

Sphingosylphosphocholine is a naturally occurring lipid mediator in blood plasma: a possible role in regulating cardiac function via sphingolipid receptors

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Blood plasma and serum contain factors that activate inwardly rectifying GIRK1/GIRK4 K⁺ channels in atrial myocytes via one or more non-atropine-sensitive receptors coupled to pertussis-toxin-sensitive G-proteins. This channel is also the target of muscarinic M₂ receptors activated by the physiological release of acetylcholine from parasympathetic nerve endings. By using a combination of HPLC and TLC techniques with matrix-assisted laser desorption ionization–time-of-flight MS, we purified and identified sphingosine 1-phosphate (SPP) and sphingosylphosphocholine (SPC) as the plasma and serum factors responsible for activating the inwardly rectifying K⁺ channel (I_K). With the use of MS the concentration of SPC was estimated at 50 nM in plasma and 130 nM in serum; those concentrations exceeded the 1.5 nM EC₅₀ measured in guinea-pig atrial myo-

cytes. With the use of reverse-transcriptase-mediated PCR and/or Western blot analysis, we detected Edg1, Edg3, Edg5 and Edg8 as well as OGR1 sphingolipid receptor transcripts and/or proteins. In perfused guinea-pig hearts, SPC exerted a negative chronotropic effect with a threshold concentration of 1 μM. SPC was completely removed after perfusion through the coronary circulation at a concentration of 10 μM. On the basis of their constitutive presence in plasma, the expression of specific receptors, and a mechanism of ligand inactivation, we propose that SPP and SPC might have a physiologically relevant role in the regulation of the heart.

Key words: Edg receptor, GIRK, lysosphingomyelin, OGR-1.

INTRODUCTION

Sphingosine 1-phosphate (SPP) is a multifunctional lipid mediator that regulates a multitude of cellular functions *in vitro* [1–3]. SPP was first identified as a Ca²⁺-mobilizing second messenger [4]. More recently, it has also been recognized as a genuine mediator that activates specific G-protein-coupled receptors of the Edg family [5]. In some cell types, sphingosylphosphocholine (SPC) has been shown to elicit similar cellular responses to those of SPP but in other cells the two sphingolipids have different effects [1,6]. For example, distinct G_i-coupled receptors have been identified for SPC in HL-60 leukaemia cells and human neutrophils [6], whereas in guinea-pig atrial myocytes both sphingolipids activate the same receptor(s) [7]. Our previous work has established that in guinea-pig atrial myocytes SPC and SPP are equipotent (EC₅₀ approx. 1.5 nM) activators of a K⁺ current, which can also be activated by acetylcholine (ACh) (I_{K(ACh)}) [7–9]. The activation of I_{K(ACh)} represents a major mechanism of vagal slowing of the cardiac frequency that includes the M₂ muscarinic ACh receptor-mediated activation of the GIRK1/GIRK4 (Kir3.1/Kir3.4) K⁺ channel complex by the βγ subunits of a pertussis-toxin-sensitive G-protein (reviewed in [10]). However, these sphingolipids do not activate I_K through

M₂ muscarinic ACh receptors because their effect is not inhibited by atropine and is not cross-desensitized with ACh [7,11]. SPP has been shown to be generated in platelets via the action of sphingosine kinase, which is stored and released on stimulation by thrombin [12]. The concentrations of SPP in human plasma and serum have been estimated to be 200 and 500 nM respectively [13].

We have reported previously that serum and plasma contain one or more albumin-associated lipid factors that activate I_K in a pertussis-toxin-sensitive fashion [11,14] and reverse the cAMP-mediated increase in L-type Ca²⁺-current in atrial and ventricular myocytes [15]. The objectives of the current investigation were (1) to purify and identify these blood factors, (2) to assess their action on the intact perfused heart and (3) to obtain further information on the identity of their G-protein-coupled receptor(s). Our results provide evidence that SPC is also present in blood at a high nanomolar concentration and is responsible in part for the activation of I_K by plasma and serum. We also describe a matrix-assisted laser desorption ionization–time-of-flight MS (MALDI–TOF–MS) method for the identification of SPC in biological fluids. The plasma concentration of SPC increased more than 2.5-fold in serum, indicating that its generation is linked to blood clotting. Moreover, circulating SPC

Abbreviations used: 2D, two-dimensional; ACh, acetylcholine; CHCA, α-cyano-4-hydroxycinnamic acid; ELSD, evaporative light-scattering detector; I_K, inwardly rectifying K⁺ channel; MALDI–TOF–MS, matrix-assisted laser desorption ionization–time-of-flight MS; RT–PCR, reverse-transcriptase-mediated PCR; SPC, sphingosylphosphocholine; SPP, sphingosine 1-phosphate.

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is effectively removed after a single passage through the coronary vasculature. In support of a physiologically relevant role, we show, with the use of an isolated perfused heart preparation, that SPC in the high nanomolar to low micromolar concentration range decreases the heart rate and synergizes with the effect of muscarinic stimulation. In agreement with these biological effects, we show that rat atrial myocytes co-express mRNA and protein for the Edg1, Edg3, Edg5 and Edg8 SPP receptors as well as mRNA for the recently identified SPC receptor OGR1. A preliminary report on this work has been published in an abstract [16].

EXPERIMENTAL

Lipid extraction

Blood was obtained by cardiac puncture of anesthetized adult New Zealand White rabbits or from the cubital vein of healthy human volunteer donors. For the isolation of plasma, blood was anticoagulated with 0.1 ml of 15% (w/v) EDTA. Blood was also collected into siliconized vials and left to clot for 1 h at 37 °C, followed by a 24 h incubation at 4 °C. Serum and plasma were separated by centrifugation for 15 min at 2000 *g*, frozen in liquid nitrogen and freeze-dried. The freeze-dried material was first extracted three times with 5 ml of chloroform/methanol (2:1, v/v) per g dry weight, and three more times with 5 ml of methanol per g dry weight. Both organic solvents contained 0.01% butylated hydroxytoluene and were concentrated *in vacuo* at ambient temperature.

Fractionation of plasma and serum lipids by HPLC

Analytical HPLC fractionation of serum and plasma lipids was performed by the method we developed earlier for the separation of SPC stereoisomers [7], using a silica-packed Microsorb column (250 mm × 4.6 mm; 5 μm particle size; Rainin Instruments, Woburn, MA, U.S.A.). Elution was monitored by an evaporative light-scattering detector (ELSD, Model IIA; Vorex, Burtonsville, MD, U.S.A.) through a metering valve, splitting the eluate 1:4 at a flow rate of 1 ml/min between the detector and a fraction collector respectively. Fractions (1 min) were collected and assayed for their ability to elicit $I_{K(ACh)}$ in guinea-pig atrial myocytes.

