British Journal of Pharmacology (2004) 143, 581–589
www.nature.com/bjp

Heterologous desensitization of the sphingosine-1-phosphate receptors by purinoceptor activation in renal mesangial cells

[1]Cuiyan Xin, [1]Shuyu Ren, [1]Josef Pfeilschifter & *,[1]Andrea Huwiler

[1]Pharmazentrum frankfurt, Klinikum der Johann Wolfgang Goethe-Universität, Theodor-Stern-Kai 7, D-60590 Frankfurt am Main, Germany

1 Sphingosine-1-phosphate (S1P) is considered a potent mitogen for mesangial cells and activates the classical mitogen-activated protein kinase (MAPK) cascade *via* S1P receptors. In this study, we show that S1P signalling is rapidly desensitized upon S1P receptor activation. A complete loss of S1P sensitivity occurs after 10 min of S1P pretreatment and remains for at least 8 h. A similar desensitization is also seen with the S1P mimetic FTY720-phosphate, but not with the nonphosphorylated FTY720, nor with sphingosine or ceramide.

2 Prestimulating the cells with extracellular ATP or UTP, which bind to and activate P2Y receptors on mesangial cells, a similar rapid desensitization of the S1P receptor occurs, suggesting a heterologous desensitization of S1P receptors by P2Y receptor activation. Furthermore, adenosine binding to P1 receptors triggers a similar desensitization. In contrast, two other growth factors, PDGF-BB and TGFβ_2, have no significant effect on S1P-induced MAPK activation.

3 S1P also triggers increased inositol trisphosphate (IP_3) formation, which is completely abolished by S1P pretreatment but only partially by ATP pretreatment, suggesting that IP_3 formation and MAPK activation stimulated by S1P involve different receptor subtypes.

4 Increasing intracellular cAMP levels by forskolin pretreatment has a similar effect on desensitization as adenosine. Moreover, a selective A_3 adenosine receptor agonist, which couples to phospholipase C and increases IP_3 formation, exerted a similar effect.

5 Pretreatment of cells with various protein kinase C (PKC) inhibitors prior to ATP prestimulation and subsequent S1P stimulation leads to a differential reversal of the ATP effect. Whereas the broad-spectrum protein kinase inhibitor staurosporine potently reverses the effect, the PKC-α inhibitor CGP41251, the PKC-δ inhibitor rottlerin and calphostin C show only a partial reversal at maximal concentrations.

6 Suramin, which is reported as a selective $S1P_3$ receptor antagonist compared to the other S1P receptor subtypes, has no effect on the S1P-induced MAPK activation, thus excluding the involvement of $S1P_3$ in this response.

7 In summary, these data document a rapid homologous and also heterologous desensitization of S1P signalling in mesangial cells, which is mechanistically triggered by PKC activation and eventually another staurosporine-sensitive protein kinase, as well as by increased cAMP formation.
British Journal of Pharmacology (2004) **143,** 581–589. doi:10.1038/sj.bjp.0705980

Keywords: EDG receptors; sphingosine-1-phosphate; ATP; P2Y receptor; mesangial cell

Abbreviations: DMEM, Dulbecco's modified Eagle medium; ECL, enhanced chemiluminescence; EDG, endothelial differentiation gene; EGF, epidermal growth factor; GPCR, G-protein-coupled receptor; GRK, G-protein-coupled receptor protein kinase; MAPK, mitogen-activated protein kinase; PDGF, platelet-derived growth factor; S1P, sphingosine-1-phosphate; SAPK/JNK, stress-activated protein kinase/N-terminal c-Jun kinase; SDS–PAGE, sodium dodecylsulphate–polyacrylamide gel electrophoresis; TGFβ, transforming growth factor-β; VEGF, vascular endothelium-derived growth factor

Introduction

Renal mesangial cells not only play an important physiological role by regulating the glomerular filtration rate, but also crucially contribute to most pathological processes of the renal glomerulus (Pfeilschifter, 1989; 1994; Kashgarian & Sterzel, 1992). Together with increased extracellular matrix production and inflammatory mediator secretion, mesangial cell proliferation is a hallmark of many forms of glomerulonephritis, and ultimately may lead to glomerulosclerosis. However, the detailed mechanisms regulating cell proliferation are still not completely understood, although the involvement of the mitogen-activated protein kinase (MAPK) cascade is generally assumed (Huwiler & Pfeilschifter, 1994; Huwiler *et al*., 1995; Pfeilschifter *et al*., 1995; Seger & Krebs, 1995; Bokemeyer *et al*., 2002).

The sphingolipid molecule sphingosine-1-phosphate (S1P) has gained increasing attention during the last few years due to its pleiotropic effects on the cell, including proliferation (Zhang *et al*., 1991; Olivera & Spiegel, 1993), differentiation (Vogler *et al*., 2003), cytoprotection (Manggau *et al*., 2001) and

*Author for correspondence; E-mail: Huwiler@em.uni-frankfurt.de
Advance online publication: 4 October 2004

cell migration (Wang *et al*., 1999). It is generated from ceramide by the action of neutral ceramidase, followed by a sphingosine kinase (for reviews, see Huwiler *et al*., 2000a; Hla, 2003; Spiegel & Milstien, 2003). So far two subtypes of sphingosine kinase have been identified, the sphingosine kinase-1 and -2, which show different substrate specificities and also different cellular localizations (Kohama *et al*., 1998; Liu *et al*., 2000). Once generated, S1P may act intracellularly by activation of still unknown targets to trigger either cell proliferation or differentiation. This intracellular action was supported by experiments microinjecting S1P into cells (Van Brocklyn *et al*., 1998; Vogler *et al*., 2003), or by using a 'caged' S1P derivative that penetrates the cell and upon photolysis is converted into mature S1P in the intracellular space (Qiao *et al*., 1998). Alternatively, S1P can be secreted by an active mechanism and acts by binding to specific cell surface S1P receptors, which trigger various signalling cascades leading to cell responses.

Such S1P receptors have been identified and belong to the endothelial differentiation gene (EDG) family (Lee *et al*., 1998), which also includes the lyso-phosphatidic acid receptors and are classical seven membrane-spanning G-protein-coupled receptors (GPCRs). Recently, the EDG receptors have been renamed to S1P receptors (Chun *et al*., 2002).

In renal mesangial cells, several S1P receptors have been reported to be expressed at least on the mRNA level, including $S1P_1$, $S1P_2$, $S1P_3$ and $S1P_5$ (formerly known as EDG-1, -5, -3 and -8 receptors; Katsuma *et al*., 2002; Gennero *et al*., 2002), which may contribute to intracellular calcium mobilization, activation of the classical MAPK cascade and cell proliferation (Gennero *et al*., 2002; Katsuma *et al*., 2002).

In this study, we show that in renal mesangial cells S1P-stimulated p42/p44-MAPK activation displays homologous desensitization. Moreover, a prior activation of ATP and adenosine receptors causes heterologous desensitization of the S1P response. Mechanistically, the desensitization involves the activation of staurosporine-sensitive protein kinases, including protein kinase C (PKC).

Methods

Chemicals

S1P, sphingosine and C6-ceramide were purchased from Biotrend Chemikalien GmbH, Köln, Germany; ATP, UTP and adenosine were from Fluka Feinchemikalien, Buchs, Switzerland; suramin, calphosin C and rottlerin were from Merck Biosciences, Schwalbach, Germany; forskolin and N^6-Bz-NECA were purchased from Sigma Aldrich Fine Chemicals, Deisenhofen, Germany; CGP41251, FTY720 and FTY-phosphate were kindly donated by Dr Fabbro and Dr Brinkmann, Novartis Pharma Inc., Basel, Switzerland; phospho-specific antibodies against p42/p44-MAPK, JNK and p38-MAPK were from Cell Signaling, Frankfurt am Main, Germany; total p42- and p44-MAPK antisera were generated and characterized as described previously (Huwiler & Pfeilschifter, 1994); anti-rabbit and anti-mouse horseradish peroxidase-linked IgGs and Hyperfilm MP were purchased from Amersham Pharmacia Biotech Europe GmbH, Freiburg, Germany; Dowex 1-X8 (100–200 mesh, formate form) was from Serva, Heidelberg, Germany; all cell culture nutrients were from Invitrogen, Karlsruhe, Germany.

