ORIGINAL ARTICLE

Sphingosine modulation of cAMP levels and beating rate in rat heart

V. Edda Benediktsdóttir[a]*, Anna M. Jónsdóttir[a], Bergthóra H. Skúladóttir[a], Alain Grynberg[b], Jón Ó. Skarphéðinsson[c], Jóhannes Helgason[c], Sigmundur Gudbjarnason[a]

[a]*Science Institute, University of Iceland, Vatnsmýrarvegur 16, IS-101, Reykjavik Iceland*
[b]*INRA-Faculté de Pharmacie, Lipides membranaires et fonctions cardiovasculaires, Batiment D1 3éme étage 5 Rue Jean, Baptiste, Clemént, 92290 Chatenay, Malabry, France*
[c]*Department of Physiology, University of Iceland, Vatnsmýrarvegur 16, IS-101, Reykjavik Iceland*

Keywords
adrenergic receptors,
cAMP,
cardiomyocytes,
heart rate,
sphingosine

Received 3 August 2001;
revised 28 November 2001;
accepted 13 May 2002

*correspondence and reprints:
eb@raunvis.hi.is

ABSTRACT

Sphingolipids, especially as elements of the sphingomyelin signal transduction cycle, are thought to play a significant role as second messengers and modulators of events in heart muscle cells. A possible modulatory role of sphingosine in signal transduction in the β-adrenergic pathway in the heart was examined. Neonatal rat cardiomyocytes were incubated with sphingosine and/or other agents after which cAMP levels and contraction rates were measured. Heart rate in anaesthetized rats was also measured before and after sphingosine injection in the jugular vein. Sphingosine caused a decrease in basal cAMP levels and diminished isoproterenol-induced increase in cAMP levels. These changes were dose- and time-dependent and showed a significant negative effect on signal transmission in the β-adrenergic pathway in cardiomyocytes. Increase in cAMP intracellular levels by forskolin, which activates adenylcyclase, was not inhibited by sphingosine. A phosphodiesterase inhibitor was used in all experiments in which cAMP was measured excluding effects on cAMP breakdown. It was also demonstrated that sphingosine caused reduction in the beating rate of cultured cardiomyocytes and a dose-dependent reduction in heart rate of anaesthetized rats. The sphingosine-induced inhibition of bradycardic response of anaesthetized rats reached a maximum about 5–10 min after the onset of sphingosine administration and returned to normal within 60 min. Sphingosine may modulate the signal transmission of the β-adrenoceptor pathway upstream of adenylcyclase in rat cardiomyocytes. This may contribute to the sphingosine-induced decrease in heart rate of rats in vivo.

INTRODUCTION

Sphingolipids are present in plasma membranes in all mammalian cells, predominantly in the outer leaflet. They are a class of lipids with common backbones termed long-chain sphingoid bases. Ceramide, which is the hydrophobic portion of the sphingolipids consists of a sphingosine base linked to a fatty acid by an amide bond.

There is growing evidence that complex sphingolipids as well as simple sphingoid molecules such as sphingosine, sphingosine-1-phosphate, ceramide and ceramide-1-phosphate participate in the intracellular transfer of molecular signals. It has been shown that sphingolipid- and sphingoid-mediated signalling is involved in important cellular events like proliferation, differentiation, apoptosis and reaction to stress conditions [1,2,3,4]. The sphingomyelin signal transduction system represents a stimulus-regulated breakdown of the membrane phospholipid sphingomyelin. A particular extracellular ligand binds to its receptor and activates a plasma membrane-bound sphingomyelinase, releasing ceramide which is the mediator of the intracellular effects of the ligand. In many cases the ceramide is further cleaved to sphingosine, which can then be phosphorylated to form

sphingosine-1-phosphate. Different stimuli applied to cultured cells or animals elicit a series of metabolic events that result in the production of distinct sphingoid molecules which, in turn, participate in the intracellular signalling network. Sphingolipid signalling is emerging as a sophisticated mechanism that is strictly integrated with other cellular signal pathways.

Endogenous sphingosine levels have been determined for rabbit cardiac and skeletal muscle which exhibited high levels of sphingosine (17 pmol/g). Isolated T-tubule membranes display high contents (1400 pmol/g) suggesting that sphingosine may be an important messenger in cardiac and striated muscle [5]. These high levels of sphingosine observed in T tubules may relate to an important regulatory role of this sphingoid molecule in cardiomyocytes.

Previous studies have shown that sphingoid molecules exert divergent effects on cellular cAMP levels, either acting in an activatory or inhibitory manner, depending on the experimental model [4]. Studies on the effects of sphingoid molecules on cultured cardiomyocytes might thus be of considerable interest.