A partial purification of the $I_{K(ACh)}$ -activating blood factors was achieved by HPLC, using a Microsorb-Si semipreparative column (250 mm × 10 mm; 5 μm particle size; Rainin Instruments). The column was eluted with a two-solvent gradient consisting of solvents A [chloroform/methanol/water/30% (v/v) NH_4OH , 60:35:4.5:0.5 by vol.] and B [chloroform/methanol/water/30% (v/v) NH_4OH , 30:60:9.5:0.5 by vol.] with a Waters HPLC system controlled by the Millennium 2010 version 2.1 software package (Waters Instruments, Milford, MA, U.S.A.). Gradient elution with a 2 ml/min flow rate was started with 100% solvent A for 10 min; the concentration of solvent B was increased linearly to 100% over a 30 min period, followed by a 60 min isocratic application of 100% solvent B. All solvents were HPLC grade (Fischer Scientific, Pittsburgh, PA, U.S.A.). Approximately 50 mg of lipid extract, dissolved in 2 ml of solvent A, was injected, and the eluate was monitored by an ELSD.

Two-dimensional (2D) TLC

Separations by 2D-TLC on K6 silica-gel plates (20 cm × 20 cm, layer thickness 250 μm; Whatman, Fairfield, NJ, U.S.A.) were performed as described previously [17]. Lipid spots were detected

after being stained with primulin (Aldrich Chemical, Milwaukee, WI, U.S.A.) [18] and in some instances were also counterstained with ninhydrin (Sigma). Each primulin-positive spot was scraped off and eluted as described [17] for bioassay in guinea-pig or rat atrial myocytes. For bioassay the lipids were dissolved in a minimal volume of methanol and diluted with recording buffer.

MALDI-TOF-MS

MALDI-TOF-MS data were acquired with a Voyager-DE RP Biospectrometry Workstation instrument (PerSeptive Biosystems, Farmingham, MA, U.S.A.) in the reflector mode. The laser energy was set at 1700 arbitrary units. Samples (in 0.5 μl) were mixed with 1.5 μl of α-cyano-4-hydroxycinnamic acid (CHCA; Sigma) matrix dissolved in 3% (v/v) trifluoroacetic acid/acetonitrile/methanol (1:5:4, by vol.) and left to dry at room temperature. For estimation of the amount of SPC, known concentrations of SPC standard ranging from 2 to 300 nM were prepared and mixed with the matrix. Each concentration of standard contained an equal amount of the CHCA matrix (50 μl) and SPC in methanol to give a total volume of 65 μl. The laser targets were loaded with 2 μl of each standard in duplicate; the solution was dried at room temperature. Each target was scanned 160 times, providing a total of 640 individual measurements for every sample for averaging. The peak heights corresponding to the matrix ($[CHCA + H]^+$, m/z 190.5) and SPC ($[SPC + H]^+$, m/z 465.3) were measured, and the logarithm of the ratio of the two peak intensities ($\log[SPC/CHCA]$) was plotted against the logarithm of the SPC concentration.

Testing of generation of SPC and SPP from sphingomyelin *ex vivo*

$[^{14}C]$ Sphingomyelin (1 μCi) (55 mCi/mmol; Amersham) was incubated with 100 μl of human serum or plasma for up to 24 h. The samples were freeze-dried and the sphingolipid fraction was extracted as described above and run on TLC. The TLC plate was exposed to ^{14}C -sensitive Super Resolution screens of a Cyclone imager (Packard Instruments). Authentic sphingomyelin, SPC and SPP standards were run alongside the samples and stained with ninhydrin.

Isolation and culture of atrial myocytes

The method for the isolation of myocytes has been described in detail elsewhere [11].

Patch clamp recording of $I_{K(ACh)}$ from atrial myocytes

Whole-cell voltage clamp recording [19] of the sphingolipid-elicited currents was performed under previously described experimental conditions [7]. Sphingolipids were applied in recording buffer and the currents were normalized to the current induced by a saturating concentration (10 μM) of ACh in the same cell.

Isolated perfused heart preparation

The animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee, and animals were maintained in the AAALAC-accredited programme (where AAALAC stands for American Association for Accreditation of Laboratory Animal Care). Experiments in Germany were performed with local ethics committee approval. The effects of SPC were tested with an isolated retrogradely perfused heart preparation. Hearts from guinea-pigs (700–900 g) anaesthetized with

sodium pentobarbital were isolated and perfused with Krebs–Henseleit buffer containing (in mM) 114 NaCl, 1.2 KH_2PO_4 , 2.5 $CaCl_2$, 1.2 $MgSO_4$, 4.7 KCl, 25 $NaHCO_3$ and 5.5 glucose, equilibrated with O_2/CO_2 (19:1), at 37 °C. Hearts were perfused at a constant rate of 18 ml/min with a peristaltic pump (Harvard Apparatus, Millis, MA, U.S.A.). The perfusate and a water jacket surrounding the heart were warmed to 37 °C. Frequency was measured by recording ventricular contractions or a ventricular surface electrocardiogram. Activation of $I_{K(ACh)}$ strongly affects sino-atrial automaticity and AV-nodal conduction. Decreases in ventricular frequency are therefore related to both parameters. Experiments were initiated after 30 min to allow mechanical function of the heart to stabilize. Thereafter, doses of ACh were injected in a 100 μ l bolus. Each dose was repeated twice at 3 min intervals before the next higher dose was injected. SPC was infused at a constant rate of 0.34 ml/min for 20 min. The SPC infusions were given consecutively in increasing order of concentration, followed by a washout until recovery to pre-exposure level occurred. To determine whether a subthreshold infusion of SPC would modify the decrease in heart rate caused by a subthreshold or larger dose of ACh, the following protocol was used: after the stabilization period and before the SPC infusion, baseline levels of ACh-induced decrease in heart rate were determined by injecting 100 μ l of 1–10 μ M ACh twice at 3 min intervals, followed by the infusion of 1 μ M SPC at 0.34 ml/min. At 3 min intervals after approx. 10 min of 1 μ M SPC infusion, 100 μ l boluses of 1–10 μ M ACh were injected to test for an ACh-induced decrease in heart rate.