Cell culture

Rat renal mesangial cells (MZB1) were cultivated and characterized as described previously (Pfeilschifter *et al*., 1986). Passages 7–18 were used for the experiments in this study.

Cell stimulation and Western blot analysis

Confluent mesangial cells in 60-mm-diameter dishes were stimulated with the indicated substances in Dulbecco's modified Eagle medium (DMEM) containing 0.1 mg ml^{-1} of fatty acid-free bovine serum albumin. Thereafter, the medium was withdrawn and the cells washed once with ice-cold phosphate-buffered saline solution. Cells were scraped into ice-cold lysis buffer (50 mM Tris/HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 2 mM EGTA, 40 mM β-glycerophosphate, 50 mM sodium fluoride, 10 μg ml^{-1} leupeptin, 10 μg ml^{-1} aprotinin, 1 μM pepstatin A, 1 mM phenylmethyl sulphonyl fluoride) and homogenized by 10 passes through a 26G needle fitted to a 1 ml syringe. Samples were centrifuged for 10 min at 14,000 $\times$ *g* and the supernatant was taken for protein determination. Cell extracts containing 50 μg of protein were subjected to sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE). Proteins were transferred onto a nitrocellulose paper as described (Huwiler *et al*., 1995). After the transfer, immunostaining was performed as described previously in detail (Huwiler *et al*., 1995). Antibodies were diluted in blocking buffer as indicated in the legends of the figures. Bands were detected by the enhanced chemiluminescence (ECL) method as recommended by the manufacturer.

Determination of inositol phosphate generation

Confluent cells in 30-mm-diameter dishes were labelled for 72 h with *myo*-[2-^{3}H]inositol (10 μCi ml^{-1}) in DMEM free of inositol, containing 2% dialysed foetal bovine serum. Thereafter, the medium was removed and cells were washed three times to remove free [^{3}H]inositol and incubated for an additional 1 h in fresh DMEM containing 0.1 mg ml^{-1} of bovine serum albumin. After stimulation, the reaction was terminated by rapid aspiration of the medium and addition of 1 ml of 20% trichloroacetic acid. The trichloroacetic acid was then removed with diethyl ether and the final extraction was neutralized and applied to anion-exchange columns containing 1 ml of Dowex 1-X8. Free inositol and the inositol phosphates were eluted sequentially as described previously (Pfeilschifter *et al*., 1986). The ratios of the phosphorylated compounds relative to total-soluble [^{3}H]inositol were calculated for each sample.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance (ANOVA). For multiple comparisons with the same control group, the limit of significance was divided by the number of comparisons according to Bonferroni. One-way ANOVA with Bonferroni's post test was performed using GraphPad InStat version 3.00 for Windows NT, GraphPad Software, San Diego, CA, U.S.A.

Results

S1P causes rapid homologous desensitization of MAPK activation

Previously, we have shown that exogenously applied S1P rapidly activates the classical MAPK cascade as detected by increased phosphorylation of the p42 and p44 isoforms of MAPK (Xin *et al*., 2004). Here, we show that pretreatment of mesangial cells with S1P leads to a rapid inhibition of MAPK activation in response to a second S1P stimulation. As seen in Figure 1, already after 5 min of S1P pretreatment, a significant reduction of the MAPK activation after a second S1P stimulus is observed. After 10 min of S1P pretreatment, desensitization is complete and cells remain desensitized upto 8 h. During the whole stimulation period, the total amounts of p42- and p44-MAPKs do not change (Figure 1, lower panel).

To see whether another synthetic agonist at the S1P receptor mediates the same effect on S1P-stimulated MAPK activation, FTY720-phosphate was tested. This compound has previously been shown to arise from the potent immunosuppressive agent FTY720 by the action of sphingosine kinases (Brinkmann *et al*., 2002; Mandala *et al*., 2002). As seen in Figure 2a, FTY-phosphate pretreatment dose-dependently decreases the S1P effect on MAPK activation, whereas the nonphosphorylated FTY720 has no effect (Figure 2b). Furthermore, short-chain C6-ceramide (Figure 2b) and long-chain C16-ceramide (data not shown) and also sphingosine (Figure 2c), which all do not bind to the S1P receptor but possess structural similarities to S1P, have no inhibiting effect. Ceramide rather increases the MAPK activation (Figure 2b), which is in line with previous data showing that ceramide directly activates c-Raf in mesangial cells and leads to MAPK activation (Huwiler *et al*., 1996).

P2Y receptor activation leads to a heterologous desensitization of the S1P receptor

Next we investigated whether the activation of other receptors causes a heterologous desensitization of the S1P receptor. Therefore, we pretreated mesangial cells for 30 min with extracellular ATP, which binds to and activates P2Y receptors prior to stimulation with S1P. As shown in Figure 3a, a concentration of 1 μM of ATP leads to a drastic reduction of the S1P-induced MAPK activation. After 3 μM of ATP pretreatment, activation of MAPK by S1P is completely lost. Furthermore, UTP exerts a similar and even more potent effect on S1P-triggered MAPK activation. At 1 μM of UTP, a complete loss of cell responsiveness towards S1P is observed (Figure 3b). Also, adenosine, which binds to P1 purinoceptors, almost completely desensitizes the cell already at 3 μM (Figure 3c). In contrast, pretreatment of cells with increasing concentrations of TGFβ_2 or with PDGF-BB hardly reduces the S1P effect (Figure 3d), suggesting that heterologous desensitization of S1P receptors is a specific response seen after activation of P1 and P2Y receptors.

Since adenosine can signal *via* different receptors and downstream signalling cascades, we probed for the receptor

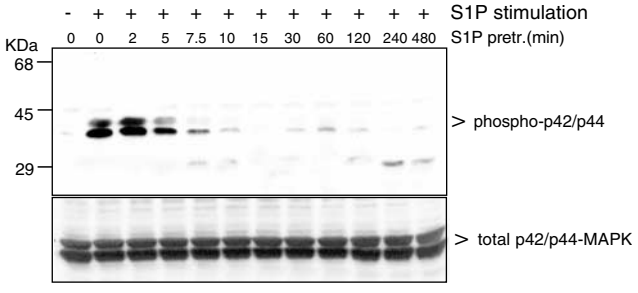

Figure 1 Effect of S1P on S1P receptor desensitization in mesangial cells. Quiescent mesangial cells were stimulated with vehicle for 10 min (Control; −) or pretreated for the indicated time periods (in min) with S1P (10 μM), prior to stimulation with a second pulse of S1P (10 μM; +) for 15 min. Thereafter, cells were harvested and Western blot analysis was performed using a specific monoclonal anti-phospho-p42/p44-MAPK antibody or polyclonal p42- and p44-MAPK antisera at a dilution of 1 : 1000 each. Bands were detected by the ECL method according to the manufacturer's recommendation. Data are representative of three independent experiments giving similar results.

