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A novel membrane receptor with high affinity for lysosphingomyelin and sphingosine 1-phosphate in atrial myocytes

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Activation of $I_{K(ACh)}$ is the major effect of the vagal neurotransmitter acetylcholine in the heart. We report that both lysosphingomyelin (D-*erythro*-sphingosylphosphorylcholine; SPC) and sphingosine 1-phosphate (SPP) activate $I_{K(ACh)}$ in guinea pig atrial myocytes through the same receptor with an EC_{50} of 1.5 and 1.2 nM, respectively. Pertussis toxin abolished the activation of $I_{K(ACh)}$ by either lipid. The putative receptor showed an exquisite stereoselectivity for the naturally occurring D-*erythro*-(2*S*,3*R*)-SPC stereoisomer, the structure of which was confirmed by mass spectroscopy and NMR. These lipids caused complete homologous and heterologous desensitization with each other but not with ACh, indicating that both act on the same receptor. This receptor displays a distinct structure–activity relationship: it requires an unsubstituted amino group because *N*-acetyl-SPC, lysophosphatidic acid and lysophosphatidylcholine were inactive. Because SPP and SPC are naturally occurring products of membrane lipid metabolism, it appears that these compounds might be important extracellular mediators acting on a family of bona fide G protein-coupled receptors. Expression of these receptors in the heart raises the possibility that sphingolipids may be a part of the physiological and/or pathophysiological regulation of the heart. Based on their ligand selectivity we propose a classification of the sphingolipid receptors.

Keywords: βγ subunit/G protein/lipid/muscarinic K^+ channel/receptor

Introduction

Recently, sphingolipids (for a review see Spiegel and Milstein, 1995), and in particular sphingosine 1-phosphate (SPP), have been shown to elicit diverse cellular responses that are best explained by a receptor-mediated action of these lipids (Bünemann *et al.*, 1995; Goodemote *et al.*, 1995; Wu *et al.*, 1995; van Koppen *et al.*, 1996). Because sphingomyelin (SM) is a ubiquitous constituent of the plasma membrane, the generation of SM metabolites with extracellular mediator function offers an attractive hypothesis which, together with the much more extensive literature on SM-derived lipid second messengers, particularly on ceramide (for reviews see Hannun *et al.*, 1993; Hannun, 1994; Hannun and Obeid, 1995; Obeid and Hannun, 1995; Pushkareva *et al.*, 1995), would place SM as an important precursor of extracellular mediators and intracellular second messengers. Lysosphingomyelin (sphingosylphosphorylcholine, SPC), a metabolite of SM, has been treated thus far primarily as a lipid second messenger (Desai and Spiegel, 1991; Desai *et al.*, 1993; Pushkareva and Hannun, 1994); only a few recent reports raise the possibility that some of its effects could be due, at least in part, to a receptor-mediated action (Okajima and Kondo, 1995; Seufferlein and Rozengurt, 1995a,b; van Koppen *et al.*, 1996). However, in contrast to SPP, which is active in the nanomolar range (Bünemann *et al.*, 1995; Postma *et al.*, 1996), micromolar concentrations of SPC are required to elicit its cellular effects, thus blurring the distinction between its proposed receptor-mediated and/or second messenger functions. Sphingosine is a potent inhibitor of protein kinase C, which has led to the hypothesis that sphingosine and some related sphingolipids fulfil second messenger functions (Hannun and Bell, 1987).

Acetylcholine (ACh) is the parasympathetic neurotransmitter that activates a K^+ conductance, designated $I_{K(ACh)}$, in supraventricular cells. Activation of $I_{K(ACh)}$ represents the major mechanism of vagal slowing of cardiac frequency. The signal transduction mechanism that couples the activation of the M_2-type muscarinic ACh receptor (M_2 mAChR) to the K^+ channel consists of a pertussis toxin (PTX)-sensitive heterotrimeric G protein that via its βγ subunits directly activates the channel encoded by the CIR and GIRK1 gene products (for a review see Clapham, 1994; Krapivinsky *et al.*, 1995). SPP (Bünemann *et al.*, 1995) and a set of serum-derived sphingolipids also activate the $I_{K(ACh)}$ via (a) receptor(s) different from the M2 mAChR (Banach *et al.*, 1993a,b; Bünemann *et al.*, 1994).