Some aspects of the effects of sphingosine in animal heart have been studied, mostly in cardiac myocytes. It was shown that sphingosine inhibits Ca^{2+} transients by inhibiting Ca^{2+} flow through the voltage-dependent Ca^{2+} channels and by direct binding to ryanodine receptors causing diminished Ca^{2+} release from the sarcoplasmic reticulum [6,7]. Furthermore it has been demonstrated that sphingosine mediates the negative inotropic effect of the cytokine tumour necrosis factor (TNF)-α [8,9].

The purpose of this study was to investigate the putative modulatory role of sphingosine in mechanisms controlling the heart function in rats, especially the β-adrenoceptor signal transduction system.

MATERIALS AND METHODS

The investigation conforms with the *Guide for the Care and Use of Laboratory Animals,* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Cell culture

Cells were cultured according to the method of Harary and Farley [10] as modified by Post et al. [11]. Neonatal Wistar rats (2–4 days old) were decapitated, and the hearts excised and minced. The mince was incubated for 10-min in a spinner flask, with a magnetic stirrer, at 37 °C with 0.6% trypsin (1:250; Difco Laboratories, Detroit, MI, USA) 100 IU/mL penicillin and 100 mg/L streptomycin in a buffered saline solution (137 mM NaCl, 5 mM KCl, 4 mM $NaHCO_2$, 5 mM glucose, pH 7.4). Nine such incubations were made with increasing the stirring rate. The supernatants from the first three incubations were discarded but after each of the remaining six incubations the supernatants were collected and by the end of the last incubation the mince was almost completely digested. The cell pellets were spun (8 min, 430 g) and resuspended in Ham's F10 growth medium from Gibco (Paisley, UK) supplemented with 10% foetal calf serum (Gibco), 10% horse serum (Gibco), 100 IU/mL penicillin, 100 mg/L streptomycin, 10 μM arabinose-C and 1 mM $CaCl_2$. The cells were plated on Falcon culture dishes (100 mm; Becton-Dickinson Labware Europe, Le Pont de Claix, France) for 2.5 h, during which time fibroblasts adhere and myocytes remain freely suspended. Finally, the myocytes were plated on 35 mm Primaria treated culture dishes (Falcon Plastics) and incubated at 37 °C with 5% CO_2. The growth medium was renewed 24 h after seeding and then every 48 h. The cells were used for experiments on day 4 when the cells were confluent and beating rhythmically.

Sphingosine supplementation and stimulation of cells

A 1 mM stock solution of D-erythro-sphingosine from bovine brain cerebrosides (Sigma-Aldrich Chemie GmbH, Tauf Kirchen, Germany), was made in chloroform. Different amounts of this solution were added to a beaker to make different final concentrations for the dose response experiment, and to a 10 μM final concentration in all other experiments. The chloroform was evaporated and the sphingosine was dissolved in the usual growth media, except that it contained 1 mM of the phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (Sigma). The same growth medium was used in parallel for the control cells. The cells were usually incubated for 15 or 30 min with sphingosine. Then the incubation was stopped and the intracellular cAMP levels were measured. When the effect of sphingosine on the cAMP levels during isoproterenol or forskolin stimulation was measured, isoproterenol or forskolin were added to the incubation medium 5 or 15 min, respectively, before the end of incubation to make a final concentration of 1 or 100 μM, respectively.

Measurement of cAMP

Incubations were stopped by removing the medium and adding 1.0 mL of cold 10% trichloroacetic acid (TCA) to

each dish. After 60 min at 2–4 °C, the contents of the dishes (except the membrane layer on the bottom) were centrifuged for 5 min at 5200 *g*. The supernatants were extracted 4x with H_2O-saturated diethylether to remove the TCA. The pH was adjusted to 7.0 by addition of 2% Tris buffer. The intracellular cAMP content was then determined by [^{3}H]cAMP protein binding method using a commercial kit (Amersham Biosciences UK Ltd, Little Chalfont, UK).

Measurements of beating rate in cardiomyocytes

The beating rate was measured as previously described [12] on cells grown in 35-mm culture dishes. The culture medium was withdrawn and replaced by a standard saline solution containing glucose and free calcium (1.2 mM). The dishes were placed on the heated stage of an inverted microscope (36 ± 1 °C) fitted with a video camera. The cells were allowed to equilibrate for 10 min on the microscope before recording. To obtain the basal contraction, recordings were made for 2 min before adding any experimental agent to the cell media. Single cell contractions were recorded by the use of custom-made online motion detector [13] and the electrical signals analysed with custom-made computer software. Sphingosine was added as 25 μL of stock solution (10 μM final concentration). After 30 min, the adrenergic system was stimulated by isoproterenol addition (5 μM).

