Effect of Sphingosine-1-Phosphate on Intracellular Free Ca\(^{2+}\) in Cat Esophageal Smooth Muscle Cells

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Abstract

A comprehensive collection of proteins senses local changes in intracellular Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_{i}\)) and transduces these signals into responses to agonists. In the present study, we examined the effect of sphingosine-1-phosphate (S1P) on modulation of intracellular Ca\(^{2+}\) concentrations in cat esophageal smooth muscle cells. To measure [Ca\(^{2+}\)]\(_{i}\) levels in cat esophageal smooth muscle cells, we used a fluorescence microscopy with the Fura-2 loading method. S1P produced a concentration-dependent increase in [Ca\(^{2+}\)]\(_{i}\) in the cells. Pretreatment with EGTA, an extracellular Ca\(^{2+}\) chelator, decreased the S1P-induced increase in [Ca\(^{2+}\)]\(_{i}\), and an L-type Ca\(^{2+}\)-channel blocker, nimodipine, decreased the effect of S1P. This indicates that Ca\(^{2+}\) influx may be required for muscle contraction by S1P. When stimulated with thapsigargin, an intracellular calcium chelator, or 2-Aminoethoxydiphenyl borate (2-APB), an InsP\(_3\) receptor blocker, the S1P-evoked increase in [Ca\(^{2+}\)]\(_{i}\) was significantly decreased. Treatment with pertussis toxin (PTX), an inhibitor of G\(_{i}\)-protein, suppressed the increase in [Ca\(^{2+}\)]\(_{i}\) evoked by S1P. These results suggest that the S1P-induced increase in [Ca\(^{2+}\)]\(_{i}\) in cat esophageal smooth muscle cells occurs upon the activation of phospholipase C and subsequent release of Ca\(^{2+}\) from the InsP\(_3\)-sensitive Ca\(^{2+}\) pool in the sarcoplasmic reticulum. These results suggest that S1P utilized extracellular Ca\(^{2+}\) via the L type Ca\(^{2+}\) channel, which was dependent on activation of the S1P\(_4\) receptor coupled to PTX-sensitive G\(_{i}\) protein, via phospholipase C-mediated Ca\(^{2+}\) release from the InsP\(_3\)-sensitive Ca\(^{2+}\) pool in cat esophageal smooth muscle cells.

Key Words: Sphingosine-1-phosphate, Calcium, Fura-2, Esophageal cells, 2-Aminoethoxydiphenyl borate, Nimodipine

INTRODUCTION

Ca\(^{2+}\) is a ubiquitous signal that regulates various cellular functions from fertilization to cell death (Bates et al., 2014). In addition to the classical Ca\(^{2+}\)-sensitive processes, such as muscle cell contraction, hormone or neurotransmitter secretion, and metabolic function regulation, intracellular free calcium ([Ca\(^{2+}\)]\(_{i}\)) elevations modulate various signaling pathways.

At rest, the esophagus is collapsed but opens readily to accept food and liquids. The upper portion of this muscular tube is composed of muscle similar to that of the arms and legs (skeletal muscle) and is therefore under voluntary control. The other two-thirds of the esophagus are composed of smooth muscle like the rest of the gut and is not under voluntary control. These muscles are arranged with an inner circular layer and outer longitudinal layer (Nishimura et al., 2017).

Recently, sphingolipids have emerged as a new class of lipid mediators. Sphingosine-1-phosphate (S1P), which is released from activated platelets, modulates a wide spectrum of biological activities, including protecting cells from apoptosis (Ahmed et al., 2015), activating calcium signaling (Ruger et al., 2014), and stimulating nitric oxide production (Cui et al., 2017). Additional effects include effects on cell proliferation (Zhai et al., 2017), regulation of adhesion molecule expression (Wetter et al., 2009), stimulation of tumor cell invasion, aggregation of platelets (Tafelmeier et al., 2017), inhibition of cell migration (Filipenko et al., 2016), and contraction of smooth muscle cells (Shaifta et al., 2015).

In many cell types, S1P is generated in response to extracellular stimuli by phosphorylation of sphingosine, which is produced from ceramide by ceramidase. Ceramide is synthesized from sphingomyelin through the action of endogenous neutral and acid sphingomyelinase, or by de novo synthesis. S1P has been identified in the human serum and plasma and...
in all rat tissues (Becker et al., 2017). However, the biological reactions induced by S1P in the gastrointestinal tract remain unclear.