Here we report that not only SPP but also SPC is capable, in nanomolar concentrations, of eliciting $I_{K(ACh)}$ in atrial myocytes. Activation of $I_{K(ACh)}$ by SPC is consistent with the PTX-sensitive G protein-coupled signaling mechanism. Isolation of *erythro*- and *threo*-stereoisomers of SPC in a highly purified form revealed that the putative receptor shows an exquisite stereoselectivity for *erythro*-SPC. SPP and SPC both showed homologous receptor desensitization, a general feature of G protein-coupled receptors. However, responses to SPP and SPC also showed a complete heterologous desensitization with each other but failed to show desensitization with ACh. Using several analogs of SPC, a distinct structure–activity relationship of the putative sphingolipid receptor was determined that showed the importance not only of stereochemical configuration but also of the substituents of the

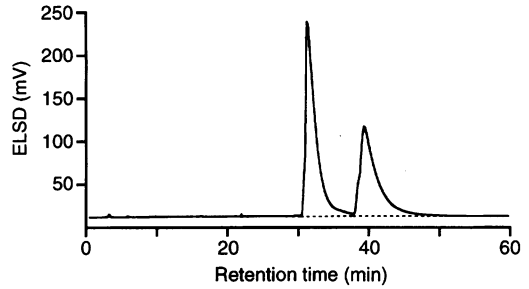


Fig. 1. Fractionation of SPC by HPLC. SPC obtained from Matreya Inc. was applied to HPLC, as described in the text, and the effluent was monitored with an evaporative light-scattering detector. The first-eluting SPC peak at 31 min was designated *erythro*-(*2S,3R*)-SPC and constituted 57% in this batch of the isomeric mix, whereas the one eluting at 39 min was designated *threo*-(*2S,3S*)-SPC.

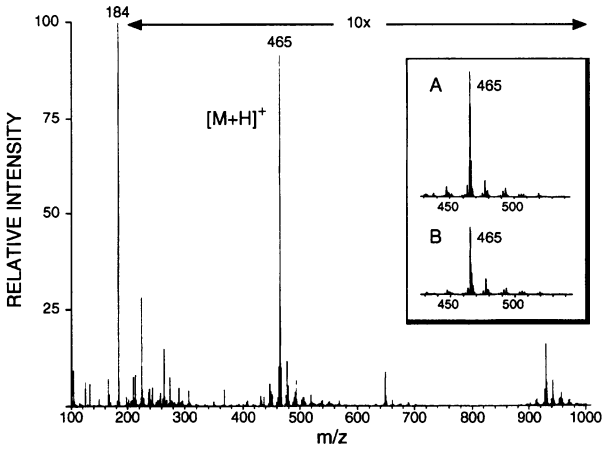


Fig. 2. FAB-MS spectrum of the isomeric mixture of SPC obtained from Matreya Inc., which was used to separate the isomers by HPLC. (**A**) The 400–600 *m/z* range for the first-eluting compound; (**B**) the 400–600 *m/z* range for the second-eluting compound. Note that all three spectra are similar, indicating the presence of a single positive $[M+H]^+$ ion at *m/z* 465 and its dimer at *m/z* 929. The abundant peak at 184 corresponds to the phosphocholine fragment of the compound. Also note that the C18 SPC, which is the predominant form in the brain SPC preparation, and the other smaller peaks spaced at multiples of ±14 around the *m/z* 465 peak represent other molecular species differing by multiples of $-CH_2$ groups.

amino, the hydroxyl and the phosphate moieties in the SM structure. Most interestingly, sphingosine, *N*-acetyl-sphingosine (C2-ceramide) and other long chain ceramides were completely inactive, indicating the pivotal role of the phosphate moiety in the lipid mediator role of sphingolipids. Based on the ligand specificity of the different sphingolipid-evoked responses, we propose a classification for this putative sphingolipid receptor family.

Results

Preparation and purification of SPC stereoisomers

The stereochemical configuration of the sphingosyl residue in SM derived from natural sources is always D-*erythro*, typically with a hydrocarbon residue of 18 carbon atoms [*trans*-D-erythro-1,3-dihydroxy-2-amino-4-octadecane, (*2S, 3R*)-C18-sphingosine; Karlsson, 1970]. However, when SPC is prepared semisynthetically by acid methanolysis of SM, acid-catalyzed conversion of the C3 chiral center occurs, resulting in the formation of *trans*(*2S,3R*)-D-*erythro*- and *trans*(*2S,3S*)-L-*threo*-SPC. HPLC analysis of SPC purchased from various manufacturers, as well as that prepared by us with acid methanolysis of bovine brain SM (Gaver and Sweeley, 1965), revealed the presence of two major components constituting ~61 ± 1 (n = 8) and 39% of the SPC (Figure 1). The first component, eluting at 31 min, comprised the more abundant species; the second component, eluting at 39 min, was less abundant. Monitoring the SM hydrolysate by HPLC at various times during the 18 h reaction showed that the faster-eluting component appeared before the later-eluting component and that its formation was followed by the gradual rise of the second component (data not shown). These timecourse data suggest that the *erythro*-isomer constituted the first peak, and that the second peak may have been formed by isomerization from the first. Because of the substantial difference in their retention times, the two components readily separated to baseline by HPLC, and were tested in atrial myocytes and subjected to structure determination by fast-atom bombardment mass spectroscopy (FAB-MS) and ^1H and ^{13}C NMR.