Reverse-transcriptase-mediated PCR (RT–PCR) analysis of the expression of sphingolipid receptor gene products

After isolation, rat atrial and ventricular myocytes (10^6 cells of each) were plated in culture dishes in the absence of serum and were incubated overnight to permit attachment. Under serum-free conditions, fibroblasts and other non-myocyte cells did not attach, providing a culture of virtually pure myocytes. The cells were scraped from the dishes, pelleted by centrifugation and placed into RNA $later$ (Ambion, Austin, TX, U.S.A.). Total RNA was isolated with TRIzol reagent (Gibco BRL, Gaithersburg, MA, U.S.A.). To prevent DNA contamination, 1 μ g of total RNA was digested with 1 μ l of DNase1 for 15 min at 37 °C, followed by inactivation with 2.5 μ l of 25 mM EDTA at 65 °C for 10 min. cDNA was obtained by reverse transcription with the use of the SUPERScript Preamplification System kit (Gibco BRL). The oligonucleotide primers were based on the rat, mouse and human receptor sequences as follows: Edg1 forward 5'-⁸¹tcacgtcctccgcattacaacta-3', reverse 5'-gagtgagctttaggtggtg-³⁵¹-3' (product size 273 bp); Edg3 forward 5'-¹³⁷cttgatcctcagcttcac-3', reverse 5'-tgctgatgcagaaggcaatga⁵⁹⁷-3' (product size 460); Edg5 forward 5'-¹¹atggcagcttctactcggag-3', reverse 5'-cagccagcagacgataaagac⁷²⁰-3' (product size 720 bp); Edg6 forward 5'-²⁸⁰tgaacatcacgctgagtgacct-3', reverse 5'-gatcatcagcaccgtcttcagc⁷⁹⁰-3' (product size 510); Edg8 forward 5'-⁸⁸¹atctgtgcctctatgcaagga-3', reverse 5'-ggtgatgatagtaggattcagca¹¹⁶¹-3' (product size 300); and OGR1 forward 5'-⁷²¹ttctgcctaccagtggtgc-3', reverse 5'-tgccagtgtaggggtctggaag¹⁰⁶²-3' (product size 341 bp). After 2 min of pre-denaturation at 94 °C, *Taq* DNA polymerase (Promega) was added, and 30 amplification cycles were run, each consisting of denaturation for 30 s at 94 °C, annealing for 1 min at 59 °C and elongation for 1 min at 72 °C. The last elongation period was 5 min at 72 °C. The products were separated on 1.2% (w/v) DNA agarose gels and stained with ethidium bromide.

Immunoprecipitation and Western blot analysis of Edg3 and Edg5 receptor proteins

Rat atrial and ventricular tissues were carefully dissected and homogenized with a Dounce homogenizer in ice-cold lysis buffer [50 mM Hepes (pH 7.4)/50 mM NaCl/1 mM $MgCl_2$ /2 mM EDTA/1 mM vanadate containing Protease Inhibitor Cocktail from Sigma]. The homogenate was centrifuged for 10 min at 4 °C and 1000 *g* to remove nuclei. To isolate the crude membrane fraction, the supernatant was centrifuged for 1 h at 4 °C and 100000 *g*. Protein concentration was determined with the Micro BCA Protein Assay kit (Pierce, Rockford, IL, U.S.A.). For immunoprecipitation, 150 μ g of protein in 250 μ l of sample volume was preincubated with Protein G PLUS-Agarose (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) for 5 h, followed by incubation overnight with monoclonal antibodies specific for a C-terminal epitope of Edg3 (2.5 μ g per sample) and Edg5 (5 μ g per sample) (Antibody Solutions, Palo Alto, CA, U.S.A.) and Protein G PLUS-Agarose. The immunocomplex was washed three times, boiled for 3 min in sample buffer, and loaded for SDS/PAGE [10% (w/v) gel]. For Western blot analysis, proteins were transferred to PVDF membranes (Bio-Rad, Hercules, CA, U.S.A.) with a semi-dry blotter (Bio-Rad). Non-specific binding was blocked overnight in TTBS [10 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.05% (v/v) Tween 20] containing 5% (w/v) non-fat dried milk (Bio-Rad). The membranes were incubated with 1 μ g/ml of either anti-Edg3 or anti-Edg5 N-terminal monoclonal antibody in TTBS for 1 h, washed three times with TTBS and incubated with a horseradish-peroxidase-labelled anti-mouse secondary antibody (Sigma) for 30 min before being developed with the SuperSignal Chemiluminescent Substrate (Pierce).

Statistical analysis

Student's *t* test was applied for the analysis of the results; differences at $P < 0.05$ were considered statistically significant.

RESULTS

We have shown previously that blood plasma or serum from various mammalian species, when applied to atrial myocytes, elicits an inwardly rectifying I_K , indistinguishable from $I_{K(ACh)}$, that is activated by ACh via M_2 receptors [7,14]. However, this plasma- or serum-activated current was not blocked by atropine, establishing that the ligand or ligands activating it were different from ACh. We therefore first focused on the characterization of this novel biological activity of serum and plasma. Independently of the source or donor species, serum and plasma at a concentration of 0.1% (v/v) elicited a saturating response of approx. 90% of the current that was activated by a saturating 10 μ M concentration of ACh. The EC_{50} dilutions for human plasma and serum were 10600 and 13200 respectively (results not shown). Delipidated human albumin at a concentration as high as 11.6 μ M was inactive. Serum albumin Cohn fraction V, at 5.8 μ M (approx. 1% of its concentration in plasma), caused a maximal activation of $I_{K(ACh)}$. The activity could be dissociated from albumin by extraction with methanol. The current–voltage relationship for the methanol extract of human serum albumin was indistinguishable from that obtained for D-erythro-SPC and ACh [7], with a reversal potential at E_K (–50 mV) and strong inward rectification, typical of $I_{K(ACh)}$. Dialysis of plasma and serum against aqueous buffers did not significantly alter their threshold concentrations (results not shown). Because albumin has been shown to bind tightly to a variety of bioactive molecules

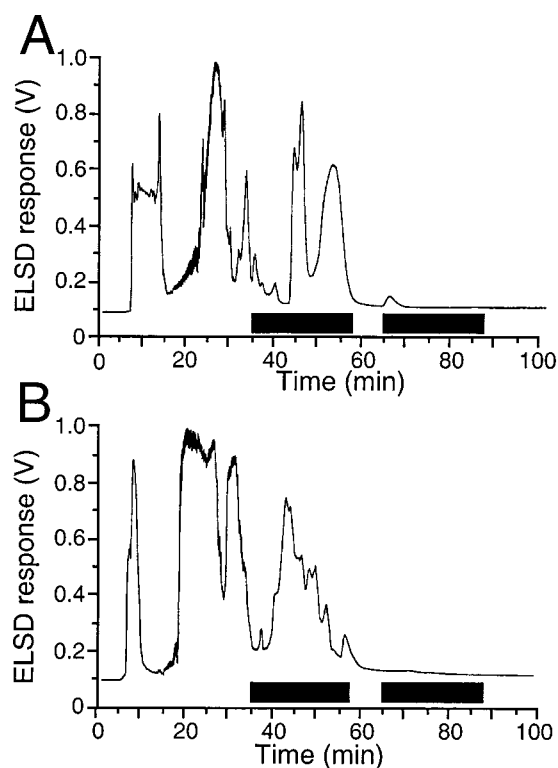


Figure 1 Semi-preparative HPLC separation of methanol-extractable polar lipids in rabbit plasma (A) and serum (B)

Approximately 50 mg of the methanol extract was loaded; the effluent was monitored with an ELSD. Black bars mark the I_K -positive fractions that were subjected to further analysis with 2D-TLC. The retention times for the SPP and SPC standards were 45 and 75 min respectively. The elution profile is highly representative of at least five separations.

and because delipidated albumin preparations were inactive, we focused our purification strategy on plasma and serum lipids.