Although some S1P-mediated responses are attributed to the action of an intracellular second messenger, most of the effects of S1P are thought to be receptor-mediated (Nema et al., 2016; Pulj et al., 2016; Ng et al., 2017; Serafimidis et al., 2017). The S1PR1-induced Rac1 activation was Ca^{2+} dependent and that the increase in intracellular Ca^{2+} was triggered by the action of PI–PLC and the IP3 receptor, it was suggested that the Ca^{2+} was released from the store in the ER (Li et al., 2015). In contrast, extracellular S1P activates specific seven transmembrane-spanning domain G-protein coupled receptors (Delgado and Martinez-Cartro, 2016). Sphingosine-1-phosphate receptor 4 (S1P4 receptor) is involved in the calcium response (Yamazaki et al., 2000).

It is known that changes in [Ca^{2+}] are involved in biological responses to stimuli. The stimulation of Ca^{2+} influx is involved in the regulation of important intracellular events triggered by the activation of receptors coupled to phospholipid hydrolysis. In smooth muscle cells, the contraction is primarily regulated by intracellular Ca^{2+} via Ca^{2+}-calmodulin-dependent myosin light chain kinase. An increase in [Ca^{2+}] resulting from Ca^{2+} influx or Ca^{2+} release from internal Ca^{2+} stores is involved in regulating intracellular events leading to the contraction of smooth muscle. S1P was reported to decrease the length of smooth muscle cells isolated from the esophagus of cat (Vyas et al., 2015). However, the role of S1P in regulating Ca^{2+} transport activity in cat esophageal smooth muscle cells has not been investigated.

Therefore, in the current study, the modification of Ca^{2+} by S1P was examined using a Fura-2 loading system to identify the related signaling pathways including G proteins and inositol triphosphate in esophageal smooth muscle cells.

**Fig. 1.** Measurements of intracellular calcium fluorescence. Fluorescence measurements were performed with a dual-wavelength spectrophotometer at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Fura-2 fluorescence signals were monitored with the use of the Felix Software from Photon Technology International (Edison, NJ, USA). Ratio fluorescence image analyzer include that Deltascan dual wavelength scanning illuminator-75 watt xenon arc lamp, Inverted fluorescence microscope of Nikon (Tokyo, Japan), Dual emission microscope photometer (model 810 photomultiplier), and Intensified CCD camera (model IC-200).

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**Fig. 2.** Fura-2/AM fluorescence microscopy of cat esophageal smooth muscle cells. (A) Images of [Ca^{2+}] from Fura-2/AM-loaded cell were obtained with an imaging microscope, with the color bar providing a linear scale for 0-255 calcium intensity. White arrow represents esophageal smooth muscle cell. (B) Image of [Ca^{2+}] from Fura-2/AM-loaded cell induced by S1P (10^{-6}). White arrow represents esophageal smooth muscle cell.

**MATERIALS AND METHODS**

**Materials**

- 4-(2-Hydroxyethyl)-1-piperazine-N’-2-ethane sulfonic acid (HEPES), sphingosine-1-phosphate (S1P), collagenase type F, bovine serum albumin, pertussis toxin (PTX), ethylene glycol-bis(-aminomethyl ether)-N,N,N’,N’-tetraacetic acid (EGTA), ethylenediamine tetraacetic acid (EDTA), pluronic F-127, thapsigargin (TG), Fura-2/AM, and other reagents were purchased from Sigma (St. Louis, MO, USA).

**Preparation of dispersed smooth muscle cells**

Single muscle cells were isolated as previously described (Biancani et al., 1987). Muscle strips were incubated overnight in normal potassium-HEPES buffer containing 1 mg/mL papain, 1 mM dithiothreitol, 1 mg/mL bovine serum albumin, and 0.5 mg/mL collagenase (type F, Sigma) and equilibrated with 95% O2-5% CO2 to maintain a pH of 7.0 at 31°C. The composition of the normal potassium-HEPES buffer was 1 mM CaCl2, 250 μM EDTA, 10 mM glucose, 10 mM HEPES, 4 mM KCl, 131 mM NaCl, 1 mM MgCl2, and 10 mM taurine. On the following day, the tissue was warmed to room temperature for 30 min and incubated the tissue in a water bath at 31°C for 30 min. After incubation, the digested tissue was poured over a 360-μm Nitex filter, rinsed with collagenase-free HEPES buffer to remove any trace of collagenase, and then incubated in this solution at 31°C and gassed with 95% O2-5% CO2. The cells were allowed to dissociate freely for 10–20 min. Suspensions of single muscle cells were harvested by filtration through 500-μm Nitex filter (Biancani et al., 1987). Before beginning the experiment, the cells were incubated at 31°C for at least 10 min to relax the cells. Throughout the entire procedure, care was taken not to agitate the fluid to avoid cell contraction in response to mechanical stress. The experiments were performed in accordance with the guidelines and approval of the Institutional Animal Care Use Committee (IACUC) of Research Institute of Pharmacy, Chung-Ang University, Korea.

