections were performed in medium with 10% csFBS using  $0.75 \mu\text{g}$  of receptor and  $0.75 \mu\text{g}$  of HA-ERK2 cDNA/well and the FuGENE 6 transfection method according to the supplier's manual. About 24 h after transfection cells were serum-starved in Ham's F12 medium containing 0.1% (m/v) FAF BSA for another 24 h until used for the experiments.

ERK activity was analysed as previously described (Blaukat *et al.*, 2000). Briefly, serum-starved cells on 6-well plates were stimulated with the indicated ligands at 37°C followed by lysis in 0.5 ml of ice-cold buffer containing (mM) HEPES 50, pH 7.2, NaCl 150, EDTA 1, NaF 20, sodium orthovanadate 2, 1% (w v<sup>-1</sup>) Triton X-100, 10% (w v<sup>-1</sup>) glycerol and protease inhibitors (1 mM Pefabloc,  $10 \mu\text{g ml}^{-1}$  leupeptin and 1% Trasylol<sup>®</sup>). Equal amounts of soluble fractions were subjected to immunoprecipitation with  $5 \mu\text{l}$  of

a polyclonal antiserum against the HA-tag (Santa Cruz Biotechnology) and  $35 \mu\text{l}$  protein A-agarose slurry for 3 h at 4°C. Resultant precipitates were washed three times with lysis buffer followed by two washes with kinase buffer (mM) (Tris 10, pH 7.5, MgCl<sub>2</sub> 10). Kinase reactions were started by addition of  $25 \mu\text{l}$  kinase buffer including  $200 \mu\text{M}$  ATP,  $1 \mu\text{Ci}$   $\gamma$ [<sup>32</sup>P]-ATP and  $10 \mu\text{g}$  myelin basic protein (MBP). After 20 min at 30°C reactions were stopped by addition of  $20 \mu\text{l}$  SDS sample buffer and boiling for 2 min. Proteins were separated by 12% SDS polyacrylamide gel electrophoresis (SDS-PAGE) and gels were cut at the 30-kDa marker band. Upper parts were transferred onto nitrocellulose membranes and probed with  $0.5 \mu\text{g ml}^{-1}$  anti-ERK2 antibodies in 5% BSA in TBS (Tris 50 mM, pH 7.6, NaCl 150 mM) to check equal immunoprecipitation of HA-ERK2. Lower parts were stained with Coomassie brilliant blue R250 to monitor amounts of MBP. Substrate phosphorylation was analysed using a PhosphoImager (BioRad, Molecular Imager FX). Western blotting of total cell lysates with monoclonal HA antibodies was performed to prove equal HA-ERK2 levels.

#### ELISA studies

To estimate cell surface expression of receptors carrying an N-terminal HA-tag, we used an indirect cellular ELISA (Schöneberg *et al.*, 1997), further referred to as 'surface ELISA'. Briefly, COS-7 cells were seeded into 48-well plates and transfected ( $0.25 \mu\text{g}$  of DNA and  $0.6 \mu\text{l}$  of Lipofect AMINE/well). Seventy-two hours after transfection cells were formaldehyde-fixed for 10 min without disrupting the cell membrane, blocked with DMEM plus 10% FBS for 1 h and incubated with a biotin-labelled anti-HA monoclonal antibody (12CA5, Boehringer Mannheim, Mannheim, Germany;  $1 \mu\text{g}$  antibody  $\text{ml}^{-1}$  DMEM plus 10% FBS) for 2 h. Incubation with S1P as indicated was performed in DMEM for 30 min at 37°C prior fixation. After three times washing with PBS bound anti-HA antibody was then detected with the help of a peroxidase-labelled streptavidin conjugate (Sigma; 1:5000 in DMEM plus 10% FBS). After removal of excess unbound conjugate, H<sub>2</sub>O<sub>2</sub> and o-phenylenediamine (2.5 mM each in 0.1 M phosphate-citrate buffer, pH 5.0) were added to serve as substrate and chromogen, respectively. After 15 min the enzyme reaction was stopped by the addition of 1 M HCl containing 0.05 M Na<sub>2</sub>SO<sub>3</sub>, and colour development was measured bichromatically at 492 and 620 nm using an ELISA reader (Titertek Multiskan MCC/340, Flow Laboratories, McLean, VA, U.S.A.).

#### Data analysis

Results are expressed as means  $\pm$  standard deviation or standard error, shown as error bars in the figures. Statistical comparisons were made by the Student's *t*-test, with significance considered when  $P < 0.05$ . One-way ANOVA with Bonferroni testing was employed in case of multiple comparisons (data of Figure 8).

#### Sources of materials

Sphingosine 1-phosphate (S1P), dihydrosphingosine 1-phosphate (dhS1P), forskolin, isobutylmethylxanthine (IBMX), staurosporine, ATP, myelin basic protein (MBP), pertussis

toxin, and the RT-PCR kit were from Sigma (St. Louis, MO, U.S.A.), HEK293 and CHO-K1 cells were from the American Type culture collection (ATCC, Manassas, VA, U.S.A.), competent *E. coli* DH5 and cell culture media and sera from GIBCO BRL (Gaithersburg, MD, U.S.A.), the enhanced green fluorescence protein (pEGFP) vector and the GC-melt PCR kit from Clontech (Palo Alto, CA, U.S.A.), FBS and basal Iscove medium was from Biochrom (Berlin, Germany), oligonucleotides from MWG-Biotech AG (Ebersberg, Germany), the expression plasmid pcDNA3.1(+), LipofectAMINE, and *E. coli* MC 1061 from Invitrogen (Carlsbad, CA, U.S.A.), restriction enzymes and T<sub>4</sub>-ligase from New England Biolabs (Schwalbach, Germany), charcoal-stripped FBS (csFBS) from PAN Biotech (Aidenbach, Germany), the homogenous Caspases Assay, L-Glutamine, Gentamicin, FuGENE and G418 solution from Roche Diagnostics (Mannheim, Germany), the alphascreen™ cAMP assay kit from Packard BioScience (Meriden, U.S.A.), 4', 6-diamino-2-phenylindole (DAPI) from Molecular Probes (Eugene, OR, U.S.A.). Polyclonal anti-HA antibodies were a gift from L. Rönstrand (Ludwig Institute for Cancer Research, Uppsala, Sweden) polyclonal ERK2 antibody was from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), protein A-HRP and protein A-sepharose was from Zymed Laboratories (San Francisco, CA, U.S.A.),  $\gamma$ -<sup>32</sup>P-ATP (6000 Ci mmol<sup>-1</sup>) was from Amersham (Braunschweig, Germany).

## Results

### *S1P<sub>5</sub> receptor expression reduces basal cAMP accumulation*

Im *et al.* (2001) have recently shown that the S1P<sub>5</sub> receptor inhibits forskolin-induced stimulation of adenylyl cyclase with nanomolar EC<sub>50</sub> values when stimulated with S1P or its reduced form, dhS1P. However, ligand-independent effects of the S1P<sub>5</sub> receptor were not addressed in this study nor reported elsewhere thus far.

We observed that stable expression of the S1P<sub>5</sub> receptor in HEK293 cells resulted in a 20% reduction of basal cAMP levels as compared with vector expressing cells (Figure 1A) when cultivated in medium containing 10% FBS, which is known to contain high levels (~400 nM) S1P (Yatomi *et al.*, 1997). To determine whether the observed effects on basal cAMP accumulation were dependent on the presence of S1P in the culture medium, cAMP assays were performed with cells grown in delipidated serum (charcoal-stripped FBS, csFBS). Intrinsic inhibition of cAMP generation did not change significantly when cells were cultured in csFBS containing medium (17%, Figure 1B vs 20%, Figure 1A). To test whether the agonist-independent effect of the S1P<sub>5</sub> receptor on adenylyl cyclase signalling is sensitive to ligand modulation, measurement of cAMP accumulation was performed in the presence of csFBS and S1P. As depicted in Figure 1C, 1  $\mu$ M S1P did not further augment S1P<sub>5</sub> receptor-mediated inhibition of basal cAMP production. Furthermore, generation of cAMP in stable S1P<sub>5</sub> receptor transfectants *via* the direct activator of adenylyl cyclase forskolin was significantly impaired in relation to vector transfected control cells (Figure 1D). To rule out non-specific effects of GPCR expression on cAMP levels, GPR63 was

included as a control as it does not show intrinsic effects on the adenylyl cyclase pathway (A. Niedernberg, manuscript in preparation). As can be seen in Figure 1A,B and D, effects on intracellular second messenger levels were specifically mediated by the S1P<sub>5</sub> receptor.