HPLC fractionation of polar serum lipids reveals two distinct peaks of biological activity

Rabbit plasma and serum was first extracted with chloroform/methanol to remove the less polar fraction of lipids, followed by extraction with methanol. The methanol extraction yielded 2.3 mg of extract per ml of plasma and 1.9 mg per ml of serum. This extraction procedure has been proved to be effective for the isolation of albumin-associated bioactive glycerolipids [20,21] from different biological fluids [17]. The methanol-extractable polar lipid fraction prepared from serum or plasma was highly active in eliciting I_K , whereas the less polar fraction extracted with chloroform/methanol was inactive (results not shown). We therefore applied an analytical HPLC to fractionate the major lipid classes present in the methanol extract. The eluate was collected in 1 min fractions and dried under N_2 ; the fractions were reconstituted in 250 μ l of methanol and applied to guinea-pig atrial myocytes at a dilution of 1:250 in recording buffer. Two completely separated peaks of activity were detected between 21 and 26 min and between 32 and 40 min. These retention times were different from those of the bioactive glycerolipids lysophosphatidic acid (16 min) and lysoplasmeyl glycerophosphate (12 min) but were similar to those of authentic standards for SPP (22 min) and SPC (33 min) [22]. These peak retention times were particularly revealing, because we have

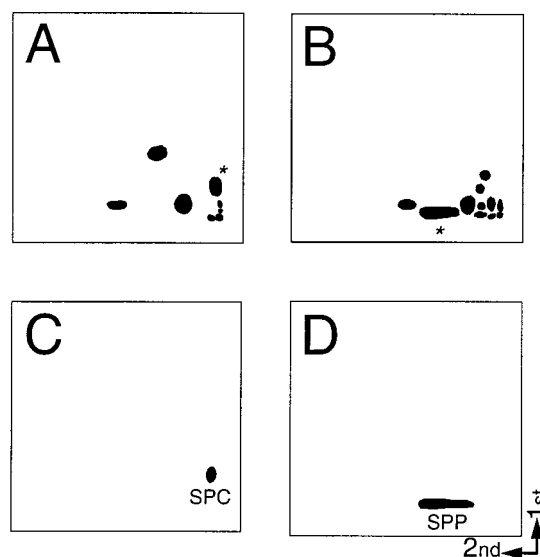


Figure 2 Two-dimensional TLC analysis of the active fractions from the semi-preparative HPLC of rabbit plasma and that of authentic SPP and SPC standards

(A, B) HPLC fractions: 65–88 min (A); 35–58 min (B). (C) SPC standard; (D) SPP standard. Separations were done first with basic (first dimension) and then with acidic (second dimension) solvents. As detected by primulin, the spot with an R_{F1} of 0.18 and an R_{F2} of 0.05 in (A) co-migrated with the SPC standard (R_{F1} 0.19; R_{F2} 0.06) and the spot with an R_{F1} of 0.03 and an R_{F2} of 0.35 in (B) co-migrated with the SPP standard (R_{F1} 0.03; R_{F2} 0.34) [denoted by asterisks in (A) and (B)]. Spots were scraped off the plates, lipids were recovered by extraction with methanol and the spots were tested for the activation of $I_{K(ACh)}$. The active spots were those denoted by asterisks in (A) and (B).

shown previously that SPP and SPC are potent activators of atrial I_K [7,9]. However, the active HPLC fractions contained a mixture of different compounds when analysed by MS; the amount of the individual components obtained from analytical HPLC was below the detection limit for 2D-TLC and staining with primulin.

A semipreparative HPLC procedure was developed for the separation of I_K -activating factors, which allowed the loading of 50 mg of methanol extract in each run (Figure 1). The eluates at 35–58 min and 65–88 min, which were co-eluted with the SPP and SPC standards, were separately collected for plasma and serum, and dried under N_2 . In the 35–58 min fraction, average quantities of 0.68 and 0.25 mg of solid material were obtained per ml of plasma and serum respectively. In the 65–88 min fraction, average quantities of 0.07 and 0.06 mg of dry material were recovered per ml of plasma and serum respectively. The material collected in the two fractions from plasma and serum was dissolved in methanol/chloroform (2:1, v/v) and further separated by 2D-TLC. Figure 2 shows representative 2D-TLC results obtained for the plasma fractions and for authentic SPC and SPP standards. In the 35–58 min fraction, 11 different lipids stained with primulin, four of which also stained with ninhydrin. In the 65–88 min fraction, eight individual spots were resolved, four of which were ninhydrin-positive. Each of these spots, detectable with primuline, was scraped off the duplicate plates that were developed in the same run and processed for bioassay at a 1:50 dilution in recording buffer containing a final concentration of 0.01% methanol. Only a single spot, eluted from a 2D-TLC separation of fraction 35–58 min, was biologically active; it co-migrated with the SPP standard (R_{F1} 0.03; R_{F2} 0.34). In the 65–88 min fraction another bioactive spot was

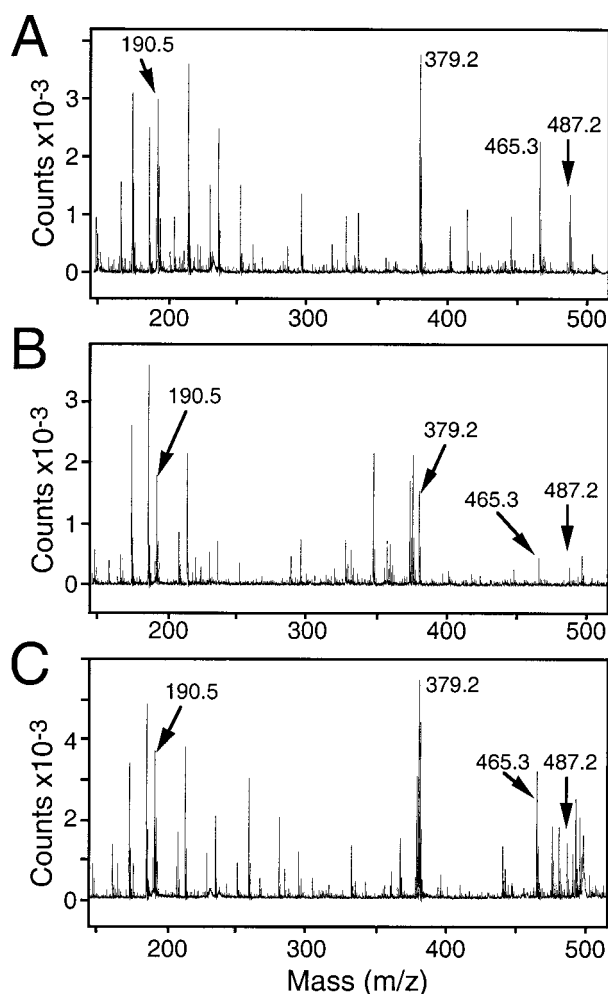


Figure 3 MALDI-TOF-MS determination of the lipids comprising the HPLC fractions with retention times between 65 and 88 min