**Fura-2 loading system**

For Fura-2 loading, the acetoxymethyl ester form of Fura-2 (Fura2/AM) was used because: 1) Fura-2/AM was shown to be loaded into muscle cells easily in its cell-permeable AM form. 2) Fura-2 alters its excitation spectrum upon binding to
Ca^{2+} (Terada et al., 2003). If we excite Fura-2 using two alternating wavelengths near 510 nm, the ratio of fluorescence intensities at the two excitation wavelengths provides a measure of the [Ca^{2+}] independently of the dye concentration.

First, Fura 2-AM was dissolved in dimethyl sulfoxide at a concentration of 2.5 mM, and then added to HEPES buffer to make the final concentration 5 μM. A noncytotoxic detergent, 0.02% pluronic F-127, was added to increase the solubility of the Fura-2/AM. The lipophilicity of fura-2 promotes the formation of micelles in aqueous media, which may impede the passage of the probe across cell membranes. The use of the nonionic detergent pluronic F-127 is recommended to prevent the formation of fura-2 micelles. The use of a detergent is not always an acceptable practice, however, especially in studies in which detergent–lipid interactions may influence membrane parameters (Yates et al., 1992). The smooth muscle cells were incubated for 45-60 min at 37°C in flasks containing 1 mL HEPES buffer with Fura-2/AM. Because Fura-2/AM is sensitive to light, the flasks were wrapped in aluminum foil.

**Fluorescence measurement**

Cat esophageal smooth muscle cells were placed in a chamber with a volume of 50 μL. The cells were superfused continuously at 1 mL/min with physiological salt solutions: NaCl 140 mM, KCl 5 mM, CaCl₂ 2 mM, MgCl₂ 1 mM, HEPES 5 mM, glucose 11 mM, pH 7.4 adjusted with NaOH. The temperature of all solutions was maintained at 37°C in a water bath.

Fluorescence measurements were performed on individual smooth muscle cells with a dual-wavelength spectrofluorometer at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Fura-2 fluorescence signals were continuously monitored and collected using the Felix Software package from Photon Technology International (Edison, NJ, USA) as shown in Fig. 1.

**Data analysis**

The data are expressed as the mean ± S.E.M. Statistical significance was estimated by Student’s t-test. p<0.05 was considered to indicate statistical significance. The measured Ca^{2+} intensity (Fig. 2) by color was compared to the control and S1P treatment or antagonist, respectively. Autofluorescence was subtracted, and the ratio (F Fura-2 340/F Fura-2 380) was calculated.

**RESULTS**

**Effects of S1P on [Ca^{2+}] in cat esophageal smooth muscle cells**

It is known that S1P induces contraction in smooth muscle cells (Shafta et al., 2015). The effect of different concentrations of S1P (10^{-10}–10^{-5}) on [Ca^{2+}] was evaluated in cat esophageal smooth muscle cells, and the red color in the cytosol caused by S1P (10^{-5} M) is shown in Fig. 2B. S1P, when added to smooth muscle cells, caused a rapid increase in the red color at peak [Ca^{2+}] response, which was followed by a slow return towards baseline over 8–10 min. S1P increased [Ca^{2+}] in a concentration-dependent manner, the maximal response was observed at 10^{-3} M (Fig. 3).

**Effects of S1P on [Ca^{2+}] after preincubation of cat esophageal smooth muscle cells with EGTA**

To evaluate whether the increase in [Ca^{2+}] induced by S1P occurred because of an influx of Ca^{2+} from an extracellular source, EGTA, an extracellular Ca^{2+} chelator, was used (Milara et al., 2009). Preincubation of smooth muscle cells for 1 min with EGTA (2 mM) led to an approximately 53% decrease in the peak [Ca^{2+}] response (Fig. 4).