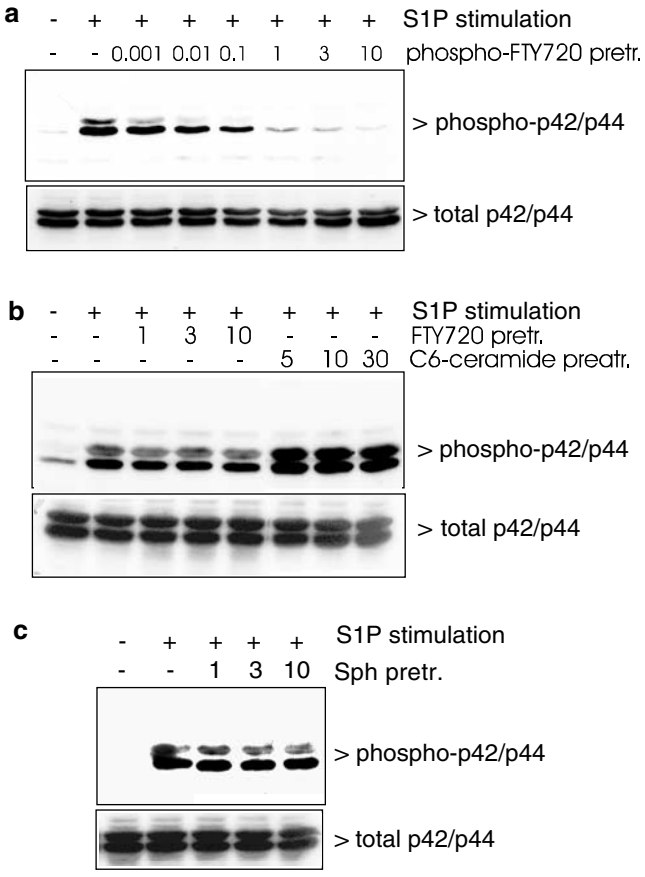

Figure 2 Effect of FTY-phosphate, FTY720, ceramide and sphingosine on S1P receptor desensitization in mesangial cells. Quiescent mesangial cells were stimulated with vehicle for 10 min (Control; −) or pretreated for 30 min with the indicated concentrations of FTY-phosphate (a; in μM), FTY720 (b; in μM), C6-ceramide (b; in μM) and sphingosine (c; in μM) prior to stimulation with S1P (10μM; +) for 15 min. Thereafter, cells were harvested and Western blot analyses were performed using a specific monoclonal anti-phospho-p42/p44-MAPK antibody (upper panels) or polyclonal p42- and p44-MAPK antisera (lower panels) at a dilution of 1 : 1000 each. Bands were detected by the ECL method according to the manufacturer's recommendation. Data are representative of three independent experiments giving similar results.

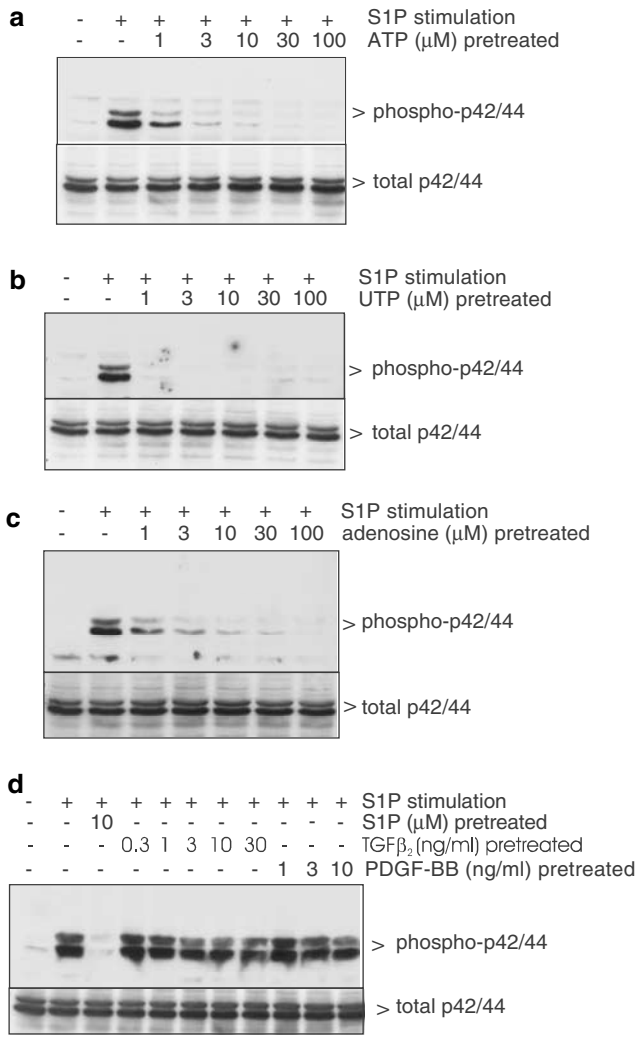

Figure 3 Effect of P2Y, PDGF and TGFβ_2 receptor activation on S1P-induced MAPK activation in mesangial cells. Quiescent mesangial cells were stimulated with vehicle for 10 min (Control; −) or pretreated for 30 min with the indicated concentrations of ATP (a; in μM), UTP (b; in μM), adenosine (c; in μM), PDGF-BB (d; in ng ml^{-1}) or TGFβ_2 (d; in ng ml^{-1}) prior to stimulation with S1P (10 μM; +) for 15 min. Thereafter, cells were harvested and Western blot analyses were performed using a specific monoclonal anti-phospho-p42/p44-MAPK antibody or polyclonal p42- and p44-MAPK antisera at a dilution of 1 : 1000 each. Bands were detected by the ECL method according to the manufacturer's recommendation. Data are representative of two (d) or three (a–c) independent experiments giving similar results.

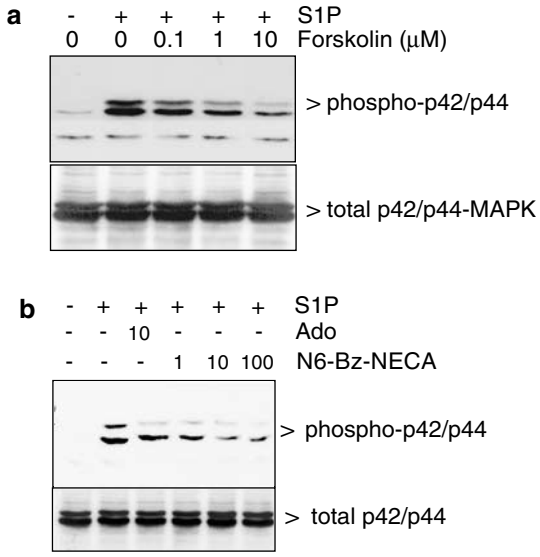

Figure 4 Effect of forskolin and an A_3 receptor agonist on S1P-induced MAPK activation in mesangial cells. Quiescent mesangial cells were stimulated with vehicle for 10 min (Control; −) or pretreated for 15 min with the indicated concentrations of forskolin (a; in μM) or the A_3 receptor agonist N^6-benzyl-5′-*N*-ethylcarboxamidoadenosine (N^6-Bz-NECA) (b; in μM) before stimulation with S1P (10 μM; +) for 15 min. Thereafter, cells were harvested and Western blot analyses were performed using a specific monoclonal anti-phospho-p42/p44-MAPK antibody or a polyclonal p42-MAPK antiserum at a dilution of 1 : 1000 each. Bands were detected by the ECL method according to the manufacturer's recommendation. Data are representative of three independent experiments giving similar results.

subtype involved, and tested which signalling cascade is involved in the S1P receptor desensitization. For this reason, cells were treated with forskolin, which is a direct activator of adenylate cyclase and thereby increases intracellular cAMP levels resembling adenosine-mediated A_2 receptor activation. As seen in Figure 4a, forskolin leads to a dose-dependent reduction of the S1P response. In addition, the selective A_3 receptor agonist N^6-benzyl-5′-*N*-ethylcarboxamidoadenosine (N^6-Bz-NECA) (Gallo-Rodriguez *et al*., 1994), which classically couples to phospholipase C and increased inositol trisphosphate (IP_3) and 1,2-diacylglycerol formation, also leads to a reduction of the S1P response (Figure 4b). These data suggest that adenosine exerts its desensitizing effect not only *via* increased cAMP generation, but also *via* enhanced IP_3 and 1,2-diacylglycerol formation.

To see whether there exists a mutual desensitization of purinoceptors and S1P receptors, we further investigated the effect of S1P pretreatment on a subsequent ATP stimulus on MAPK activation. This regimen also leads to a reduction of ATP-induced MAPK phosphorylation, although the effect is less potent. At 1 μM of S1P pretreatment, which fully activates all S1P receptors, only a 48% reduction of MAPK phosphorylation is seen (data not shown).

In order to get a hint on the S1P receptor subtype involved in the S1P-induced MAPK activation, cells were pretreated with suramin prior to S1P stimulation. Suramin displays an interesting panel of specificities. Although being a rather unselective antagonist at various purinoceptors (Ralevic & Burnstock, 1998), it has been reported to be a selective $S1P_3$ receptor antagonist compared to the other S1P receptor subtypes *in vitro* (Ancellin & Hla, 1999). Figure 5 shows that even very high concentrations of suramin (up to 300 μM) have no effect on S1P-induced MAPK activation, thus excluding the involvement of the $S1P_3$ receptor subtype.