Measurements of heart rate in anaesthetized rats

Ten Wistar rats at the age of 6 months were used. The rats were initially anaesthetized with methohexital sodium (70 mg/kg i.p.; Brietal, Lilly, IN, USA), and then received two boluses with 100 mg/kg thiobutabarbital sodium, (Inactin; Research Biochemicals International, Natic, MA, USA) with 1/2–1-h interval. The rats were tracheotomized and prepared with catheters in the tail artery [PE25] for arterial pressure recording and the right jugular vein (PE25) for i.v. injections. Rectal temperature was continuously recorded and maintained at 37.5 °C by external heating. Arterial pressure was measured from the tail artery with a Micro Switch 156PC pressure transducer (Micro Switch, Freeport, IN, USA). Heart rate was measured from the pulsating arterial pressure signal with a rate meter and the mean arterial pressure obtained by electronic filtering. The variables were recorded on a Grass polygraph (Grass Instrument Division, Astro-Med Inc., West Warwick, RI, USA). After completion of surgery, at least 1 h was allowed with no disturbance to the animal and if the animal was stable in heart rate and pressure' the recordings and injections were started.

A 1 or 2.5 mg/mL sphingosine were dissolved in 2% Tween-80 enabling adjustments of injection volume according to body weight to give doses of 1 or 2.5 mg/kg sphingosine. The same volume of vehicle (2% Tween-80) was first injected into the jugular vein of the rats. After 1 h, 1 and 2.5 mg/kg sphingosine were injected in random order with 1 h recovery between the injections. The rat weight was 0.306 ± 0.007 kg (mean ± SE) ($n = 10$).

Protein measurements

After addition of TCA and removal of the supernatant from the culture dishes the cellular proteins on the culture dishes were dissolved in 1 M NaOH and measured with the method of Zaman et al. [14] using bovine serum albumin (Sigma) as a standard.

Statistics

The results are reported as mean ± SE. ANOVA and Scheffe *F*-test or paired Student's *t*-test was used for statistical comparations. Significant differences were considered present at $P < 0.05$.

RESULTS

Effect on intracellular cAMP levels

Stimulation of the β-adrenergic receptor with 1 μM isoproterenol resulted in a significant increase in the intracellular cAMP levels in the cardiomyocytes. A dose–response curve that shows the effect of isoproterenol concentration on cAMP levels is shown in *Figure 1*.

Figure 2a shows the dose-dependent decrease in basal (open symbols) and isoproterenol-stimulated (filled

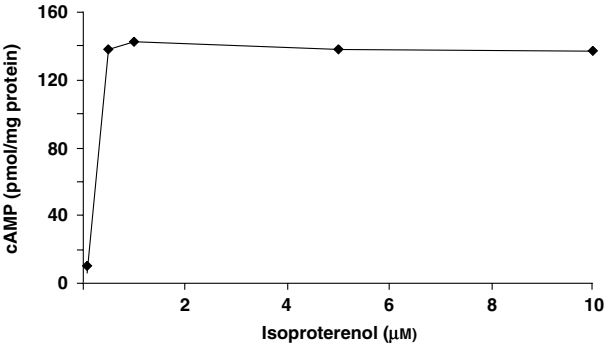

Figure 1 Intracellular cAMP levels in neonatal rat cardiomyocytes after stimulation with isoproterenol in the indicated concentrations for 5 min. The dose–response curve is representative of three experiments.

