

Comparison of contractile mechanisms of sphingosylphosphorylcholine and sphingosine-1-phosphate in rabbit coronary artery

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[Ca²⁺]_i;
RhoA/RhoA-associated kinase;
Rabbit coronary artery

Aims Although stimulation with sphingosylphosphorylcholine (SPC) or sphingosine-1-phosphate (S1P) generally leads to similar vascular responses, the contractile patterns and their underlying signalling mechanisms are often distinct. We investigated the different reliance upon Ca²⁺-dependent and Ca²⁺-sensitizing mechanisms of constriction in response to SPC or S1P in coronary arteries.

Methods and results Contractile responses, changes in [Ca²⁺]_i, and phosphorylation of myosin light chain phosphatase-targeting subunit (MYPT1) were measured. SPC induced a concentration-dependent sustained contraction. S1P evoked a rapid rise in force (initial transient), which was followed by a secondary sustained force. In the absence of extracellular Ca²⁺, the concentration dependency of constriction to SPC was shifted to the right, but with no change in maximum force, whereas S1P-induced contraction was significantly blunted. Cyclopiazonic acid (CPA) significantly decreased the initial transient force induced by S1P. In isolated single cells, S1P markedly increased [Ca²⁺]_i, whereas only a modest elevation was noted with SPC. The S1P-induced elevation of [Ca²⁺]_i was abolished by pre-treatment with CPA and was significantly reduced in the absence of extracellular Ca²⁺. In β-escin-permeabilized strips, SPC augmented pCa 6.3-induced force; this was significantly inhibited by fasudil hydrochloride. S1P induced little or no augmentation of pCa 6.3-induced force. In intact arteries, SPC-induced contraction was completely inhibited by fasudil hydrochloride. Fasudil hydrochloride had no effect on the initial transient force induced by S1P but significantly inhibited the secondary sustained force. SPC induced a several-fold increase in Thr⁶⁹⁶ and Thr⁸⁵³ phosphorylation of MYPT1, but S1P did not affect phosphorylation of MYPT1.

Conclusion Our results suggest that constriction of coronary arteries in response to the bioactive lipid S1P or SPC occurs by distinct signalling pathways. Activation of the RhoA/RhoA-associated kinase pathway and subsequent phosphorylation of MYPT1 play a key role in SPC-induced coronary contraction, whereas elevation of [Ca²⁺]_i is crucial for S1P-induced coronary constriction.

1. Introduction

The biologically active sphingomyelin metabolites, sphingosylphosphorylcholine (SPC), sphingosine-1-phosphate (S1P), sphingosine, and ceramide are generated upon cell activation from membrane phospholipids as parts of the sphingomyelin cycle.^{1,2} They are present in plasma at high nanomolar concentrations, released from activated platelets,^{3,4} and increased in inflammation and atherosclerosis.⁵ The sphingomyelin pathway is emerging as an important regulator of membrane signal transduction and a variety of cellular functions.^{6,7} In particular, SPC and S1P have recently been added to the list of vascular modulators.^{8–11}

Numerous studies have confirmed a modulation of vascular tone by SPC and S1P, but have yielded equivocal results. Several investigators have reported vasoconstriction responses for SPC and S1P,^{12–15} whereas other studies have found the opposite.^{16–19} The mechanisms involved in SPC- and S1P-induced contractions also have not been fully identified. Recently, evidence for the involvement of the elevation of intracellular free Ca²⁺ concentration ([Ca²⁺]_i)²⁰ and Ca²⁺-sensitization mechanisms, especially phosphorylation of myosin light chain phosphatase (MLCP)-targeting subunit (MYPT1) by RhoA/RhoA-associated kinase (ROCK) pathway,^{15,16,21–23} was reported from several investigations. Taken together, these results suggest that the degree and/or type of vascular response to SPC or S1P differs between vessel types, particularly with respect to the vessel size, involvement of

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endothelium, and different expression patterns of their receptors.¹⁰

Vasospasm is a sustained abnormal contraction of the artery.^{24–26} Several spasmogenic substances have been suggested. These include oxyhaemoglobin, endothelin-1, thrombin, serotonin, noradrenalin, and thromboxane.^{27–29} Sphingolipids have been suggested as candidates of spasmogenic substances because they are released from activated platelets and increase in inflammation, predisposing situations for vasospasm. The cause and pathophysiology of vasospasm have been intensively investigated but not fully understood. Recent investigations suggested that Ca^{2+} -sensitization mechanisms, especially PKC and RhoA/ROCK pathways, are involved in vasospasm rather than increase in $[\text{Ca}^{2+}]_i$.^{21,27,30,31} To determine whether sphingolipids operate as spasmogenic substances, and whether different sphingolipids exert different potencies, studies to compare the patterns and specific mechanisms between SPC- and S1P-induced contractions in the same artery are needed.

In this study, we investigated the differences in patterns between SPC- and S1P-induced contractions in rabbit coronary arteries by measuring contractile responses. We also investigated underlying mechanisms involved in SPC- and S1P-induced contractions by measuring contractile responses in normal and permeabilized strips, changes in $[\text{Ca}^{2+}]_i$, and phosphorylation of MYPT1.