Identification of the SPC isomers by mass spectroscopy

FAB-MS in the positive mode of the peak material eluting at 31 min showed that this fraction contained a single major protonated molecule ion, $[M+H]^+$, at *m/z* 465 (Figure 2A). This is consistent with the calculated mass of $[SPC+H]^+$ containing an 18:1 carbon chain sphingoid base, the most abundant species found in brain SM (Karlsson, 1970), which was the starting material used in the chemical synthesis of all SPC used here. FAB-MS of the second component with a peak retention time of 39 min yielded a mass spectrum indistinguishable from that of the first peak (Figure 2A and B). Consistent with the mass spectra of these two HPLC fractions, FAB-MS of the isomeric mixture also showed the presence of a single major protonated molecule ion at *m/z* 465 (Figure 2). Because the components resolved by HPLC yielded identical mass spectra (Figure 2A and B) that were also indistinguishable from that of high-purity nominal SPC obtained from Matreya (Figure 2), it was concluded that the two peaks correspond to the *erythro*- and *threo*-SPC.

Identification of erythro- and threo-isomers of SPC by ^1H and ^{13}C NMR

The ^1H NMR and ^{13}C NMR spectra of the two compounds separated by HPLC, dissolved in deuterated chloroform, were obtained on a Bruker-300 spectrometer. ^1H chemical shifts (p.p.m.) were calibrated against $CDCl_3$ at δ =7.24, whereas ^{13}C chemical shifts were measured against $CDCl_3$ at δ =77.00. The ^1H NMR spectrum of the first-eluting compound, believed to be *erythro*-(*2S,3R*)-SPC, was consistent with that of D-*erythro*-SM, which has been reported in the literature (Cohen *et al.*, 1984). The coupling constants $J_{4,5}$ = 15.2 Hz suggested that the two protons on the double bond between carbon atoms 4 and 5 are *trans* to each other. The proton on C-4 appears as a double doublet at 5.43 p.p.m. ($J_{4,5}$ = 15.1 Hz; $J_{5,6}$ = 7.6 Hz). The ^1H NMR spectrum of the second-eluting compound, resulting from the conformational reversion of the C-3 atom of D-*erythro*-SPC during the acid-catalyzed methanolysis, was assigned as *threo*-(*2S,3S*)-SPC and showed a

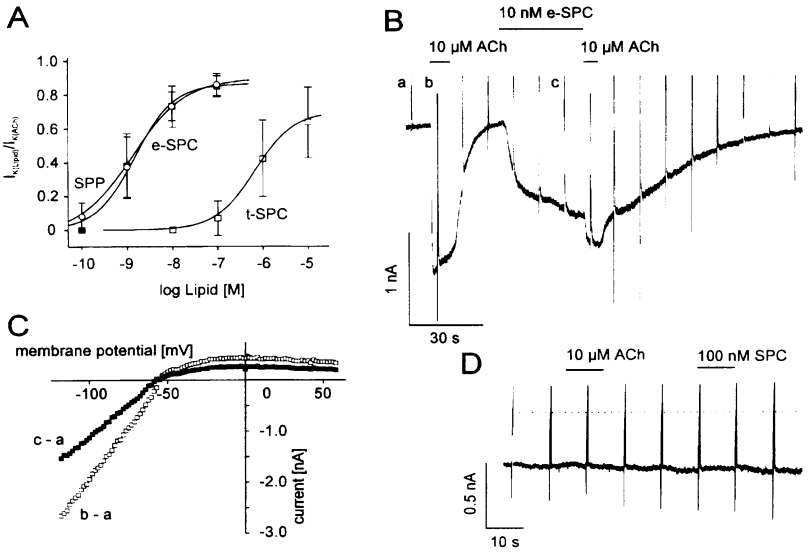


Fig. 3. Electrophysiological recording of K^+ currents in atrial myocytes elicited by SPC, SPP and ACh. (**A**) Dose–response relationship of currents elicited by SPP, *erythro*-(*2S,3R*)-SPC and *threo*-(*2S,3S*)-SPC. Note that SPP (○) and *erythro*-(*2S,3R*)-SPC (■) are approximately three orders of magnitude more potent than the second-eluting component from HPLC mostly comprising the *threo*-(*2S,3S*)-SPC isomer (□). (**B**) $I_{K(ACh)}$ and $I_{K(Lipid)}$ are nonadditive and converge on the same K^+ channel. A saturating 10 µM concentration of ACh is considered to activate 100% of the current. A 10 nM saturating concentration of SPC activates only 85% of the current; however, coapplication of 10 µM ACh results in the activation of 'only' 15% of the current. (**C**) Current–voltage curves of $I_{K(ACh)}$ and $I_{K(Lipid)}$, showing a strong inwardly rectifying current. (**D**) $I_{K(Lipid)}$ is abolished by PTX treatment. A brief 2 h treatment of the cells with 2 µg/ml PTX abolishes the current, suggesting the role of a PTX-sensitive heterotrimeric G protein in mediating the activation of K^+ channels.