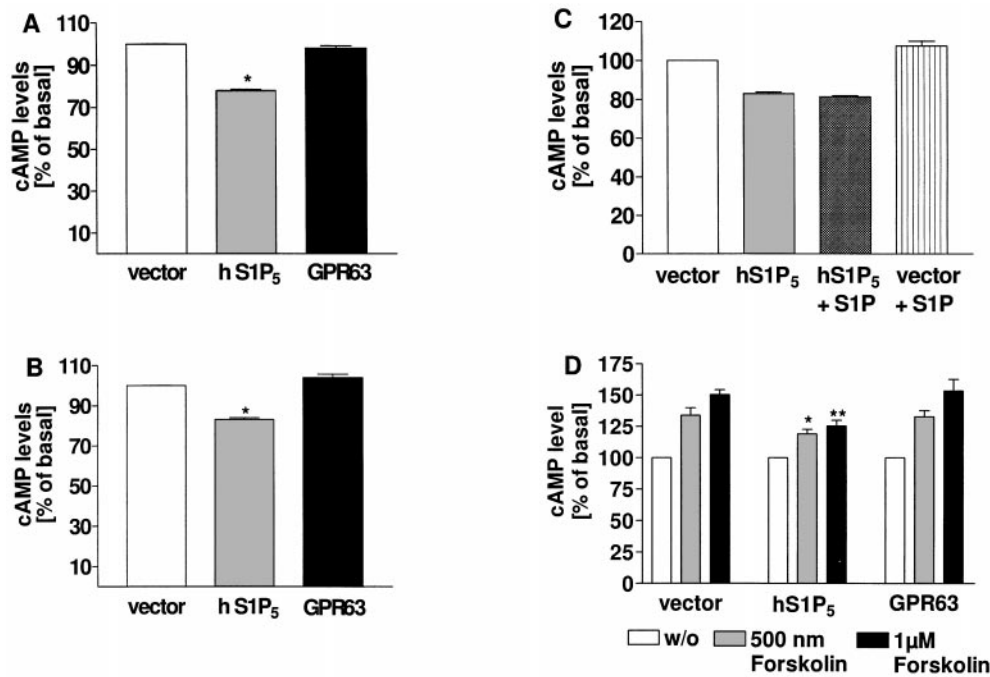
To investigate whether S1P may enhance S1P<sub>5</sub> receptor-mediated inhibition of forskolin-stimulated adenylyl cyclase, *i.e.* inhibit the enzyme when cAMP levels are elevated as opposed to the conditions in Figure 1, various concentrations of S1P were tested for agonism in S1P<sub>5</sub>-HEK293 cells, exposed to 1000 nM of forskolin during 30 min (Figure 2). Unlike in non-forskolin treated S1P<sub>5</sub>-HEK293 cells (Figure 1C), S1P now dose dependently inhibits forskolin-stimulated adenylyl cyclase (EC<sub>50</sub> = 173 nM, log EC<sub>50</sub> = -6.76 + 0.15; *n* = 4, Figure 2). Collectively, our adenylyl cyclase data show that S1P acting through the S1P<sub>5</sub> receptor further enhances basal inhibition of forskolin-stimulated but not of non-stimulated adenylyl cyclase.

### *Human S1P<sub>5</sub> receptor inhibits basal ERK activity in vitro kinase assays*

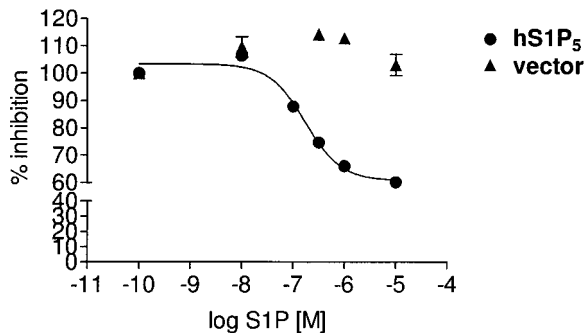
Little is known about the different signalling cascades initiated by the human S1P<sub>5</sub> receptor in a ligand-dependent or -independent manner. So far, the only measured endpoints have been G $\alpha_i$ -mediated responses such as inhibition of cAMP production and stimulation of GTP- $\gamma$ -S binding (Im *et al.*, 2001). For the rat S1P<sub>5</sub> receptor orthologue, dose-dependent inhibition of adenylyl cyclase and ERK activity by S1P have been shown (Malek *et al.*, 2001) but ligand-independent effects on these pathways have not been described so far. Therefore, we tested the hypothesis whether the human S1P<sub>5</sub> receptor also modulates MAP-kinase signalling and whether the effect is ligand-dependent or not. ERK activity was determined *via* phosphorylation of the ERK substrate myelin basic protein (MBP) such that the phosphorylation status of MBP directly reflects ERK activity. As shown in Figure 3A expression of S1P<sub>5</sub> receptor in CHO-K1 cells grown in medium supplemented with 10% FBS significantly reduced the kinase activity of ERK in comparison to mock transfected cells. Serum-induced ERK1/2 phosphorylation was not inhibited by S1P in mock transfected cells (Figure 3A, left panel). Moreover, 1  $\mu$ M S1P did not modify the intrinsic effects of the S1P<sub>5</sub> receptor on ERK activity (Figure 3A, right panel). Notably, the inhibition of ERK activity in cells expressing the S1P<sub>5</sub> receptor cultivated in medium containing csFBS (Figure 3B, right panel) was as prominent as in cells grown with FBS (Figure 3A, right panel) and again, addition of S1P did not modify the inhibitory effects of the S1P<sub>5</sub> receptor on ERK activation (Figure 3B, right panel). Collectively, our data suggest that inhibition of ERK activity through the S1P<sub>5</sub> receptor is constitutive and insensitive to the ligand S1P.

### *Human S1P<sub>5</sub> receptor overexpression induces S1P-dependent cell rounding*

To test whether the S1P<sub>5</sub> receptor-mediated basal activity is correlated with intrinsic effects on cell morphology, the S1P<sub>5</sub> receptor in combination with GFP at a 4:1 ratio was transiently transfected into mammalian CHO-K1 cells. The rat S1P<sub>2</sub> (rS1P<sub>2</sub>, formerly referred to as H218) receptor was



**Figure 1** cAMP accumulation in HEK 293 cells stably expressing the human S1P<sub>5</sub> receptor, GPR63 or vector. Cells stably expressing the indicated receptors or pcDNA3.1(+) vector DNA as a control were grown in medium containing 10% FBS (A) or charcoal-stripped FBS (csFBS) (B) for 24 h and cAMP levels quantitated in the presence of 500 μM IBMX with the alpha-screen<sup>®</sup> kit as described in Methods. (C) Transfected cells were cultivated in csFBS for 24 h and stimulated with 1 μM S1P for 30 min prior to quantification of cAMP levels. (D) Transfected cells were stimulated with forskolin for 30 min in the presence of 500 μM IBMX and cAMP levels determined as described above. In cells expressing the human S1P<sub>5</sub> receptor basal cAMP levels were significantly lower than in vector or GPR63 expressing cells, whether they were cultivated in the presence (A) or absence (B) of lipids: \* $P < 0.05$  indicates significant difference versus cAMP levels in vector transfected cells. Addition of S1P did not significantly affect intrinsic S1P<sub>5</sub> receptor-mediated cAMP inhibition (C). Forskolin stimulation of S1P<sub>5</sub> receptor transfected cells resulted in significant lower cAMP levels as compared with vector or GPR63 transfectants (D): \* $P < 0.05$  and \*\* $P < 0.01$  indicate significant differences versus cAMP levels in vector transfected cells under corresponding conditions. Data are means  $\pm$  s.e.mean ( $n = 4-10$ ).

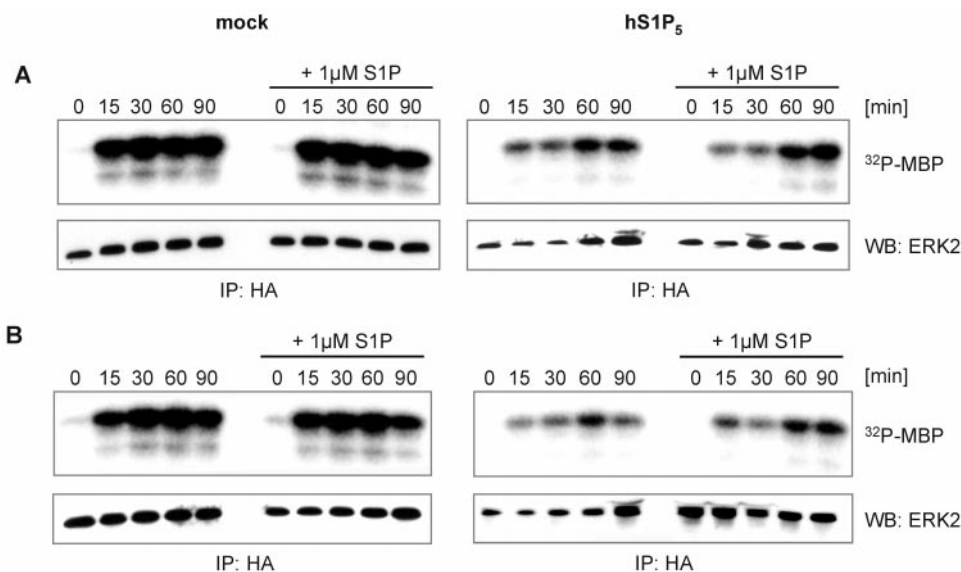


**Figure 2** S1P-mediated inhibition of forskolin-stimulated adenylyl cyclase in S1P<sub>5</sub>-HEK293 cells. Cells stably expressing the S1P<sub>5</sub> receptor were grown in medium containing 10% charcoal-stripped FBS (csFBS) for 24 h and cAMP levels were quantitated upon challenge with increasing concentrations of S1P with the alpha-screen<sup>®</sup> kit as described in Methods. S1P dose dependently inhibits forskolin-stimulated adenylyl cyclase activity in S1P<sub>5</sub> receptor but not in vector transfected cells. Data are means  $\pm$  s.e.mean of 3-4 independent experiments, performed in quintuplets. Where not shown error bars lie within the dimensions of the symbols.

included as a positive control as a recent study by van Brocklyn *et al.* (1999) showed that transfection of rS1P<sub>2</sub> receptor into HEK293 cells leads to cell rounding followed by apoptotic cell death.

