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Evidence for Edg-3 Receptor-Mediated Activation of $I_{K.ACh}$ by Sphingosine-1-Phosphate in Human Atrial Cardiomyocytes

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ABSTRACT

Sphingosine-1-phosphate (SPP) and sphingosylphosphorylcholine (SPPC) have been reported to activate muscarinic receptor-activated inward rectifier K^+ current ($I_{K.ACh}$) in cultured guinea pig atrial myocytes with similar nanomolar potency. Members of the endothelial differentiation gene (Edg) receptor family were recently identified as receptors for SPP; however, these receptors respond only to micromolar concentrations of SPPC. Here we investigated the sphingolipid-induced activation of $I_{K.ACh}$ in freshly isolated guinea pig, mouse, and human atrial myocytes. SPP activated $I_{K.ACh}$ in atrial myocytes from all three species with a similar nanomolar potency (EC_{50} values: 4–8 nM). At these low concentrations, SPPC also activated $I_{K.ACh}$ in guinea pig myocytes. In contrast, SPPC was almost ineffective in mouse and human myocytes, thus resembling the pharmacology of the Edg receptors. Transcripts of *Edg-1*, *Edg-3*, and *Edg-5* were detected in human atrial cells. Moreover, activation of $I_{K.ACh}$ by SPP was blocked by the Edg-3-selective antagonist suramin, which did not affect basal or carbachol-stimulated K^+ currents. In conclusion, these data indicate that $I_{K.ACh}$ activation by SPP and SPPC exhibits large species differences. Furthermore, they suggest that SPP-induced $I_{K.ACh}$ activation in human atrial myocytes is mediated by the Edg-3 subtype of SPP receptors.

The lysosphingolipid, sphingosine-1-phosphate (SPP), has been identified as a signaling molecule in a large number of different cell types. Major cellular effects reported for SPP include cell proliferation and differentiation, suppression of apoptosis, and cytoskeleton-dependent responses such as contraction, adhesion, and chemotaxis (for reviews, see Spiegel and Milstien, 1995; Meyer zu Heringdorf et al., 1997; Goetzl and An, 1998). Extracellularly applied SPP is able to activate or to inhibit adenylyl cyclase, to increase cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), and to stimulate mitogen-activated protein kinase. Signal transduction occurs via G protein-coupled receptors encoded by the recently identified endothelial differentiation gene (Edg) family. Edg-1, -3, and -5 receptors respond to nanomolar concentrations of SPP but have a micromolar affinity for sphingosylphosphorylcholine (SPPC). Edg-2, -4, and -7 receptors are dedicated to lysophosphatidic acid (LPA). Some effects induced by SPP are attenuated by pertussis toxin, suggesting the involvement of G_i/G_o-type G proteins in the signaling cascade.

Some sphingolipid-mediated cellular responses possess a pharmacological profile that differs markedly from those of the known Edg receptors, suggesting the existence of as-yet-unidentified lysosphingolipid receptors. For example, in human leukemia HL-60 cells, only SPPC at micromolar concentrations increased $[Ca^{2+}]_i$, whereas SPP was ineffective (van Koppen et al., 1996b). Furthermore, in RINm5F insulinoma cells, micromolar concentrations of the sphingolipids SPPC and glucopsychosine attenuated depolarization-induced increase in $[Ca^{2+}]_i$ and L-type Ca^{2+} current, whereas SPP had no effect (Himmel et al., 1998). Finally, in cultured guinea pig atrial cardiomyocytes, the muscarinic receptor-activated inward rectifier K^+ current ($I_{K.ACh}$) was stimulated by both SPP and SPPC at nanomolar concentrations (Bünemann et al., 1995, 1996), thus being the only cellular effect activated by nanomolar SPPC.

The findings that SPP is a normal constituent of human plasma and can be released by activated platelets (Yatomi et al., 1997) raise the questions of whether SPP activates $I_{K.ACh}$

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ABBREVIATIONS: SPP, sphingosine-1-phosphate; $[Ca^{2+}]_i$, cytosolic free Ca^{2+} concentration; C8-ceramide-1-P, *N*-octanoyl ceramide-1-phosphate; E_{rev}, reversal potential; dihydro-SPP, dihydrosphingosine-1-phosphate; I_{K1}, inward rectifier K^+ current; $I_{K.ACh}$, muscarinic receptor-activated inward rectifier K^+ current; LPA, lysophosphatidic acid; SPPC, sphingosylphosphorylcholine; PCR, polymerase chain reaction; bp, base pairs.

also in human cardiomyocytes and, if so, which receptor could mediate this effect. In atrial cardiomyocytes, $I_{K.ACh}$ is the major effector of vagal stimulation, which occurs via M_2 muscarinic acetylcholine receptors and G_i protein $\beta\gamma$-subunits (Yamada et al., 1998). $I_{K.ACh}$ is an inwardly rectifying K^+ current with a voltage dependence very similar to the inward rectifier K^+ current (I_{K1}), from which it cannot be distinguished at the whole-cell level. Both channels are of clinical significance because they contribute to the atrial resting membrane potential and determine the shape of cardiac action potentials during the final phase of repolarization. This investigation was aimed at extending the data from guinea pig to human atrial myocytes and at identifying the receptor mediating $I_{K.ACh}$ activation by SPP. Our results demonstrate that SPP activates $I_{K.ACh}$ in human, mouse, and guinea pig atrial myocytes with similar potency but different efficacy. SPPC, however, is ineffective in human and mouse but equipotent to SPP in guinea pig atrial cells, suggesting considerable species differences. Evidence is provided that, in human atrial myocytes, the action of SPP may be mediated by Edg-3 receptors. Preliminary results were published previously in abstract form (Himmel et al., 2000).