(A) Mass spectrum of 2 nmol of *o*-erythro-SPC standard; (B, C) spectra of the 65–88 min semipreparative HPLC fractions of methanol extracts of plasma (B) and serum (C). Labelled peaks: $[\text{CHCA} + \text{H}]^+$ at m/z 190.5 is the protonated molecule ion of the CHCA matrix; $[2 \times \text{CHCA} + \text{H}]^+$ at m/z 379.2 is the dimer of the CHCA matrix; $[\text{SPC} + \text{H}]^+$ at m/z 465.3 is the protonated molecule ion of SPC; and $[\text{SPC} + \text{Na}]^+$ at m/z 487.2 is the sodium adduct ion of SPC. The logarithm of the peak intensity ratio of $[\text{SPC} + \text{H}]^+$ at m/z 465.3 and that of $[\text{CHCA} + \text{H}]^+$ at m/z 190.5 gives a straight line if plotted against the logarithm of SPC concentration, allowing the estimation of the concentration of SPC.

detected; this co-migrated with the SPC standard (R_{F1} 0.19; R_{F2} 0.06). The corresponding spots in the duplicate plates were ninhydrin-positive, lending strong support to the notion that these spots did indeed correspond to SPP and SPC respectively. Similarly, only two active lipids were detected in the methanol extract from serum (results not shown).

Identification of SPC and SPP by MALDI-TOF-MS

The two eluted bioactive spots that co-migrated with the SPP and SPC standards were analysed by MALDI-TOF-MS. The spot in the plasma fraction that co-migrated with the SPC standard was dominated by molecular ions at m/z 465.3 and 487.2, corresponding to $[\text{SPC} + \text{H}]^+$ and $[\text{SPC} + \text{Na}]^+$. The active spot recovered from the SPP region of the plasma chromatogram showed three major molecular ions at m/z 380.3, 402.5 and 424.5, corresponding to $[\text{SPP} + \text{H}]^+$, $[\text{SPP} + \text{Na}]^+$ and

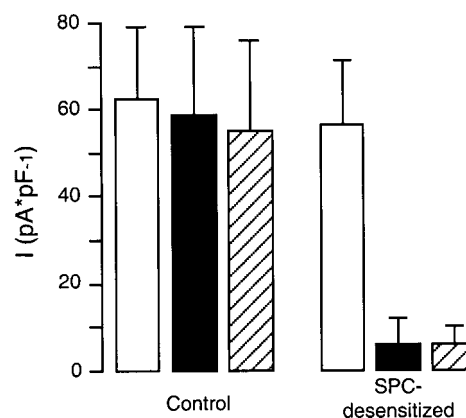


Figure 4 SPC and serum activate the same sphingolipid receptor or receptors

Columns show the average currents measured in control guinea-pig atrial myocytes and in myocytes desensitized with 100 nM SPC (mean \pm S.E.M. for 5–8 cells for each condition). Desensitization led to a substantial decrease in responsiveness of myocytes to serum (filled columns) and SPC (hatched columns) without a significant decrease in the response to ACh (open columns).

$[\text{SPP} + 2\text{Na}]^+$ respectively. Identical mass spectra were found for the two corresponding spots isolated from serum (results not shown). Figure 3 shows the mass spectrum of an SPC standard and mass spectra for the 65–88 min HPLC fractions of methanol extracts of plasma and serum. The two peaks at m/z 465.3 ($[\text{SPC} + \text{H}]^+$) and 487.2 ($[\text{SPC} + \text{Na}]^+$) indicate the presence of SPC in the plasma and serum samples. The intensity of the $[\text{SPC} + \text{H}]^+$ peak at m/z 465.3 showed a strict concentration dependence compared with the m/z 190.5 peak of the $[\text{CHCA} + \text{H}]^+$ matrix. The logarithm of the peak intensity ratio of $[\text{SPC} + \text{H}]^+$ to $[\text{CHCA} + \text{H}]^+$ plotted against the logarithm of SPC concentration yielded a straight line with a correlation coefficient of 0.978. This tight relationship between the SPC concentration and the normalized MS signal intensity over the range 2–300 nM enabled us to estimate the plasma and serum concentrations of SPC as 50 ± 15 nM and 130 ± 25 nM ($n = 4$) respectively (means \pm S.E.M.). Unfortunately, this method could not be applied for the quantification of SPP because $[\text{SPP} + \text{H}]^+$ at m/z 380.3 could not be clearly resolved from the CHCA matrix dimer at m/z 379.2.

To exclude the possibility that SPC or SPP was generated *ex vivo*, ^{14}C sphingomyelin was incubated with plasma and serum for up to 24 h. The lipid extracts prepared from these samples with chloroform/methanol and with methanol showed no detectable generation of SPP and SPC or breakdown of sphingomyelin (results not shown).

SPC and SPP desensitizes I_K elicited by serum but not by ACh

We have previously shown that preincubation of guinea-pig myocytes with SPC causes the complete heterologous desensitization of I_K elicited by SPP, whereas the sensitivity of I_K to stimulation with M_2 muscarinic ACh receptors was not affected [7]. This result indicated that SPP and SPC activate the same population of receptors. To establish whether the I_K -activating serum factors activate the same subset of sphingolipid receptors, we preincubated guinea-pig atrial myocytes with 100 nM SPC for ≥ 10 h and tested their responsiveness to 1:100-diluted rabbit serum and 10 μM ACh. Cells from untreated (non-desensitized) cultures served as controls. Figure 4 shows