ATP-induced desensitization of the S1P receptor involves protein kinase C

To further unravel the mechanism of desensitization of the S1P-induced MAPK activation by ATP, various protein kinase inhibitors were tested. In the presence of staurosporine, which is a potent broad-spectrum protein kinase inhibitor

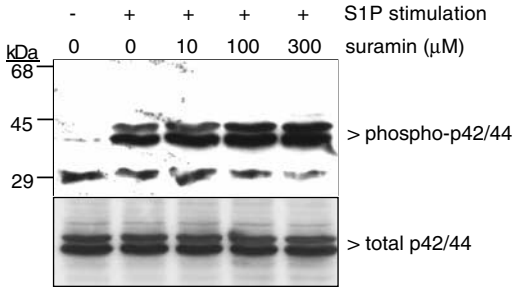

Figure 5 Effect of suramin on S1P-induced MAPK activation in mesangial cells. Quiescent mesangial cells were stimulated with vehicle for 10 min (Control; −) or pretreated for 15 min with the indicated concentrations of suramin (in μM) before stimulation with S1P (10 μM; +) for 15 min. Thereafter, cells were harvested and Western blot analyses were performed using a specific monoclonal anti-phospho-p42/p44-MAPK antibody at a dilution of 1 : 1000 each. Bands were detected by the ECL method according to the manufacturer's recommendation. Data are representative of at least four independent experiments giving similar results.

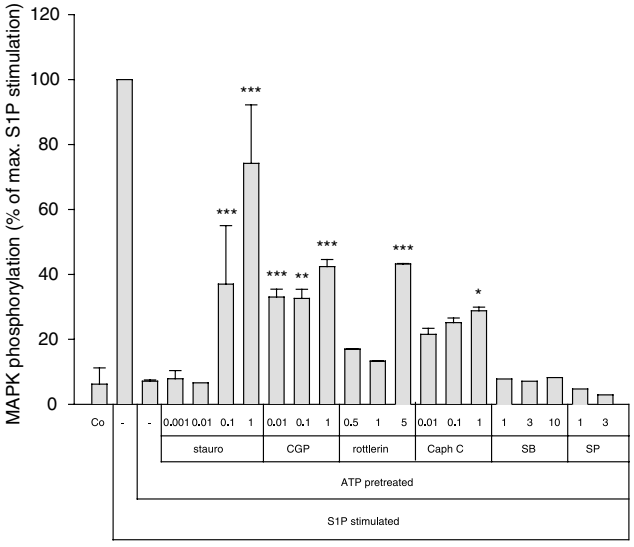

Figure 6 Effect of different protein kinase inhibitors on ATP-induced desensitization of S1P-induced MAPK activation in mesangial cells. Quiescent mesangial cells were stimulated with vehicle for 10 min (Co) or incubated for 30 min with the indicated concentrations of staurosporine (stauro; in μM), CGP41251 (CGP; in μM), rottlerin (in μM), calphostin C (Calph C; in μM), SB203580 (SB; in μM) or SP600125 (SP; in μM) before being treated with ATP (30 min; 100 μM) prior to stimulation with S1P (10 μM; +) for 15 min. Thereafter, cells were harvested and Western blot analyses were performed using a specific monoclonal anti-phospho-p42/p44-MAPK antibody at a dilution of 1 : 1000 each. Bands were detected by the ECL method according to the manufacturer's recommendation. Bands were densitometrically evaluated and data were expressed as % of maximal S1P-stimulated MAPK phosphorylation, and are means ± s.d. ($n = 2$–3). $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, considered statistically significant compared to the ATP-pretreated control values.

but shows selective inhibition of the Ca^{2+}-dependent PKC isoenzymes when compared to the Ca^{2+}-independent novel PKC isoenzymes (Tamaoki *et al*., 1986; McGlynn *et al*., 1992; Geiges *et al*., 1997), a dose-dependent reversal of the ATP response is observed (Figure 6). CGP41251, which potently blocks the Ca^{2+}-dependent PKC isoenzymes (Meyer *et al*., 1989; Geiges *et al*., 1997), that is, PKC-α in mesangial cells, is able to partially reverse the desensitization effect of ATP (Figure 6). Rottlerin, reported to selectively inhibit PKC-δ among the PKC isoenzymes (Gschwendt *et al*., 1994), also partially reverses the ATP effect (Figure 6). A similar partial effect is obtained with calphostin C (Figure 6), which interacts with the regulatory domain of PKC isoenzymes (Kobayashi *et al*., 1989; Pfeilschifter & Huwiler, 1993; Mizuno *et al*., 1995).

Since ATP is also activating the stress-activated protein kinases SAPK/JNK (Huwiler *et al*., 1997) and p38-MAPK (Huwiler *et al*., 2000b), we tested whether these two cascades are involved in the desensitization process by using specific inhibitors of both enzymes. However, neither SB203580 (Cuenda *et al*., 1995) nor SP600125 (Bennett *et al*., 2001) have the potential to reverse the ATP-triggered desensitization (Figure 6).

S1P-mediated IP_3 formation is also desensitized by S1P, but only partially by ATP

To see whether the rapid desensitization of S1P response on MAPK activation is also reflected on another very early level of S1P receptor activation, we investigated the generation of IP_3. As seen in Figure 7a, stimulation of mesangial cells for 30 s with either a low concentration (100 nM) or a high concentration (3 μM) of S1P leads to a significant 2–3-fold increase of IP_3 formation. By pretreating cells for 30 min with S1P, a slightly increased IP_3 level is detected, which, however, is completely insensitive to a second exposure to S1P for 30 s. When pretreating cells for 30 min with ATP, no change in IP_3 level is detected compared to control, and a second stimulation with S1P can still partially increase IP_3 generation, suggesting an incomplete desensitization of the S1P receptor signalling cascade by purinoceptor activation. Pretreatment of cells with either PDGF or TGFβ_2 has no desensitizing effect on S1P-triggered IP_3 generation. Rather, PDGF pretreatment leads to a further increase of the S1P-stimulated IP_3 formation, consistent with the fact that IP_3 can be formed by two independent pathways, on one side by PLC-β activation and on the other side by growth factor-mediated PLC-γ activation. In line with this, S1P pretreatment, which can lead to a transactivation of the PDGF receptor (Katsuma *et al*., 2002), is not able to desensitize ATP-stimulated IP_3 formation, but rather enhances the response (Figure 7b).

Discussion

Exogenous S1P has been shown to trigger many important cellular responses, including proliferation (Zhang *et al*., 1991; Olivera & Spiegel, 1993), differentiation (Vogler *et al*., 2003), cytoprotection (Manggau *et al*., 2001) and cell migration (Wang *et al*., 1999). Mechanistically, it has been extensively documented that S1P binds to specific cell surface receptors, which have been discovered as EDG receptors (Lee *et al*., 1998) and have now been renamed to $S1P_{1-5}$ receptors (Chun *et al*., 2002). Previously, it was reported that mesangial cells express mRNA of $S1P_1$ (EDG-1), $S1P_2$ (EDG-5), $S1P_3$ (EDG-3) and $S1P_5$ (EDG-8) (Gennero *et al*., 2002; Katsuma *et al*., 2002), and eventually also $S1P_4$ (Gennero *et al*., 2002). Whether the corresponding receptor is also expressed on the