Materials and Methods

Chemicals. SPP, SPPC, dihydro-SPP, *N*-octanoyl ceramide-1-phosphate (C8-ceramide-1-P) (all D-*erythro* conformation), LPA, and sphingosine were purchased from Biomol (Hamburg, Germany). The sphingolipids were dissolved in methanol and stored at −20°C. Aliquots were dried in a SpeedVac concentrator (Bachofer, Reutlingen, Germany) before use and redissolved in H_2O with 1 mg/ml BSA. Alternatively, the methanol-dissolved stock solutions were diluted directly into the bath solution to achieve the final concentration. Neither of the solvents influenced current measurements. Collagenase (type 1, 254 U/mg) was from Worthington (Freehold, NJ), and pronase E (4000 proteolytic U/mg) was from Merck (Darmstadt, Germany). Protease (type 24), carbachol, suramin, GTP, and other chemicals were obtained from Sigma (Deisenhofen, Germany) or other commercial suppliers at the highest available purity.

Isolation of Human Atrial Myocytes. Right atrial specimens were obtained from 32 patients (22 males, 10 females) who underwent open-heart coronary artery bypass grafting surgery. Each patient gave written informed consent for the investigation, which was approved by the local ethics committee. The procedure for isolating human cardiac myocytes has been described previously in detail (Amos et al., 1996). Briefly, right atrial specimens were placed immediately in chilled Ca^{2+}-free solution (100 mM NaCl, 10 mM KCl, 1.2 mM KH_2PO_4, 5 mM $MgSO_4$, 50 mM taurine, 5 mM MOPS, 20 mM D-glucose, adjusted to pH 7.0 with NaOH) supplemented with 30 mM 2,3-butanedione monoxime and were transported to the laboratory within 1 h. Small tissue chunks were then washed three times with Ca^{2+}-free Tyrode's solution. At all steps, the solutions were oxygenated with 100% O_2 at 35°C. Tissue pieces were transferred into Ca^{2+}-free Tyrode's solution containing 254 U/ml collagenase and 0.5 mg/ml protease and gently stirred for 10 min. Then, the Ca^{2+} concentration was increased to 20 μM, and the tissue was stirred for an additional 35 min. Stirring was continued with Tyrode's solution (20 μM Ca^{2+}) containing collagenase only until the yield of rod-shaped striated myocytes reached a maximum (10–30 min, cell yield 15–25%). The enzyme solution was removed by centrifugation, and the myocytes were stored until use at room temperature either in Ca^{2+} (0.5 mM)-containing Tyrode's solution or in a Kraftbrühe-like solution (Feng et al., 1996).

Isolation of Guinea Pig and Mouse Atrial Myocytes. All studies complied with the German Home Office Regulations Governing the Care and Use of Laboratory Animals. Guinea pig atrial myocytes were isolated by pronase perfusion of a Langendorff heart preparation according to previously described methods (Wettwer et al., 1991; Bünemann et al., 1995). Hearts of adult Dunkin-Hartley guinea pigs (280–320 g) were perfused for 3 min at 37°C with oxygenated preparation buffer of the following composition: 5.4 mM KCl, 150 mM NaCl, 10 mM HEPES, 10 mM D-glucose, 2 mM $MgCl_2$, 1.8 mM $CaCl_2$ (pH 7.4, adjusted with NaOH). This initial period was followed by 5 min of perfusion with nominally Ca^{2+}-free preparation buffer. Subsequently, the hearts were perfused for a period of 4 to 6 min with Ca^{2+}-free preparation buffer containing 1 mg/ml BSA, 125 U/ml collagenase, and 0.034 mg/ml pronase E. This procedure was followed by a 2 min wash period with Ca^{2+}-free preparation buffer. The atria were then chopped into small chunks that were gently stirred for 5 min in storage buffer of the following composition: 70 mM KOH, 50 mM L-glutamic acid, 40 mM KCl, 20 mM taurine, 20 mM KH_2PO_4, 3 mM $MgCl_2$, 10 mM HEPES, 10 mM D-glucose, 0.5 mM EGTA (pH 7.4, adjusted with KOH). Single myocytes were harvested by decanting the supernatant. The cells were allowed to settle under gravity for 15 min and were washed once with storage buffer.