double doublet at 5.45 p.p.m. ($J_{4,5}$ = 15.3 Hz; $J_{3,4}$ = 5.3 Hz) for the proton on C-4 and a double triplet at 5.71 p.p.m. ($J_{4,5}$ = 15.2 Hz; $J_{5,6}$ = 7.6 Hz) for the proton on C-5. Other parts of the ^1H NMR spectra of the two isomers were very similar. The ^{13}C NMR spectra of the two isomers, although highly similar, also showed remarkable differences between two pairs of peaks. The first-eluting compound gave peaks at 133.93 (C-5) and 73.09 (C-3) p.p.m., whereas the later-eluting isomer gave peaks at 132.55 (C-5) and 70.63 (C-3) p.p.m. The assignment of the chemical shifts to the specific carbon atoms were based on calculations by the ACD/CMR 1.0 computer program. Molecular modeling by the SYBYL/6.2 program demonstrated that the *threo*-SPC tends to form a stable intramolecular hydrogen bond between the amino group on the C-2 carbon atom and the hydroxyl group on the C-3 carbon atom, which may explain the shift to lower frequency (up-field shift) for the C-3 carbon atom. In contrast, *erythro*-SPC cannot form such a bond. This calculation, combined with the experimental results, indicates that the structure of the first-eluting compound is consistent with that of *erythro*-(*2S,3R*)-SPC, whereas the second is consistent with that of *threo*-(*2S,3S*)-SPC.

SPC activates $I_{K(ACh)}$ in atrial myocytes: evidence for stereoselective action

The measurement of $I_{K(ACh)}$ in atrial myocytes provides the most sensitive on-line assay for activation of G_i (for a review see Kurachi, 1995). We have shown recently that atrial $I_{K(ACh)}$ is activated by SPP with an EC_{50} of 1.2 nM (Bünemann *et al.*, 1995). The maximum current elicited by a saturating concentration of the phospholipid was identical to steady-state current in the presence of a saturating 10 µM concentration of ACh (Figure 3). Figure 3A compares the dose–response curves of SPP and both stereoisomers of SPC. SPP and *erythro*-SPC activated the current with similar potency (EC_{50} 1.2 versus 1.5 nM). The formal Hill coefficient fitting the curves was 1.0 for *erythro*-SPC and 0.8 for SPP. The other stereoisomer, *threo*-SPC, was only able to activate $I_{K(ACh)}$ with an EC_{50} of 0.65 µM, which is 430 times higher than that measured for the *erythro*-stereoisomer. This difference is likely to be due to a trace contaminant of *erythro*-SPC eluting with the *threo*-SPC peak that is virtually impossible to detect by our analytical methods. Given the very high affinity of the sphingolipid receptor for the *erythro*-isomer (EC_{50} 1.5 nM), a contaminant of as little as 0.2% present in the *threo*-SPC fraction could account for the responses seen with the *threo*-SPC fraction. Alternatively, the stereoselectivity of this sphingolipid receptor might not be absolute, and *threo*-SPC might indeed be a true ligand with a 430 times lower EC_{50}.

The almost saturating 10 nM concentration of *erythro*-SPC activates ~85% of the current available to activation by M_2 mAChR. The effects of ACh and SPC were nonadditive, i.e. in the presence of SPC at a concentration resulting in the activation of $I_{K(ACh)}$ to 80%, coapplication of ACh at a concentration of 10 µM results in 100% activation of $I_{K(ACh)}$. Thus, coapplication of saturating concentrations of ACh and SPC will only yield a current that equals that elicited by the full activation of the M_2 receptors (Figure 3B). The nonadditive nature of the current activated by the two ligands clearly demonstrates that the effector systems activated by either ligand converge on the same population of K^+ channels, a finding that is also supported by the identical strongly inward-rectifying current–voltage relationship characteristic of $I_{K(ACh)}$ (Figure 3C). The simultaneous application of 100 nM *threo*-SPC had no inhibitory effect on the response to 1 nM *erythro*-SPC, indicating that there was no partial antagonism of the *threo*-stereoisomer, and that the differences in sensitivity are likely to reflect different affinities

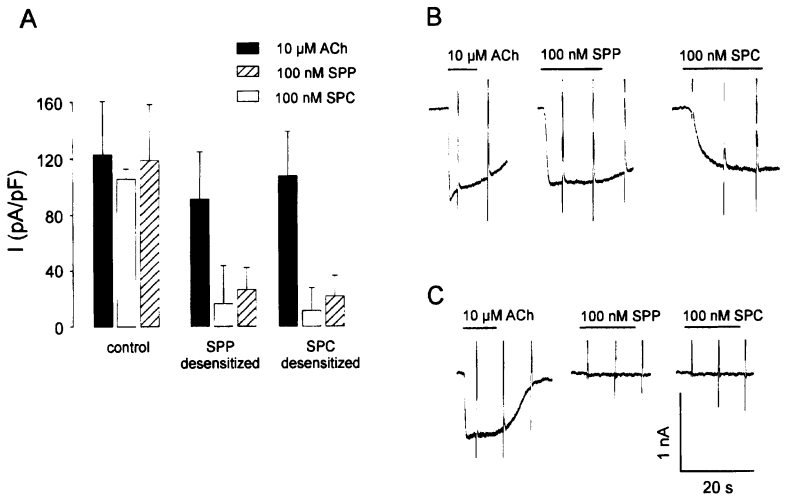


Fig. 4. Heterologous desensitization reveals that SPP and SPC activate the same receptors. (**A**) Statistical evaluation of heterologous desensitization experiments with SPC- and SPP-desensitized atrial myocytes. Note that, whereas desensitization with either sphingolipid did not affect the response to ACh, the responses to the both lipids were greatly diminished. (**B**) Typical current traces to ACh, SPP and SPC, which all activate I_K in atrial myocytes. (**C**) In cells that have been desensitized by prolonged application (2 h) to 100 nM SPP, SPC is no longer capable of activating $I_{K(ACh)}$.