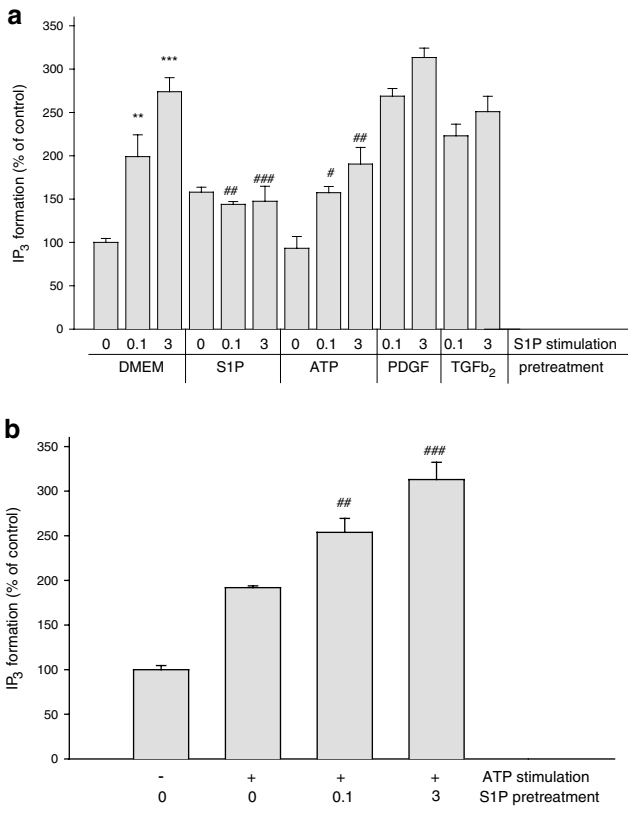

Figure 7 Effect of S1P, P2Y, PDGF and TGFβ_2 receptor activation on S1P-induced IP_3 generation in mesangial cells. (a) [^{3}H]inositol-labelled cells were pretreated for 30 min with either vehicle (DMEM) or S1P (3 μM), ATP (30 μM), PDGF-BB (30 ng ml^{-1}) or TGFβ_2 (10 ng ml^{-1}) prior to stimulation for 30 s with either DMEM (0) or the indicated concentrations of S1P (in μM). (b) Labelled cells were pretreated for 30 min with either DMEM (0) or the indicated concentrations of S1P (in μM) prior to stimulation for 30 s with DMEM (−) or ATP (30 μM; +). IP_3 was analysed as described in the Methods section. Results are expressed as % of control values and are means ± s.d. ($n=3$). $^{**}P<0.01$, $^{***}P<0.001$, considered statistically significant compared to the unstimulated control; $^{\#}P<0.05$, $^{\#\#}P<0.01$, $^{\#\#\#}P<0.001$, considered statistically significant compared to the respective non-pretreated stimulation values.

protein level has not been investigated. This is mainly due to the fact that specific antibodies are not yet available. However, using antisense oligonucleotides against $S1P_2$ and $S1P_3$ in mesangial cells, the mitogenic effect of S1P was abrogated, suggesting that these two receptors are indeed expressed and contribute to the proliferative response of mesangial cells induced by S1P. We have analyzed our mesangial cell line (MZB1) (Pfeilschifter & Vosbeck, 1991) for S1P receptor expression and could confirm the presence of $S1P_1$, $S1P_2$ and $S1P_3$ on the mRNA level (data not shown). In contrast to Gennero *et al*. (2002), but in line with Katsuma *et al*. (2002), we cannot detect $S1P_5$ in our mesangial cells. Previously, Terai *et al*. (2003) reported that $S1P_5$ is preferentially expressed in oligodendrocytes in the central nervous system.

In the present study, we show for the first time that the S1P receptors in mesangial cells are subject not only to homologous desensitization, but also to heterologous desensitization triggered by activation of P2Y purinoceptors and P1 adenosine receptors, but not by PDGF and TGFβ receptors. Desensitization and subsequent resensitization of S1P receptors may be very critical in the regulation of S1P-mediated cell responses. Such a cross-desensitization of S1P receptors by other mitogenic factors like ATP or adenosine (Huwiler & Pfeilschifter, 1994) would guarantee that, once the cell is activated by one stimulus, it is desensitized to protect the cell from an overshooting stimulation by other synergistically acting agonists, especially those agonists that use the same signalling devices, for example, the heterotrimeric G proteins. This notion is supported by our data that receptor systems not involving heterotrimeric G proteins, such as PDGF and TGFβ, do not uncouple the S1P signalling cascade.

Moreover, it has become evident that different types of receptors including GPCRs and tyrosine kinase receptors undergo not only homodimerization but also heterodimerization or oligomerization with other receptor classes, and thereby trigger a positive forward cross-talk between different signalling cascades (Devi, 2001). In this context, it is worth mentioning that the S1P receptors cross-activate especially other growth factor signalling pathways. Recently, it was shown that vascular endothelium-derived growth factor (VEGF), epidermal growth factor (EGF) or PDGF receptors all can be transactivated by S1P stimulation, and this leads to an enhanced mitogenic response in various cell types (Rani *et al*., 1997; Kim *et al*., 2000; Endo *et al*., 2002; Tanimoto *et al*., 2002).

There are various known and suspected mechanisms by which GPCRs are desensitized, which may also occur in combination. These mechanisms include the uncoupling of the receptor from heterotrimeric G proteins through receptor phosphorylation, or the internalization of receptors from the cell surface to intracellular compartments, or even a depletion of the receptor due to reduced mRNA and protein synthesis or lysosomal and other degradation processes by proteases. Thus, receptor desensitization can occur over a wide time frame, ranging from seconds (phosphorylation) to minutes (endocytosis) to hours (protein depletion).

Although many GPCRs are subjected to phosphorylation, this is not an absolute requirement for their desensitization since phosphorylation-independent desensitization has also been reported (Pao & Benovic, 2002). Phosphorylation of GPCRs is mainly carried out by the G-protein-coupled receptor protein kinases (GRK), but other kinases like PKC or MAPK can also phosphorylate certain receptors, like the α1B-adrenergic receptor (Diviani *et al*., 1997) and the angiotensin AT_1 receptor (Smith *et al*., 1998). However, this direct PKC- or MAPK-mediated phosphorylation may also play a role in other aspects of receptor functioning different from desensitization. Several subtypes of GRKs have been identified to date, denoted as GRK_{1-6}. These GRKs are themselves activated upon phosphorylation by protein kinases like PKC and c-Src (Pronin & Benovic, 1997; Sarnago *et al*., 1999). Krasel *et al*. (2001) reported that, in HEK293 cells, especially the Ca^{2+}-dependent PKC-α, -β and -γ isoenzymes directly phosphorylate GRK_2 and thereby release GRK_2-bound calmodulin, which acts as an inhibitor of GRK, thus resulting in GRK activation and receptor desensitization. These findings are consistent with our data, in that PKC is at least partially involved in the desensitization event. Due to the differential potencies of staurosporine and CGP41251, both of which inhibit PKC-α with IC_{50}'s of 2 and 30 nM, respectively (Geiges *et al*., 1997), it is concluded that staurosporine additionally affects other protein kinases which actively contribute to S1P receptor desensitization. It is tempting to speculate that staurosporine also blocks GRK activity. Yet, no

data are available on the inhibition of GRK catalytic activity by staurosporine. However, previous *in vitro* studies using preparations of GRK_5 and GRK_6 showed an inhibition of the catalytic activity by GF 109203X, another PKC inhibitor (Zhou *et al*., 2001). Furthermore, it was reported that GRK_2 is also directly regulated by $\beta\gamma$ subunits of G proteins (Lodowski *et al*., 2003). This critical regulatory role of $\beta\gamma$ subunits may explain why PDGF receptor activation, which classically activates PKC isoenzymes also in mesangial cells (Choudhury *et al*., 1993), has no effect on $\beta\gamma$ activation and consequently is not sufficient to desensitize S1P receptors.

Our data further suggest that cAMP is also involved in activation of GRKs and consequently in desensitization of S1P receptors. Similarly, it has been reported that cAMP is involved in desensitization of the β_1-adrenergic receptor (Freedman *et al*., 1995) as well as the β_2-adrenergic receptor (Pitcher *et al*., 1992), probably *via* direct phosphorylation of the receptors by the cAMP-dependent protein kinase.

Desensitization of S1P receptors has recently been suggested to be the critical mechanism of action of the immunosuppressive agent FTY720. FTY720 shows an immunosuppressive potency that is 1000-fold higher than that of other immunosuppressive drugs such as cyclosporine, although, until recently, the mechanism of action remained unclear. A characteristic depletion of T cells in the periphery is observed in response to FTY720, with an accumulation of T cells in the lymph nodes, suggesting an inhibitory mechanism somehow affecting the T-cell homing process. Recently, Brinkmann *et al*. (2002) and Mandala *et al*. (2002) demonstrated that FTY720, which possesses a sphingosine-like structure, serves as a good substrate for the sphingosine kinases and becomes phosphorylated. This FTY-phosphate is now mimicking S1P action and is able to bind to S1P receptors, thereby inducing the downstream signalling cascades. More important, it was shown that FTY720 and FTY-phosphate downregulate specifically the $S1P_1$ receptor on T cells, suggesting a key role for the $S1P_1$ in immunological responses like allograft rejection (Matloubian *et al*., 2004). This was further confirmed by $S1P_1$-deficient mice which show the same phenotype as FTY720-treated mice, that is, an accumulation of T cells in lymph nodes with a concomitant peripheral depletion (Matloubian *et al*., 2004).