This method was modified as follows to isolate mouse atrial myocytes (strain c57bl6). After preparation buffer (3 min) and Ca^{2+}-free buffer (5 min), mouse hearts were perfused for 10 to 15 min with Ca^{2+}-free preparation buffer containing 1 mg/ml BSA and 125 U/ml collagenase. Atria were then cut off and chopped into small chunks that were gently stirred and triturated for 3 to 4 min in Ca^{2+}-free preparation buffer containing 1 mg/ml BSA, 65 U/ml collagenase, and 0.5 mg/ml pronase E. The supernatant was harvested into Dulbecco's minimal essential medium supplemented with 10% fetal bovine serum and centrifuged gently, and the cell pellet was resuspended in storage buffer.

Measurement of Membrane Currents. The single-electrode whole-cell voltage-clamp method was applied using a List EPC-7 amplifier (List, Darmstadt, Germany) to measure membrane currents. pCLAMP 5.5 software (Axon Instruments, Foster City, CA) was used for data acquisition and analysis. Currents were filtered at 2 kHz and digitized at 1 kHz with a TL-125 A/D interface (Axon Instruments) for off-line analysis. Electrodes were fabricated from filamented borosilicate glass (Hilgenberg Co., Malsfeld, Germany) using a programmable horizontal puller (DMZ universal puller; Zeitz, München, Germany). When filled with electrode solution (100 mM potassium aspartate, 10 mM NaCl, 40 mM KCl, 5.0 mM magnesium ATP, 2.0 mM EGTA, 0.1 mM GTP-Tris, 10 mM HEPES, pH adjusted to 7.4 with KOH), the microelectrodes had tip resistances of 2 to 3 MΩ. Seal resistances were usually 5 to 10 GΩ.

Membrane capacitance was routinely measured using depolarizing ramps (1 V/s) from −40 to −35 mV (Amos et al., 1996) and was compensated up to 100 pF. Series resistance compensation was set to 70%. The liquid junction potential between electrode and standard bath solution amounted to −12 mV (software JPCalc; Barry, 1994). The mean resting potential was -23 ± 1 mV ($n = 54$). Taking into account the calculated liquid junction potential, the resting membrane potential was −35 mV, which is positive to the K^+ equilibrium potential of −50 mV. The data presented are corrected neither for the calculated junction potential nor for leak currents.

From the holding potential of −80 mV, the command voltage was stepped for 50 ms to −100 mV, followed by a depolarizing ramp to −10 mV (800 ms), a 100 ms step to −50 mV, and back to the holding potential. The pulse protocol was elicited at a rate of 0.5 Hz. Inward rectifier current I_{K1} was quantified as either inward current at −100 mV or outward current at −10 mV corrected for cell capacitance (in pA/pF). Once current traces had stabilized (usually 3–5 min after membrane rupture), $I_{K.ACh}$ was elicited by applying either carbachol or various sphingolipids to the bath solution (120 mM NaCl, 20 mM KCl, 1 mM $MgCl_2$, 2.0 mM $CaCl_2$, 10 mM D-glucose, 10 mM HEPES, adjusted to pH 7.4 with NaOH). Drug application was performed with a gravity-driven, eight-channel rapid solution exchanger (SPS-8; List). All experiments were conducted at 22–24°C.

Extraction of mRNA and Reverse Transcriptase Polymerase Chain Reaction (PCR). After removal of collagenase, isolated myocytes were resuspended in storage solution supplemented with 0.1% BSA and layered on top of 6% BSA in storage solution. This procedure allowed us to remove cell debris, nonmyocytes, and undigested tissue fragments from the atrial myocytes within 45 min as checked by light microscopy. The myocytes were washed in Ca^{2+}/Mg^{2+}-containing phosphate-buffered saline and resuspended in RNAzol to extract total cellular RNA according to the manufacturer's specifications (peqLab, Erlangen, Germany). Reverse transcription was performed with a kit from Superscript (Life Technologies, Eggenstein, Germany) using 1 μg of RNA, 600 ng of random hexanucleotide primers, and 0.5 mM dNTP. PCR was performed with a *Taq* polymerase kit (MBI Fermentas, St. Leon-Rot, Germany). Oligonucleotide primers were chosen as follows: Edg-1 (GenBank accession no. M31210), 5′-GCAATCTGGCCCTCTCAGAC-3′ and 5′-CGCCACATTCTCAGAGCTGC-3′; Edg-2 (GenBank accession no. Y09479), 5′-CATGGTGGCAATCTATGTCAAC-3′ and 5′-TTCAGAAGACTCATCATGGTATC-3′; Edg-3 (GenBank accession no. X83864), 5′-TGCAGCTTACTGG CCATCGC-3′ and 5′-AACACG ATGAACCACTGAGCC-3′; Edg-4 (GenBank accession no. NM 004720), 5′-TGGTCATCATGGGCCAGTGC-3′ and 5′-GGTGCCATGCGTGAGCAGC-3′; Edg-5 (GenBank accession no. NM 004230), 5′-ACGCCTGTGCAGTGGTTTGC-3′ and 5′-GACGATAAAGACGCCTAGCAC-3′. Thermocycler steps were 3 min at 95°C, then 35 cycles of 1 min at 95°C, 1 min at 59°C, and 1 min at 72°C (Edg-1, Edg-2, Edg-3) or 35 cycles of 1 min at 95°C, 1 min at 62°C, and 1 min at 72°C (Edg-4, Edg-5). PCR products were separated by electrophoresis in 1% agarose with *Alu*I-digested pBR322 plasmid as marker. Control reactions were performed using RNA that had not been reverse transcribed to check for contaminating genomic DNA.