2. Methods

This 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). All procedures were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee.

2.1 Organ bath study

New Zealand white rabbits weighing 1.7–2.2 kg were anaesthetized with pentobarbital sodium (50 mg/kg) containing anticoagulant, heparin (2000 IU/kg). The heart was excised and placed in a modified Krebs–Henseleit (KH) solution composed of (in mM): NaCl, 119; KCl, 4.6; CaCl_2 , 2.5; KH_2PO_4 , 1.2; NaHCO_3 , 25; MgSO_4 1.5; glucose 11. The KH solution was continuously aerated with a mixture of 95% O_2 and 5% CO_2 . The second branch of left descending coronary arteries was dissected out from the heart and ring segments were prepared (4–5 mm in length). To avoid the possible influences of endothelium-derived factors, the endothelium of the arterial ring was removed by gently rubbing the endothelial surface with forceps. Removal of endothelium was confirmed by observing the absence of relaxation on challenge with acetylcholine when 70 mM K^+ solution (equimolar substitution of Na^+ with K^+)-induced contraction had reached plateau.

Force was measured as described previously.³² Briefly, arterial rings were held horizontally in a temperature-controlled, 15 mL organ chamber. The KH solution was maintained at 37°C and was continuously aerated with 95% O_2 and 5% CO_2 . One end of the arterial ring was anchored to a stationary support and the other end was connected to a force-displacement transducer to monitor the muscle contraction. Muscle strips were stretched passively by imposing an optimal resting force of 5 mN (which was evaluated as maximum tension developed by 70 mM K^+ solution), which was maintained throughout the experiment. Each preparation was equilibrated for more than 60 min before starting the experiment. In some preparations, force measurements were recorded in the absence of external Ca^{2+} (0 Ca^{2+} -1 mM EGTA solution). The 0 Ca^{2+} -1 mM EGTA solution was prepared by omission of Ca^{2+} from

KH solution and addition of 1 mM EGTA. Care was taken to ensure the pH remained buffered to 7.3.

SPC or S1P was added cumulatively to construct concentration–response curves. Incubation time of each concentration for SPC and S1P was 20 min and 15 min, respectively. A maximal concentration of SPC (5 μM) or S1P (15 μM) was selected for further force experiments. In Ca^{2+} -imaging experiments, 5 μM SPC or 1 μM S1P was selected. The effects of several pharmacological inhibitory agents were examined following a 30 min pre-incubation period and continued exposure thereafter to the drug during the application of SPC or S1P.

2.2 Single-cell isolation and Ca^{2+} imaging

Single smooth muscle cells were enzymatically isolated from the rabbit coronary arteries. After coronary artery was isolated from the heart, it was moved to a Ca^{2+} -free Tyrode's solution (0 Ca^{2+} PSS) of following composition (in mM): NaCl, 140; KCl, 5.4; MgCl_2 , 1.0; HEPES, 10; glucose, 5.5; pH was adjusted to 7.4 with NaOH. At first, the vessel was incubated in 0 Ca^{2+} PSS for 15 min at 37°C. Secondly, it was incubated in 0 Ca^{2+} PSS with collagenase (2 mg/mL, 190 U; Wako, Richmond, VA, USA), papain (0.0165 mg/mL, Sigma, St Louis, MO, USA), dithiothreitol (0.165 mg/mL, Sigma), trypsin inhibitor (2 mg/mL, Sigma) for 60 min at 37°C. After enzymatic digestion, segments were transferred to 0 Ca^{2+} PSS and single cells were dispersed by gentle agitation with glass pipette. Isolated single cells were stored in 1 mM Ca^{2+} Tyrode's solution at 4°C until use.

Changes in $[\text{Ca}^{2+}]_i$ were measured in isolated single cells loaded with the Ca^{2+} -sensitive dye fura-2/AM (Molecular Probes, Eugene, OR, USA) using a dual excitation digital Ca^{2+} -imaging system (Till Photonics, Munich, Germany) equipped with an intensified CCD camera (Roper Scientific, Tucson, AZ, USA). The imaging system was mounted on an inverted microscope (Olympus, Tokyo, Japan). The strips of rabbit coronary arteries were loaded with 1 μM fura-2/AM in Tyrode's solution for 30 min at room temperature. Cells were illuminated with a xenon arc lamp at 340 and 380 nm and emitted light collected at 510 nm with a CCD camera from regions that encompassed single cells. The ratio of F_{340} to F_{380} [$R(F_{340}/F_{380})$] was calculated as an indicator of $[\text{Ca}^{2+}]_i$. The absolute Ca^{2+} concentration was not calculated in this experiment because the dissociation constant of fluorescence indicator for Ca^{2+} in cytosol may be different from that obtained *in vitro*.