**Effects of S1P on [Ca^{2+}] after preincubation of cat esophageal smooth muscle cells with nimodipine**

To examine if the S1P-induced increase in [Ca^{2+}] occurs because an influx of Ca^{2+} from an extracellular source via the L-type Ca^{2+} channel, nimodipine, an L-type Ca^{2+} channel blocker, was used. Preincubation for 5 min with nimodipine (100 nM) reduced the immediate [Ca^{2+}] response induced by S1P (10^{-5} M) (Fig. 5).

**Effects of S1P on [Ca^{2+}] after preincubation of cat esophageal smooth muscle cells with PTX**

It has been shown that S1P has its own PTX-sensitive G-protein coupled receptor (Fuhrmann et al., 2015; Aoyama et al., 2017). The cells were preincubated for 60 min with PTX (400 ng/mL). PTX inhibited S1P-induced Ca^{2+} signaling.

![Fig. 3. Dose-dependent increases in Ca^{2+} signaling induced by S1P in cat esophagus. Freshly isolated smooth muscle cells were stimulated for 60 s with the indicated concentration of sphingosine-1-phosphate. Values are expressed as the means ± SEM. *p<0.05 vs. control.](https://doi.org/10.4062/biomolther.2018.053)

![Fig. 4. Effect of EGTA on intracellular Ca^{2+} level induced by S1P (10^{-5} M) Representative images of autocalculating digitalized diagram (top panels) and bar diagram of [Ca^{2+}] (bottom panels). The cells were preincubated for 1 min with EGTA (2 mM) in Ca^{2+}-free buffer. Intracellular Ca^{2+} level was decreased by EGTA. Values are expressed as the means ± SEM. **p<0.01 vs. control.](https://doi.org/10.4062/biomolther.2018.053)
Intracellular Ca\(^{2+}\) mobilization are mediated by receptors coupled to a PTX-sensitive G-protein (Fig. 6).

**Effects of S1P on [Ca\(^{2+}\)], after preincubation of cat esophageal smooth muscle cells with thapsigargin and APB**

To investigate if the S1P-induced increase in [Ca\(^{2+}\)] occurs because of release of Ca\(^{2+}\) from the sarcoplasmic reticulum (SR), an inhibitor of the SR Ca\(^{2+}\)-ATPase (Choi et al., 2009) was used. Preincubation of the cells for 5 min with thapsigargin (10 \(\mu\)M) resulted in a significant change in the [Ca\(^{2+}\)] response. Thapsigargin nearly abolished the increase in the [Ca\(^{2+}\)] response evoked by S1P (10\(^{-7}\) M) (Fig. 7). The cells were preincubated for 5 min with 200 \(\mu\)M 2-APB, an InsP\(_3\) receptor antagonist. As shown in Fig. 6, 2-APB significantly inhibited S1P-induced Ca\(^{2+}\) mobilization, indicating that S1P caused the release of Ca\(^{2+}\) from the SR through InsP\(_3\) receptors (Fig. 8).

**Summarized Ca\(^{2+}\) Signaling pathway by S1P**

S1P utilized extracellular Ca\(^{2+}\) via an L type Ca\(^{2+}\) channel, which depended on the PTX-sensitive G protein. This occurred via a phospholipase C (PLC)-mediated Ca\(^{2+}\) release from the InsP\(_3\)-sensitive Ca\(^{2+}\) pool in the SR of cat esophageal smooth muscle cells (Fig. 9).

**DISCUSSION**

In the present study, we examined the effect of S1P on intracellular Ca\(^{2+}\) mobilization in cat esophageal smooth muscle cells. S1P is a polar sphingolipid metabolite that has been proposed to act both as an extracellular mediator and intracellular second messenger (Gomez-Munoz et al., 2010; Matula et al., 2015; Badawy et al., 2017; Patmanathan et al., 2017). A wide variety of stimuli have been shown to increase sphingosine kinase activity and elevate intracellular S1P levels (Bates et al., 2014; Sysol et al., 2016; Kanemura et al., 2017). For example, platelet-derived growth factor stimulated rapid activation of sphingosine kinase and transient production of S1P in Swiss 3T3 fibroblasts (Qiu and Steinberg, 2016) and airway smooth muscle cells (Candalija et al., 2014). Elevated S1P acts as an intracellular second messenger. In contrast, extracellular S1P activates specific G-protein coupled receptors, which belong to the family of S1P\(_r\) receptors (Yu et al., 2011; Hohenhaus et al., 2013; Archbold et al., 2014; Dyckman 2017; Vestri et al., 2017). The above findings suggest that S1P functions as a messenger proposed to act in both extracellular and intracellular Ca\(^{2+}\) mobilization.

