



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

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## Abstract

A comprehensive collection of proteins senses local changes in intracellular  $\text{Ca}^{2+}$  concentrations ( $[\text{Ca}^{2+}]_i$ ) 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  $\text{Ca}^{2+}$  concentrations in cat esophageal smooth muscle cells. To measure  $[\text{Ca}^{2+}]_i$  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  $[\text{Ca}^{2+}]_i$  in the cells. Pretreatment with EGTA, an extracellular  $\text{Ca}^{2+}$  chelator, decreased the S1P-induced increase in  $[\text{Ca}^{2+}]_i$ , and an L-type  $\text{Ca}^{2+}$ -channel blocker, nimodipine, decreased the effect of S1P. This indicates that  $\text{Ca}^{2+}$  influx may be required for muscle contraction by S1P. When stimulated with thapsigargin, an intracellular calcium chelator, or 2-Aminoethoxydiphenyl borate (2-APB), an  $\text{InsP}_3$  receptor blocker, the S1P-evoked increase in  $[\text{Ca}^{2+}]_i$  was significantly decreased. Treatment with pertussis toxin (PTX), an inhibitor of  $G_i$ -protein, suppressed the increase in  $[\text{Ca}^{2+}]_i$  evoked by S1P. These results suggest that the S1P-induced increase in  $[\text{Ca}^{2+}]_i$  in cat esophageal smooth muscle cells occurs upon the activation of phospholipase C and subsequent release of  $\text{Ca}^{2+}$  from the  $\text{InsP}_3$ -sensitive  $\text{Ca}^{2+}$  pool in the sarcoplasmic reticulum. These results suggest that S1P utilized extracellular  $\text{Ca}^{2+}$  via the L type  $\text{Ca}^{2+}$  channel, which was dependent on activation of the  $\text{S1P}_4$  receptor coupled to PTX-sensitive  $G_i$  protein, via phospholipase C-mediated  $\text{Ca}^{2+}$  release from the  $\text{InsP}_3$ -sensitive  $\text{Ca}^{2+}$  pool in cat esophageal smooth muscle cells.

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

## INTRODUCTION

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

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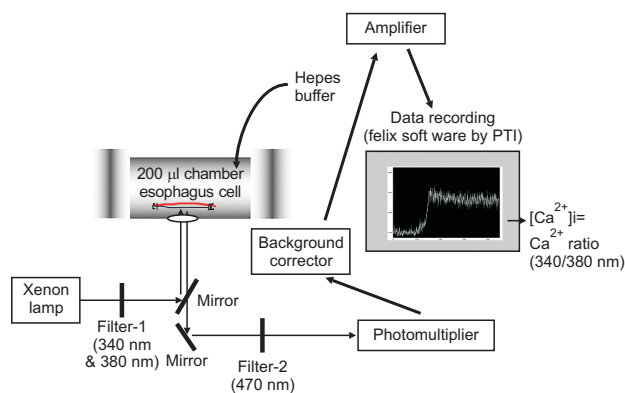
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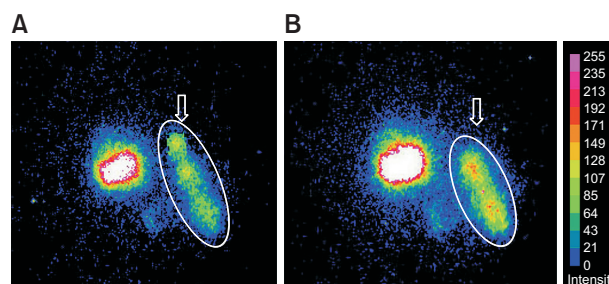
**Fig. 1.** Measurements of intracellular calcium fluorescence. Fluorescence measurements were performed with a dual-wavelength spectrofluorometer at excitation wavelengths of 340 and 380 nm and an emission wavelength of 510 nm. Fura-2 fluorescence signals were monitored with the use of the Felix Software 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).

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; Puli *et al.*, 2016; Ng *et al.*, 2017; Serafimidis *et al.*, 2017). The S1PR1-induced Rac1 activation was Ca<sup>2+</sup> dependent and that the increase in intracellular Ca<sup>2+</sup> was triggered by the action of PI-PLC and the IP<sub>3</sub> receptor, it was suggested that the Ca<sup>2+</sup> 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-Castro, 2016). Sphingosine-1-phosphate receptor 4 (S1P<sub>4</sub> receptor) is involved in the calcium response (Yamazaki *et al.*, 2000).