Statistical Analysis. Results were expressed as mean values ± S.E of n experiments. Statistical differences were analyzed by means of Welch's approximate t test or ANOVA followed by Dunnett's test. To establish concentration-response relations, a standard four parameter logistic function was fitted to individual concentration-response curves with minimum = 0 as constant and with maximum, log EC_{50}, and slope as variables. Curve fitting was performed using pCLAMP software (Clampfit) or Prism (Graphpad Software, San Diego, CA).

Results

The lysosphingolipids, SPP and SPPC, have been reported to induce activation of $I_{K.ACh}$ in cultured guinea pig atrial myocytes (Bünemann et al., 1996). We have used freshly isolated guinea pig cells to confirm these results and to exclude putative cell culture-mediated alterations. Freshly isolated mouse atrial myocytes were studied in comparison. In both guinea pig and mouse atrial myocytes, voltage ramps from −100 to −10 mV elicited inwardly rectifying currents, which were rapidly and reversibly increased by SPP (100 nM) and by carbachol (1 μM) and which were blocked by Ba^{2+} (1 mM; Fig. 1, A and B). In both species, basal as well as agonist-activated currents reversed at approximately −40 mV, i.e., slightly positive to the K^+ equilibrium potential. The potencies of SPP and carbachol were similar in guinea pig and mouse; $I_{K.ACh}$ activation was half-maximal with 6.3 nM SPP and 148 nM carbachol in guinea pig and with 8.1 nM SPP and 115 nM carbachol in mouse atrial myocytes (Fig. 1, C and D). As reported before (Bünemann et al., 1996), SPPC activated $I_{K.ACh}$ to the same extent and with the same potency (EC_{50}: 4.7 nM) as SPP in guinea pig atrial myocytes (Fig. 1C). In contrast, SPPC up to 10 μM was ineffective in mouse atrial myocytes (Fig. 1D). LPA (1 μM) did not activate $I_{K.ACh}$, neither in guinea pig (Bünemann et al., 1996) nor in mouse atrial myocytes (data not shown).

In freshly isolated human atrial cardiomyocytes, inwardly rectifying current was of smaller amplitude and had a more positive reversal potential (E_{rev}) than that in guinea pig and mouse myocytes (Fig. 2A). Under control conditions, E_{rev} was −24.8 ± 1.4 mV (n = 32) and current amplitudes were −6.57 ± 0.74 and +0.35 ± 0.07 pA/pF at potentials of −100 and −10 mV, respectively (Fig. 2A). Inward rectifier currents were rapidly and reversibly increased by the muscarinic receptor agonist carbachol and by the lysosphingolipid SPP (Fig. 2, B and C). Basal as well as agonist-activated inward rectifier currents were almost abolished by Ba^{2+} (1 mM, Fig. 2A). The Ba^{2+}-insensitive current amounted to −1.27 ± 0.18 pA/pF at −100 mV and reversed at −21.8 ± 1.6 mV. The Ba^{2+}-sensitive basal current had amplitudes of −5.42 ± 0.78 pA/pF at −100 mV and of +0.08 ± 0.04 pA/pF at −10 mV; E_{rev} was −30.6 ± 2.4 mV. Application of SPP (100 nM, n = 28) and carbachol (1 μM, n = 32) significantly increased inward rectifier current at −100 and −10 mV by −5.16 ± 0.45 and +0.78 ± 0.10 pA/pF (SPP) and by −9.45 ± 0.71 and +1.46 ± 0.13 pA/pF (carbachol), respectively. The E_{rev} values of the currents stimulated by either SPP or carbachol were indistinguishable from E_{rev} of the Ba^{2+}-sensitive basal current. The velocities of onset and decay of $I_{K.ACh}$ activation differed between SPP and carbachol (Fig. 2, B and C). Both onset and decay of effects were slower with SPP than with carbachol, which is reflected in the different time scales in