S1P (10\(^{-10}\)–10\(^{-5}\) M) was found to produce a concentration-dependent increase in [Ca\(^{2+}\)] in cat esophageal smooth muscle cells. We used 10\(^{-7}\) M S1P to study its mechanism of action because the maximal response was observed at 10\(^{-7}\) M and similar concentrations of S1P have been used by other investigators in previous studies (Adamson et al., 2012).
Interestingly, preincubation of cells with EGTA lowered the S1P-induced increase in the intracellular Ca²⁺ response; this finding indicates that the S1P-induced increase in [Ca²⁺], is partially dependent on the influx of extracellular Ca²⁺.

S1P has been previously suggested to mobilize [Ca²⁺] by stimulating L-type Ca²⁺ channels in renovascular cells (Shaifta et al., 2015). Similar abolishment was observed for nimodipine, suggesting that Ca²⁺ influx from the extracellular environment occurs via L-type Ca²⁺ channels in cat esophageal smooth muscle cells. While S1P effects on renovascular cells were fully blocked by chelation of extracellular Ca²⁺ and inhibited by Ca²⁺ entry blockers (Li and Zhang, 2016), incubation with the Ca²⁺-channel blocker nimodipine partially reduced the S1P-evoked increase in [Ca²⁺] in esophageal smooth muscle cells. The discrepancy in these results may be related to differences in the cell types employed in these studies. Thus, it is reasonable to assume that the S1P-induced increase in [Ca²⁺], in the esophageal smooth muscle cells is linked to opening the L-type Ca²⁺ channels in the sarcenlolem membrane. Because EGTA also partially decreased in the levels of [Ca²⁺], and influx of Ca²⁺ is obligatory to replenish SR Ca²⁺ stores, intracellular Ca²⁺ stores may become depleted by the use of EGTA, resulting in decreased S1P-induced Ca²⁺ release from the SR in esophageal smooth muscle cells treated with EGTA. However, the role of the store-operated Ca²⁺ channel (Simo-Cheyou et al., 2017) in S1P-induced mobilization in esophageal smooth muscle cells requires further investigation.

To understand the exact mode of action of S1P on esophageal smooth muscle cells, we incubated the cells with TG, which depletes SR Ca²⁺ stores by inhibiting SR Ca²⁺-ATPase (Feuerborn et al., 2017). It was observed that the S1P-induced increase in [Ca²⁺] was depressed by TG. Such observations indicated that Ca²⁺ release from the SR is involved in S1P-induced Ca²⁺ mobilization. Thus, the S1P-evoked increase in [Ca²⁺] may be associated with the release of Ca²⁺ from SR Ca²⁺ stores.

To further confirm the role of S1P in the release of Ca²⁺ from the SR through the involvement of InsP₃-sensitive SR Ca²⁺ pools, the cells were preincubated with the IP₃ receptor blocker, 2-APB (Selli and Tosun, 2016). The S1P-induced increase in [Ca²⁺], in cells preincubated with 2-APB was decreased compared to in the control, suggesting that InsP₃-sensitive Ca²⁺ channels are involved in the S1P-induced increase in [Ca²⁺]. It was reported that vasopressin-induced Ca²⁺ mobilization in cardiomyocytes is depressed by PLC inhibitors, but not by ryanodine or caffeine, suggesting that there are two Ca²⁺ pools in the SR, namely ryanodine-sensitive and InsP₃-sensitive pools (Liu et al., 1999). Our studies using different agents to modify the action of S1P suggested that S1P releases Ca²⁺ from the InsP₃-sensitive Ca²⁺ pool in the SR; further studies are required to investigate the role of the ryanodine-sensitive Ca²⁺ pool in the SR in S1P-induced Ca²⁺ mobilization in esophageal smooth muscle cells. Our finding was consistent with other experiment that the S1PR1-induced Rac1 activation was triggered by the action of PI-PLC and the IP3 receptor, it was produced from the store in the ER (Li et al., 2015).