It is known that changes in [Ca<sup>2+</sup>]<sub>i</sub> are involved in biological responses to stimuli. The stimulation of Ca<sup>2+</sup> 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<sup>2+</sup> via Ca<sup>2+</sup>-calmodulin-dependent myosin light chain kinase. An increase in [Ca<sup>2+</sup>]<sub>i</sub> resulting from Ca<sup>2+</sup> influx or Ca<sup>2+</sup> release from internal Ca<sup>2+</sup> 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 the cat (Vyas *et al.*, 2015). However, the role of S1P in regulating Ca<sup>2+</sup> transport activity in cat esophageal smooth muscle cells has not been investigated.

Therefore, in the current study, the modification of Ca<sup>2+</sup> 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. 2.** Fura-2/AM fluorescence microscopy of cat esophageal smooth muscle cells. (A) Images of [Ca<sup>2+</sup>]<sub>i</sub> 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<sup>2+</sup>]<sub>i</sub> from Fura-2/AM-loaded cell induced by S1P (10<sup>-7</sup>). 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-β-aminoethyl 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% O<sub>2</sub>-5% CO<sub>2</sub> to maintain a pH of 7.0 at 31°C. The composition of the normal potassium-HEPES buffer was 1 mM CaCl<sub>2</sub>, 250 µM EDTA, 10 mM glucose, 10 mM HEPES, 4 mM KCl, 131 mM NaCl, 1 mM MgCl<sub>2</sub>, 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% O<sub>2</sub>-5% CO<sub>2</sub>. 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 (Fura-2/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<sup>2+</sup> (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<sup>2+</sup>]<sub>i</sub> 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.

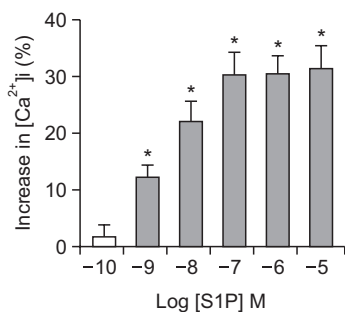
**Fluorescence measurement**

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, CaCl<sub>2</sub> 2 mM, MgCl<sub>2</sub> 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<sup>2+</sup> 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.



**Fig. 3.** Dose-dependent increases in Ca<sup>2+</sup> 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.

**RESULTS**

**Effects of S1P on [Ca<sup>2+</sup>]<sub>i</sub> in cat esophageal smooth muscle cells**

It is known that S1P induces contraction in smooth muscle cells (Shaifita *et al.*, 2015). The effect of different concentrations of S1P (10<sup>-10</sup>–10<sup>-5</sup>) on [Ca<sup>2+</sup>]<sub>i</sub> was evaluated in cat esophageal smooth muscle cells, and the red color in the cytosol caused by S1P (10<sup>-7</sup> M) is shown in Fig. 2B. S1P, when added to smooth muscle cells, caused a rapid increase in the red color at peak [Ca<sup>2+</sup>]<sub>i</sub> response, which was followed by a slow return towards baseline over 8–10 min. S1P increased [Ca<sup>2+</sup>]<sub>i</sub> in a concentration-dependent manner, the maximal response was observed at 10<sup>-7</sup> M (Fig. 3).

**Effects of S1P on [Ca<sup>2+</sup>]<sub>i</sub> after preincubation of cat esophageal smooth muscle cells with EGTA**

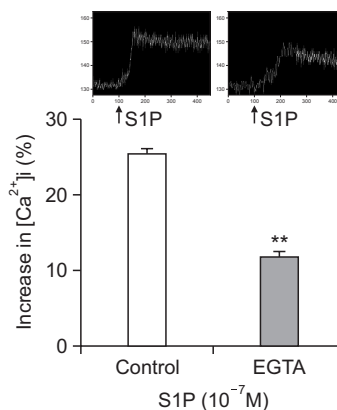
To evaluate whether the increase in [Ca<sup>2+</sup>]<sub>i</sub> induced by S1P occurred because of an influx of Ca<sup>2+</sup> from an extracellular source, EGTA, an extracellular Ca<sup>2+</sup> chelator, was used (Milara *et al.*, 2009). Preincubation of smooth muscle cells for 1 min with EGTA (2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

**Effects of S1P on [Ca<sup>2+</sup>]<sub>i</sub> after preincubation of cat esophageal smooth muscle cells with nimodipine**

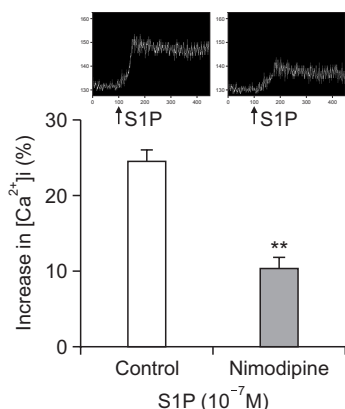
To examine if the S1P-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> occurs because an influx of Ca<sup>2+</sup> from an extracellular source via the L-type Ca<sup>2+</sup> channel, nimodipine, an L-type Ca<sup>2+</sup> channel blocker, was used. Preincubation for 5 min with nimodipine (100 nM) reduced the immediate [Ca<sup>2+</sup>]<sub>i</sub> response induced by S1P (10<sup>-7</sup> M) (Fig. 5).