CHO-K1 cells transfected with the S1P<sub>5</sub> receptor, rS1P<sub>2</sub> receptor (not shown) or pcDNA3.1(+) vector cDNA and GFP were cultured for 34 h in medium containing FBS (Figure 4). Under these conditions most of the cells expressing the S1P<sub>5</sub> receptor displayed a rounded morphology. Expression of the rS1P<sub>2</sub> receptor caused cell shape changes that were less pronounced than those seen with the S1P<sub>5</sub> receptor (not shown). It is evident that morphological changes are correlated with receptor expression levels, as cells with intense GFP fluorescence, and thus presumably also high S1P receptor levels, exhibit most prominent cell shape changes. Vector-transfected cells displayed the normal CHO cell morphology. As shown in Figure 4, S1P<sub>5</sub> receptor-transfected cells exposed to csFBS had ~30-40% round morphology compared to ~90% in FBS. S1P<sub>5</sub> receptor expressing cells exposed to csFBS containing 1 μM S1P showed a completely round phenotype, comparable to that observed in FBS containing medium. These data suggest that S1P<sub>5</sub> receptor-mediated cell rounding does not require, but is enhanced by, S1P stimulation. It should be noted that cell rounding responses were not only observed in CHO cells but also in HEK293, NIH-3T3, COS7, and RH7777 cells (not shown).

As regulation of the actin cytoskeleton and cell rounding responses are frequently mediated *via* Gα<sub>12/13</sub> and the small G protein Rho, we tested whether S1P<sub>5</sub> expressing cells activate Rho signalling in HEK293 cells in a reporter gene assay, that



**Figure 3** The  $SIP_5$  receptor displays ligand-independent inhibition of ERK activity. (A) CHO cells were cotransfected with human  $SIP_5$  receptor cDNA or vector (mock) and HA-ERK2 cDNA. After incubation in serum-free medium for 24 h, cells were stimulated with medium containing 10% FBS with or without addition of  $1 \mu\text{M}$  SIP. After cell lysis, immunoprecipitation, *in vitro* kinase reactions and separation by SDS page (see Methods), myelin basic protein (MBP) phosphorylation was quantified for measurement of ERK activity. HA-ERK2 was quantified *via* Western blot for confirmation of equal expression levels. (B) The assay was essentially carried out as described above but stimulation was performed in medium containing 10% csFBS with or without SIP. Data shown are representative for 2–4 independent experiments.

translates receptor-mediated Rho activation into stimulation of a serum responsive element, which in turn promotes transcription of luciferase. The neurotensin receptor served as a positive control in this assay and caused an 8 fold increase of luciferase activity upon 4 h of ligand stimulation. In contrast, the  $SIP_5$  receptor did neither display agonist-independent nor SIP-induced modulation of the Rho signalling pathway in the respective reporter gene assay (data not shown).

#### *Human $SIP_5$ receptor does not lead to apoptosis in transfected CHO-K1 cells*

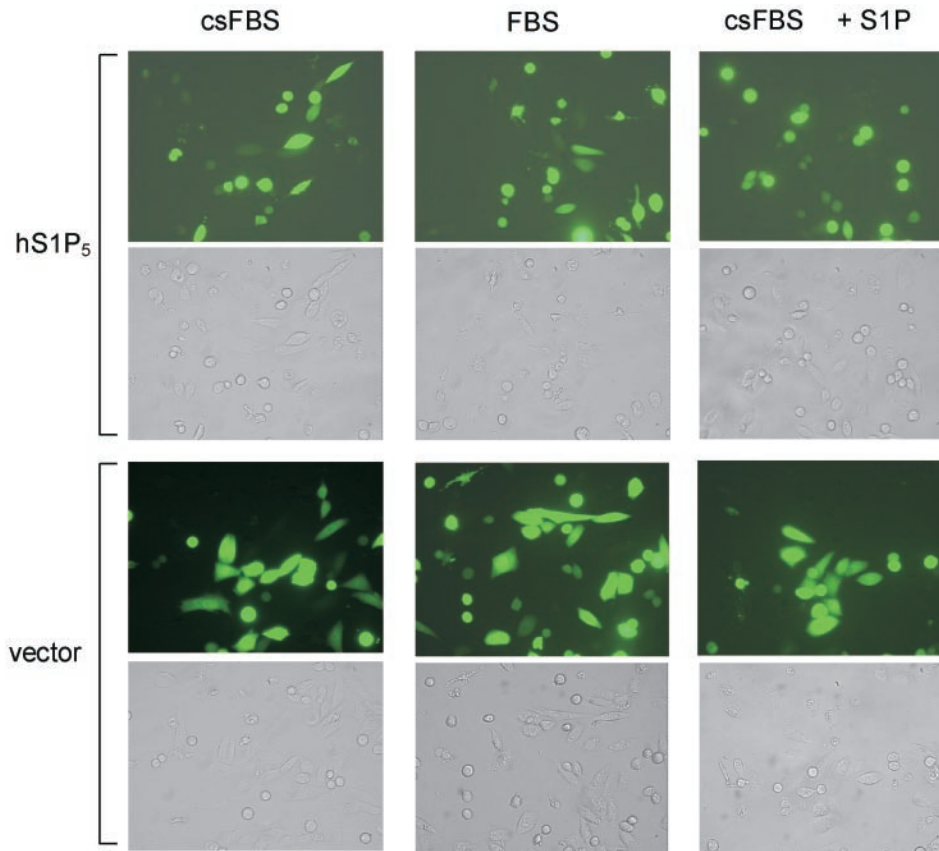
To determine whether cell rounding is correlated with apoptotic cell death, nuclei were stained with DAPI. As seen in Figure 5, none of the rounded cells contained fragmented DNA in their nuclei, which would indicate apoptosis. To determine whether  $G\alpha_i$  proteins are involved in the cell rounding response, experiments were performed in the presence of PTX which specifically inactivates  $G\alpha_i$  proteins. However, PTX did not affect  $SIP_5$  receptor-mediated cell rounding (data not shown), indicating a role of either  $G\alpha_{q/11}$  or  $G\alpha_{12/13}$ . Very similar phenotypical observations were made when the  $SIP_5$  receptor was transfected into HEK293, COS-7, RH7777 or NIH-3T3 cells. In all cases cell rounding was induced in the presence as well as in the absence of SIP in the medium (data not shown).

Although DAPI staining did not indicate that the  $SIP_5$  receptor activates the cell death machinery, we wanted to confirm this finding with a second independent assay. A caspase assay was chosen as caspase activation is one of the cellular processes triggered during apoptosis (Nicholson & Thornberry, 1997; Andersson *et al.*, 2000), and precedes complete fragmentation of the nuclei and cell death.

As shown in Figure 6, CHO-K1 cells transfected with the  $SIP_5$  receptor neither showed intrinsic nor ligand-induced induction of caspase activity when incubated for 2 h without serum or in the presence of various concentrations of SIP. In contrast, addition of 10% FBS clearly inhibits starvation-induced caspase activation, whereas staurosporin promotes cell death in all transfectants tested. As recent literature indicated that caspases are not necessarily involved in the very early events of apoptosis (Dumont *et al.*, 2000) we repeated the assay with 7 h incubation. Again, staurosporin induced caspase activation (4–5 fold in comparison to no serum) but no pro-apoptotic effect could be detected in  $SIP_5$  transfected cells treated with different concentrations of SIP (data not shown). It appeared however, that  $SIP_5$  receptor expression unexpectedly exerted a protective effect, as evidenced by (i) lack of induction of caspases by 300 and 1000 nM SIP in  $SIP_5$  transfectants as compared with vector transfected cells and (ii) decreased caspase activity upon staurosporin treatment in  $SIP_5$  as compared with vector transfected cells (Figure 6). Our data suggest that the  $SIP_5$  receptor does not promote but constitutively protects CHO cells from cell death as determined in caspase assays.