To examine the role of G-proteins in S1P-induced Ca²⁺ mobilization, the cells were preincubated pertussis toxin. [Ca²⁺] increases by the S1P₁ receptor were caused by PLC-mediated Ca²⁺ mobilization, which was fully sensitive to PTX and thus mediated by G proteins (Germinario et al., 2016). In the present study, PTX inhibited S1P-induced Ca²⁺ mobilization, indicating that the effects of S1P on intracellular Ca²⁺ mobilization are mediated by receptors coupled to a PTX-sensitive G-protein. This result was similar to previous findings in which muscarinic M2 coupled to Gᵢ or rho protein was involved in mediating contraction and [Ca²⁺] increase, as they were blocked by pertussis and C3 toxin incubation (Sohn et al., 2000). It is known that the Gi family contains sites susceptible to modification by PTX and can mediate the activation of PTX-sensitive signaling.

In summary, S1P utilized extracellular Ca²⁺ via an L type Ca²⁺ channel, which depended on the PTX-sensitive Gi protein; this response occurred because of PLC-mediated Ca²⁺ release from the InsP₃-sensitive Ca²⁺ pool in the SR of cat
esophageal smooth muscle cells.

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REFERENCES

Adamson, R. H., Sarai, R. K., Clark, J. F., Altangerel, A., Thirkill, T. L. and Curry, F. E. (2012) Attenuation by sphingosine-1-phosphate of rat microvessel acute permeability response to bradykinin is rapidly reversible. *Am. J. Physiol. Heart Circ. Physiol.* 302, H1929-H1935.

Ahmed, D., de Verdier, P. J., Ryk, C., Lunq, O., Stal, P. and Flygare, J. (2015) FTY720 (Fingolimod) sensitizes hepatocellular carcinoma cells to sodium-meditated cytotoxicity. *Pharmacol. Res. Perspect.* 3, e00171.

Aoyama, Y., Sobue, S., Mizutani, N., Inoue, C., Kawamoto, Y., Nishizawa, Y., Ichihara, M., Kyogashima, M., Suzuki, M., Nozawa, Y. and Murate, T. (2017) Modulation of the sphingolipid osteostat is involved in paclitaxel resistance of the human prostate cancer cell line PC3-PR. *Biochem. Biophys. Res. Commun.* 486, 551-557.

Archbold, J. K., Martin, J. L. and Sweet, M. J. (2014) Towards selective lysophospholipid GPCR modulators. *Trends Pharmacol.* 35, 219-226.

Badawy, S. M. M., Okada, T., Kajimoto, T., Ijuin, T. and Nakamura, S. I. (2014) Towards selective lysophospholipid GPCR modulators. *Trends Pharmacol.* 35, 219-226.

Bates, R. C., Fees, C. P., Holland, W. L., Winger, C. C., Batbayar, K., Adamson, R. H., Sarai, R. K., Clark, J. F., Altangerel, A., Thirkill, T. L. and Curry, F. E. (2012) Attenuation by sphingosine-1-phosphate of rat microvessel acute permeability response to bradykinin is rapidly reversible. *Am. J. Physiol. Heart Circ. Physiol.* 302, H1929-H1935.

Arnold, A. (2016) Comparative immunohistochemical evaluation of the zonal differentiation and inflammation markers in human meniscus in osteoarthritis and rheumatoid arthritis. *Acta Histochem.* 117, 243-254.

Biancani, P., Hillemeier, C., Bitar, K. N. and Makhlouf, G. M. (1987) Calcium influx in esophageal muscle and by contraction mediated by Ca2+ uptake in esophageal muscle and by Ca2+ release in the LES. *Am. J. Physiol.* 253, G760-G766.

Candalija, A., Cubi, R., Ortega, A., Aguilera, J. and Gil, C. (2014) Trk receptor need neutral sphingomyelinase activity to promote cell viability. *FEBS Lett.* 588, 167-174.

Choi, S. K., Ahn, D. S. and Lee, Y. H. (2009) Comparison of contractile mechanisms of sphingosine-1-phosphate and sphingosine-1-phosphate in rabbit coronary artery. *Cardiovasc. Res.* 82, 324-332.

Cui, K., Ruan, Y., Wang, T., Rao, K., Chen, Z., Wang, S. and Liu, J. (2017) FTY720 supplementation partially improves erectile dysfunction in rats with streptozotocin-induced type 1 diabetes through inhibition of endothelial dysfunction and corporal fibrosis. *J. Sex. Med.* 14, 323-335.