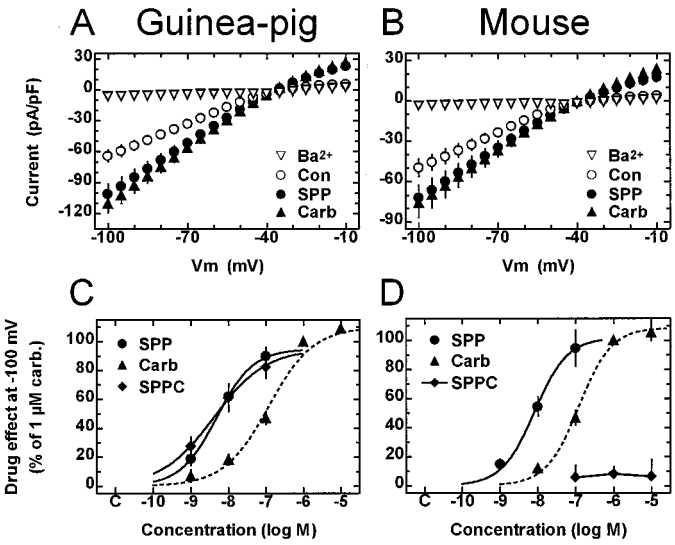

Fig. 1. Effects of SPP, SPPC, and carbachol on inward rectifier current in guinea pig (A, C) and mouse atrial cardiomyocytes (B, D). A and B, average current-voltage relations of inward rectifier current under control conditions (Con) and in the presence of SPP (100 nM), carbachol (Carb; 1 μM), or Ba^{2+} (1 mM). Mean values ± S.E.; guinea pig: n = 25 for control, carbachol, and Ba^{2+}; n = 15 for SPP; mouse: n = 18 for control, carbachol, and Ba^{2+}; n = 13 for SPP. Voltage ramps as described under *Materials and Methods*. C and D, concentration-response curves for the effects of SPP, SPPC, and carbachol on inward rectifier current. Drug effects were evaluated at −100 mV and were expressed as percentage of the effect of 1 μM carbachol from the same cell. Curve fitting in guinea pig atrial myocytes yielded respective values for maximum, log EC_{50}, and slope of 98.1 ± 6.4%, −8.20 ± 0.16, and 1.07 ± 0.10 for SPP (11 cells/4 preparations); 100.3 ± 4.3%, −8.33 ± 0.22, and 0.97 ± 0.13 for SPPC (11 cells/5 preparations); and 115.4 ± 5.5%, −6.83 ± 0.10, and 0.88 ± 0.12 for carbachol (12 cells/5 preparations). Respective values in mouse atrial myocytes were 106.3 ± 14.9%, −8.09 ± 0.09, and 1.05 ± 0.12 for SPP (13 cells/5 preparations) and 110.3 ± 5.3%, −6.94 ± 0.11, and 1.25 ± 0.15 for carbachol (10 cells/6 preparations); no curve fitting was performed for SPPC (11 cells/9 preparations).

Fig. 2, B and C. The onset of effects could be fitted with a sigmoidal function reaching half-maximal values after 1770 ± 409 ms (n = 6) for SPP and 104 ± 7 ms (n = 11) for carbachol. When the stimulus was discontinued, activated current returned to baseline values; this decay was described by a monoexponential function with time constants of 10.9 ± 2.1 s and 2.0 ± 0.2 s for SPP and carbachol, respectively. The current response decreased again in the continued presence of SPP and carbachol; this rapid desensitization process was both faster and more pronounced with carbachol than with SPP, as reported before for guinea pigs (Bünemann et al., 1995).

Concentration-response curves for SPP, SPPC, and carbachol were constructed by evaluating drug-sensitive currents at −100 mV in relation to the effect of 1 μM carbachol, which is an almost maximally effective concentration (Fig. 3). SPP activated $I_{K.ACh}$ to a maximum of 64.7 ± 4.2%, which was significantly less than the maximal stimulation obtained with carbachol (106.9 ± 5.7%). Half-maximal activation of $I_{K.ACh}$ was observed with 4.2 nM SPP and 63.1 nM carbachol. The slopes of the concentration-response curves were 1.19 ± 0.14 for SPP and 1.44 ± 0.23 for carbachol. These values were not significantly different from unity, suggesting a stoichiometric drug-receptor interaction. The SPP analog dihydro-SPP (1 μM, n = 8) activated $I_{K.ACh}$ to 58.2 ± 9.3% of the effect of 1 μM carbachol and was thus as effective as SPP. In contrast to SPP, SPPC hardly activated $I_{K.ACh}$ in the concentration range from 10 nM to 1 μM. At 0.1 μM SPPC, $I_{K.ACh}$ activation amounted to −1.74 ± 0.55 pA/pF (n = 18, $P < .05$) at −100 mV. Sphingosine (1 μM, 0.8 ± 5.3%, n = 5), C8-ceramide-1-P (1 μM, 3.0 ± 3.3%, n = 8), and LPA (1 μM, −5.8 ± 7.3%, n = 9) were ineffective with respect to $I_{K.ACh}$ activation by 1 μM carbachol.