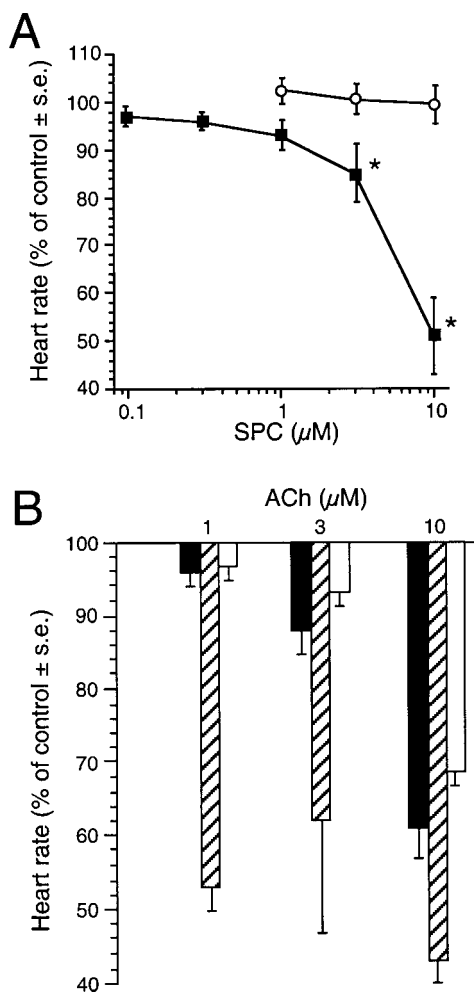


Figure 5 SPC modifies cardiac function in a concentration-dependent manner in the isolated heart

(A) The naturally occurring *D-erythro*-SPC (■) caused a concentration-dependent decrease in heart rate, similar to that caused by ACh (results not shown). *L-threo*-SPC (○) was ineffective up to 10 μM. * $P < 0.05$ compared with control (by Student's *t* test). (B) *D-erythro*-SPC at its threshold concentration of 1 μM potentiates the effect of ACh, whereas *L-threo*-SPC at the same concentration does not. Filled columns, ACh alone; hatched columns, 1 μM *D-erythro*-SPC plus ACh; open columns, 1 μM *L-threo*-SPC plus ACh. Values are presented as means ± S.E.M.

that the sensitivity of I_{K} to 100 nM SPC and 1:100-diluted serum was abolished by pretreatment with SPC; these saturating concentrations of SPC or serum evoked only a minimal current in SPC-desensitized cells, whereas the response in the same cells to 10 μM ACh was unaffected. Comparable results were obtained when 100 nM SPP was used for desensitization (results not shown).

SPC decreases cardiac frequency in the isolated heart

ACh decreases spontaneous beating frequency in the isolated perfused heart, which is due at least partly to the activation of $I_{K(ACh)}$ [23,24]. In the isolated guinea-pig heart the naturally occurring stereoisomer *D-erythro*-SPC decreased the heart rate in a concentration-dependent manner (Figure 5A). However, the active concentration range in the intact heart was three orders of magnitude larger than for the activation of I_{K} in isolated myocytes. The *L-threo*-SPC stereoisomer, at concentrations up to

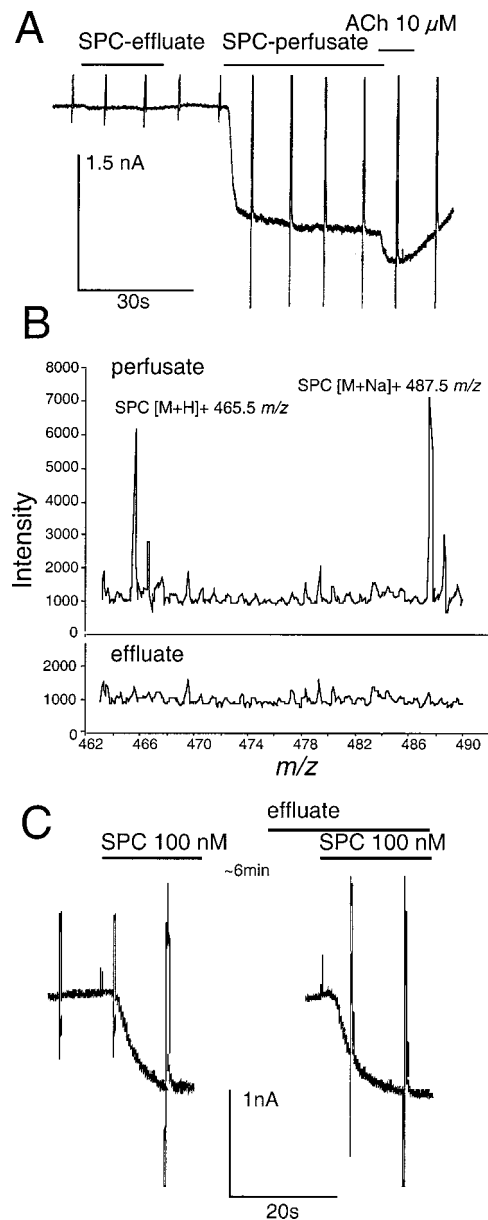


Figure 6 Inactivation of SPC by the coronary vasculature

(A) The current trace shows that SPC was inactivated during the coronary passage, as the 10 μM SPC included in the perfusate exerted its maximal effect on myocytes, whereas the effluate was devoid of the biological activity. Both the perfusate and the effluate were tested at a 50-fold dilution in guinea-pig atrial myocytes for the activation of $I_{K(ACh)}$. (B) MALDI-TOF spectrum for the methanol-soluble lipids prepared from the perfusate and the effluate. Note the clear presence of the SPC molecular ions (m/z 465.5 and 487.5) in the perfusate and its complete absence from the effluate. (C) I_{K} activated by SPC diluted in the effluate is not attenuated compared with SPC diluted in recording buffer, suggesting that the effluate did not contain inhibitory factors that diminish its effect.

10 μM, was ineffective in the perfused isolated heart (Figure 5A); those results duplicate our earlier findings with this compound, which was ineffective in activating I_{K} in isolated myocytes [7]. Only *D-erythro*-SPC caused an increase in coronary perfusion pressure and a decrease in ventricular contractility parameters (results not shown), properties that are likely to reflect actions different from the activation of $I_{K(ACh)}$.

SPC and ACh elicited similar physiological responses in the

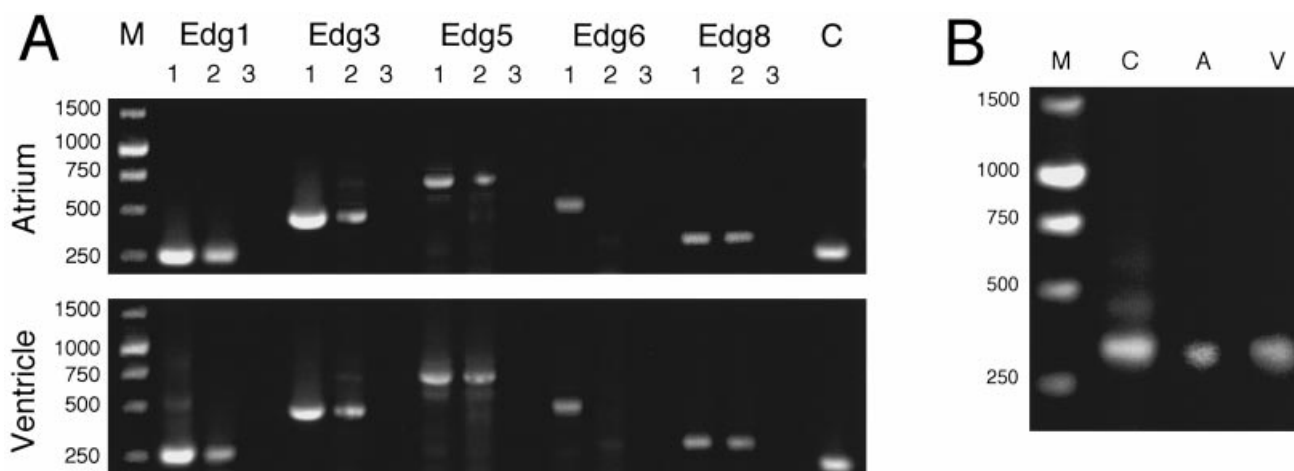


Figure 7 RT-PCR analysis detects the presence of messages for Edg1, Edg3, Edg5, Edg8 and OGR1 receptors