Delgado, A. and Martinez-Cartro, M. (2016) Therapeutic potential of the modulation of sphingosine-1-phosphate receptors. *Curr. Med. Chem.* 23, 242-264.

Dyckman, A. J. (2017) Modulators of sphingosine-1-phosphate pathway biology: recent advances of Sphingosine-1-phosphate Receptor 1 (S1P1) agonists and future perspectives. *J. Med. Chem.* 60, 5287-5289.

Feuerborn, R., Becker, S., Poti, F., Nagel, P., Brodde, M., Schmidt, H., Christoffersen, C., Ceglarek, U., Burkhardt, R. and Nofer, J.R. (2017) High density lipoprotein (HDL)-associated sphingosine-1-phosphate (S1P) inhibits macrophage apoptosis by stimulating STAT3 activity and survivin expression. *Atherosclerosis* 257, 29-37.

Filipenko, I., Schwalm, S., Reali, L., Pfeilschifter, J., Fabbro, D., Hulwiler, A. and Zangemeister-Wittke, U. (2016) Upregulation of the S1P3 receptor in metastatic breast cancer cells increases migration and invasion by induction of PGE2 and EP2/EP4 activation. *Biochim. Biophys. Acta* 1861, 1840-1851.

Fuhrmann, I. K., Steinhaegen, J., Ruther, W. and Schumacher, U. (2015) Comparative immunohistochemical evaluation of the zonal distribution of extracellular matrix and inflammation markers in human meniscus in osteoarthritis and rheumatoid arthritis. *Acta Histochem.* 117, 243-254.

Germaine, E., Bondi, M., Cencetti, F., Donati, C., Nocella, M., Colombini, B., Betto, R., Bruni, P., Baggi, M. A. and Danieli-Betto, D. (2016) S1P3 receptor influences key physiological properties of fast-twitch extensor digitorum longus muscle. *J. Appl. Physiol.* 120, 1288-1300.

Gomez-Munoz, A., Gangolli, P., Granado, M. H., Arana, L. and Ouro, A. (2010) Ceramide-1-phosphate in cell survival and inflammatory signaling. *Adv. Exp. Med. Biol.* 688, 118-130.

Hohenhaus, D. M., Schaele, K., La Cao, K. A., Seow, V., Iyer, A., Fairlie, D. P. and Sweet, M. J. (2013) An mRNA atlas of G protein-coupled receptor expression during primary human monocyte/macrophage differentiation and lipopolysaccharide-mediated activation identifies targetable candidate regulators of inflammation. *Immunobiology* 218, 1345-1353.

Kanemura, N., Shibata, R., Ohashi, K., Ogawa, H., Hiramatsu-Ito, M., Enomoto, T., Yusa, D., lto, M., Hayakawa, S., Otaka, N., Murohara, T. and Ouchi, N. (2017) C1q/TNF-related protein 1 prevents neointimal formation after arterial injury. *Atherosclerosis* 257, 138-145.

Li, N. and Zhang, F. (2016) Implication of sphingosine-1-phosphate in cardiovascular regulation. *Front. Biosci. (Landmark Ed.)* 21, 1296-1313.

Li, Q., Chen, B., Zeng, C., Fan, A., Yuan, Y., Guo, X., Huang, X. and Huang, Q. (2015) Differential activation of receptors and signal pathways upon stimulation by different doses of sphingosine-1-phosphate in endothelial cells. *Exp. Physiol.* 100, 95-107.

Li, S., Chen, J., Fang, X. and Xia, X. (2017) Sphingosine-1-phosphate activates the AKT pathway to inhibit chemotherapy induced human granulosa cell apoptosis. *Gynecol. Endocrinol.* 33, 476-479.

Liu, P., Hopfner, R. L., Xu, Y. J. and Golapkalirshnan, V. (1999) Vasopressin-evoked [Ca2+]i responses in neonatal rat cardiomyocytes. *J. Cardiovasc. Pharmacol.* 34, 540-546.

Matula, K., Collie-Duguid, E., Murray, G., Park, K., Grabsh, H., Tan, P., Lalwani, S., Garau, R., Ong, Y., Bain, G., Smith, A. D., Urquhart, G., Bielawski, J., Finnegan, M. and Petty, R. (2015) Regulation of cellular sphingosine-1-phosphate by sphingosine kinase 1 and sphingosine-1-phosphate lyase determines chemotherapy resistance in gastrointestinal cancer. *BMC Cancer* 15, 762.