#### *A $SIP_5$ -green fluorescent protein (GFP) fusion protein is mainly located in intracellular compartments*

As the  $SIP_5$  receptor does not require any ligand to stimulate most cellular responses such as inhibition of adenylyl cyclase and ERK activity, we wondered whether internalization of the  $SIP_5$  receptor could be achieved by addition of the activating ligand SIP, or whether the  $SIP_5$  receptor population may already be completely internalized. To this end, a  $SIP_5$  receptor-GFP fusion protein was transfected into HEK293 cells and images were analysed with confocal



**Figure 4**  $S1P_5$  receptor expression in CHO-K1 cells causes cell rounding. CHO cells transiently transfected with  $S1P_5$  or pcDNA3.1(+)-vector and GFP (4:1) cDNA were grown for 34 h in Iscove supplemented with FBS, csFBS, or csFBS +  $1 \mu\text{M}$   $S1P$ . Plates were then analysed by fluorescence microscopy. The human  $S1P_5$  receptor induced cell rounding that was more pronounced in transfected cells cultivated in medium containing 10% FBS as compared with medium containing csFBS. Addition of  $1 \mu\text{M}$   $S1P$  to csFBS containing medium led to a complete round morphology of  $S1P_5$  transfected cells. Vector transfected cells displayed the normal CHO cell morphology.

microscopy. As seen in Figure 7 (A1–3), most of the transfected cells showed cell rounding, whereas non-transfected cells displayed the characteristic HEK293 cell morphology Figure 7 (A2–3). Frequently, images as in B1–3 were observed with a significant amount of GFP fluorescence in intracellular compartments, some cells (Figure 7, C1–3) displayed GFP fluorescence at the plasma membrane. Cell rounding *per se* complicates an analysis of ligand-induced internalization, and indeed  $10 \mu\text{M}$   $S1P$  stimulation for 10 min (Figure 7, D1, 2) or 20 min (Figure 7, E1, 2) did not lead to a detectable internalization in this assay. Identical cell rounding responses were obtained upon transfection of  $S1P_5$ -GFP into COS-7 and CHO-K1 cells (data not shown) and prevented application of this technique for characterization of internalization responses.

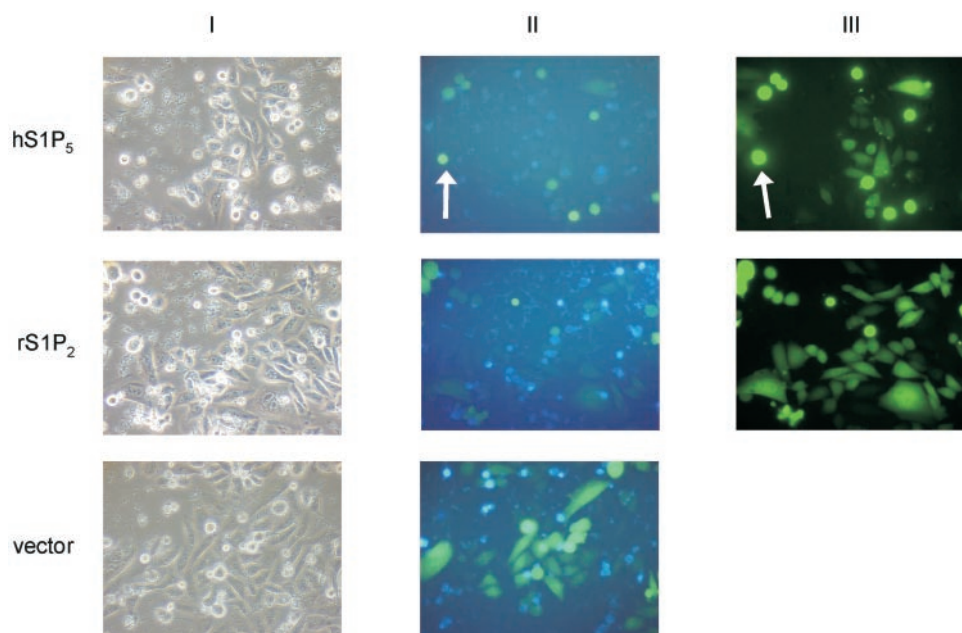
#### *S1P induces $S1P_5$ receptor internalization in a cell surface ELISA assay*

We constructed an N-terminally tagged version of the  $S1P_5$  receptor carrying the influenza virus haemagglutinin epitope (HA) sequence immediately after the initiating methionine codon, and applied a cell surface ELISA technique to verify the cell surface expression of the  $S1P_5$  receptor and to monitor potential disappearance of cell surface receptors to intracellular

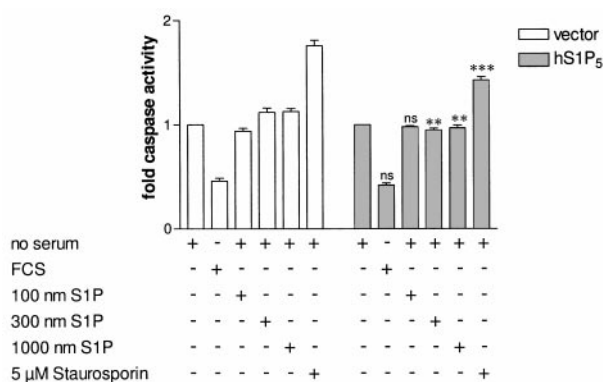
compartments upon stimulation with  $S1P$ . As depicted in Figure 8, the  $S1P_5$  receptor dose-dependently internalized with  $S1P$  to a significant extent when cultivated in medium containing FBS: 20 nM  $S1P$  internalized 11% and 200 nM  $S1P$  internalized 18% of receptors present on the plasma membrane. Interestingly, ligand-induced internalization did not change when cells were kept in csFBS containing medium. It should be noted that our cell surface ELISA was performed in COS7 cells and not with HEK293 or CHO cells used throughout the study, as this was the only cell type that allowed to apply the ELISA protocol without significant detachment of cells.

## Discussion

Constitutive activity of GPCRs has been extensively examined in recent years to understand conformational changes imparted on GPCRs upon ligand binding, or distinct features of ligand-induced internalization, trafficking or inverse agonist-induced up-regulation (McCune *et al.*, 2000; Stevens *et al.*, 2000; Miserey-Lenkei *et al.*, 2002; Chen *et al.*, 2002). Some studies are available on the influence of constitutive activity imbued by mutational alteration at different receptor sites on distinct signalling cascades (e.g. Perez *et al.*, 1996). These and related



**Figure 5** The S1P<sub>5</sub> receptor causes cell rounding but not cell death in transiently transfected CHO-K1 cells. CHO cells transiently transfected with the cDNAs of human S1P<sub>5</sub> receptor (A), rS1P<sub>2</sub> receptor (B), pcDNA3.1(+) (C) and GFP (4:1) were grown for 34 h in Iscove supplemented with FBS after transfection and images recorded after 4', 6-diamino-2-phenylidole (DAPI) nuclear staining. No signs of apoptosis could be seen in S1P<sub>5</sub> or rS1P<sub>2</sub> receptor transfected cells with rounded morphology (II + III). White arrows highlight selected cells that are round but not apoptotic.



**Figure 6** Caspase activity assay in CHO cells transiently transfected with S1P<sub>5</sub> receptor or pcDNA3.1(+) cDNAs. Thirty-six hours after transfection, CHO-K1 cells were grown in no serum containing medium for 6 h prior to the assay. Cells were then incubated for 2 h in serum-free medium, 10% FBS containing medium, or the indicated S1P concentration or staurosporin in serum-free medium. Fluorescence was measured using the Homogeneous Caspase Assay<sup>®</sup> kit according to the manufacturer's instructions. Data are means  $\pm$  s.e.mean of eight independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.005$  indicate significant differences versus fold caspase activity in vector transfected cells under the corresponding conditions, ns = non-significant.

findings lead to the hypothesis that constitutively active mutants and the conformational alterations they produce in a given receptor are unlikely to be equivalent (Stevens *et al.*, 2000). Hence, very little is known whether constitutive activity of wild-type GPCRs that mediate various cellular responses differentially affects those responses.