The amplification was performed with both atrial and ventricular cultured myocytes separately; the products were analysed by agarose-gel electrophoresis. **(A)** Amplification for the Edg family of sphingolipid receptors. Lane M, DNA standards; lane C, β -actin control (220 bp). For all Edg receptors, lanes were as follows: lane 1, positive control (plasmid); lane 2, sample; lane 3, negative control (no transcription). **(B)** Amplification for the SPC-specific receptor OGR1. Lane M, DNA standards; lane C, positive control (plasmid); lane A, atrial myocytes; lane V, ventricular myocytes.

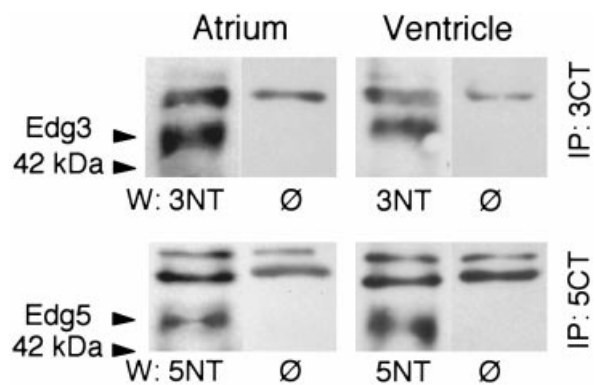


Figure 8 Co-expression of the Edg3 and Edg5 receptor proteins in ventricular and atrial myocytes

Membrane preparations from carefully dissected rat atria and ventriculi were first immunoprecipitated (IP) with anti-Edg3 and anti-Edg5 monoclonal antibodies recognizing the C-terminus of the receptors (3CT and 5CT respectively), followed by Western blots (W) with monoclonal antibodies against an N-terminal epitope (3NT and 5NT respectively). Western blots performed without the primary antibodies served as negative controls (\emptyset). Identical results were obtained when the N-terminal-specific antibodies were used for immunoprecipitation (results not shown).

isolated heart preparation and in atrial myocytes, raising the possibility that they might jointly modulate the heart rate. To test this hypothesis, SPC was perfused at a threshold concentration and ACh was applied at 1–10 μ M, around its own threshold concentration of 1 μ M. As depicted in Figure 5(B), the ACh-elicited decrease in heart rate was significantly augmented in the presence of 1 μ M *D-erythro*-SPC, whereas it was unchanged when the perfusate contained the inactive *L-threo*-SPC stereoisomer.

The micromolar concentration required in the perfused heart to decrease heart rate is difficult to reconcile with the 1.5 nM EC_{50} found for SPP and SPC in isolated myocytes [7,9]. One explanation for this discrepancy could be a rapid inactivation by

degradation and/or cellular uptake during coronary perfusion. To test this hypothesis, SPC was monitored in the coronary effluate of the perfused heart preparation by using the atrial myocyte I_K as a highly sensitive bioassay and by MALDI-TOF-MS. In these experiments, 10 μ M SPC was infused at 0.34 ml/min through the perfusion cannula; samples were taken from both the coronary perfusate and the effluate. After 1:50 dilution (approx. 200 nM) the aliquots were tested on atrial myocytes as well as by MALDI-TOF-MS. As shown by the representative trace in Figure 6(A), the perfusate induced an inward I_K corresponding to 90% of the maximum current that could be activated by ACh. In contrast, the effluate from the coronary sinus at the same dilution was completely inactive (Figure 6A). Because the detection threshold for SPC in the guinea-pig atrial myocyte assay is between 0.1 and 1 nM [7], this demonstrates that more than 99% of the SPC had been removed during coronary perfusion by a hitherto unknown mechanism. This clearance mechanism was independent of the duration of perfusion of SPC between the durations of 30 s and 9 min tested (results not shown). MALDI-TOF analysis of the lipid extract prepared from the effluate showed no detectable amount of SPC molecular ion, whereas the ion was clearly detectable in the perfusate (Figure 6B). In addition, to exclude the generation of inhibitory factors in the coronary vasculature, fresh SPC was perfused in the effluate collected from the coronary sinus and tested with SPC made up in buffer. These two samples showed activity that was equally effective in the myocyte assay activation of I_K (Figure 6C).

Atrial myocytes express multiple subtypes of sphingolipid receptors

Edg1, Edg3, Edg5, Edg6 and Edg8 are G-protein-coupled receptors that are specifically activated by SPP and/or SPC [25–30]. RT-PCR analysis detected the presence of Edg1, Edg3, Edg5 and Edg8 mRNA in isolated atrial and ventricular myocytes (Figure 7A). Transcripts for Edg6 receptor could not be detected

(Figure 7A). It has recently been shown that SPC, but not SPP, is a high-affinity ligand for the ovarian cancer orphan receptor OGR1 [31]. OGR1 transcripts were detectable in atrial and ventricular myocytes (Figure 7B).

To ascertain the expression of Edg3 and Edg5 protein in the atrium and ventricle, we applied monoclonal antibodies raised against the N-terminal and C-terminal epitopes. The immunoprecipitated protein was probed by using Western blots with a second monoclonal antibody specific for N-terminal epitopes of the receptors. Blots prepared from either tissue showed the presence of a single band at approx. 42 kDa that was not present when the blots were developed without the primary antibodies (Figure 8). This 42 kDa antigen has been previously identified as the Edg3 and Edg5 receptor protein [32].