**Effects of S1P on [Ca<sup>2+</sup>]<sub>i</sub> after preincubation of cat esophageal smooth muscle cells with PTX**

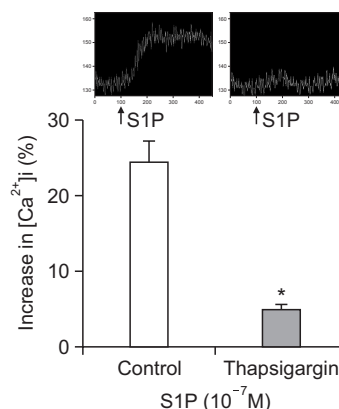
It has been shown that S1P has its own PTX-sensitive G-protein coupled receptor (Fuhrmann *et al.*, 2015; Aoyama *et al.*, 2017; Li *et al.*, 2017). The cells were preincubated for 60 min with PTX (400 ng/mL). PTX inhibited S1P-induced Ca<sup>2+</sup>



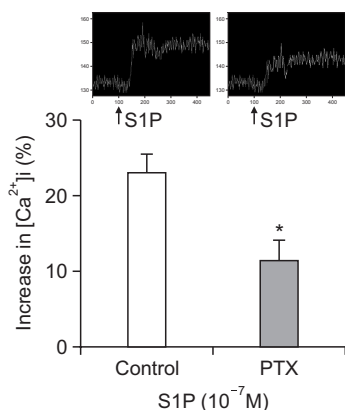
**Fig. 4.** Effect of EGTA on intracellular Ca<sup>2+</sup> level induced by S1P (10<sup>-7</sup> M) Representative images of autocalculating digitalized diagram (top panels) and bar diagram of [Ca<sup>2+</sup>]<sub>i</sub> (bottom panels). The cells were preincubated for 1 min with EGTA (2 mM) in Ca<sup>2+</sup>-free buffer. Intracellular Ca<sup>2+</sup> level was decreased by EGTA. Values are expressed as the means ± SEM. \*\**p*<0.01 vs. control.



**Fig. 5.** Effect of nimodipine on intracellular Ca<sup>2+</sup> level induced by S1P (10<sup>-7</sup> M). Representative images of autocalculating digitalized diagram (top panels) and bar diagram of [Ca<sup>2+</sup>]<sub>i</sub> (bottom panels). The cells were preincubated for 5 min with nimodipine (100 nM). Intracellular Ca<sup>2+</sup> level was decreased by nimodipine. Values are expressed as the means ± SEM. \*\**p*<0.01 vs. control.



**Fig. 7.** Effect of thapsigargin on intracellular Ca<sup>2+</sup> level induced by S1P (10<sup>-7</sup> M). Representative images of autocalculating digitalized diagram (top panels) and bar diagram of [Ca<sup>2+</sup>]<sub>i</sub> (bottom panels). The cells were preincubated for 5 min with TG (10 μM). Intracellular Ca<sup>2+</sup> level was decreased by TG. Values are expressed as the means ± SEM. \**p*<0.05 vs. control.



**Fig. 6.** Effect of PTX on intracellular Ca<sup>2+</sup> level induced by S1P (10<sup>-7</sup> M). Representative images of autocalculating digitalized diagram (top panels) and bar diagram of [Ca<sup>2+</sup>]<sub>i</sub> (bottom panels). The cells were preincubated for 60 min with PTX (400 ng/mL). Intracellular Ca<sup>2+</sup> level was decreased by PTX. Values are expressed as the means ± SEM. \**p*<0.05 vs. control.

mobilization, indicating that the effects of S1P on intracellular Ca<sup>2+</sup> mobilization are mediated by receptors coupled to a PTX-sensitive G-protein (Fig. 6).