Since our data in mesangial cells do not reveal a desensitization of S1P-induced MAPK activation upon FTY720 treatment but do desensitize in response to FTY720-phosphate, this suggests a cell-type specificity of the cells' capacity to phosphorylate FTY720 and to trigger MAPK activation.

Our data further show that suramin has no inhibitory effect on S1P-induced MAPK activation. In view of recent publications which manifest suramin as an antagonist at the mouse and human $S1P_3$ receptors when overexpressed in oocytes (Ancellin & Hla, 1999), we have to conclude that the $S1P_3$ receptor is not involved in S1P-mediated MAPK activation in mesangial cells. However, at present, it cannot be excluded that the rat $S1P_3$ in a cellular environment is not sensitive to suramin.

Furthermore, the data of Katsuma *et al*. (2002) suggested the involvement of $S1P_2$ and/or $S1P_3$ in the mitogenic response of mesangial cells based on antisense oligonucleotide treatment. However, it is worth mentioning that the $S1P_2$ is the only receptor lacking affinity for phospho-FTY (Mandala *et al*., 2002). Furthermore, it was shown that $S1P_2$ is coupled to the small G proteins Rho and Rac in receptor-overexpressing Chinese hamster ovary cells (Sugimoto *et al*., 2003). Whereas $S1P_2$ positively couples to Rho, Rac activity is depleted by $S1P_2$. Both mechanisms involve the activation of $G_{12/13}$ heterotrimeric G proteins (Sugimoto *et al*., 2003). Regarding the S1P-triggered MAPK activation in mesangial cells, we have previously shown that this is completely dependent on a pertussis toxin-sensitive $G_{i/o}$ protein (Xin *et al*., 2004). Since the activation of $G_{i/o}$ proteins is a hallmark of S1P stimulation, it may be possible that $G_{i/o}$ proteins are also involved in the desensitization mechanism. However, presently, it remains difficult to address this question since inhibition of $G_{i/o}$ proteins by pertussis toxin also depletes the primary response of S1P-stimulated MAPK activation and thereby masks any additional event that may occur.

The coupling of $S1P_2$ to G_i has also been reported by Ancellin & Hla (1999) in receptor-transfected oocytes. In view of all these data and our own results, it is presently difficult to pinpoint the S1P receptor subtype involved in MAPK activation in mesangial cells, and further studies are required to identify the receptor subtype involved.

Moreover, it is unknown whether all S1P receptors show the same mechanism of desensitization, and only biochemical studies using highly specific S1P receptor antibodies and antagonists will be able to unravel subtype-specific behaviours in terms of phosphorylation, internalization and degradation.

As an additional readout for S1P receptor activation, we have measured IP_3 formation, which is a very early event preceding intracellular Ca^{2+} mobilization. Comparable to the S1P-triggered desensitization of S1P-stimulated MAPK activation, IP_3 formation was also completely abolished by S1P pretreatment. However, after ATP pretreatment, the S1P-stimulated IP_3 formation is only partially reduced, which may be explained by the contribution of different S1P receptor subtypes with different sensitivities to ATP desensitization.

Moreover, the ATP-stimulated IP_3 formation was even enhanced by S1P pretreatment (Figure 7b). This latter observation may be due to the transactivating capacity of S1P on the PDGF receptor signalling cascade. In this context, Katsuma *et al*. (2002) reported that S1P stimulation had additive effects on PDGF-induced mesangial cell proliferation. Furthermore, our own data reveal that S1P stimulation leads to an increased phosphorylation of the PDGF receptor-β at Tyr^{716} (data not shown). Moreover, we have previously shown that PDGF leads to a potent and sustained increase of IP_3 formation in mesangial cells *via* PLC-γ activation (Pfeilschifter & Hosang, 1991). Nevertheless, a direct proof for the involvement of PDGF in the increased ATP-mediated IP_3 formation by S1P pretreatment still needs to be shown.

In summary, we have shown that S1P-induced MAPK activation is rapidly desensitized not only in a homologous but also in a heterologous fashion by activation of P2Y and P1 receptors. Mechanistically, PKC and possibly other staurosporine-sensitive protein kinases are involved in the P2Y-mediated response, whereas the P1-mediated response additionally involves cAMP.

We gratefully acknowledge the receipt of FTY720 and FTY-phosphate from Dr Volker Brinkmann, Novartis Pharma Inc., Basel, Switzerland. This work was supported by grants from the Deutsche Forschungsgemeinschaft (HU 842/2-3 and PF361/2-1) and the August-Scheidel Stiftung.

References

ANCELLIN, N. & HLA, T. (1999). Differential pharmacological properties and signal transduction of the sphingosine 1-phosphate receptors. EDG-1, EDG-3, and EDG-5. *J. Biol. Chem.,* **274,** 18997–19002.

BENNETT, B.L., SASAKI, D.T., MURRAY, B.W., O'LEARY, E.C., SAKATA, S.T., XU, W., LEISTEN, J.C., MOTIWALA, A., PIERCE, S., SATOH, Y., BHAGWAT, S.S., MANNING, A.M. & ANDERSON, D.W. (2001). SP600125, an anthrapyrazolone inhibitor of Jun N-terminal kinase. *Proc. Natl. Acad. Sci. U.S.A.,* **98,** 13681–13686.

BOKEMEYER, D., PANEK, D., KRAMER, H.J., LINDEMANN, M., KITAAHARA, M., BOOR, P., KERJASCHKI, D., TRZASKOS, J.M., FLOEGE, J. & OSTENDORF, T. (2002). *In vivo* identification of the mitogen-activated protein kinase cascade as a central pathogenic pathway in experimental mesangioproliferative glomerulonephritis. *J. Am. Soc. Nephrol.,* **13,** 1473–1480.

BRINKMANN, V., DAVIES, M.D., HEISE, C.E., ALBERT, R., COTTENS, S., HOF, R., BRUNS, C., PRIESCHL, E., BAUMRUKER, T., HIESTAND, P., FOSTER, C.A., ZOLLINGER, M. & LYNCH, K.R. (2002). The immune modulator FTY720 targets sphingosine 1-phosphate receptors. *J. Biol. Chem.,* **277,** 21453–21457.

CHOUDHURY, G.G., BISWAS, P., GRANDALIANO, G. & ABBOUD, H.E. (1993). Involvement of PKC-α in PDGF-mediated mitogenic signalling in human mesangial cells. *Am. J. Physiol.,* **265,** F634–F642.

CHUN, J., GOETZL, E.J., HLA, T., IGARASHI, Y., LYNCH, K.R., MOOLENAAR, W., PYNE, S. & TIGYI, G. (2002). International Union of Pharmacology. XXXIV. Lysophospholipid receptor nomenclature. *Pharmacol. Rev.,* **54,** 265–269.

CUENDA, A., ROUSE, J., DOZA, Y.N., MEIER, R., COHEN, P., GALLAGHER, T.F., YOUNG, P.R. & LEE, J.C. (1995). SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1. *FEBS Lett.,* **364,** 229–233.

DEVI, L.A. (2001). Heterodimerization of G-protein-coupled receptors: pharmacology, signaling and trafficking. *Trends Pharmacol. Sci.,* **22,** 532–537.

DIVIANI, D., LATTION, A.L. & COTECCHIA, S. (1997). Characterization of the phosphorylation sites involved in G protein-coupled receptor kinase- and protein kinase C-mediated desensitization of the α1B-adrenergic receptor. *J. Biol. Chem.,* **272,** 28712–28719.

ENDO, A., NAGASHIMA, K., KUROSE, H., MOCHIZUKI, S., MATSUDA, M. & MOCHIZUKI, N. (2002). Sphingosine 1-phosphate induces membrane ruffling and increases motility of human umbilical vein endothelial cells *via* vascular endothelial growth factor receptor and CrkII. *J. Biol. Chem.,* **277,** 23747–23754.

FREEDMAN, N.J., LIGGETT, S.B., DRACHMAN, D.E., PEI, G., CARON, M.G. & LEFKOWITZ, R.J. (1995). Phosphorylation and desensitization of the human β1-adrenergic receptor. Involvement of G protein-coupled receptor kinases and cAMP-dependent protein kinase. *J. Biol. Chem.,* **270,** 17953–17961.