2.3 Permeabilization with β -escin and force recording

Force in permeabilized strips was measured as described previously.³³ Briefly, thin strips (200 μm wide and 2–3 mm long) of coronary artery were dissected and stretched to a resting force of 5 mN. Strips were incubated in relaxing solution containing 4.5 mM MgATP, 1 mM EGTA, 2 mM magnesium methanesulfonate, 74.1 mM potassium methanesulfonate, 30 mM piperazine-*N*, *N*-bis[2-ethanesulfonic acid] (PIPES), 10 mM creatine phosphate neutralized to pH 7.1 with KOH at 20°C for 20 min. Cell permeabilization was achieved by incubation at room temperature (20°C) in β -escin (30 μM , Sigma) in the relaxing solution for 25 min. In the pCa solution, 10 mM EGTA was used to give a desired concentration of free Ca^{2+} . Ionic strength was constant at 0.2 M by adjusting the concentration of potassium methanesulfonate. The SPC- or S1P-induced contraction was examined at pCa 6.3 including 10 μM of GTP γ S (Sigma).

2.4 Western blot

Phosphorylation of the total MYPT, p(696)MYPT, and p(853)MYPT was measured by using western blot. After SPC- and S1P-induced tension was stable, the vessels were immediately frozen by immersion in liquid nitrogen. For each preparation, vessels from 20 animals were pooled. Protein-matched samples (40 μg protein/lane) were

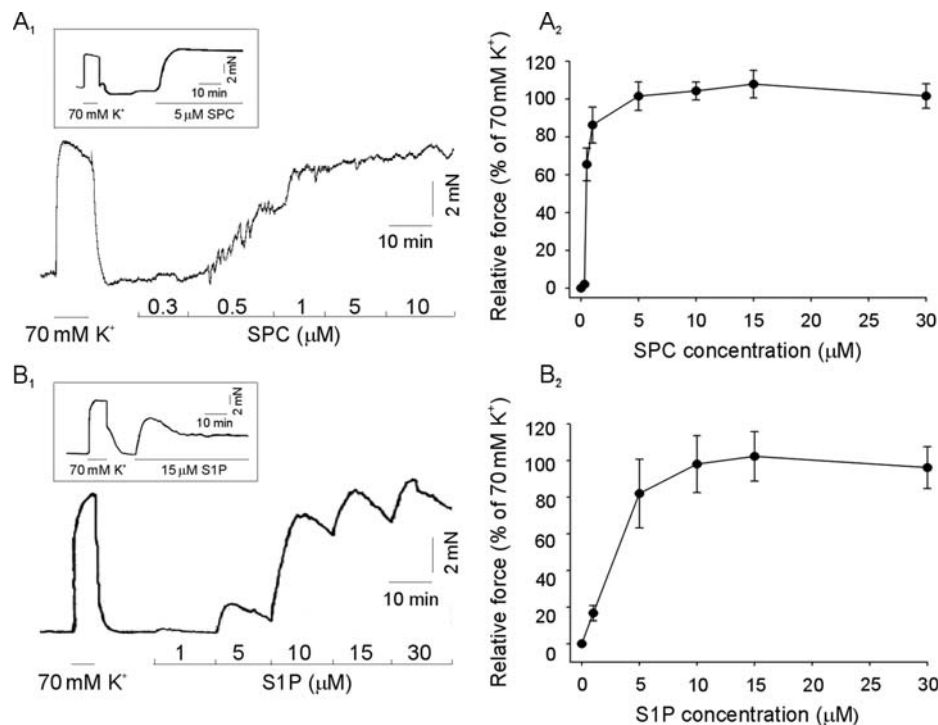


Figure 1 Effects of sphingosylphosphorylcholine (SPC) and sphingosine-1-phosphate (S1P) on force in rabbit coronary artery. (A_1 and A_2) Typical recording (A_1) and statistical evaluation (A_2) showing the concentration–response curve obtained by cumulative addition of sphingosylphosphorylcholine. The inset illustrates the effect of $5\ \mu\text{M}$ sphingosylphosphorylcholine on the force. (B_1 and B_2) Typical recording (B_1) and statistical evaluation (B_2) showing the concentration–response curve obtained by cumulative addition of sphingosine-1-phosphate. The inset illustrates the effect of $15\ \mu\text{M}$ sphingosine-1-phosphate on the force. Data are expressed as relative percentage of $70\ \text{mM}\ \text{K}^+$ solution-induced contraction ($n = 6$).

subjected to electrophoresis on 7.5% SDS–polyacrylamide gels and then transferred to nitrocellulose membranes. Reversible Ponceau S staining of the membranes was performed to confirm the equal loading of protein. Membranes were incubated in 5% skim milk in PBS-Tween buffer for 2 h at room temperature and then were incubated overnight at 4°C in the presence of primary antibodies to MYPT-total (1:500; BD Bioscience, San Jose, CA, USA; Cat No. 612164), MYPT-696 (1:500; Upstate, Charlottesville, VA, USA; Cat No. 07-251), MYPT-853 (1:500; Upstate; Cat No. 36-003). Membranes were washed and then incubated with horseradish peroxidase-conjugated secondary antibody (1:10 000; Calbiochem, Darmstadt, Germany) for 1 h at room temperature. Immunoreactive bands were visualized by enhanced chemiluminescence (ECL; Amersham, Uppsala, Sweden). Developed films from ECL were scanned, and MYPT phosphorylation was quantitated by densitometry of X-ray films using Fuji Photo Film Image with Fuji film image gauge program (ver 2.54; Fuji Photo Film Co, Tokyo, Japan). Care was taken to avoid saturation of the signal at any step in the process.