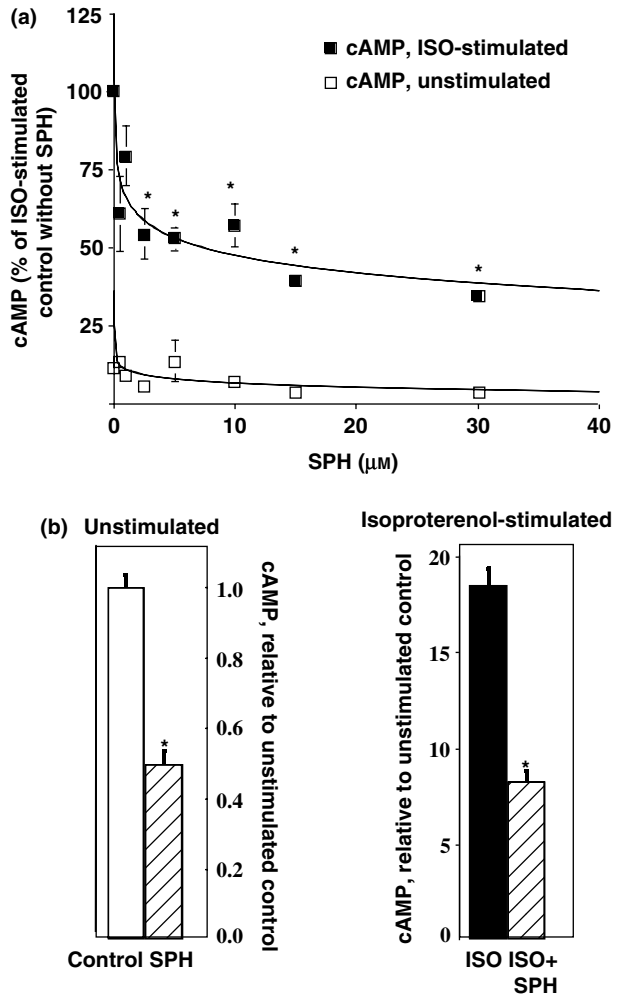

Figure 2 (a) Intracellular levels of cAMP in unstimulated (open symbols) or isoproterenol (ISO) stimulated (filled symbols) cardiomyocytes after 30-min incubation with the indicated concentrations of sphingosine (SPH). The results are expressed as cAMP levels with SPH as percentage of cAMP level of control in the absence of SPH, mean ± SE., $n = 3–5$, originally measured as pmol cAMP/mg protein. (b) The effect of 10 μM sphingosine incubation on cAMP levels in unstimulated and isoproterenol (ISO, 1 μM) stimulated cardiomyocytes. The cAMP levels are shown as fold stimulation compared with unstimulated control (means ± SE, $n = 4$).
* $P < 0.05$ compared with control (without sphingosine), ANOVA and Scheffe *F*-test. See Materials and Methods for further information on experimental detail.

symbols) intracellular cAMP levels in the rat cardiomyocytes after a 30-min incubation of the cells with sphingosine in the concentration ranged 0.5–30 μM. Isoproterenol stimulated cAMP levels in the cardiomyocytes were lowered significantly after incubation with 2.5, 5, 10, 15, and 30 μM sphingosine compared with control. The experiment shown in *Figure 2b* confirms a significant difference between cAMP levels in the presence and absence of 10 μM sphingosine both in unstimulated cells and cells stimulated with isoproterenol.

The decrease of cAMP levels in the cardiomyocytes induced by sphingosine was rapid both in isoproterenol-stimulated and unstimulated cells. After 5–15-min incubation with sphingosine, the cAMP levels were decreased by 40–50% and after 60 min the cAMP levels were still decreased. This time course of the decrease in cAMP intracellular levels by sphingosine was similar in unstimulated and isoproterenol-stimulated cardiomyocytes.

The effect of sphingosine on cAMP levels in cardiomyocytes could have been the result of direct or indirect inhibition of adenyl cyclase. To investigate this, cells were stimulated with different concentrations of forskolin with or without a 15-min incubation with 10 μM sphingosine as seen in *Figure 3a*. This experiment did not indicate inhibition of forskoline stimulation of cAMP levels by sphingosine. In the next experiment one concentration, 100 μM, of forskolin was used to stimulate the cells in the presence or absence of sphingosine (*Figure 3b*). The forskolin- stimulated cAMP levels were not lowered by sphingosine. This shows that sphingosine does not affect the adenyl cyclase activity in rat cardiomyocytes.

Effect on the beating rate in cardiomyocytes

The basal beating rate of neonatal cardiomyocytes was 151 ± 19.6 (mean ± SE) in the range of 102–261 beats/min. After sphingosine addition to the incubation medium, a sharp decrease in the frequency of contractions was observed. It was 83 ± 2.9 beats/min after 30-min incubation with sphingosine (*Figure 4*). The chronotropic response to isoproterenol stimulation in the presence of sphingosine was positive (+18%) but failed to restore the initial frequency of contractions measured before the addition of sphingosine (*Figure 4*, inset).

Effect on heart rate in anaesthetized rats

The results showing that sphingosine causes a decrease in intracellular cAMP levels and a decrease in frequency of contractions in cardiomyocytes in vitro raised questions about the effects of sphingosine in vivo. Therefore, the heart rate in anaesthetized rats was measured before and after sphingosine administration. The average heart rate before i.v. sphingosine injection was 403 ± 13 beats/min, ($n = 10$). The sphingosine injection caused a significant bradycardic response. The time course of the alterations in heart rate in one represen-