GALLO-RODRIGUEZ, C., JI, X.D., MELMAN, N., SIEGMAN, B.D., SANDERS, L.H., ORLINA, J., FISCHER, B., PU, O., OLAH, M.E., VAN GALEN, P.J., STILES, G.L. & JACOBSON, K.A. (1994). Structure–activity relationships of N^6-benzyladenosine-5′-uronamides as A_3-selective adenosine agonists. *J. Med. Chem.,* **37,** 636–646.

GEIGES, D., MEYER, T., MARTE, B., VANEK, M., WEISSGERBER, G., STABEL, S., PFEILSCHIFTER, J., FABBRO, D. & HUWILER, A. (1997). Activation of protein kinase C subtypes α, γ, δ, ε, ζ, and η by tumor-promoting and nontumor-promoting agents. *Biochem. Pharmacol.,* **53,** 865–875.

GENNERO, I., FAUVEL, J., NIETO, M., CARIVEN, C., GAITS, F., BRIAND-MESANGE, F., CHAP, H. & SALLES, J.P. (2002). Apoptotic effect of sphingosine 1-phosphate and increased sphingosine 1-phosphate hydrolysis on mesangial cells cultured at low cell density. *J. Biol. Chem.,* **277,** 12724–12734.

GSCHWENDT, M., MÜLLER, H.J., KIELBASSA, K., ZANG, R., KITTSTEIN, W., RINCKE, G. & MARKS, F. (1994). Rottlerin, a novel protein kinase inhibitor. *Biochem. Biophys. Res. Commun.,* **199,** 93–98.

HLA, T. (2003). Signaling and biological actions of sphingosine-1-phosphate. *Pharmacol. Res.,* **47,** 401–407.

HUWILER, A., BRUNNER, J., HUMMEL, R., VERVOORDELDONK, M., STABEL, S., VAN DEN BOSCH, H. & PFEILSCHIFTER, J. (1996). Ceramide-binding and activation defines protein kinase c-Raf as a ceramide-activated protein kinase. *Proc. Natl. Acad Sci. U.S.A.,* **93,** 6959–6963.

HUWILER, A., KOLTER, T., PFEILSCHIFTER, J. & SANDHOFF, K. (2000a). Physiology and pathophysiology of sphingolipid metabolism and signalling. *Biochim. Biophys. Acta,* **1485,** 63–99.

HUWILER, A. & PFEILSCHIFTER, J. (1994). Stimulation by extracellular ATP and UTP of the mitogen-activated protein kinase cascade and proliferation of rat renal mesangial cells. *Br. J. Pharmacol.,* **113,** 1455–1463.

HUWILER, A., STABEL, S., FABBRO, D. & PFEILSCHIFTER, J. (1995). Platelet-derived growth factor and angiotensin II stimulate the mitogen-activated protein kinase cascade in renal mesangial cells: comparison of hypertrophic and hyperplastic agonists. *Biochem. J.,* **305,** 777–784.

HUWILER, A., VAN ROSSUM, G., WARTMANN, M. & PFEILSCHIFTER, J. (1997). Stimulation by extracellular ATP and UTP of the stress-activated protein kinase cascade in rat renal mesangial cells. *Br. J. Pharmacol.,* **120,** 807–812.

HUWILER, A., WARTMANN, M., VAN DEN BOSCH, H. & PFEILSCHIFTER, J. (2000b). Extracellular ATP and UTP activate the p38stress-activated protein kinase cascade in glomerular mesangial cells. *Br. J. Pharmacol.,* **129,** 612–618.

KASHGARIAN, M. & STERZEL, R.B. (1992). The pathobiology of the mesangium. *Kidney Int.,* **41,** 524–529.

KATSUMA, S., HADA, Y., UEDA, T., SHIOJIMA, S., HIRASAWA, A., TANOUE, A., TAKAGAKI, K., OHGI, T., YANO, J. & TSUJIMOTO, G. (2002). Signalling mechanisms in sphingosine 1-phosphate-promoted mesangial cell proliferation. *Genes Cells,* **7,** 1217–1230.

KIM, J.H., KIM, J.H., SONG, W.K., KIM, J.H. & CHUN, J.S. (2000). Sphingosine 1-phosphate activates Erk-1/-2 by transactivating epidermal growth factor receptor in rat-2 cells. *IUBMB Life,* **50,** 119–124.

KOBAYASHI, E., NAKANO, H., MORIMOTO, M. & TAMAOKI, T. (1989). Calphostin C (UCN-1028C), a novel microbial compound, is a highly potent and specific inhibitor of protein kinase C. *Biochem. Biophys. Res. Commun.,* **159,** 548–553.

KOHAMA, T., OLIVERA, A., EDSALL, L., NAGIEC, M.M., DICKSON, R. & SPIEGEL, S. (1998). Molecular cloning and functional characterizataion of murine sphingosine kinase. *J. Biol. Chem.,* **273,** 23722–23728.

KRASEL, C., DAMMEIER, S., WINSTEL, R., BROCKMANN, J., MISCHAK, H. & LOHSE, M.J. (2001). Phosphorylation of GRK2 by protein kinase C abolishes its inhibition by calmodulin. *J. Biol. Chem.,* **276,** 1911–1915.

LEE, M.J., VAN BROCKLYN, J.R., THANGADA, S., LIU, C.H., HAND, A.R., MENZELEEV, R., SPIEGEL, S. & HLA, T. (1998). Sphingosine-1-phosphate as a ligand for the G protein-coupled receptor EDG-1. *Science,* **279,** 1552–1555.

LIU, H., SUGIURA, M., NAVA, V.E., EDSALL, L.C., KONO, K., POULTON, S., MILSTIEN, S., KOHAMA, T. & SPIEGEL, S. (2000). Molecular cloning and functional characterization of a novel mammalian sphingosine kinase type 2 isoform. *J. Biol. Chem.,* **275,** 19513–19520.

LODOWSKI, D.T., PITCHER, J.A., CAPEL, W.D., LEFKOWITZ, R.J. & TESMER, J.J.G. (2003). Keeping G proteins at bay: a complex between G protein-coupled receptor kinase 2 and G$\beta\gamma$. *Science,* **300,** 1256–1262.

MANDALA, S., HAJDU, R., BERGSTROM, J., QUACKENBUSH, E., XIE, J., MILLIGAN, J., THORNTON, R., SHEI, G.J., CARD, D., KEOHANE, C., ROSENBACH, M., HALE, J., LYNCH, C.L., RUPPRECHT, K., PARSONS, W. & ROSEN, H. (2002). Alteration of lymphocyte trafficking by sphingosine-1-phosphate receptor agonists. *Science,* **296,** 346–349.

MANGGAU, M., KIM, D.S., RUWISCH, L., VOGLER, R., KORTING, H.C., SCHÄFER-KORTING, M. & KLEUSER, B. (2001). 1α,25-dihydroxyvitamin D_3 protects human keratinocytes from apoptosis by the formation of sphingosine-1-phosphate. *J. Invest. Dermatol.,* **117,** 1241–1249.

MATLOUBIAN, M., LO, C.G., CINAMON, G., LESNESKI, M.J., XU, Y., BRINKMANN, V., PROIA, R.L. & CYSTER, J.G. (2004). Lymphocyte egress from thymus and peripheral lymphoid organs is dependent on S1P receptor 1. *Nature,* **427,** 355–360.

MCGLYNN, E., LIEBETANZ, J., REUTENER, S., WOOD, J., LYDON, N.B., HOFSTETTER, H., VANEK, M., MEYER, T. & FABBRO, D. (1992). Expression and partial characterization of rat protein kinase C-δ and protein kinase C-ζ in insect cells using recombinant baculovirus. *J. Cell. Biochem.,* **49,** 239–250.

MEYER, T., REGENASS, U., FABBRO, D., ALTERI, E., RÖSEL, J., CARAVATTI, G. & MATTER, A. (1989). A derivative of staurosporine (CGP 41 251) shows selectivity for protein kinase C inhibition and *in vitro* anti-proliferative as well as *in vivo* anti-tumor activity. *Int. J. Cancer,* **43,** 851–856.