2.5 Drugs and chemicals

The following drugs were used: S1P (Sigma), SPC (Sigma), cyclopiazonic acid (CPA; TOCRIS, Ellisville, MO, USA), and fasudil hydrochloride (TOCRIS). General laboratory reagents used were of analytical grade or better. S1P was dissolved in 100% methanol. SPC was dissolved in 100% ethanol.

2.6 Statistics

All values given in the text are mean \pm SEM and were analysed by ANOVA, followed by Tukey's test. Significant differences were taken at the $P < 0.05$ level. Force was expressed as a relative percentage of the amplitude of the $70\ \text{mM}\ \text{K}^+$ solution in organ bath study, $50.5\ \text{mM}\ \text{K}^+$ solution in Ca^{2+} imaging, and pCa 4.5 in

permeabilization study, respectively. New strips of coronary artery were used for each separate experiment.

3. Results

3.1 SPC- and S1P-induced contractions in rabbit coronary artery

SPC ($5\ \mu\text{M}$) induced a sustained contraction in rabbit coronary arteries, which reached a maximum plateau level within 10 min (*Figure 1*, inset of A_1). SPC-induced contraction was maintained for at least 1 h. As shown in *Figure 1A*, the SPC-induced contraction was concentration-dependent. The maximum response was obtained at $5\ \mu\text{M}$. The magnitude of the $5\ \mu\text{M}$ SPC-induced contraction was $101.81 \pm 5.92\%$ ($n = 6$) of the contraction produced by $70\ \text{mM}\ \text{K}^+$ solution. We also tested the effect of the same volume of vehicle (ethanol) used at $30\ \mu\text{M}$ SPC on the resting and $70\ \text{mM}\ \text{K}^+$ solution-induced contraction. Treatment with vehicle had no effect (data not shown).

S1P ($15\ \mu\text{M}$) evoked a rapid rise in force (initial transient), which was followed by a secondary sustained force. (*Figure 1*, inset of B_1). S1P-induced contraction was concentration-dependent (*Figure 1*, B_1 and B_2). The magnitude of the $15\ \mu\text{M}$ S1P-induced contractions was $103.62 \pm 15.65\%$ (initial transient force, $n = 6$) of the contraction produced by $70\ \text{mM}\ \text{K}^+$ solution. We also tested the effect of the same volume of vehicle (methanol) used at $30\ \mu\text{M}$ S1P on the resting and $70\ \text{mM}\ \text{K}^+$ solution-induced contraction, respectively. Treatment with vehicle had no effect (data not shown).