DISCUSSION

The primary focus of our study was to identify blood-derived mediators that affect the heart through a K^+ conductance channel in a membrane-delimited mechanism similar to the vagal neurotransmitter ACh. $I_{K(ACh)}$ is important for the physiological regulation of cardiac parameters such as sino-atrial frequency, duration of the atrial action potential and AV-nodal conduction [10]. Using the myocyte I_K as a bioassay, we found that the active mediators were SPP and SPC on the basis of (1) co-migration with authentic SPP and SPC standards in 2D-TLC and (2) MALDI-TOF-MS analysis. In contrast with SPP, which has been found in high nanomolar concentrations in blood plasma [13], no results were previously available on the natural occurrence of SPC. Using MALDI-TOF-MS, we estimated the SPC concentration in plasma and serum as 50 ± 15 and 130 ± 25 nM respectively. Thus our data establish that SPC is also a naturally occurring lipid mediator in blood, with a slightly lower concentration than SPP, which was previously estimated as 200 and 500 nM in plasma and serum respectively [13]. Quantification of SPC should be treated as an estimate because a quantitative determination could only be derived from experiments that include a mass-tagged internal standard that was not available to us. Our estimate for the concentration of SPC and the previously reported concentration of SPP yield an approx. 250 nM combined plasma concentration for the two sphingolipids. Given the 1:10000 dilution as the EC_{50} plasma concentration for the activation of I_K and that the EC_{50} for SPP and SPC was approx. 1.5 nM in this assay, one would expect a combined plasma concentration of the two mediators of approx. 15 μ M. The reason for the substantial difference between the estimated and expected concentrations remains unclear and will be further investigated. Serum or plasma might contain other factors that enhance solubilization, potentiate and/or synergize with the effect of the sphingolipids as well as other non-lipid factors that have eluded our purification strategy. Nevertheless, we tested each HPLC fraction and primulin-positive lipid spot in the 2D-TLC separations for bioactivity in the I_K assay. Thus, any active lipid compound(s) other than SPP and SPC, if present, was in a concentration that was below the threshold sensitivity of our detection and bioassay systems.

Numerous studies *in vitro* with established cell lines have reported biological effects elicited by SPP and SPC [1,2,33]. Despite the fact that SPP is known as a platelet-derived sphingolipid mediator [12,13], only a few recent studies have reported on the cardiac effects of SPP in complex models such as the canine isolated, blood-perfused sinoatrial node and papillary muscle preparation [34] and a rat model *in vivo* [35]. In those experiments SPP elicited species-dependent effects. In the canine model, it evoked positive chronotropic and coronary vasocon-

strictor effects [34], whereas in the rat model circulating SPP decreased the heart rate, ventricular contraction and blood pressure [35]. However, there is a complete lack of information on whether SPC has any acute effects *in vivo* at the organ level. In the guinea-pig intact heart preparation we found that SPC decreased cardiac frequency in a dose-dependent manner, with a similar efficiency to that of ACh. Furthermore, as in isolated atrial myocytes, so in the whole heart too was only the naturally occurring *D-erythro* stereoisomer of SPC active. In a limited number of experiments, SPP showed similar effects in the guinea-pig heart preparation (results not shown).

Because SPC and ACh both activate I_K , and because both compounds are capable of decreasing the cardiac frequency in the intact heart preparation, we investigated whether their effect is additive at the organ level. We found that when the perfusate contained 1 μ M *D-erythro*-SPC, ACh was approximately an order of magnitude more potent in decreasing the heart rate. This modulation was specific for the *D-erythro*-SPC stereoisomer and was absent with *L-threo*-SPC; such stereospecificity is the same as that found previously for the activation of I_K [7]. Together, these observations raise the possibility that SPC and SPP, in concert with other mediators, contribute to the regulation of heart rate and possibly to other cardiovascular parameters.

The genuine mediator role of SPC is supported not only by its increased concentration after blood clotting but also by the discovery of its inactivation during the coronary passage (Figure 6). The heart, and possibly other organs, might contain a quantitative clearance mechanism for SPC. Because SPC is present in blood at a high nanomolar steady-state concentration and it is not generated *ex vivo* from sphingomyelin (results not shown), we hypothesize that SPC, like SPP, could be released into the bloodstream at a constant rate. Whereas plasma SPP has been shown to originate from platelets, the source of SPC remains to be identified.

Which receptor or receptors mediate the effect of SPC in the heart? Recently, five G-protein-coupled receptors of the Edg family, Edg1, Edg3, Edg5, Edg6 and Edg8, have been identified that are selectively activated by SPP [25–30], whereas these Edg receptors bind SPC about two orders of magnitude less potently than SPP, the ovarian cancer orphan receptor OGR1 has been shown recently to be a high-affinity receptor for SPC [31]. RT-PCR analysis, with mRNA purified from isolated atrial and ventricular rat myocyte cultures, confirmed the expression of four Edg sphingolipid receptors with the exception of Edg6, as well as OGR1 receptors. Further evidence was obtained by Western blot analysis, showing that both Edg3 and Edg5 proteins are expressed in atrial as well as ventricular membranes. The antibodies currently available could not be used for immunocytochemical localization of the antigen. Thus we could not obtain direct evidence for the co-expression of these receptors in the same cell. Nevertheless, because atrial myocytes form a functionally coupled syncytium, the simultaneous detection of Edg3 and Edg5 proteins lends strong support for a joint role of these receptors in mediating responses to lysosphingolipids.

Because of the simultaneous presence of these receptors in cardiac cells, we could not reach a definitive conclusion about which (sub)types mediate each of the different sphingolipid effects, which include activation of I_K , inhibition of L-type Ca^{2+} current, enhancement of ACh response, effects on cardiac contractility and coronary perfusion. The identification of the receptor(s) and the signalling pathways involved in mediating these effects must await a future study. Recently, Himmel et al. [36] confirmed our previous results [7] reporting that SPC and SPP are equipotent activators of I_K in guinea-pig atrial myocytes, whereas in mouse and human atrial myocytes SPC was less

potent, suggesting substantial interspecies differences in either the ligand specificity of the sphingolipid receptor(s) or the expression of a specific receptor subtype responsible for the high potency of SPC in guinea-pigs. These authors proposed that the non-specific pharmacological agent suramin potently and selectively inhibits the activation of $I_{K(ACh)}$ by SPP in human cardiomyocytes via the Edg3 receptor subtype. Their concept was based on a report by Ancellin and Hla [37] illustrating a differential inhibition of the SPP-mediated activation of Edg7, but not Edg1 or Edg5, by suramin. However, Ancellin and Hla used heterologous co-expression of Edg1 and Edg5 with the chimaeric $G_{\alpha_{i1}}$ protein in *Xenopus* oocytes, whereas for Edg3 they expressed the receptor only. Furthermore, the IC_{50} for suramin in oocytes treated with 5 nM SPP was approx. 50 μ M [37], which is consistent with the concentration range at which suramin usually exerts its non-specific pharmacological effects in a variety of systems. In contrast, Himmel et al. [36] reported a 0.2 nM IC_{50} for suramin against 1 μ M SPP. These contradictions must be resolved in further studies with knock-out animal models and/or anti-sense oligonucleotide-mediated inhibition studies.

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