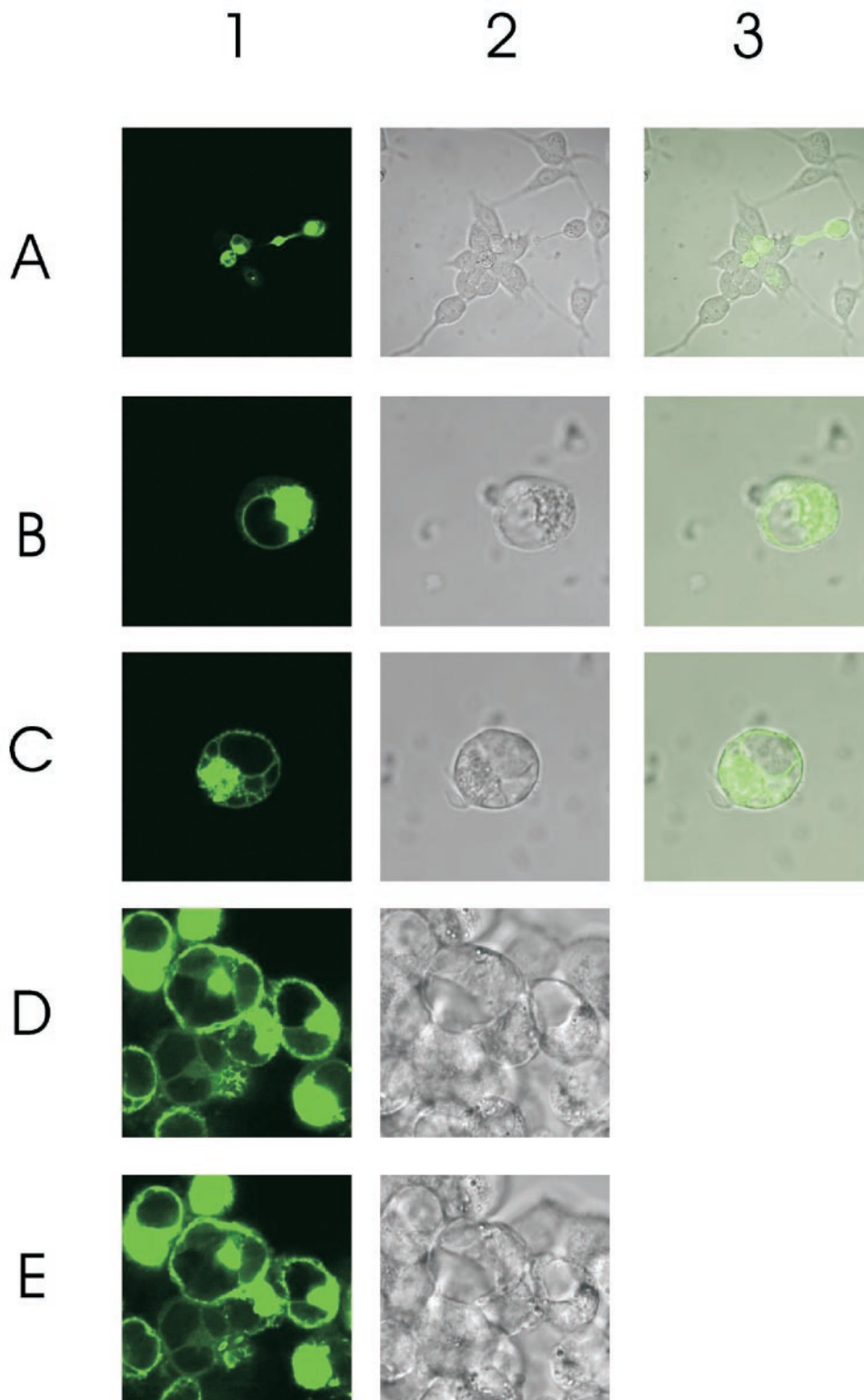
A family of GPCRs reported to exert intrinsic effects on cells are the members of the sphingosine 1-phosphate family

S1P<sub>1,2,3,4</sub> (Lee *et al.*, 1998; van Brocklyn *et al.*, 1999). However, in most cases their 'constitutive activity' is a consequence of the high S1P levels in the cell culture medium. Presence of the agonist S1P in the medium facilitated identification of its cognate S1P<sub>1</sub> receptor (Lee *et al.*, 1998). HEK293 cells stably expressing the S1P<sub>1</sub> receptor exhibited enhanced adherens junction-based cell-cell aggregation, exaggerated cadherin expression and were phenotypically differentiated. Removal of lipids from FBS by charcoal-stripping completely removed the factor responsible for morphogenetic differentiation. Subsequently, S1P was identified as the serum factor responsible for the phenotypic changes. Therefore, in case of the S1P<sub>1</sub> receptor, morphogenetic differentiation of cells does not seem to be an intrinsic feature of the receptor due to agonist-independent signalling but a consequence of S1P presence in the medium.

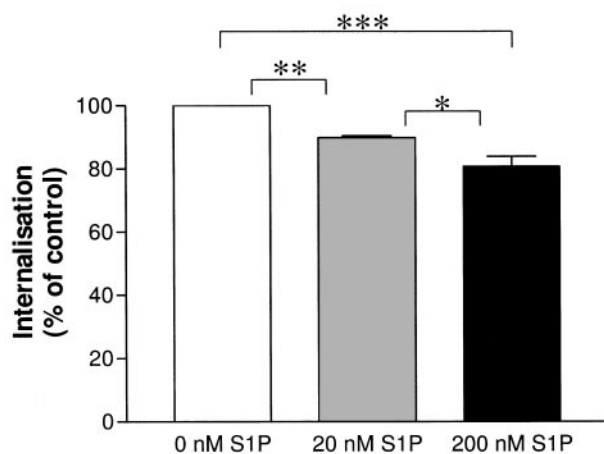
Meanwhile, many S1P receptor subtypes have been reported to cause phenotypical changes (morphology, proliferation, stress fibre formation, cytoskeletal reorganization, etc) when transfected in various cell types (Lee *et al.*, 1998; 1999; Moolenaar, 1999; van Brocklyn *et al.*, 1999; Robert *et al.*, 2001); in all cases, however, changes were dependent on the presence of lipids in the medium, and no reports are available so far about ligand-independent effects of S1P receptor subtypes. We tested several S1P receptors and chose the poorly characterized human S1P<sub>5</sub> receptor as a model to study a possible link between activation of various cellular events and their responsiveness to ligand modulation.

Initially, we observed that expression of the S1P<sub>5</sub> receptor in HEK293 cells led to a significant reduction of basal cAMP production. Basal cAMP levels decreased when cells were cultured in medium containing FBS as well as in delipidated medium (i.e. medium supplemented with csFBS). This finding indicates that the S1P<sub>5</sub> receptor is constitutively active in the





**Figure 7** Cellular distribution pattern of GFP-tagged wild type S1P<sub>5</sub> receptor. (A) C-terminally GFP-tagged S1P<sub>5</sub> receptor was expressed transiently in HEK293 cells and images were analysed confocally. A1–3: the majority of transfected cells are round whereas non transfected cells display the characteristic HEK293 cell morphology (A2,3). B1–3: images frequently observed showing a significant amount of GFP fluorescence in intracellular compartments. Some cells (C1–3) display GFP fluorescence at the plasma membrane. D: cells were stimulated with 10  $\mu$ M S1P for 10 min. E: cells were stimulated with 10  $\mu$ M S1P for 20 min. Cell rounding does not allow evaluation of internalization responses.



**Figure 8** SIP-mediated internalization of a HA-tagged wild-type  $SIP_5$  receptor. COS-7 cells transiently expressing the HA-tagged  $SIP_5$  receptor were grown and transfected in 48-well plates. Seventy-two hours after transfection cells were fixed and surface expressed receptors detected with a surface ELISA as described in detail in the Method section. SIP concentration-dependently internalizes a certain percentage of  $SIP_5$  receptors. Data are shown as mean  $\pm$  s.e. mean of three independent experiments performed in quadruplicates. \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  indicate significant differences of the respective multiple comparisons.

absence of the stimulating ligand. In order to test whether its agonist SIP may modulate the  $SIP_5$  receptor effects on basal cAMP production, cells were cultivated in csFBS and then stimulated with  $1 \mu M$  SIP. We did not observe a further decrease in cAMP levels under these conditions (Figure 1C). It cannot unequivocally be stated that constitutive inhibition of adenylyl cyclase *via* the  $SIP_5$  receptor is insensitive to ligand modulation as vector transfected cells displayed a small but significant increase in basal cAMP production. This increase may have counteracted the SIP effects on  $SIP_5$  receptor-mediated inhibition of adenylyl cyclase. SIP action on endogenously present  $SIP_2$  and  $SIP_3$  receptor may have caused this increase, as to the best of our knowledge no other SIP receptor subtype is positively linked to adenylyl cyclase. Alternatively, increase of cAMP levels in vector transfected cells may be due to an as yet unidentified SIP-responsive receptor. On the other hand, basal cAMP levels in FBS and csFBS were about identical, suggesting that SIP present in FBS at a concentration of  $\sim 400$  nM does indeed not affect the intrinsic adenylyl cyclase inhibition. Consistent with the hypothesis of constitutive activity is the finding that stimulation with the direct activator of adenylyl cyclase forskolin induced significantly lower cAMP levels in  $SIP_5$  receptor expressing cells as compared to vector transfected cells (Figure 1D). Under these conditions, however, i.e. when cAMP levels of forskolin-stimulated cyclase are increased, the agonist ligand SIP further enhances basal inhibition of adenylyl cyclase *via* the  $SIP_5$  receptor (Figure 2). One may speculate that in living cells the  $SIP_5$  receptor fine tunes adenylyl cyclase activity: it acts as a ligand-independent brake of adenylyl cyclase when cAMP levels are not elevated but converts to a potent ligand-dependent inhibitor of adenylyl cyclase when the enzyme is activated and/or intracellular cAMP levels are increased. This mechanism even raises the possibility that cAMP itself may be an intracellular allosteric

modulator of various active conformations of the  $SIP_5$  receptor and merits further investigation.