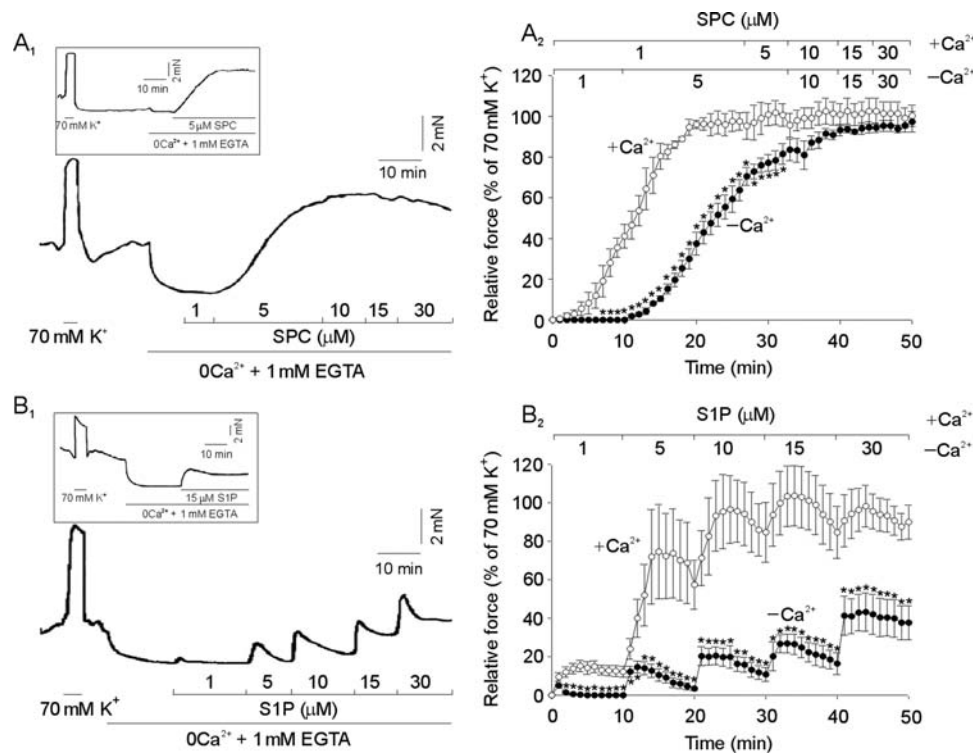


Figure 2 Effects of sphingosylphosphorylcholine (SPC) and sphingosine-1-phosphate (S1P) on force in the absence of extracellular Ca^{2+} . (A_1 and A_2) Typical recording (A_1) and statistical evaluation (A_2) showing the concentration-response curve obtained by cumulative addition of sphingosylphosphorylcholine in the absence of extracellular Ca^{2+} . The inset illustrates the effect of $5 \mu\text{M}$ sphingosylphosphorylcholine on the force. (B_1 and B_2) Typical recording (B_1) and statistical evaluation (B_2) showing the concentration-response curve obtained by cumulative addition of sphingosine-1-phosphate in the absence of extracellular Ca^{2+} . The inset illustrates the effect of $15 \mu\text{M}$ sphingosine-1-phosphate on the force. Data are expressed as relative percentage of 70 mM K^+ solution-induced contraction ($n = 6$). $*P < 0.05$ compared in the presence of extracellular Ca^{2+} .

3.2 Role of Ca^{2+} influx through plasma membrane and/or Ca^{2+} release from sarcoplasmic reticulum on the SPC- and S1P-induced contractions in rabbit coronary artery

To clarify the role of Ca^{2+} influx through plasma membrane in the SPC- and S1P-induced contractions in the rabbit coronary artery, we tested the effect of SPC and S1P on contractile force in the absence of extracellular Ca^{2+} . In the absence of extracellular Ca^{2+} , SPC ($5 \mu\text{M}$) still evoked a gradual and sustained contraction in rabbit coronary artery, which reached a maximum plateau level within 20 min (Figure 2, inset of A_1). As shown in Figure 2A₂, the SPC-induced contraction was concentration-dependent, but this was shifted to the right in the absence of extracellular Ca^{2+} compared with the presence of extracellular Ca^{2+} . The maximum response of SPC-induced contraction was slightly decreased by the absence of extracellular Ca^{2+} . The magnitude of the $5 \mu\text{M}$ SPC-induced contraction was $83.60 \pm 5.34\%$ ($n = 6$) of the contraction produced by 70 mM K^+ solution. In contrast, as shown in Figure 2B, S1P-induced contraction was smaller in the absence of extracellular Ca^{2+} compared with the presence of extracellular Ca^{2+} . At the $15 \mu\text{M}$ S1P, the initial peak magnitude of S1P-induced contraction in the absence and presence of extracellular Ca^{2+} was 26.88 ± 4.91 and $103.62 \pm 15.65\%$ ($P < 0.05$, $n = 6$), respectively.

To determine the involvement of Ca^{2+} release from sarcoplasmic reticulum (SR) in SPC- and S1P-induced contractions, the effect of $20 \mu\text{M}$ CPA, a sarcoplasmic reticular

Ca^{2+} ATPase (SERCA) inhibitor, was examined on SPC- or S1P-induced contractions. In the absence of extracellular Ca^{2+} , SPC ($5 \mu\text{M}$)-induced contraction was partially reduced, but not significantly, by pre-treatment of arterial rings with $20 \mu\text{M}$ CPA. The magnitude of contraction responses to SPC without and with CPA was 89.66 ± 4.68 and $65.53 \pm 6.56\%$ ($n = 6$), respectively (Figure 3A₁ and A₂). In contrast, pre-treatment of arterial rings with $20 \mu\text{M}$ CPA significantly decreased the initial transient force induced by S1P ($15 \mu\text{M}$). The magnitude of S1P-induced contractions in the absence and presence of CPA was 33.52 ± 5.29 and $16.72 \pm 4.71\%$ ($P < 0.05$, $n = 6$), respectively. However, the secondary sustained force of S1P-induced contraction was not affected by CPA pre-treatment (Figure 3B₁ and B₂).

To confirm the role of Ca^{2+} influx through plasma membrane and/or Ca^{2+} release from SR in the SPC- and S1P-induced contractions, we measured changes in intracellular Ca^{2+} transients in single isolated coronary arterial cells using Ca^{2+} -imaging techniques. As shown in Figure 4A and B, in the presence of extracellular Ca^{2+} , SPC ($5 \mu\text{M}$) barely elevated $[\text{Ca}^{2+}]_i$ ($2.95 \pm 1.38\%$, $n = 5$, relative to percentage of 50.5 mM K^+ solution, Figure 4E), but S1P ($1 \mu\text{M}$) significantly increased $[\text{Ca}^{2+}]_i$ ($65.52 \pm 5.47\%$, $n = 5$, relative to percentage of 50.5 mM K^+ solution, Figure 4E). In the absence of extracellular Ca^{2+} , S1P-induced increase in $[\text{Ca}^{2+}]_i$ was significantly inhibited (Figure 4C). The magnitude of S1P-induced increase in $[\text{Ca}^{2+}]_i$ in the absence of extracellular Ca^{2+} was $16.74 \pm 2.80\%$ ($n = 5$) of the $[\text{Ca}^{2+}]_i$ produced by 50.5 mM K^+ solution