to a single distinct binding site. As shown previously for SPP, activation of $I_{K(ACh)}$ by SPC was blocked completely by pretreatment of the cells with PTX. In six cells from three cultures tested, treatment with PTX at a concentration of 2 μg/ml for 2 h abolished responses to ACh, SPP and *erythro*-SPC (Figure 3D). In untreated cultures, every cell responded with a current of at least 0.5 nA upon exposure to either of these agonists.

The question of whether SPP and SPC act via a common sphingolipid receptor was investigated using a heterologous desensitization protocol. Cultures were treated either with 100 nM SPP or SPC for 2 h with a change of the ligand-containing medium after 1 h, or with 100 μM of the stabile muscarinic agonist carbamylcholine for 24 h. Thereafter, responses to ACh and both sphingolipids were recorded. Untreated cultures served as controls. Representative traces of membrane current recordings caused by saturating concentrations of the three ligands are illustrated in Figure 4. The response to ACh was characterized by a small acutely desensitizing component (Bünemann *et al.*, 1995) which was not present in the sphingolipid responses. However, the steady-state currents were identical for all three ligands in the control situation (Figure 4B). In cells treated with SPP, the response to ACh was unaffected; in contrast, responses to SPP and SPC were practically abolished (Figure 4C). Data from this series of experiments (Figure 4A) clearly demonstrated that pretreatment of the cells with either SPC or SPP desensitizes the cells for both sphingolipids without significantly affecting the M_2 receptor-mediated response to ACh.

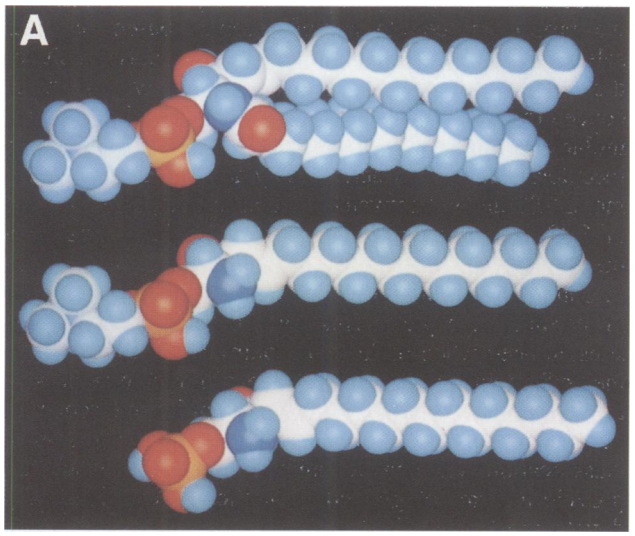
An analysis of several SPC derivatives and glycerolipids for their efficacy in activating $I_{K(ACh)}$ in atrial myocytes showed a strict structure–activity relationship of the sphingolipid receptor (Table I). Glycerolipid mediators and second messengers, including lysophosphatidic acid, lysophosphatidyl choline, platelet-activating factor (PAF), lyso-PAF, phosphatidic acid and diacylglycerol, were all inactive, indicating that the receptor expressed in myocytes is different from those activated by these glycerolipids and justifying the terminology of a putative sphingolipid mediator receptor.

Four major observations were derived from these experiments. First, the receptor showed an absolute requirement for the phosphate moiety on C-1, because SPP was highly active but sphingosine was completely inactive. The observation that a phosphocholine substitution on C-1 is fully active (SPC) is physiologically relevant because SPC was detectable in serum by HPLC and confirmed by FAB-MS as well as tandem mass spectroscopy (MS/MS; K.Liliom *et al.*, manuscript in preparation), providing strong evidence that this sphingolipid, like SPP (Yatomi *et al.*, 1995), is a normal constituent of a major body fluid. Second, alteration to the double bond between C-4 and C-5 has been shown to have a moderate effect on the activity, as the saturated or the triple bond-containing analogs were less effective than the naturally occurring one with a double bond. Third, the importance of the C-4, C-5 hydrocarbon region is supported by the observation that the analog with a hydroxyl group on C-4 in SPP (4-hydroxy-sphinganine) was inactive. Fourth, the fact that none of the N-substituted compounds—ceramides, ceramide phosphates, methyl- and acetyl-SPC—activated the receptor pinpoints the essential difference between the prototypical structures of sphingolipid mediators (SPP, SPC) and sphingolipid second messengers (ceramides). These data suggest the pivotal role of the free amino group in the interaction with the binding site of the putative sphingolipid mediator receptor. Moreover, because sphingosine was inactive, the unmodified sphingoid base structure is insufficient for activating the response, underlining the necessity of the phosphate moiety for lipid mediator activity.