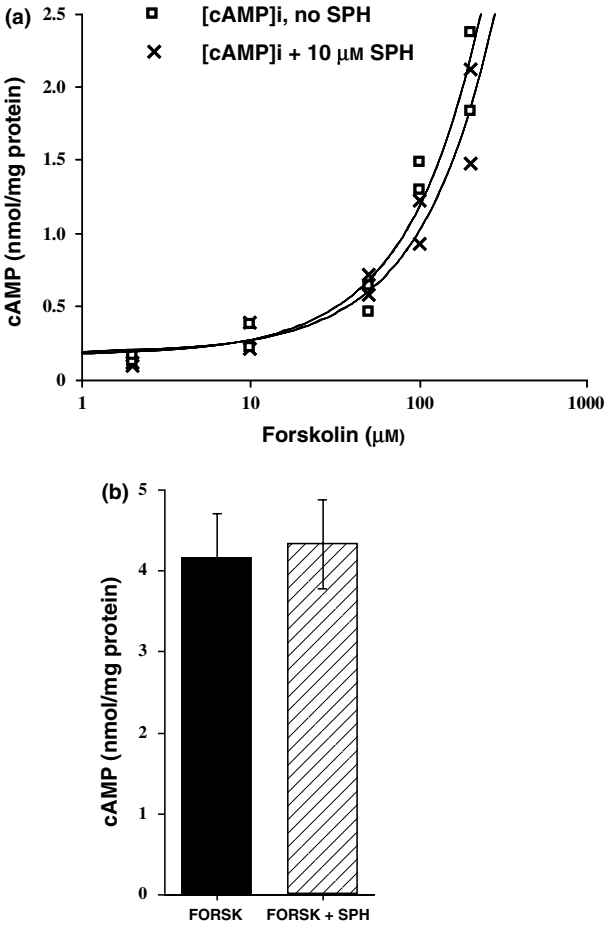

Figure 3 (a) cAMP levels in neonatal rat cardiomyocytes after stimulation with the indicated concentrations of forskolin (FORSK) (dose–response) for 5 min with or without a 15-min preincubation with 10 μM sphingosine (SPH). (b) The cAMP as in A, but stimulated only with 100 μM forskolin. The results are expressed as nmol cAMP/mg protein (means ± SE, $n = 11$).

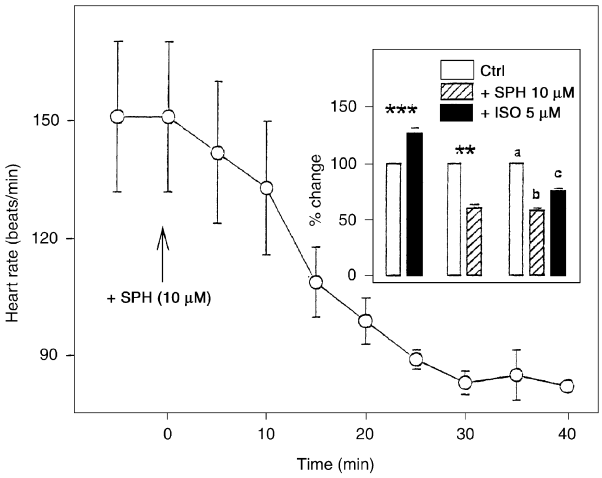

Figure 4 Decrease in the beating rate of rat cardiomyocytes during the first 40 min of incubation with 10 μM sphingosine (SPH). The results are expressed as beats/min (means ± SE, $n = 4$–8 for each measurement).

Inset: Effects of sphingosine and isoproterenol on contraction rate of rat cardiomyocytes. From left: effect of 5 μM isoproterenol (Iso) alone, ($n = 14$), effect of 10 μM sphingosine (SPH) incubation for 30 min alone ($n = 8$), effect of sphingosine and sphingosine + isoproterenol ($n = 4$). The results are given as beats/min, percentage compared with control (Ctrl, white bars) (means ± SE; **, $P < 0.01$; ***, $P < 0.001$); (ANOVA). The bars affected with different letters (**a**, **b** and **c**) are significantly different ($P < 0.01$); (ANOVA).

tative experiment of 10, which all gave similar results, is shown in *Figure 5a*. In all the experiments a maximal heart rate response was usually reached within 5 min and the heart rate returned to normal within 1 h.

The decrease in heart rate in response to sphingosine was dose-dependent (*Figure 5b*). The heart rate decreased by about 30 and 60 beats/min after injection of 1.0 and 2.5 mg/kg sphingosine, respectively.

DISCUSSION

The results of this study suggest that sphingosine may be a negative modulator of a signal transduction system in the rat heart, possibly the β-adrenergic signalling system. This would explain the diminished basal and isoproterenol-stimulated cAMP levels, reduced frequency of contractions of cardiomyocytes and the bradycardic response in anaesthetized rats after exposure to sphingosine.

The direct intracellular targets of sphingoid biomediators, identified so far, are enzymes or receptors specifically activated by those molecules. Sphingoid-mediated signals bring about activation of enzymes by phosphorylation or dephosphorylation and there seems to be extensive cross-talk between the sphingoid molecule mediated system and other signalling systems [15]. Previous studies have shown that sphingoid molecules exert different effects on intracellular cAMP levels depending on the cell type and type of molecule. It has been shown that sphingosine reduces the basal production of cAMP in Swiss 3T3 fibroblasts [16], whereas it activates adenylate cyclase and stimulates cAMP formation in airway smooth muscle cells [17]. In the latter, potential interconversion of sphingosine and sphingosine-1-phosphate was suggested as a switch that can elicit reciprocal changes in the intracellular levels of cAMP. According to studies summarized by Riboni et al. [4], sphingoid molecules affect