MIZUNO, K., NODAA, K., UEDA, Y., HANAKI, H., SAIDO, T.C., IKUTA, T., KUROKI, T., TAMAOKI, T., HIRAI, S. & OSADA, S. (1995). UCN-01, an anti-tumor drug, is a selective inhibitor of the conventional PKC subfamily. *FEBS Lett.,* **359,** 259–261.

OLIVERA, A. & SPIEGEL, S. (1993). Sphingosine-1-phosphate as second messenger in cell proliferation induced by PDGF and FCS mitogens. *Nature,* **365,** 557–560.

PAO, C.S. & BENOVIC, J.L. (2002). Phosphorylation-independent desensitization of G protein-coupled receptors? *Sci STKE,* **153,** PE42.

PFEILSCHIFTER, J. (1994). Mesangial cells orchestrate inflammation in the renal glomerulus. *News Physiol. Sci.,* **9,** 271–276.

PFEILSCHIFTER, J. (1989). Cross-talk between transmembrane signalling systems: a prerequisite for the delicate regulation of glomerular haemodynamics by mesangial cells. *Eur. J. Clin. Invest.,* **19,** 347–361.

PFEILSCHIFTER, J. & HOSANG, M. (1991). Effects of homo- and heterodimeric isoforms of PDGF on signalling events in rat renal mesangial cells. *Cell. Signal.,* **3,** 413–424.

PFEILSCHIFTER, J. & HUWILER, A. (1993). A role for protein kinase C-ε in angiotensin II stimulation of phospholipase D in rat renal mesangial cells. *FEBS Lett.,* **331,** 267–271.

PFEILSCHIFTER, J., HUWILER, A. & BRINER, V.A. (1995). Proliferative mechanisms in kidney cells. *Curr. Opin. Nephrol. Hypertens.,* **4,** 98–103.

PFEILSCHIFTER, J., KURTZ, A. & BAUER, C. (1986). Role of phospholipase C and protein kinase C in vasoconstrictor-induced prostaglandin synthesis in cultured rat renal mesangial cells. *Biochem. J.,* **234,** 125–130.

PFEILSCHIFTER, J. & VOSBECK, K. (1991). Transforming growth factor β2 inhibits interleukin 1β- and tumour necrosis factor zα-induction of nitric oxide synthase in rat renal mesangial cells. *Biochem. Biophys. Res. Commun.,* **175,** 372–379.

PITCHER, J., LOHSE, M.J., CODINA, J., CARON, M.G. & LEFKOWITZ, R.J. (1992). Desensitization of the isolated beta 2-adrenergic receptor by beta-adrenergic receptor kinase, cAMP-dependent protein kinase, and protein kinase C occurs *via* distinct molecular mechanisms. *Biochemistry,* **31,** 3193–3197.

PRONIN, A.N. & BENOVIC, J.L. (1997). Regulation of the G protein-coupled receptor kinase GRK5 by protein kinase C. *J. Biol. Chem.,* **272,** 3806–3812.

QIAO, L., KOZIKOWSKI, A.P., OLIVERA, A. & SPIEGEL, S. (1998). Synthesis and evaluation of a photolyzable derivative of sphingosine 1-phosphate-caged SPP. *Bioorg. Med. Chem. Lett.,* **8,** 711–714.

RALEVIC, V. & BURNSTOCK, G. (1998). Receptors for purines and pyrimidines. *Pharmacol. Rev.,* **50,** 413–492.

RANI, C.S., WANG, F., FUIOR, E., BERGER, A., WU, J., STURGILL, T.W., BEITNER-JOHNSON, D., LEROITH, D., VARTICOVSKI, L. & SPIEGEL, S. (1997). Divergence in signal transduction pathways of platelet-derived growth factor (PDGF) and epidermal growth factor (EGF) receptors. Involvement of sphingosine 1-phosphate in PDGF but not EGF signaling. *J. Biol. Chem.,* **272,** 10777–10783.

SARNAGO, S., ELORZA, A. & MAYOR, F.JR. (1999). Agonist-dependent phosphorylation of the G protein-coupled receptor kinase 2 (GRK2) by Src tyrosine kinase. *J. Biol. Chem.,* **274,** 34411–34416.

SEGER, R. & KREBS, E.G. (1995). The MAPK signaling cascade. *FASEB J.,* **9,** 726–735.

SMITH, R.D., BAUKAL, A.J., ZOLYOMI, A., GABORIK, Z., HUNYADY, L., SUN, L., ZHANG, M., CHEN, H.C. & CATT, K.J. (1998). Agonist-induced phosphorylation of the endogenous AT1 angiotensin receptor in bovine adrenal glomerulosa cells. *Mol. Endocrinol.,* **12,** 634–644.

SPIEGEL, S. & MILSTIEN, S. (2003). Sphingosine-1phosphate: an enigmatic signalling lipid. *Nat. Rev.,* **4,** 397–407.

SUGIMOTO, N., TAKUWA, N., OKAMOTO, H., SAKURADA, S. & TAKUWA, Y. (2003). Inhibitory and stimulatory regulation of Rac and cell motility by the G12/13-Rho and Gi pathways integrated downstream of a single G protein-coupled sphingosine-1-phosphate receptor isoform. *Mol. Cell. Biol.,* **23,** 1534–1545.

TAMAOKI, T., NOMOTO, H., TAKAHASHI, I., KATO, Y., MORIMOTO, M. & TOMITA, F. (1986). Staurosporine, a potent inhibitor of phospholipid/Ca 2+-dependent protein kinase. *Biochem. Biophys. Res. Commun.,* **135,** 397–402.

TANIMOTO, T., JIN, Z.G. & BERK, B.C. (2002). Transactivation of vascular endothelial growth factor (VEGF) receptor Flk-1/KDR is involved in sphingosine 1-phosphate-stimulated phosphorylation of Akt and endothelial nitric-oxide synthase (eNOS). *J. Biol. Chem.,* **277,** 42997–43001.

TERAI, K., SOGA, T., TAKAHASHI, M., KAMOHARA, M., OHNO, K., YATSUGI, S., OKADA, M. & YAMAGUCHI, T. (2003). Edg-8 receptors are preferentially expressed in oligodendrocyte lineage cells of the rat CNS. *Neuroscience,* **116,** 1053–1062.

VAN BROCKLYN, J.R., LEE, M.J., MENZELEEV, R., OLIVERA, A., EDSALL, L., CUVILLIER, O., THOMAS, D.M., COOPMAN, P.J., THANGADA, S., LIU, C.H., HLA, T. & SPIEGEL, S. (1998). Dual actions of sphingosine-1-phosphate: extracellular through the Gi-coupled receptor Edg-1 and intracellular to regulate proliferation and survival. *J. Cell. Biol.,* **142,** 229–240.

VOGLER, R., SAUER, B., KIM, D.S., SCHÄFER-KORTING, M. & KLEUSER, B. (2003). Sphingosine-1-phosphate and its potentially paradoxical effects on critical parameters of cutaneous wound healing. *J. Invest. Dermatol.,* **120,** 693–700.

WANG, F., VAN BROCKLYN, J.R., HOBSON, J.P., MOVAFAGH, S., ZUKOWSKA-GROJEC, Z., MILSTIEN, S. & SPIEGEL, S. (1999). Sphingosine-1-phosphate stimulates cell migration through a G(i)-coupled cell surface receptor. Potential involvement in angiogenesis. *J. Biol. Chem.,* **274,** 35343–35350.

XIN, C., REN, S., KLEUSER, B., SHABAHANG, S., EBERHARDT, W., RADEKE, H., SCHAFER-KORTING, M., PFEILSCHIFTER, J. & HUWILER, A. (2004). Sphingosine-1-phosphate cross-activates the Smad signaling cascade and mimics transforming growth factor-b-induced cell responses. *J. Biol. Chem.,* **279,** 35255–35280.

ZHANG, H., DESAI, N.N., OLIVERA, A., SEKI, T., BROOKER, G. & SPIEGEL, S. (1991). Sphingosine-1-phosphate, a novel lipid, involved in cellular proliferation. *J. Cell. Biol.,* **114,** 155–167.

ZHOU, H., YAN, F. & TAI, H.H. (2001). Phosphorylation and desensitization of the human thromboxane receptor-alpha by G protein-coupled receptor kinases. *J. Pharmacol. Exp. Ther.,* **298,** 1243–1251.

(Received May 11, 2004
Revised August 18, 2004
Accepted August 20, 2004)