So far, a subset of GPCRs, including those for lysophosphatidic acid (LPA), endothelin, angiotensin II, bombesin and thrombin, have been reported to induce stress fibres, cytoskeletal re-arrangement and cell rounding (Hecht *et al.*, 1996; Ishida *et al.*, 1999; Sinnott-Smith *et al.*, 2001; Kawanabe *et al.*, 2002). These cellular responses are well recognized to be mediated *via* the  $G\alpha_{12}/G\alpha_{13}$ -Rho signalling pathway. Surprisingly, we could not detect Rho activation in a reporter gene assay. Moreover, insensitivity of cell rounding responses to PTX pretreatment clearly rules out involvement of  $G\alpha_i$  proteins. Irrespective of the pathway that is responsible for the phenotypic changes, it is intriguing to note that cell rounding is an intrinsic ability of the  $SIP_5$  receptor that can be further modulated by the ligand SIP. In contrast, intrinsic effects on the  $G\alpha_i$ -mediated events such as inhibition of non-stimulated adenylyl cyclase or ERK activity are insensitive to ligand stimulation. Our findings suggest that different intracellular effector systems triggered by a given constitutively active receptor are not equally activated and support the notion that inverse agonist ligands will be useful tools to dissect the pharmacology of individual GPCR-mediated signal transduction events.

Apoptotic cell death is often preceded by cell rounding. van Brocklyn *et al.* (1999) have reported that HEK293 cells transfected with the  $rSIP_2$  receptor, and to a lesser extent the  $SIP_3$  receptor, display rounded cell morphology and signs of apoptosis as evidenced by staining of fragmented DNA in the nuclei of the cells. To determine whether the related  $SIP_5$  receptor also causes cell death, DAPI staining was performed in  $SIP_5$  receptor transfected cells. Our findings clearly show that cell rounding does not correlate with cell death (Figure 5). In addition, caspase assays that are routinely used to determine activation of cell death suggest that the  $SIP_5$  receptor does not activate the cell death machinery. It should be noted that Rho activation has also been associated with dynamic membrane blebbing during the final stages of apoptotic cell death (Mills *et al.*, 1998). Moreover, the Rho effector protein kinase N (PKN) is another possible mediator of apoptosis, because it is proteolytically cleaved by caspases to generate a constitutively active kinase fragment (Takahashi *et al.*, 1998). It has to be emphasized, however, that there is as yet no direct evidence implicating specific signalling molecules downstream of Rho in the apoptotic signalling cascade. Nevertheless, the negative outcome of the caspase assay, together with our inability to demonstrate involvement of the  $G\alpha_{12}/G\alpha_{13}$ -Rho pathway in  $SIP_5$  receptor-mediated signalling, and the fact that Rho activation may lead to apoptosis (Mills *et al.*, 1998; Takahashi *et al.*, 1998, for review see Sah *et al.*, 2000) collectively support the idea that the  $SIP_5$  receptor may not utilize the  $G\alpha_{12}/G\alpha_{13}$  pathway to transmit signals to the cell interior. It should be noted, that in our study the  $rSIP_2$  receptor only led to modest cell rounding but not to apoptosis as described by van Brocklyn *et al.* (1999). Different cell types – HEK293 cells used by van Brocklyn *et al.* (1999) *versus* CHO-K1 cells in our study – may be the reason for the observed discrepancy. As stated by the authors in van Brocklyn *et al.* (1999), HEK293 cells are much easier to detach from the culture dishes than other cell types, and loss of cell attachment may have been a possible cause for the observed cell death in their study. Whereas our

data clearly support the concept that the  $SIP_5$  receptor does not act pro-apoptotic, it appears that it even exerts a small but significant protective effect. As can be seen in Figure 6,  $SIP$ -dependent stimulation of caspase activity in vector transfected cells is abolished in  $SIP_5$ -transfected cells and the fold caspase activation induced by staurosporin is decreased in  $SIP_5$ -transfected cells as compared with vector transfected cells. The  $SIP_5$  receptor therefore represents a receptor protecting cells in a constitutive manner against noxious stimuli such as staurosporin (Figure 6).

It is of interest to note that receptors with primarily intracellular localization are still signalling-competent. This raises the question whether activation of cellular signalling cascades requires presence of the receptor on the cell surface or whether internalized receptors are still signalling-competent. It is a general view that upon activation of cellular signalling cascades GPCR kinases are recruited to the plasma membrane to phosphorylate the activated GPCR, which in turn promotes arrestin binding (Lohse *et al.*, 1990; Vilardaga *et al.*, 2002).

Arrestin binding is then thought to uncouple the receptor from the G protein and to initiate a process of internalization. Traditionally, internalization has been thought of as a way to terminate cellular signalling. Recently, however, studies using transfected  $\beta_2$ -adrenoceptors or endogenous opioid receptors indicate that internalization of certain GPCRs can also activate cellular signalling. Daaka *et al.* (1998) and Ignatova *et al.* (1999) showed that receptor internalization was necessary to activate ERK. In these studies, dominant negative mutants of  $\beta$ -arrestin or dynamin prevented receptor internalization and most importantly, receptor-stimulated ERK activity. It is therefore intriguing to ask whether  $SIP_5$  receptor-mediated agonist-independent effects require internalization or whether the observed internalization is the consequence of constitutive signalling. Future studies will address this question in more detail. Irrespective of the order of the cellular events, our data further support the concept that wild-type receptors with agonist independent signalling are mainly located in

intracellular compartments of the cell as evidenced by the  $SIP_5$ -GFP fusion construct. This notion is further underscored by our finding that  $SIP$  only internalizes a small percentage of  $SIP_5$  receptors which may reflect a high basal internalization rate rather than the inability of  $SIP$  to internalize the  $SIP_5$  receptor. Availability of  $SIP_5$  receptor inverse agonists will help to shed more light on the links between constitutive activity and internalization.

$SIP$  acts both as an extracellular ligand *via* GPCRs, and as an intracellular mediator *via* as yet unidentified molecular targets (Spiegel & Milstien, 2000; Pyne & Pyne, 2000). Recently, it has been hypothesized that signal transduction cascades are not only initiated at the plasma membrane but also at nuclear membranes (Malviya & Rogue, 1998), as several studies have disclosed that the nuclear envelope plays a major role in signal transduction cascades and a novel nuclear envelope signal transduction (NEST) hypothesis has been put forward (Baldassare *et al.*, 1997; Raben & Baldassare, 2000). Moreover, both heterotrimeric and small G proteins (Baldassare *et al.*, 1997; Saffitz *et al.*, 1994), adenylate cyclase (Yamamoto *et al.*, 1998), phospholipase C (Malviya & Rogue, 1998; Cocco *et al.*, 1999), phospholipase D (Baldassare *et al.*, 1997), and several GPCRs such as the muscarinic M3 receptor (Lind & Cavanagh, 1993), the angiotensin II AT1 receptor (Booz *et al.*, 1992) and prostaglandin EP3 and EP4 receptors (Bhattacharya *et al.*, 1999) have been shown to be localized at the nucleus. It is therefore tempting to speculate that a possible intracellular target for  $SIP$  action is represented by a GPCR such as the  $SIP_5$  receptor. Clearly, future studies are required to address functionality of GPCRs with intracellular localization.

In summary, our data demonstrate that  $SIP_5$  is a constitutively active receptor with the ability to intrinsically stimulate various effector pathways albeit to a different extent. We suggest that different signal transduction pathways are not equally activated through constitutively active GPCRs with promiscuous signalling characteristics and highlight the importance of inverse agonist tools for the understanding of CAR pharmacology.