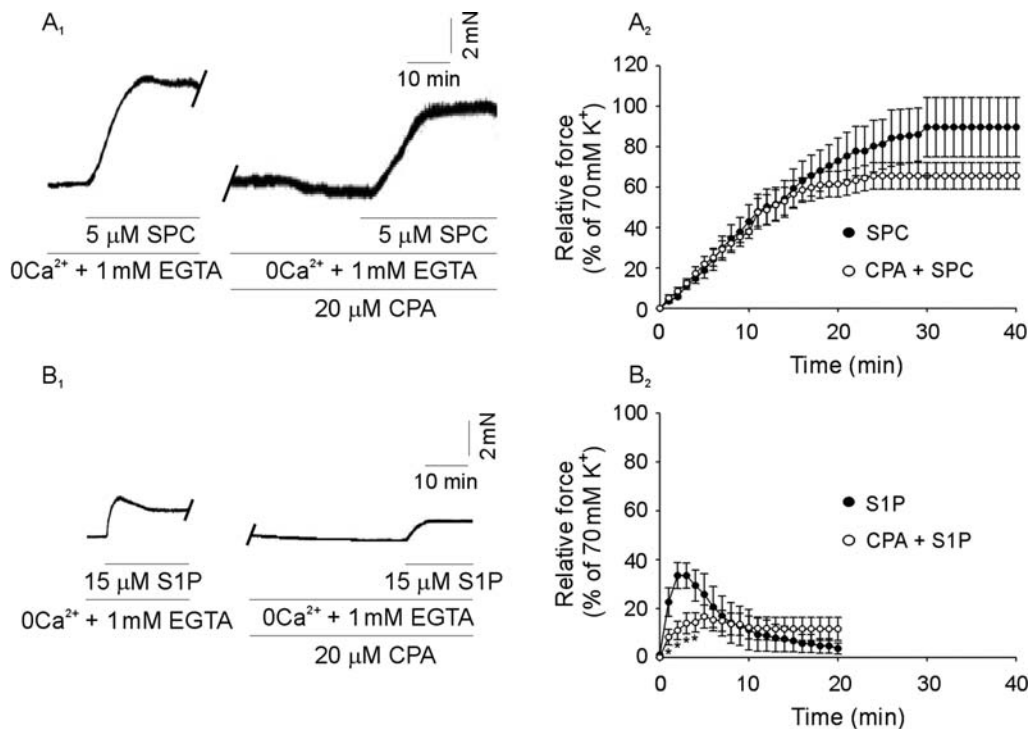


Figure 3 Effect of cyclopiazonic acid (CPA) (20 μM) on the sphingosylphosphorylcholine (SPC)- (A_1 and A_2) and sphingosine-1-phosphate (S1P) (B_1 and B_2)-induced contraction in the absence of extracellular Ca^{2+} . Cyclopiazonic acid (20 μM) was present 30 min before and after application of sphingosylphosphorylcholine (5 μM) or sphingosine-1-phosphate (15 μM). Data are expressed as relative percentage of 70 mM K^+ solution-induced contraction ($n = 6$). * $P < 0.05$ compared with control (in the absence of cyclopiazonic acid).

(Figure 4E). In contrast, pre-treatment of single cells with 5 μM CPA completely abolished the S1P-induced increase in $[\text{Ca}^{2+}]_i$ (Figure 4D).

To investigate the nature of channel(s) involved in the extracellular Ca^{2+} influx induced by S1P, we tested the effect of nifedipine, a voltage-operated Ca^{2+} channel blocker, on the S1P-induced contraction. Pre-treatment with 1 μM nifedipine did not evoke significant changes in S1P-induced contraction (data not shown). The effect of S1P on the Ca^{2+} entry through store-operated Ca^{2+} channels was assessed by evaluating the effect of SKF-96365 on the S1P-induced contraction. Pre-treatment of arterial rings with 10 μM SKF-96365 significantly decreased the force induced by 15 μM S1P (data not shown).

3.3 Role of Ca^{2+} -sensitization mechanisms on the SPC- and S1P-induced contractions in rabbit coronary artery

To determine the role of Ca^{2+} -sensitization mechanisms in the SPC- and S1P-induced contractions in the rabbit coronary artery, we observed the effect of SPC and S1P on contractility at a constant supra-basal but sub-maximal activating $[\text{Ca}^{2+}]_i$ level using β -escin permeabilized strips. As shown in Figure 5, SPC (5 μM) induced significant augmentation of the pCa 6.3-induced force ($46.97 \pm 8.47\%$, $n = 5$, relative to the percentage of pCa 4.5) but this SPC-induced force was abolished by treatment of 5 μM fasudil hydrochloride, a ROCK inhibitor. On the other hand, S1P (15 μM) induced little or no augmentation of force induced by pCa 6.3.

To further determine whether RhoA/ROCK pathway-mediated Ca^{2+} sensitization may play a role in the SPC- and S1P-induced contractions, the effect of 5 μM fasudil hydrochloride was examined on SPC- and S1P-induced responses of intact arteries. As shown in Figure 6A₁ and A₂, fasudil hydrochloride (5 μM) significantly blunted SPC (5 μM)-induced contraction. The magnitude of contractions in response to SPC without and with fasudil hydrochloride was 101.04 ± 9.93 and $12.50 \pm 7.90\%$ ($P < 0.05$, $n = 5$). In contrast, pre-treatment of arterial rings with 5 μM fasudil hydrochloride had no effect on the initial transient force induced by S1P (15 μM). The magnitude of S1P-induced initial transient contractions in the absence and presence of fasudil was 73.99 ± 3.40 and $70.91 \pm 5.30\%$ ($n = 5$), respectively. However, fasudil hydrochloride significantly inhibited the secondary sustained force of S1P-induced contraction (Figure 6B₁ and B₂). The magnitude of S1P-induced secondary sustained contractions in the absence and presence of fasudil was 51.01 ± 5.84 and $15.15 \pm 5.12\%$ ($P < 0.05$, $n = 5$), respectively.