Discussion

Experiments presented here establish that SPC activates a current indistinguishable from that activated by ACh in primary cultures of terminally differentiated supraventricular myocytes. However, SPC and ACh showed no cross-

Table I. Structure–activity relationship of the putative sphingolipid receptor expressed in atrial myocytes (**A**) Space-filling molecular model of SM (top), the putative precursor of SPP (middle) and SPC (bottom). (**B** and **C**) I_K responses to different sphingo- and glycerolipids structurally related to SPC.



Compound	I_K responses	
	Concentration	Response (% of SPP current)
B Sphingolipids		
erythro-SPC	100 nM	100
threo-SPC	100 nM	5
Dihydro-*erythro*-SPC	100 nM	100
N-Methyl-*erythro*-SPC	100 nM	0
N-Acetyl-*erythro*-SPC	10 μM	0
N-Acetyl-*threo*-SPC	10 μM	0
SPP	100 nM	100
Dihydro-SPP	100 nM	80
SPP-(4-5, triple-bond)	1 μM	75
4-Hydroxy-sphinganine-1-phosphate	100 nM	0
Sphingosine	1 μM	0
Sphingomyelin	10 μM	0
Ceramides (brain)	10 μM	0
Ceramides (C8)	10 μM	0
N-Acetylsphingosine (C2-ceramide)	10 μM	0
Dihydro-*N*-acetylsphingosine	10 μM	0
Ceramide-1-phosphate (C2)	10 μM	0
Ceramide-1-phosphate (C16)	10 μM	0
C Glycerolipids		
Lysophosphatidic acid (18:1)	10 μM	0
Phosphatidic acid (18:1)	10 μM	0
Diacylglycerol (18:1)	10 μM	0
Monoacylglycerol (18:1)	10 μM	0
Lysophosphatidylcholine	10 μM	0
Phosphatidylcholine	10 μM	0
Lysophosphatidylethanolamine	10 μM	0
Lysophosphatidylserine	10 μM	0
Lysophospahtidylglycerol	10 μM	0

desensitization, indicating that these compounds activated different receptors that converge to the same effector system, namely the K^+ channel encoded by the GIRK1 and CIR gene products (Krapivinsky *et al.*, 1995). SPC action was blocked completely by PTX, indicating that its signal transduction mechanism involves a heterotrimeric G protein, most probably of the G_i family. This receptor channel-coupling mechanism is identical to that activated by not only the M_2 AChR (Wickman *et al.*, 1994) but also another lipid mediator, SPP (Bünemann *et al.*, 1995).

Because SPC and SPP are structurally related, the possibility arose that they might activate the identical receptor. To test this possibility, we used heterologous desensitization techniques. Ligand activation of the receptor makes the activated receptor a highly specific target of a G protein-coupled receptor kinase, which in turn phosphorylates the receptor rendering it unresponsive to further stimulation by the ligand (Dohlman *et al.*, 1991; Takano *et al.*, 1994; Ablas *et al.*, 1995). The exquisite selectivity of the G protein-coupled kinases for the receptors that lead to their activation, which is the likely mechanism in our system, makes the desensitization technique a highly effective tool for distinguishing whether two ligands activate the same or different receptors (Liliom *et al.*, 1996). First, we have provided evidence that SPC and SPP both cause homologous desensitization of the $I_{K(Lipid)}$. Using heterologous desensitization between ACh, SPP and SPC, a clear pattern emerged, indicating that whereas neither lipid desensitized the response to ACh, they completely desensitized the responses to each other. This latter result provides an important line of evidence that SPP and SPC activate the same receptor(s). The short latency and comparable kinetics of the activation of the $I_{K(Lipid)}$ also argue against SPC being converted by some enzyme expressed on the cell surface that not only must display an extremely high turnover number but should convert any applied SPC into SPP with a nearly 100% efficiency. However, the expression of such a putative ecto-esterase enzyme in those cell types that respond to SPC and SPP remains to be demonstrated, as does the lack of its expression in other types of cell in which SPP but not SPC activates responses when applied in nanomolar concentrations. Lastly, the hypothesis of such an ecto-esterase is hardly consistent with data obtained from cell lines, e.g. HL60 and K563, that respond to SPC but not to SPP (van Koppen *et al.*, 1996).

The atrial sphingolipid receptor showed a high degree of specificity for the *erythro*-stereoisomer of SPC, whereas the *threo*-isomer was 430 times less active. Such unique preference for one stereoisomer over the other isomer has already been shown in the case of the PAF receptor (Wykle *et al.*, 1981), which can only be activated by the 1-*O*-hexadecyl-2-acetyl-*sn*-glycero-3-phosphocholine (L-PAF) stereoisomer and not by the 3-*O*-hexadecyl-2-acetyl-*sn*-glycero-1-phosphocholine (D-PAF). Moreover, the lack of inhibitory action of a 100-fold excess of *threo*-SPC on the response evoked by *erythro*-SPC provides further support for the chiral selectivity of the putative sphingolipid receptor.