## References

- ANDERSSON, M., SJOSTRAND, J., PETERSEN, A., HONARVAR, A.K. & KARLSSON, J.O. (2000). Caspase and proteasome activity during staurosporin-induced apoptosis in lens endothelial cells. *Invest. Ophthalmol. Vis. Sci.*, **41**, 2623–2632.
- BALDASSARE, J.J., JARPE, M., ALFERES, L. & RABEN, D.M. (1997). Nuclear translocation of RhoA mediates the mitogen-induced activation of phospholipase D involved in nuclear envelope signal transduction. *J. Biol. Chem.*, **272**, 4911–4914.
- BHATTACHARYA, M., PERI, K., RIBEIRO-DA-SILVA, A., ALMAZAN, G., SHICHI, H., HOU, X., VARMA, D.R. & CHEMTOB, S. (1999). Localization of functional prostaglandin E2 receptors EP3 and EP4 in the nuclear envelope. *J. Biol. Chem.*, **274**, 15719–15724.
- BLAUKAT, A., BARAC, A., CROSS, M.J., OFFERMANN, S. & DIKIC, I. (2000). G protein-coupled receptor-mediated mitogen-activated protein kinase activation through cooperation of  $G\alpha_q$  and  $G\alpha_i$  signals. *Mol. Cell. Biol.*, **20**, 6837–6848.
- BOCKAERT, J. & PIN, J.P. (1999). Molecular tinkering of G protein-coupled receptors: an evolutionary success. *EMBO J.*, **18**, 1723–1729.
- BOOZ, G.W., CONRAD, K.M., HESS, A.L., SINGER, H.A. & BAKER, K.M. (1992). Angiotensin-II-binding sites on hepatocyte nuclei. *Endocrinology*, **130**, 3641–3649.
- BROWN, E.M. (1999). Physiology and pathophysiology of the extracellular calcium-sensing receptor. *Am. J. Med.*, **106**, 238–253.
- CHEN, G., WAY, J., ARMOUR, S., WATSON, C., QUEEN, K., JAYAWICKREME, C.K., CHEN, W.J. & KENAKIN, T. (2000). Use of constitutive G protein coupled receptor activity for drug discovery. *Mol. Pharmacol.*, **57**, 125–134.
- CHEN, S., LIN, F., XU, M. & GRAHAM, R.M. (2002). Phe(303) in TMVI of the alpha(1B)-adrenergic receptor is a key residue coupling TM helical movements to G-protein activation. *Biochemistry*, **41**, 588–596.
- CHUN, J., GOETZL, E.J., HLA, T., IGARASHI, Y., LYNCH, K.R., MOOLENAAR, W.H., PYNE, S. & TIGYI, G. (2002). International Union of Pharmacology. XXXIV. Lysophospholipid Receptor Nomenclature. *Pharmacol. Rev.*, **54**, 265–269.
- COUGHLIN, S.R. (1994). Expanding horizons for receptors coupled to G proteins: diversity and disease. *Curr. Opin. Cell. Biol.*, **6**, 191–197.
- COCCO, L., RUBBINI, S., MANZOLI, L., BILLI, A.M., FAENZA, I., PERUZZI, D., MATTEUCCI, A., ARTICO, M., GILMOUR, R.S. & RHEE, S.G. (1999). Inositides in the nucleus: presence and characterisation of the isozymes of phospholipase beta family in NIH 3T3 cells. *Biochem. Biophys. Acta*, **1438**, 295–299.

- DAAKA, Y., LUTTRELL, L.M., AHN, S., DELLA ROCCA, G.J., FERGUSON, S.S., CARON, M.G. & LEFKOWITZ, R.J. (1998). Essential role for G protein-coupled receptor endocytosis in the activation of mitogen-activated protein kinase. *J. Biol. Chem.*, **273**, 685–688.
- DUMONT, C., DURRBACH, A., BIDERE, N., ROULEAU, M., KREOMER, G., BERNARD, G., HIRSCH, F., CHARPENTIER, B., SUSIN, S.A. & SENIK, A. (2000). Caspase-independent commitment phase to apoptosis in activated blood t lymphocytes: reversibility at low apoptotic insult. *Blood*, **93**, 1030–1038.
- GREASLEY, P.J., FANELLI, F., SCHEER, A., ABUIN, L., NENNIGERTOSATO, M., DEBENEDETTI, P.G. & COTECCHIA, S. (2001). Mutational and computational analysis of the alpha(1b)-adrenergic receptor. Involvement of basic and hydrophobic residues in receptor activation and G protein coupling. *J. Biol. Chem.*, **276**, 46485–46494.
- HECHT, J.H., WEINER, J.A., POST, S.R. & CHUN, J. (1996). Ventricular zone gene-1 (vzq-1) encodes a lysophosphatidic acid receptor expressed in neurogenic regions of the developing cerebral cortex. *J. Cell. Biol.*, **135**, 1071–1083.
- IGNATOVA, E.G., BELCHEVA, M.M., BOHN, L.M., NEUMAN, M.C. & COSCIA, C.J. (1999). Requirement of receptor internalisation for opioid stimulation of mitogen-activated protein kinase: biochemical and immunofluorescence confocal microscopic evidence. *J. Neurosci.*, **19**, 56–63.
- IM, D.-S., CLEMENS, J., MACDONALD, T.L. & LYNCH, K.R. (2001). Characterization of the human and mouse sphingosine 1-phosphate receptor,  $SIP_5$  (EDG8): Structure-activity relationship of sphingosine 1-phosphate receptors. *Biochem.*, **40**, 14053–14060.
- IIRI, T., FARFEL, Z. & BOURNE, H.R. (1998). G-protein diseases furnish a model for the turn-on switch. *Nature*, **394**, 35–38.
- ISHIDA, T., ISHIDA, M., SUERO, J., TAKAHASHI, M. & BERK, B.C. (1999). Agonist-stimulated cytoskeletal reorganization and signal transduction at focal adhesions in vascular smooth muscle cells require c-Src. *J. Clin. Invest.*, **103**, 789–797.
- JENSEN, A.A., SPALDING, T.A., BURSTEIN, E.S., SHEPPARD, P.O., O'HARA, P.J., BRANN, M.R., KROGSGAARD-LARSEN, P. & BRAUNER-OSBORNE, H. (2000). Functional importance of the Ala(116)-Pro(136) region in the calcium-sensing receptor. Constitutive activity and inverse agonism in a family C G-protein-coupled receptor. *J. Biol. Chem.*, **275**, 29547–29555.
- KAWANABE, Y., OKAMOTO, Y., NOZAK, I.K., HASHIMOTO, N., MIWA, S. & MASAKI, T. (2002). Molecular mechanism for endothelin-1-induced stress-fiber formation: analysis of G proteins using a mutant endothelin(A) receptor. *Mol. Pharmacol.*, **61**, 277–284.
- KENAKIN, T.P. (1996). The classification of seven transmembrane receptors in recombinant expression systems. *Pharmacol. Rev.*, **48**, 413–463.
- LEE, M.J., THANGADA, S., CLAFFEY, K.P., ANCELLIN, N., LIU, C.H., KLUK, M., VOLPI, M., SHA'AFI, R.I. & HLA, T. (1999). Vascular endothelial cell adherens junction assembly and morphogenesis induced by sphingosine-1-phosphate. *Cell*, **99**, 301–312.
- LEE, M.J., VAN BROCKLYN, J.R., THANGADA, S., LIU, C.H., HAND, A.R., MENZELLEEV, R., SPIEGEL, S. & HLA, T. (1998). Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science*, **279**, 1552–1555.
- LEFKOWITZ, R.J., COTECCHIA, S., SAMAMA, P. & COSTA, T. (1993). Constitutive activity of receptors coupled to guanine nucleotide regulatory proteins. *Trends Pharmacol. Sci.*, **14**, 303–307.
- LEURS, R., SMIT, M.J., ALEWIJNSE, A.E. & TIMMERMAN, H. (1998). Agonist-independent regulation of constitutively active G-protein-coupled receptors. *Trends Biochem. Sci.*, **23**, 418–422.
- LIND, G.J. & CAVANAGH, H.D. (1993). Nuclear muscarinic acetylcholine receptors in corneal cells from rabbit. *Invest. Ophthalmol. Vis. Sci.*, **34**, 2943–2952.
- LOHSE, M.J., BENOVIĆ, J.L., CODINA, J., CARON, M.G. & LEFKOWITZ, R.J. (1990). beta-Arrestin: a protein that regulates beta-adrenergic receptor function. *Science*, **248**, 1547–1550.
- MALEK, R.L., TOMAN, R.E., EDSALL, L.C., WONG, S., CHIU, J., LETTERLE, C.A., VAN BROCKLYN, J.R., MILSTEIN, S., SPIEGEL, S. & LEE, N.H. (2001). Nrg-1 belongs to the endothelial differentiation gene family of G protein-coupled sphingosine-1-phosphate receptors. *J. Biol. Chem.*, **276**, 5692–5699.
- MALVIYA, A.N. & ROGUE, P.J. (1998). 'Tell me where is calcium bred': clarifying the roles of nuclear calcium. *Cell*, **92**, 17–23.
- MARINISSEN, M.J. & GUTKIND, J.S. (2001). G-protein-coupled receptors and signalling networks: emerging paradigms. *Trends Pharmacol. Sci.*, **22**, 368–376.
- MCCUNE, D.F., EDELMANN, S.E., OLGES, J.R., POST, G.R., WALDROP, B.A., WAUGH, D.J., PEREZ, D.M. & PIASCIK, M.T. (2000). Regulation of the cellular localization and signalling properties of the alpha(1B)- and alpha(1D)-adrenoceptors by agonists and inverse agonists. *Mol. Pharmacol.*, **57**, 659–666.
- MENG, E.C. & BOURNE, H.R. (2001). Receptor activation: what does the rhodopsin structure tell us? *Trends Pharmacol. Sci.*, **22**, 587–593.
- MILLIGAN, G., BOND, R.A. & LEE, M. (1995). Inverse agonism: pharmacological curiosity or potential therapeutic strategy? *Trends Pharmacol. Sci.*, **16**, 10–13.
- MILLS, J.C., STONE, N.L., ERHARDT, J. & PITTMAN, R.N. (1998). Apoptotic membrane blebbing is regulated by myosin light chain phosphorylation. *J. Cell Biol.*, **140**, 627–636.
- MISEREY-LENKEI, S., PARNOT, C., BARDIN, S., CORVOL, P. & CLAUSER, E. (2002). Constitutive internalisation of constitutively active angiotensin II AT(1A) receptor mutants is blocked by inverse agonists. *J. Biol. Chem.*, **277**, 5891–5901.
- MOOLENAAR, W.H. (1999). Bioactive lysophospholipids and their G protein-coupled receptors. *Exp. Cell Res.*, **253**, 230–238.
- NICHOLSON, D.W. & THORNBERRY, N.A. (1997). Caspases: killer proteases. *Trends Biochem. Sci.*, **22**, 299–306.
- PARMA, J., DUPREZ, L., VAN SANDE, J., COCHAUX, P., GERVY, C., MOCKEL, J., DUMONT, J. & VASSART, G. (1993). Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. *Nature*, **365**, 649–651.
- PAUWELS, P.J. & WURCH, T. (1998). Review: amino acid domains involved in constitutive activation of G-protein-coupled receptors. *Mol. Neurobiol.*, **17**, 109–135.
- PEI, G., SAMAMA, P., LOHSE, M., WANG, M., CODINA, J. & LEFKOWITZ, R.J. (1994). A constitutively active mutant beta 2-adrenergic receptor is constitutively desensitized and phosphorylated. *Proc. Natl. Acad. Sci.*, **91**, 2699–2702.
- PEREZ, D.M., HWA, J., GAVIN, R., MATHUR, M., BROWN, F. & GRAHAM, R.M. (1996). Constitutive activation of a single effector pathway: evidence for multiple activation states of a G protein-coupled receptor. *Mol. Pharmacol.*, **49**, 112–122.
- PYNE, S. & PYNE, N.J. (2000). Sphingosine 1-phosphate signalling in mammalian cells. *Biochem. J.*, **349**, 385–402.
- RABEN, D.M. & BALDASSARE, J.J. (2000). Nuclear envelope signalling-role of phospholipid metabolism. *Eur. J. Histochem.*, **44**, 67–80. Review.
- RAMSAY, D., BEVAN, N., REES, S. & MILLIGAN, G. (2001). Detection of receptor ligands by monitoring selective stabilization of a Renilla luciferase-tagged, constitutively active mutant, G-protein-coupled receptor. *Br. J. Pharmacol.*, **133**, 315–323.
- RAO, V.R., COHEN, G.B. & OPRIAN, D.D. (1994). Rhodopsin mutation G90D and a molecular mechanism for congenital night blindness. *Nature*, **367**, 639–642.
- ROBERT, P., TSUI, P., LAVILLE, M.P., LIVI, G.P., SARAU, H.M., BRIL, A. & BERREBI-BERTRAND, I. (2001). EDG1 receptor stimulation leads to cardiac hypertrophy in rat neonatal myocytes. *J. Mol. Cell. Cardiol.*, **33**, 1589–1606.
- SAFFITZ, J.E., NASH, J.A., GREEN, K.G., LUKE, R.A., RANSNAS, L.A. & INSEL, P.A. (1994). Immunoelectron microscopic identification of cytoplasmic and nuclear Gs alpha in S49 lymphoma cells. *FASEB J.*, **8**, 252–258.
- SAH, V.P., SEASHOLTZ, T.M., SAGI, S.A., & HELLER BROWN, J. (2000). The role of rho in G protein-coupled receptor signal transduction. *Annu. Rev. Pharmacol. Toxicol.*, **40**, 459–489.
- SAMAMA, P., COTECCHIA, S., COSTA, T. & LEFKOWITZ, R.J. (1993). A mutation induced activated state of the beta<sub>2</sub>-adrenergic receptor. Extending the ternary complex model. *J. Biol. Chem.*, **268**, 4625–4636.
- SCHEER, A., COSTA, T., FANELLI, F., DE BENEDETTI, P.G., MHAOUTY-KODJA, S., ABUIN, L., NENNIGERTOSATO, M. & COTECCHIA, S. (2000). Mutational analysis of the highly conserved arginine within the Glu/Asp-Arg-Tyr motif of the alpha(1b)-adrenergic receptor: effects on receptor isomerization and activation. *Mol. Pharmacol.*, **57**, 219–231.