#### Effects of S1P on [Ca<sup>2+</sup>]<sub>i</sub> after preincubation of cat esophageal smooth muscle cells with thapsigargin and APB

To investigate if the S1P-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> occurs because of release of Ca<sup>2+</sup> from the sarcoplasmic reticulum (SR), an inhibitor of the SR Ca<sup>2+</sup>-ATPase (Choi *et al.*, 2009) was used. Preincubation of the cells for 5 min with thapsigargin (10 μM) resulted in a significant change in the [Ca<sup>2+</sup>]<sub>i</sub> response. Thapsigargin nearly abolished the increase in the [Ca<sup>2+</sup>]<sub>i</sub> response evoked by S1P (10<sup>-7</sup> M) (Fig. 7). The cells were preincubated for 5 min with 200 μM 2-APB, an InsP<sub>3</sub> receptor antagonist. As shown in Fig. 6, 2-APB significantly inhibited S1P-induced Ca<sup>2+</sup> mobilization, indicating that S1P

caused the release of Ca<sup>2+</sup> from the SR through InsP<sub>3</sub> receptors (Fig. 8).

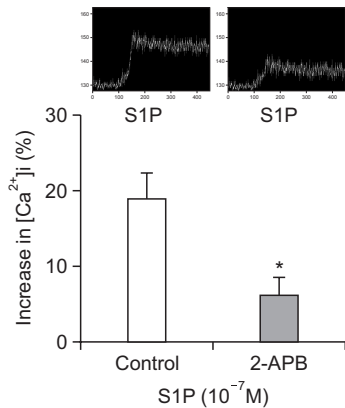
#### Summarized Ca<sup>2+</sup> Signaling pathway by S1P

S1P utilized extracellular Ca<sup>2+</sup> via an L type Ca<sup>2+</sup> channel, which depended on the PTX-sensitive G<sub>i</sub> protein. This occurred via a phospholipase C (PLC)-mediated Ca<sup>2+</sup> release from the InsP<sub>3</sub>-sensitive Ca<sup>2+</sup> 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<sup>2+</sup> modification 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; Ng *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<sub>4</sub> 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<sup>2+</sup> mobilization.

S1P (10<sup>-10</sup>–10<sup>-5</sup> M) was found to produce a concentration-dependent increase in [Ca<sup>2+</sup>]<sub>i</sub> in cat esophageal smooth muscle cells. We used 10<sup>-7</sup> M S1P to study its mechanism of action because the maximal response was observed at 10<sup>-7</sup> M and similar concentrations of S1P have been used by other investigators in previous studies (Adamson *et al.*, 2012).



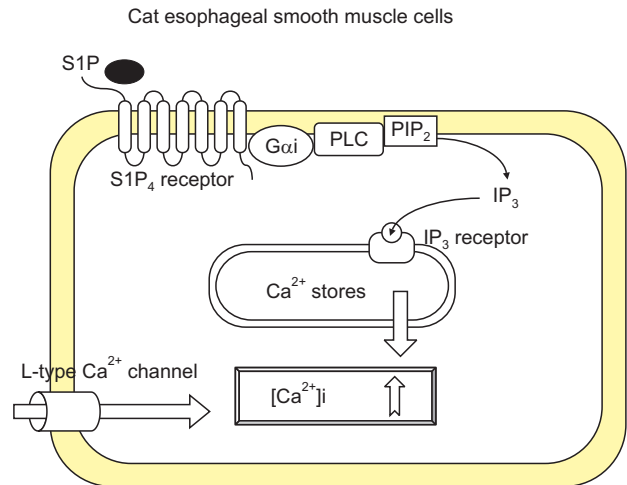
**Fig. 8.** Effect of 2-APB on intracellular Ca<sup>2+</sup> level induced by S1P (10<sup>-7</sup> M). Representative images of autocalculating digitalized diagram (top panels) and bar diagram of [Ca<sup>2+</sup>]<sub>i</sub> (bottom panels). The cells were preincubated for 5 min with 2-APB (200 μM). Intracellular Ca<sup>2+</sup> level was decreased by 2-APB. Values are expressed as the means ± SEM. \*p<0.05 vs. control.

Interestingly, preincubation of cells with EGTA lowered the S1P-induced increase in the intracellular Ca<sup>2+</sup> response; this finding indicates that the S1P-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> is partially dependent on the influx of extracellular Ca<sup>2+</sup>.