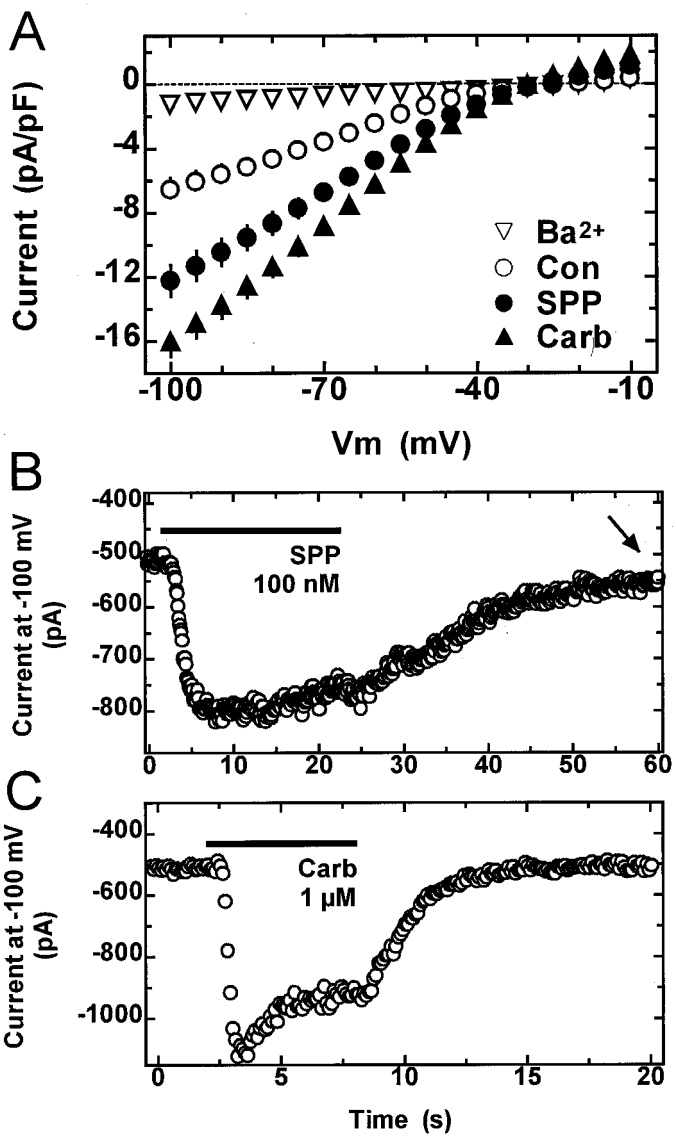

Fig. 2. A, average current-voltage relations of inward rectifier current in human atrial myocytes under control conditions (Con; n = 32) and in the presence of SPP (100 nM, n = 28), carbachol (Carb; 1 μM, n = 32), or Ba^{2+} (1 mM, n = 29). Mean values ± S.E.; voltage ramps as described under *Materials and Methods*. B and C, onset and decay of effects of SPP (100 nM, B) and carbachol (1 μM, C) on human atrial inward rectifier current measured at −100 mV. Please note that both time scale and ordinate differ between B and C. The SPP-stimulated inward rectifier current has not yet reached baseline values at t = 60 s (arrow in B), whereas the effect of carbachol has completely disappeared at t = 20 s (C).

Thus, the concentration-response relations of SPP and SPPC in human and mouse myocytes are in accordance with the activities of SPP and SPPC at transfected Edg receptors (Ancellin and Hla, 1999; Okamoto et al., 1999). Therefore, we have analyzed human atrial myocytes for expression of Edg receptor subtypes and have detected transcripts for *Edg-1*, *Edg-3*, and *Edg-5* (Fig. 4). Expression of *Edg-2*, which is activated by LPA, was also observed. Thus, all three presently known SPP/Edg receptors are expressed by human atrial myocytes.

To investigate which of the expressed SPP/Edg receptors could be involved in activation of $I_{K.ACh}$ in atrial myocytes, selective receptor antagonists are required. Although such antagonists are not available for Edg-1 and Edg-5 receptors, the polycyclic anionic compound, suramin, appears to be selective for Edg-3 receptors. It was recently reported that suramin blocks SPP-induced $[Ca^{2+}]_i$ increase in Edg-3 receptor-transfected *Xenopus* oocytes (Ancellin and Hla, 1999). Therefore, we have tested whether the SPP-mediated activation of $I_{K.ACh}$ could be antagonized by suramin. To avoid desensitization-biased results, one particular myocyte from a cell preparation was exposed to agonists only once, i.e., either to SPP (1 μM) or to suramin followed by SPP (1 μM). In a typical control cell, SPP activated $I_{K.ACh}$ to approximately half of the effect of carbachol (Fig. 5A, left panel). When pretreated for 60 to 120 s with suramin (1 μM) in the bath

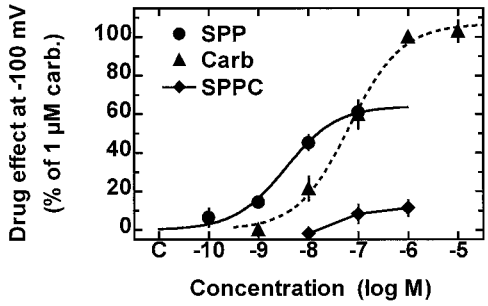

Fig. 3. Concentration-response curves for the effects of SPP, SPPC, and carbachol on inward rectifier current in human atrial cardiomyocytes. Curve fitting yielded respective values for maximum, log EC_{50}, and slope of 64.7 ± 4.2%, −8.38 ± 0.10, and 1.19 ± 0.14 for SPP (19 cells/7 patients) and 106.9 ± 5.7%, −7.20 ± 0.13, and 1.44 ± 0.23 for carbachol (17 cells/6 patients). No curve fitting was performed for SPPC (17 cells/6 patients).

solution, the subsequent exposure to SPP in the continued presence of suramin did not result in any stimulation of $I_{K.ACh}$. In contrast, the carbachol response was fully preserved (Fig. 5A, right panel). On average, neither the carbachol-activated $I_{K.ACh}$ nor the control current measured at −100 mV in the absence of carbachol were significantly affected by suramin (1 μM; Fig. 5B). However, the SPP-induced stimulation of $I_{K.ACh}$ was potently inhibited by suramin in a concentration-dependent manner (IC_{50} ~0.2 nM; Fig. 5C).