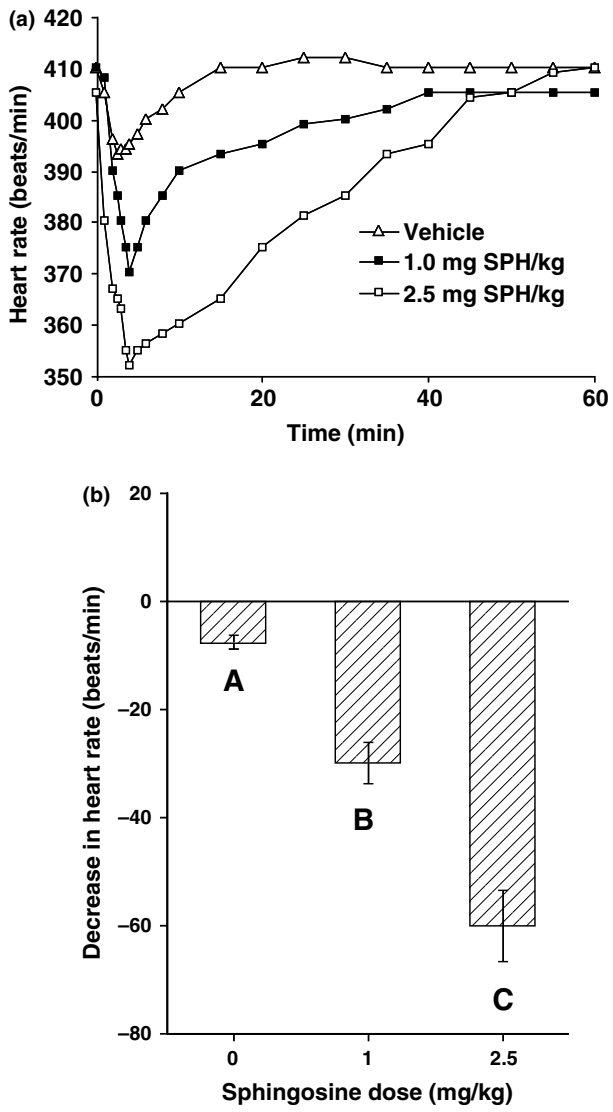

Figure 5 (a) Time course of alterations in heart rate (beats/min) in anaesthetized rats after injection of vehicle or sphingosine (SPH) in the indicated doses into vena jugularis. The figure shows results of one representative experiment of 10 with similar results. (b) Decrease in heart rate (HR) of anasthetized rats after injection of vehicle only (2–4% Tween-80) or sphingosine in the indicated doses into vena jugularis. The results are expressed as means ± SE ($n = 10$). Significant difference between bars with different letters (**A**, **B** and **C**) ($P < 0.02$); (ANOVA-repeated measures, Scheffe F-test). For further information on experimental details, see Materials and Methods.

cellular cAMP levels by modification of ligand-dependent signal systems and both basal levels and levels after ligand stimulation are affected. Furthermore, in these studies on different cell types, sphingoid molecules have been observed to act on adenylcyclase, phosphodiesterase or upstream of adenylcyclase.

The present study showed that, in cardiomyocytes, sphingosine decreased basal levels of cAMP and also decreased cAMP levels in the presence of the β1-adrenoceptor agonist isoproterenol in a concentration-dependent way, which indicates direct or indirect sphingosine modulation of the β-adrenoceptor pathway. Forskolin stimulation was not inhibited by sphingosine which shows that sphingosine acts upstream of adenyl cyclase and may be a negative modulator of the β-adrenoceptor itself or its Gs-protein.

Webster et al. [7] observed that sphingosine reduced the rate of contraction in skinned rat cardiomyocytes. It inhibited Ca^{2+} release from sarcoplasmic reticulum (SR; the intracellular Ca^{2+} store) by directly affecting the ryanodine receptor which consists of SR–Ca^{2+}channel and a 'footprotein'. When the sarcolemma in the cardiac muscle cell becomes depolarized, the inducer for the SR–Ca^{2+} release is thought to be 'trigger' Ca^{2+} which enters the cell from the T-tubule lumen through the voltage sensitive L–Ca^{2+}channel in the sarcolemma. Further studies showed that sphingosine inhibited exitation–contraction coupling in cardiomyocytes in two ways, by reducing the amount of entering 'trigger' Ca^{2+} and by simultaneously raising the threshold of the ryanodine receptor for Ca^{2+}-induced Ca^{2+} release [6].

The decrease in cAMP levels observed in this study may contribute to the decrease in the beating rate in rat cardiomyocytes and in vivo. Reduction of the amount of entering 'trigger' Ca^{2+} could result in lower frequency of contractions because of the decrease in cAMP level in the cell. A rise in cAMP level in the cell after β-adrenoceptor stimulation leads to activation of the L–Ca^{2+}channel and an increase in calcium flow through the channel after depolarization [18]. This is considered to be the main cause of the inotropic and chronotropic effects of β-adrenergic agonists such as isoproterenol and would be reversed by the decrease of cAMP caused by sphingosine.