SM is a ubiquitous constituent of the plasma membrane with preferential localization in the outer leaflet of the bilayer. The importance of glycerophospholipids, particularly phosphatidylinositol-*bis*-phosphate, as precursors of many important lipid second messengers and lipid mediators is overwhelming (for reviews see Nishizuka, 1992; Divecha and Irvine, 1995). SM has been proposed to fulfil a somewhat similar function, giving rise to ceramide, which has been shown to function as an important second messenger within the cell (Hannun *et al.*, 1993; Hannun, 1994; Hannun and Obeid, 1995).

This report, together with a few others (Bünemann *et al.*, 1995; Goodemote *et al.*, 1995; Wu *et al.*, 1995; Postma *et al.*, 1996; van Koppen *et al.*, 1996) on the receptor-mediated actions of SPP or SPC, indicates that these SM metabolites are highly potent lipid mediators acting on specific target cells expressing cognate receptors. The structural criteria delineated by the structure–activity studies indicate the requirement of the phosphate ester moiety on C-1, the unsubstituted amino group on C-2 and the chiral selectivity for the *erythro*-(*2S,3R*)-SPC analog. This set of structural features appears to define the prototypic structure of the SM-derived lipid mediator family and distinguishes it from the structural features of ceramide, an SM-derived second messenger.

Very little is known about the biochemical pathways generating SPC (cf. Bell *et al.*, 1993). The activation of sphingomyelinase, which breaks down SM into ceramide, has been shown to be coupled to the activation of growth factor receptors (Yang *et al.*, 1993; Dobrowsky *et al.*, 1994, 1995). It is conceivable that the generation of SPC is also receptor regulated via an as yet uncharacterized SM-breakdown mechanism (acyl-esterase/transferase) or, alternatively, from ceramide (CDP-choline transferase). The enzymatic pathways—remodeling and/or turnover type—and the stimuli activating/regulating the enzymes generating these lipid mediators should now become an important focus of investigation. SPP is generated from activated platelets (Yatomi *et al.*, 1995), and SPC is one of several plasma sphingolipids (Banach *et al.*, 1993a,b; Bünemann *et al.*, 1994; K.Liliom *et al.*, manuscript in preparation) that activate $I_{K(ACh)}$, suggesting the existence of enzymatic mechanisms generating these highly potent mediators. Whether SPP, SPC or these other yet unidentified plasma sphingolipids (Banach *et al.*, 1993a,b; Bünemann *et al.*, 1994; K.Liliom *et al.*, manuscript in preparation) all represent physiologically relevant ligands of the sphingolipid receptors also remains to be investigated. Hannun and Bell (1987) proposed that lysosphingolipids play a pathogenic role in the inborn metabolic disorders known as sphingolipidoses. In light of our findings, we propose to expand this hypothesis to accommodate the receptor-mediated effects of lysosphingolipids as a part of this pathogenic process.

SPC and SPP appear to possess lipid mediator functions in several types of cell, whereas there are cell types in which no ion current responses are evoked by either sphingolipid (e.g. *Xenopus* oocytes; G.Tigyi, unpublished observation). SPP in nanomolar concentrations has been shown to activate Ca^{2+} transients in A431 carcinoma cells (Jalink *et al.*, 1995) and in Swiss 3T3 fibroblasts (Olivera *et al.*, 1994). In contrast, micromolar concentrations of SPC were required to elicit Ca^{2+} mobilization in Swiss 3T3 fibroblasts and human embryonic kidney HEK 293 cells that also responded to nanomolar concentrations of SPP (van Koppen *et al.*, 1996). Interestingly, there are several cell types, including HL60 promyelocytic leukemia, K563 erythroleukemia cells (van Koppen *et al.*, 1996) and human tracheal epithelial cells (L.Pott, unpublished observations), in which SPP is inactive, even if applied at a very high 10 μM concentration.

The lack of responses in some cells and the G protein-mediated action in other cells responding to SPP provide the foundation for a novel hypothesis that SPP and SPC might act through several subtypes of bona fide G protein-coupled plasma membrane receptors. It is already quite clear from the data available in the literature that the selectivity to SPP and SPC of the putative sphingolipid receptors might provide the basis of a pharmacological classification of the sphingolipid receptor family. Here, we propose three classes of receptor based on their selectivity to SPC and SPP. The first class includes those receptors that are only activated by nanomolar concentrations of SPP and not activated by high micromolar concentrations of SPC. The second class includes receptors that are only activated by nanomolar–low micromolar SPC and not activated by micromolar concentrations of SPP. The third class of receptor is characterized by their comparable sensitivity to nanomolar concentrations of both SPP and SPC, like the one described in this study in cardiac myocytes. This classification can only be substantiated when molecular cloning techniques identify the individual genes encoding the different receptors in the different cell types. However, until such sequence data are available, we believe that this pharmacological classification will help to build a simple consensus and provide a terminology widely applicable to this rapidly developing field of lipid mediator research.