To determine the potential downstream effectors of SPC and S1P, we measured MYPT1 phosphorylation in SPC- and S1P-treated rabbit coronary arterial strips. As shown in Figure 7, SPC (5 μM) induced a several-fold increase in Thr⁶⁹⁶ (to 2.92 ± 0.93 -fold) and Thr⁸⁵³ (to 4.13 ± 1.55 -fold) phosphorylation of MYPT1 in the rabbit coronary artery. However, S1P did not significantly affect phosphorylation of MYPT1 either at Thr⁶⁹⁶ (to 1.19 ± 0.56 -fold) or at Thr⁸⁵³ (to 1.15 ± 0.33 -fold).

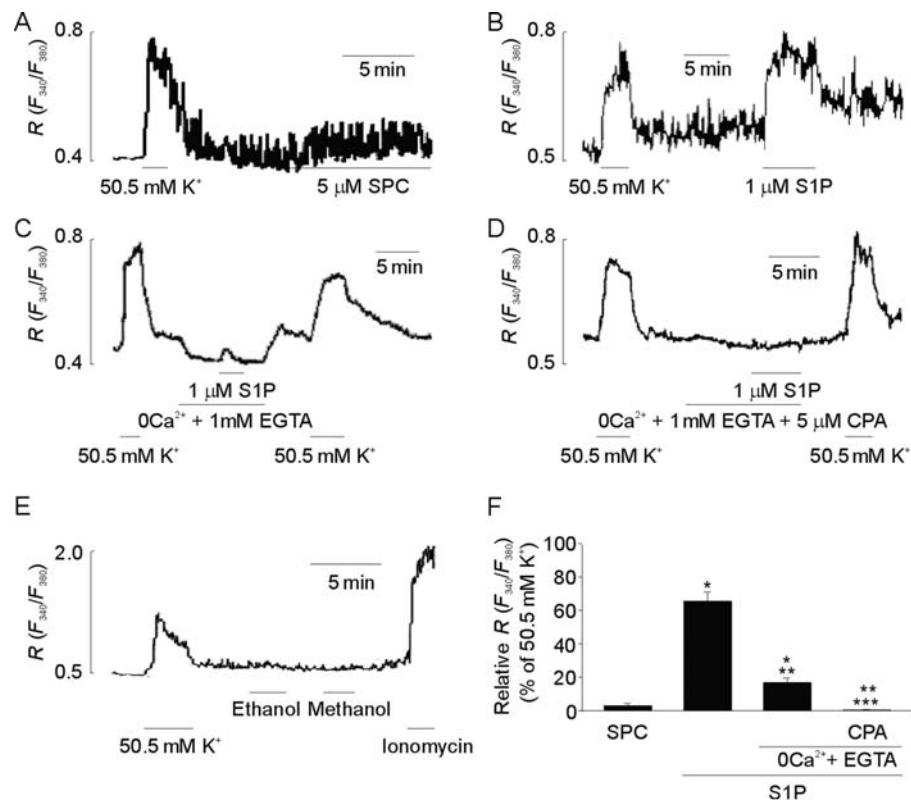


Figure 4 Effects of sphingosylphosphorylcholine (SPC) and sphingosine-1-phosphate (S1P) on intracellular Ca^{2+} transient in isolated single-coronary artery smooth muscle cells. (A) Ca^{2+} transient evoked by sphingosylphosphorylcholine (5 μM) in the presence of extracellular Ca^{2+} in isolated single-coronary artery smooth muscle cells. (B and C) Ca^{2+} transient evoked by sphingosine-1-phosphate (1 μM) in the presence (B) and absence (C) of extracellular Ca^{2+} . (D) The effect of cyclopiazonic acid (CPA) on the sphingosine-1-phosphate-induced increase in Ca^{2+} transient in the absence of extracellular Ca^{2+} . Cyclopiazonic acid (5 μM) was present 15 min before and after the application of sphingosine-1-phosphate (1 μM). (E) The effect of the same volume of solvent, ethanol, or methanol on the resting Ca^{2+} ratio. (F) Statistical evaluation showing the sphingosine-1-phosphate-induced increase in Ca^{2+} transient at the different conditions. Data are expressed as relative percentage of 50.5 mM K^+ solution-induced response ($n = 5$). * $P < 0.05$ compared with resting; ** $P < 0.05$ compared with sphingosine-1-phosphate only; $P < 0.05$ compared with sphingosine-1-phosphate under 0 Ca^{2+} +EGTA.