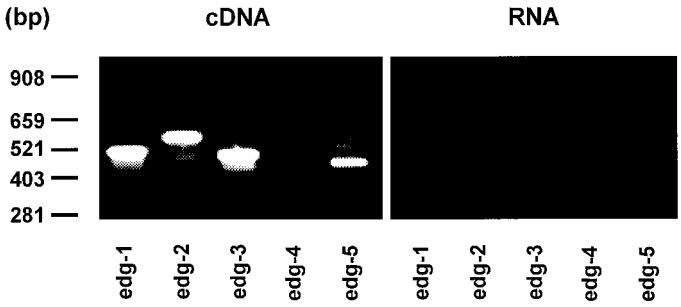

Fig. 4. Edg receptor expression in human atrial cardiomyocytes. Reverse transcriptase PCR was performed with cDNA (lanes 1–5) or RNA (lanes 6–10) that had not been reverse-transcribed. Calculated length of the fragments is 502 base pairs (bp) (Edg-1), 562 bp (Edg-2), 478 bp (Edg-3), 546 bp (Edg-4), and 431 bp (Edg-5). Identical results were obtained with myocytes of five different human subjects.

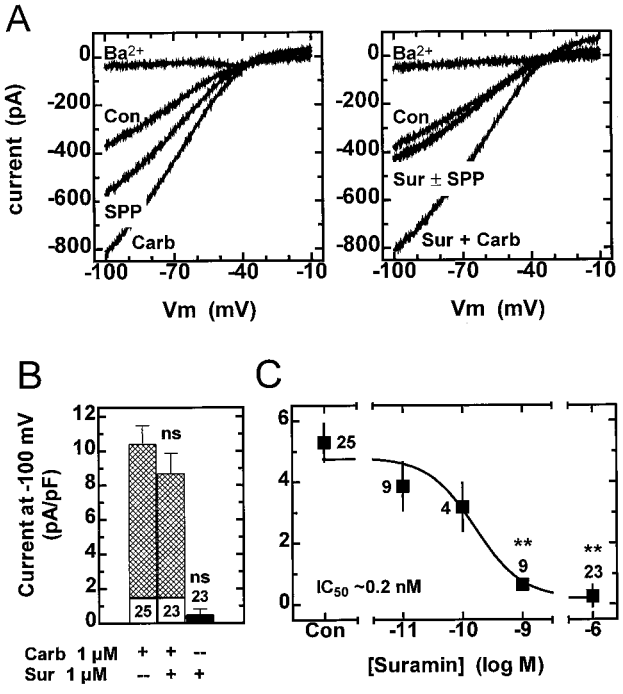

Fig. 5. Suramin selectively blocks SPP-induced activation of $I_{K.ACh}$ in human atrial cardiomyocytes. A, superimposed current-voltage relations before (Con) and after exposure to 1 μM SPP, 1 μM carbachol (Carb), or 1 mM Ba^{2+}. Left panel, control cell (30 pF); right panel, cell (40 pF) with SPP and carbachol exposure in the presence of 1 μM suramin (Sur) in the bath. Clamp protocol as under *Materials and Methods*. B, mean amplitudes of current activated by carbachol (1 μM) without and with suramin (1 μM). Mean values ± S.E.; suramin affected neither the effect of carbachol (Welch's approximate *t* test) nor basal current (one sample *t* test). C, current at −100 mV activated by SPP (1 μM) as a function of the concentration of suramin. Mean values ± S.E., *n* as indicated at symbols; $^{**}P < .01$, ANOVA followed by Dunnett's test versus control. Curve fitting yielded an IC_{50} value of ~0.2 nM and a slope of −1.1.

Discussion

This study has three major results: 1) previous reports on SPP-mediated activation of $I_{K.ACh}$ in cultured guinea pig atrial myocytes (Bünemann et al., 1995; van Koppen et al., 1996a) were confirmed and extended to freshly isolated atrial myocytes from guinea pigs, mice, and human subjects; 2) considerable pharmacological differences were detected between lysosphingolipid-induced $I_{K.ACh}$ activation in humans and mice compared with guinea pigs; and 3) SPP-mediated activation of $I_{K.ACh}$ in human myocytes was ascribed to the action of SPP/Edg receptors, most likely Edg-3.