Milan, J., Mata, M., Mauricio, M. D., Donet, E., Morzillo, E. J. and Corr, J. (2009) Sphingosine-1-phosphate increases human alveolar epithelial IL-8 secretion, proliferation and neutrophil chemotaxis. *Eur. J. Pharmacol.* 609, 132-139.

Nema, R., Vishwakarma, S., Agarwal, R., Panday, R. K. and Kumar, A. (2016) Emerging role of sphingosine-1-phosphate signaling in head and neck squamous cell carcinoma. *Onco Targets Ther.* 9, 3269-3280.

Ng, M. L., Wadham, C. and Sukocheva, O. A. (2017) The role of sphingolipid signalling in diabetes associated pathologies (Review). *Int. J. Mol. Med.* 39, 243-252.

Nishimura, N., Endo, S., Ueno, S., Ueno, N., Tatetsu, H., Hirata, S., Hata, H., Komohara, Y., Takeya, M., Mitsuya, H. and Okuno, Y. (2017) A xenograft model reveals that PU.1 functions as a tumor suppressor for multiple myeloma in vivo. *Biochem. Biophys. Res. Commun.* 486, 916-922.

Patmanathan, S. N., Wang, W., Yap, L. F., Herr, D. R. and Paterson, I. C. (2017) Mechanisms of sphingosine 1-phosphate receptor signalling in cancer. *Cell Signal.* 34, 86-75.

Pill, M. R., Rajsheel, P., Aswani, V., Agurla, S., Kuchitsu, K. and Raghavendra, A. S. (2016) Stomatial closure induced by phyto-sphingosine-1-phosphate and sphingosine-1-phosphate depends
on nitric oxide and pH of guard cells in Pisum sativum. *Plant* 244, 831-841.

Qiu, W. and Steinberg, S. F. (2016) Phos-tag SDS-PAGE resolves agonist- and isoform-specific activation patterns for PKD2 and PKD3 in cardiomyocytes and cardiac fibroblasts. *J. Mol. Cell. Cardiol.* 99, 14-22.

Ruger, K., Ottenfinger, F., Schroder, M., Zivkovic, A., Stark, H., Pfeilschifter, J. M. and Radeke, H. H. (2014) Modulation of IL-33/ST2-TIR and TLR signalling pathway by fingolimod and analogues in immune cells. *Scand. J. Immunol.* 80, 398-407.

Selli, C. and Tosun, M. (2016) Effects of cyclopiazonic acid and dexamethasone on serotonin-induced calcium responses in vascular smooth muscle cells. *J. Physiol. Biochem.* 72, 245-253.

Serafimidis, I., Rodriguez-Aznar, E., Lesche, M., Yoshioka, K., Takuwa, Y., Dahl, A., Pan, D. and Gavalas, A. (2017) Pancreas lineage allocation and specification are regulated by sphingosine-1-phosphate signalling. *PLoS Biol.* 15, e2000949.

Shaifta, Y., Snetkov, V. A., Prieto-Lloret, J., Knock, G. A., Smirnov, S. V., Aaronson, P. I. and Ward, J. P. (2015) Sphingosylphosphorylcholine potentiates vasoreactivity and voltage-gated Ca2+ entry via NOX1 and reactive oxygen species. *Cardiovasc. Res.* 106, 121-130.

Yamazaki, Y., Kon, J., Sato, K., Tomura, H., Sato, M., Yoneya, T., Okazaki, H., Okajima, F. and Ohta, H. (2000) Edg-6 as a putative sphingosine 1-phosphate receptor coupling to Ca2+ signaling pathway. *Biochem. Biophys. Res. Commun.* 268, 583-589.

Yu, X., Wang, X., Huang, X., Buchenauer, H., Han, Q., Guo, J., Zhao, J., Qu, Z., Huang, L. and Kang, Z. (2011) Cloning and characterization of a wheat neutral ceramidase gene Ta-CDase. *Mol. Biol. Rep.* 38, 3447-3454.

Zhai, L., Wu, R., Han, W., Zhang, Y. and Zhu, D. (2017) miR-127 enhances myogenic cell differentiation by targeting S1PR3. *Cell Death Dis.* 8, e2707.