The inhibition of the SR–Ca^{2+}release channel (ryanodine receptor) and voltage-dependent L–Ca^{2+} channel caused by sphingosine has also been reported to have negative inotropic effects in isolated mammalian myocytes. It was shown that sphingosine mediated the negative inotropic effect of the cytokine TNF-α which caused an increase in sphingosine production [9, 19]. These effects were not caused by sphingosine-1-phosphate or ceramide [9,20].

Sphingosine may be converted to sphingosine-1-phosphate after uptake into the myocyte. Sphingosine-

1-phosphate would then act intracellularly to reduce cAMP levels and decrease contraction frequency. Sphingosine-1-phosphate has been reported to have negative chronotropic effects in guinea-pig atrial myocytes [21] and rabbit sino atrial node cells [22] mainly because of activation of a background, inwardly rectifying K^+ channel. In the former case it was shown that this occurred strictly when sphingosine-1-phosphate was applied at the extracellular surface of the plasma membrane [21] and the effects were attributed to sphingosine-1-phosphate activation of a Gi-protein coupled receptors. Other experiments have shown opposite effects of sphingosine-1-phosphate: it increased the sinoatrial node and decreased coronary blood flow (coronary vasoconstriction) in dog papillary muscle [23], which points to species variation. In a rat in vivo model it was shown that i.v. administration of sphingosine-1-phosphate decreased the heart rate, ventricular contraction and blood pressure [24]. Later reports confirmed the extracellular action of sphingosine-1-phosphate. It has been demonstrated that sphingosine-1-phosphate is a ligand for the Gi-protein coupled EDG-1 receptors in rat cardiomyocytes. It induced hypertrophy of the cells via the EDG-1 receptors and subsequently via the Gi-protein through ERKs, p38 MAPK, JNK, P13K and the Rho pathway [25]. Nakajima et al. [26] demonstrated that rat cardiomyocytes express EDG-1 receptors that respond to sphingosine-1-phosphate and mediated calcium overload in the myocytes.

In the present study, sphingosine was added to the growth medium or administrated intravenously and it is expected to easily pass the cell membrane which is consistent with internal action of sphingosine, i.e. decreasing the intracellular cAMP level.

As mentioned above, other reports have shown that sphingosine decreases the contraction rate in spontaneously contracting cardiac cells [6,7] in agreement with this study. The effect of sphingosine on the heart rate in animals in vivo reported here has to our knowledge not been shown before. The decrease in heart rate in the rats was dependent on the dose of sphingosine in the range 0–2.5 mg/kg (*Figure 5b*). The bradycardic response to sphingosine in vivo was transient (*Figure 5a*) as can be expected if sphingosine serves as a regulatory function. The sphingosine- induced reduction in contraction frequency of cardiac myocytes in vitro persisted for more than 30 min (*Figure 4*). The rate of removal of sphingosine and conversion to other sphingolipids can be expected to be quite different in the animal and in myocytes in culture.

CONCLUSION

The results show that sphingosine decreases heart rate in vivo and the frequency of contractions in rat neonatal cardiomyocytes. It also decreases the intracellular cAMP levels both time and dose dependently in the presence or absence of isoproterenol stimulation without direct effects on adenylcyclase. This may relate to a negative modulatory role of sphingosine in a signal transduction system stimulated by isoproterenol such as the β-adrenergic receptor pathway.

ACKNOWLEDGEMENTS

The authors wish to thank Professor Jan A. Post, Department of Molecular Biology, University Utrect, for help and guidance in isolation of cardiomyocytes; and Mr Richard Kletzkine for his determinant contribution to the computer software. This work was supported by the Research Fund of the University of Iceland and the Icelandic Research Council.

REFERENCES

1 Spiegel S., Olivera A., Carlson R.O. The role of sphingosine in cell growth regulation and transmembrane signaling. Adv. Lipid Res. (1993) **25** 105–129.
2 Hakomori S., Igarashi Y. Functional role of Glycosphingolipids in Cell Recognition and Signaling, review. J. Biochem. (1995) **118** 1091–1103.
3 Merrill A.H. Jr., Hannun Y.A., Bell R.M. Introduction: sphingolipids and their metabolites in cell regulation. Adv. Lipid Res. (1993) **25** 1–24.
4 Riboni L., Viani P., Bassi R., Prinetti A., Tettamanti G. The role of sphingolipids in the process of signal transduction, review. Progr. Lip. Res. (1997) **36** 153–195.
5 Sabbadini R., McNutt W., Jenkins G., Betto R., Salviati G. Sphingosine is endogenous to cardiac and skeletal muscle. Biochem. Biophys. Res. Commun. (1993) **193** 752–758.
6 McDonough P.M., Yasui K., Betto R. et al. Control of Cardiac Ca^{2+} Levels. Inhibitory actions of sphingosine on Ca^{2+} transients and L-type Ca^{2+} channel conductance. Circ. Res. (1994) **75** 981–989.
7 Webster R.J., Sabbadini R.A., Dettbarn C.A., Paolini P.J. Sphingosine effects on the contractile behavior of skinned cardiac myocytes. J. Mol. Cell. Cardiol. (1994) **26** 1273–1290.
8 Meldrum D.L. Tumor necrosis factor in the heart. Am. J. Physiol. (1998) **274** R577–R595.
9 Oral H., Dorn G.W. II, Mann D.L. Sphingosine mediates the immediate negative inotropic effects of tumor necrosis factor-alpha in the adult mammalian cardiac myocyte. J. Biol. Chem. (1997) **272** 4836–4842.