Studies in guinea pig atrial myocytes had demonstrated for the first time that SPP can act via G protein-coupled receptors. Activation of $I_{K.ACh}$ by SPP was fully pertussis toxin-sensitive and could only be induced in isolated membrane patches when SPP was added to the extracellular face of the plasma membrane in the presence of intracellular GTP (van Koppen et al., 1996a). Subsequently, many other actions of SPP, such as increase in $[Ca^{2+}]_i$, neurite retraction, or cell migration, were found to be mediated by G protein-coupled receptors (Meyer zu Heringdorf et al., 1996; Postma et al., 1996; Yamamura et al., 1997). However, $I_{K.ACh}$ activation in guinea pig atrial myocytes differed markedly from the other SPP effects in that SPP and SPPC activated $I_{K.ACh}$ with equal potency (EC_{50} values: SPP, 1.3 nM and D-*erythro*-SPPC, 1.5 nM; Bünemann et al., 1996). In contrast, in eliciting other cellular responses, SPPC was 2 to 3 orders of magnitude less potent than SPP. For example, in bovine aortic endothelial cells, SPP increased $[Ca^{2+}]_i$ with a nanomolar potency (EC_{50}: 0.8 nM), whereas D-*erythro*-SPPC exhibited a micromolar potency (EC_{50}: 0.4 μM; Meyer zu Heringdorf et al., 1998). The high sensitivity to SPPC observed in guinea pig atrial myocytes led to the assumption that $I_{K.ACh}$ activation may involve a lysosphingolipid receptor subtype distinct from that found in other cell types, such as endothelial cells and fibroblasts (Meyer zu Heringdorf et al., 1997).

G protein-coupled SPP receptors are widely expressed and have recently been identified on the molecular level as members of the Edg family of lipid receptors (for review, see Goetzl and An, 1998). The pharmacological profile of the presently characterized SPP/Edg receptors, Edg-1, Edg-3, and Edg-5, strongly resembles that of SPP receptors described functionally in various cell types. Activation of these three SPP/Edg receptors expressed in different cell types was achieved by SPP at nanomolar concentrations, whereas SPPC activated these receptors at micromolar concentrations (Ancellin and Hla, 1999; Kon et al., 1999; Okamoto et al., 1999). These data suggested that activation of $I_{K.ACh}$ in guinea pig atrial myocytes may not be mediated by one of these SPP/Edg receptors.

Here, we demonstrate that SPP activates $I_{K.ACh}$ in freshly isolated guinea pig, mouse, and human atrial cardiomyocytes with a similar potency in the nanomolar range (EC_{50} values of 4.2–8.1 nM). SPP was nearly as efficient as the muscarinic receptor agonist carbachol to activate $I_{K.ACh}$ in guinea pig and mouse cardiomyocytes, whereas it caused maximally 65% of the effect of carbachol in human myocytes. Most important, although SPPC activated $I_{K.ACh}$ in *freshly isolated* guinea pig atrial myocytes with a similar potency and efficiency as SPP, as reported before for *cultured* guinea pig myocytes (Bünemann et al., 1996), SPPC was much less

potent and hardly activated $I_{K.ACh}$ at all in human and mouse cardiomyocytes. These data indicate that the lysosphingolipid receptor activating $I_{K.ACh}$ in guinea pig myocytes differs from that found in other species. However, the missing or weak activity of SPPC observed in mouse and human cardiomyocytes suggested that $I_{K.ACh}$ activation by SPP in myocytes of these species may be mediated by one of the SPP/Edg receptors. Furthermore, $I_{K.ACh}$ in human atrial myocytes was also activated by dihydro-SPP, which binds to and activates all three above-mentioned SPP/Edg receptors (van Brocklyn et al., 1998, 1999). In contrast, both LPA and C8-ceramide-1-P did not stimulate $I_{K.ACh}$ (see *Results*) and neither bound to nor activated SPP/Edg receptors (Goetzl and An, 1998; van Brocklyn et al., 1998, 1999; Ancellin and Hla, 1999). Therefore, contribution of SPP/Edg receptors to SPP-induced $I_{K.ACh}$ activation was studied in more detail in human cardiomyocytes.

Human atrial myocytes express at least three Edg receptor subtypes, i.e., Edg-1, Edg-3, and Edg-5, which have similar affinity for SPP (Kon et al., 1999). Therefore, association of a cellular effect of SPP with any one receptor subtype cannot be achieved by order of potency but requires selective antagonists. Although antagonists for Edg-1 and Edg-5 receptors are not available, suramin is an antagonist selective for Edg-3 receptors (Ancellin and Hla, 1999). We demonstrate here that nanomolar concentrations of suramin abolish the effect of SPP on $I_{K.ACh}$ in human atrial myocytes. Despite being notorious for its nonspecific pharmacological properties (for review, see Voogd et al., 1993), suramin did not affect basal current or carbachol-induced current activation. Because Edg-1 and Edg-5 are not affected by suramin (Ancellin and Hla, 1999), it is hypothesized that the Edg-3 receptor mediates SPP-induced activation of $I_{K.ACh}$ in human atrial myocytes. In a manner similar to the LPA receptor Edg-2, which is expressed in human atrial myocytes but does not lead to $I_{K.ACh}$ activation, the SPP receptors Edg-1 and Edg-5 may mediate other cellular actions of SPP not studied here.

The muscarinic receptor-activated potassium channel has been implicated in vagally mediated regulation of heart rate (Wickman et al., 1998; Yamada et al., 1998). Although SPP has recently been demonstrated to reduce pacemaker activity in single sinoatrial node cells (Guo et al., 1999), the precise physiological and pathophysiological roles of SPP remain a matter of speculation. Because SPP is released by activated platelets (Yatomi et al., 1997), local release of this sphingolipid may induce cardioprotective bradycardia during scenarios of ischemia and platelet aggregation.

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