Materials and methods

Materials

Isomeric mixtures of D-*erythro*/L-*threo*-SPC used here were either purchased from commercial suppliers (Matreya Inc., Pleasant Gap, PA or Sigma Chemical Co., St Louis, MO) or prepared by acid methanolysis of bovine brain SM purchased from Sigma or Avanti Polar Lipids (Alabaster, AL), according to the method of Gaver and Sweeley (1965). D-*Erythro*-SPP and ceramide were purchased from Biomol Fine Chemicals (Plymouth Meeting, PA). D-*Erythro/threo*-acetyl-SPC, D-dihydro-*erythro*-SPC and D-*erythro*-sphingosine were obtained from Matreya. The saturated and triple bond-containing analogs of SPP, and the ceramide phosphate, were generously provided by Dr Robert Bittman (Queens College, City University of New York); 4-hydroxy sphinganine 1-phosphate was isolated from the polar lipid extract of bovine brain (Avanti Polar Lipids). The purity ($[M+H]^+$) of the compounds used in this study has been confirmed in every case by FAB-MS.

HPLC of SPC stereoisomers

Racemic mixtures of SPC were chromatographed on a 5 μm silica-packed 250.0×4.6 mm Microsorb column (Rainin Instruments, Woburn, MA). To separate the isomers, a gradient of two solvents was developed. Solvent A was made of 60% chloroform, 35% methanol, 4.5% deionized water and 0.5% ammonium hydroxide; solvent B consisted of the same ingredients with proportions of 30:60:9.5:0.5, respectively. A gradient of the two solvents was applied by increasing solvent B to 100% over 25 min, followed by a 30 min isocratic period at a flow rate of 1 ml/min, using a Waters pump hardware controlled by the Milleneum software package (Waters Instruments, Milford, MA). Elution of the lipids was monitored by a Varex evaporative light scattering detector (Burtonsville, MD) through a metering valve, splitting the effluent 1:4 between the detector and a fraction collector, respectively. HPLC-grade solvents were obtained from J.T.Baker Chemical Co. (Phillipsburg, NJ).

Analytical methods

Mass spectroscopy was performed with an AutoSpecQ (E1BE2qQ) tandem mass spectrometer (VG Fisions, Altrincham, UK) using the VG Opus level 1.7f software package for data analysis. A glycerol matrix (1 μl) was deposited on the sample holder, and CsI was used for the calibration of the instrument.

Space-filling molecular models of the various sphingolipids were built by using the SYBYL software package (v.6.2, Tripos Associates Inc., St Louis, MO) implemented on a Sun 4-260 workstation and displayed on an Evans & Sutherland PS390 graphics display terminal.

Isolation and culture of atrial myocytes

The method of enzymatic isolation of atrial myocytes from guinea pig heart has been described in detail elsewhere (Banach *et al.*, 1993a). Isolated myocytes were cultured in a chemically defined M199 medium (Life Technologies, Dreicheich, Germany) without serum, containing gentamycin and kanamycin (10 μg/ml; Sigma Chemical Co., Deisenhofen, Germany). Myocytes were maintained in this medium for up to 8 days, with medium replacement every other day. The culture medium was replaced 30 min prior to electrophysiological recording by a solution containing (in mM) NaCl 120, KCl 20, $CaCl_2$ 2.0, $MgCl_2$ 1.0 and HEPES–NaOH 10.0, pH 7.4.

Electrophysiological recording

Membrane currents were measured with whole-cell voltage clamp recording (Hamill *et al.*, 1981). Pipettes were pulled from borosilicate glass with filament (Clark Ltd, Pangbourne, UK) on a horizontal puller (DMZ, Munich, Germany). The resistance of the pipettes ranged from 2 to 6 MΩ. The solution for filling the patch pipettes contained (in mM) K-aspartate 100, KCl 40; Mg-ATP 5.0; EGTA 2.0; GTP 0.01; HEPES–KOH 10.0, pH 7.4. Dialysis of the cells with the above solution resulted in ACh-evoked currents with a highly reproducible steady-state amplitude throughout the recording session. Currents were measured by an LM/EPC7 patch clamp amplifier (List, Darmstadt, Germany). Signals were analog filtered with a corner frequency of 1–3 kHz and stored digitally on an IBM-compatible computer. Voltage control, data acquisition, storage and evaluation were performed by an ISO2 software package (MFK, Frankfurt-on-Main, Germany). Experiments were performed at ambient temperature (22–24°C). Cells were voltage-clamped at a –90 mV holding potential, negative to E_K (–50 mV). K^+ conductances under these conditions result in currents in the inward direction. This experimental condition was chosen because of the strong inward-rectifying properties of $I_{K(ACh)}$.

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