S1P has been previously suggested to mobilize [Ca<sup>2+</sup>]<sub>i</sub> by stimulating L-type Ca<sup>2+</sup> channels in renovascular cells (Shaifita *et al.*, 2015). Similar abolishment was observed for nimodipine, suggesting that Ca<sup>2+</sup> influx from the extracellular environment occurs via L-type Ca<sup>2+</sup> channels in cat esophageal smooth muscle cells. While S1P effects on renovascular cells were fully blocked by chelation of extracellular Ca<sup>2+</sup> and inhibited by Ca<sup>2+</sup> entry blockers (Li and Zhang, 2016), incubation with the Ca<sup>2+</sup>-channel blocker nimodipine partially reduced the S1P-evoked increase in [Ca<sup>2+</sup>]<sub>i</sub> 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 [Ca<sup>2+</sup>]<sub>i</sub> in the esophageal smooth muscle cells is linked to opening of the L-type Ca<sup>2+</sup> channels in the sarcolemmal membrane. Because EGTA also partially decreased in the levels of [Ca<sup>2+</sup>]<sub>i</sub> and influx of Ca<sup>2+</sup> is obligatory to replenish SR Ca<sup>2+</sup> stores, intracellular Ca<sup>2+</sup> stores may become depleted by the use of EGTA, resulting in decreased S1P-induced Ca<sup>2+</sup> release from the SR in esophageal smooth muscle cells treated with EGTA. However, the role of the store-operated Ca<sup>2+</sup> channel (Simocheyou *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 Ca<sup>2+</sup> stores by inhibiting SR Ca<sup>2+</sup>-ATPase (Feuerborn *et al.*, 2017). It was observed that the S1P-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> was depressed by TG. Such observations indicated that Ca<sup>2+</sup> release from the SR is involved in S1P-induced Ca<sup>2+</sup> mobilization. Thus, the S1P-evoked increase in [Ca<sup>2+</sup>]<sub>i</sub> may be associated with the release of Ca<sup>2+</sup> from SR Ca<sup>2+</sup> stores.

To further confirm the role of S1P in the release of Ca<sup>2+</sup> from the SR through the involvement of InsP<sub>3</sub>-sensitive SR Ca<sup>2+</sup> pools, the cells were preincubated with the IP<sub>3</sub> recep-



**Fig. 9.** Summarized Ca<sup>2+</sup> Signaling pathway by S1P. S1P utilizes extracellular Ca<sup>2+</sup> via an L type Ca<sup>2+</sup> channel, which continuously depends on PTX-sensitive Gi protein. This occurs due to PLC-mediated Ca<sup>2+</sup> release from the InsP<sub>3</sub>-sensitive Ca<sup>2+</sup> pool in the SR of cat esophageal smooth muscle cells.

tor blocker, 2-APB (Selli and Tosun, 2016). The S1P-induced increase in [Ca<sup>2+</sup>]<sub>i</sub> in cells preincubated with 2-APB was decreased compared to in the control, suggesting that InsP<sub>3</sub>-sensitive Ca<sup>2+</sup> channels are involved in the S1P-induced increase in [Ca<sup>2+</sup>]<sub>i</sub>. It was reported that vasopressin-induced Ca<sup>2+</sup> mobilization in cardiomyocytes is depressed by PLC inhibitors, but not by ryanodine or caffeine, suggesting that there are two Ca<sup>2+</sup> pools in the SR, namely ryanodine-sensitive and InsP<sub>3</sub>-sensitive pools (Liu *et al.*, 1999). Our studies using different agents to modify the action of S1P suggested that S1P releases Ca<sup>2+</sup> from the InsP<sub>3</sub>-sensitive Ca<sup>2+</sup> pool in the SR; further studies are required to investigate the role of the ryanodine-sensitive Ca<sup>2+</sup> pool in the SR in S1P-induced Ca<sup>2+</sup> 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 IP<sub>3</sub> receptor, it was produced from the store in the ER (Li *et al.*, 2015).

To examine the role of G-proteins in S1P-induced Ca<sup>2+</sup> mobilization, the cells were preincubated pertussis toxin. [Ca<sup>2+</sup>]<sub>i</sub> increases by the S1P<sub>1</sub> receptor were caused by PLC-mediated Ca<sup>2+</sup> mobilization, which was fully sensitive to PTX and thus mediated by G<sub>i</sub> proteins (Germinario *et al.*, 2016). In the present study, PTX inhibited S1P-induced Ca<sup>2+</sup> mobilization, indicating that the effects of S1P on intracellular Ca<sup>2+</sup> mobilization are mediated by receptors coupled to a PTX-sensitive G<sub>i</sub>-protein. This result was similar to previous findings in which muscarinic M2 coupled to G<sub>i</sub> or rho protein was involved in mediating contraction and [Ca<sup>2+</sup>]<sub>i</sub> 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 Ca<sup>2+</sup> via an L type Ca<sup>2+</sup> channel, which depended on the PTX-sensitive Gi protein; this response occurred because of PLC-mediated Ca<sup>2+</sup> release from the InsP<sub>3</sub>-sensitive Ca<sup>2+</sup> pool in the SR of cat

esophageal smooth muscle cells.

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