- SCHEER, A. & COTECCHIA, S. (1997). Constitutively active G protein-coupled receptors: potential mechanisms of receptor activation. *J. Recept. Signal. Transduct.*, **17**, 57–73.
- SCHÖNEBERG, T., SANDIG, V., WESS, J., GUDERMANN, T. & SCHULTZ, G. (1997). Reconstitution of mutant V2 vasopressin receptors by adenovirus-mediated gene transfer. Molecular basis and clinical implication. *J. Clin. Invest.*, **100**, 1547–1556.
- SHENKER, A., LAUE, L., KOSUGI, S., MERENDINO, JR. J.J., MINEGISHI, T. & CUTLER, JR. G.B. (1993). A constitutively activating mutation of the luteinizing hormone receptor in familial male precocious puberty. *Nature*, **365**, 652–654.
- SINNETT-SMITH, J., LUNN, J.A., LEOPOLDT, D. & ROZENGURT, E. (2001). Y-27632, an inhibitor of Rho-associated kinases, prevents tyrosine phosphorylation of focal adhesion kinase and paxillin induced by bombesin: dissociation from tyrosine phosphorylation of p130(CAS). *Exp. Cell. Res.*, **266**, 292–302.
- SPIEGEL, S. & MILSTIEN, S. (2000). Sphingosine-1-phosphate: signalling inside and out. *FEBS Lett.*, **6**, 55–57.
- STEVENS, P.A., BEVAN, N., REES, S. & MILLIGAN, G. (2000). Resolution of inverse agonist-induced up-regulation from constitutive activity of mutants of the alpha(1b)-adrenoceptor. *Mol. Pharmacol.*, **58**, 438–448.
- TAKAHASHI, M., MUKAI, H., TOSHIMORI, M., MIYAMOTO, M. & ONO, Y. (1998). Proteolytic activation of PKN by caspase-3 or related protease during apoptosis. *Proc. Nat. Acad. Sci.*, **95**, 11566–11571.
- VAN BROCKLYN, J.R., TU, X., EDSALL, L.C., SCHMIDT, R.R. & SPIEGEL, S. (1999). Sphingosine 1-phosphate-induced cell rounding and neurite retraction are mediated by the G protein-coupled receptor H218. *J. Biol. Chem.*, **274**, 4626–4632.
- VAN SANDE, J., SWILLENS, S., GERARD, C., ALLGEIER, A., MASSART, C., VASSART, G. & DUMONT, J.E. (1995). In Chinese hamster ovary K1 cells dog and human thyrotropin receptors activate both the cyclic AMP and phosphatidylinositol 4,5-bisphosphate cascades in the presence of thyrotropin and cyclic AMP cascade in its absence. *Eur. J. Biochem.*, **229**, 338–343.
- VILARDAGA, J.P., KRASEL, C., CHAUVIN, S., BAMBINO, T., LOHSE, M.J. & NISSENSON, R.A. (2002). Internalization determinants of the parathyroid hormone receptor differentially regulate beta-arrestin/receptor association. *J. Biol. Chem.*, **277**, 8121–8129.
- WEISS, J.M., MORGAN, P.H., LUTZ, M.W. & KENAKIN, T.P. (1996). The cubic ternary complex receptor. Occupancy model. III. Resurrecting efficacy. *J. Theor. Biol.*, **181**, 381–397.
- YAMAMOTO, S., KAWAMURA, K. & JAMES, T.N. (1998). Intracellular distribution of adenylate cyclase in human cardiocytes determined by electron microscopic cytochemistry. *Microsc. Res. Tech.*, **40**, 479–487.
- YATOMI, Y., IGARASHI, Y., YANG, L., HISANO, N., QI, R., ASAZUMA, N., SATOH, K., OZAKI, Y. & KUME, S.P. (1997). Sphingosine 1-phosphate, a bioactive sphingolipid abundantly stored in platelets, is a normal constituent of human plasma and serum. *J. Biochem.*, **121**, 969–973.

(Received September 11, 2002

Accepted October 18, 2002)