10 Harary L., Farley B. In vitro studies of single rat heart cells. I. Growth and organization. Exp. Cell. Res. (1963) **29** 451–465.
11 Post J.A., Langer G.A., Op den Kamp J.A., Verklej A.J. Phospholipid asymmetry in cardiac sarcolemma. Analysis of intact cells and "gas-dissected" membranes. Biochim. Biophys. Acta (1988) **943** 256–266.
12 Grynberg A., Fournier A., Sergiel J.P., Athias P. Effect of docosahexaenoic acid and eicosapentaenoic acid in the phospholipids of rat heart muscle cells on adrenoceptor responsiveness and mechanism. J. Mol. Cell. Cardiol. (1995) **27** 2607–2520.
13 Cortois M., Katami S., Fantini E., Athias P., Mielle P., Grynberg A. Polyunsaturated Fatty Acids in Cultured Cardiomyocytes: Effect on Physiology and β-Adrenoceptor Function. Am. J. Physiol. (1992) **262** H451–H456.
14 .Zaman Z., Verwilghen R.L. Quantitation of proteins solubilized in sodium dodecyl sulphate-mercaptoethanol-tris electroforesis buffer. Analytical Biochem. (1979) **100** 64–69.
15 Merrill A.H., Sweeley C.C. Sphingolipids: metabolism and cell signaling, in: Vance D.E. & Vance J.E. (Eds.), Biochemistry of lipids, lipoproteins and membranes. Elsevier Science B.V. Amsterdam, the Netherlands. 1996, pp. 309–339.
16 Zhang H., Desai N.N., Murphy J.M., Spiegel S. Increases in phosphatitic acid levels accompany sphingosine-stimulated proliferation of quiscent Swiss 3T3 cells. J. Biol. Chem. (1990) **265** 21309–21316.
17 Pyne S., Pyne N.J. The differential regulation of cyclic AMP by sphingomyelin-derived lipids and the modulation of sphingolipid-stimulated extracellular signal regulated kinase-2 in airway smooth muscle. Biochem. J. (1996) **315** 917–923.
18 Rosenthal W., Hescheler J., Trautwein W., Schultz G. Receptor and G-protein-mediated modulation of voltage-dependent calcium channels. Cold Spring Harbor Symposia on Quantitative Biology. (1988) **LIII** 247–254.
19 Sugishita K., Kinugawa K., Shimizu T. et al. Cellular basis for the acute inhibitory effects of IL-6 and TNF-α on excitation contraction coupling. J. Mol. Cell. Cardiol. (1999) **31** 1457–1467.
20 Sharma C., Smith T., Li S., Schroepfer G.J. Jr, Needleman D.H. Inhibition of Ca^{2+} release channel (ryanodine receptor) activity by sphingolipid bases: mechanism of action. Chem. Phys. Lipids (2000) **104** 1–11.
21 Van Koppen C., Meyer zu Heringdorf M., Laser K.T. et al. Activation of a high affnity Gi protein-coupled plasma membrane receptor by sphingosine-1-phosphate. J. Biol. Chem. (1996) **271** 2082–2087.
22 Guo J., MacDonnell K.L., Giles W.R. Effects of sphingosine 1-phosphate on pacemaker activity in rabbit sino-atrial node cells. Pflugers Arch. (1999) **438** 642–648.
23 Shugyama A., Yatomi Y., Ozaki Y., Hashimoto K. Sphingosine 1-phosphate induces sinus tachicardia and coronary vasoconstriction in the canine heart. Card. Res. (2000) **46** 119–125.
24 Sugiyama A., Aye N. N., Yatomi Y., Ozaki Y., Hashimoto K. Effects of sphingosine-1-phosphate, a naturally occurring biologically active lysophospholipid, on the rat cardiovascular system. Jpn. J. Pharmacol. (2000) **4** 338–342.
25 Robert P., Tsui P., Laville M.P. et al. EDG-1 receptor stimulation leads to cardiac hypertrophy in rat neonatal myocytes. J. Mol. Cell. Cardiol. (2001) **33** 1589–1606.
26 Nakajima N., Cavalli A. L., Biral D. et al. Expression and characterization of Edg-1 receptors in rat cardiomyocytes: calcium deregulation in response to sphingosine-1-phosphate. Eur. J. Biochem. (2000) **267** 5679–5686.