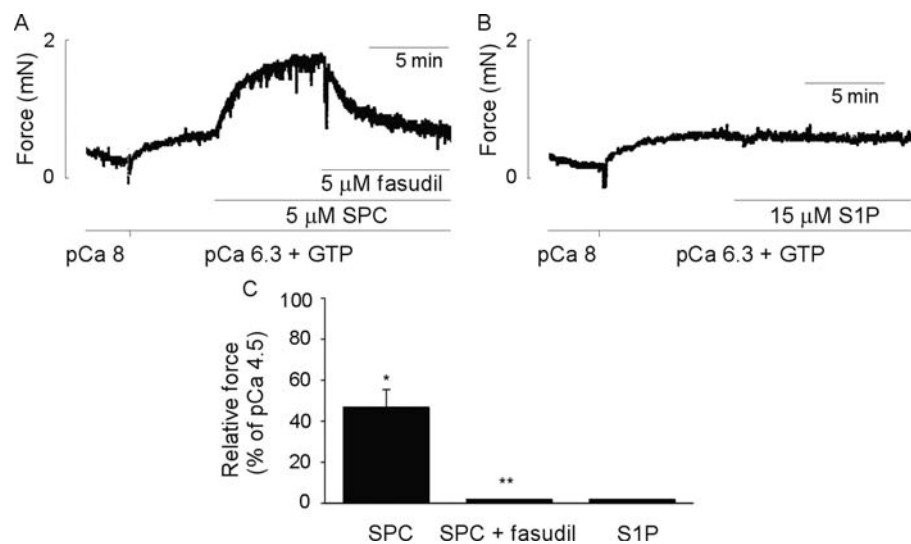


Figure 5 Effects of sphingosylphosphorylcholine (SPC) and sphingosine-1-phosphate (S1P) on the contractility in β -escin permeabilized rabbit coronary artery. (A and B) Typical records for sphingosylphosphorylcholine- (A) and sphingosine-1-phosphate (B)-induced force in β -escin permeabilized rabbit coronary artery. Sphingosylphosphorylcholine or sphingosine-1-phosphate was added when pCa 6.3-induced force was stable, and fasudil hydrochloride was added after sphingosylphosphorylcholine-induced force was stable. (C) Statistical evaluation showing the sphingosylphosphorylcholine- and sphingosine-1-phosphate-induced changes in force at the different conditions. Data are expressed as relative percentage of pCa 4.5-induced response ($n = 5$). * $P < 0.05$ compared with pCa 6.3; ** $P < 0.05$ compared between sphingosylphosphorylcholine and sphingosylphosphorylcholine+fasudil.

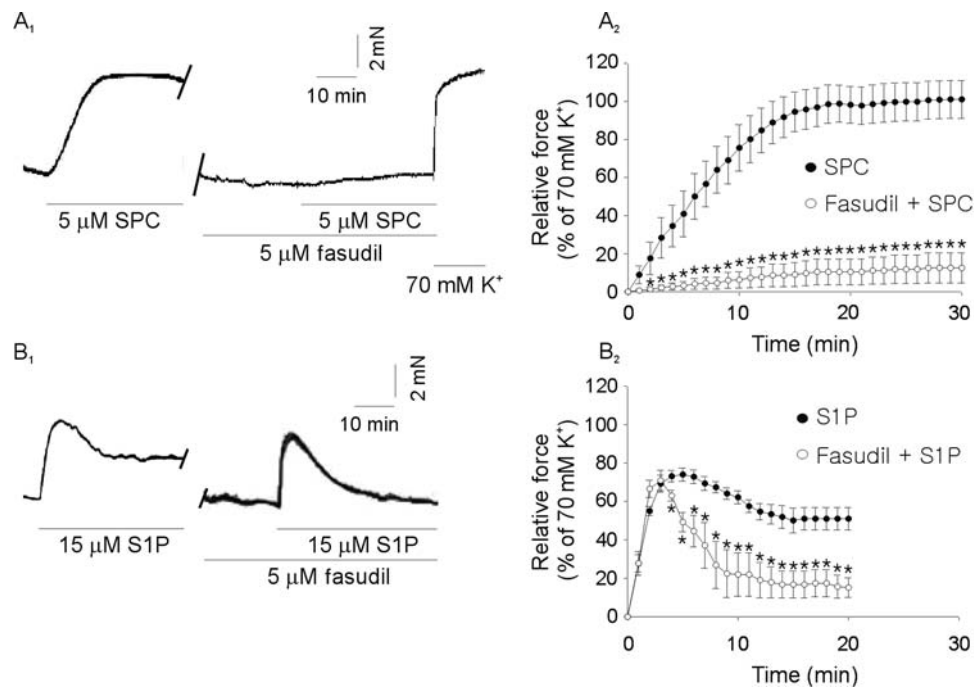


Figure 6 Effects of fasudil hydrochloride (5 μM) on the sphingosylphosphorylcholine (SPC)- (A₁ and A₂) and sphingosine-1-phosphate (B₁ and B₂)-induced contraction. Fasudil hydrochloride (5 μM) was present 30 min before and after the application of sphingosylphosphorylcholine (5 μM) or sphingosine-1-phosphate (15 μM). Data are expressed as relative percentage of 70 mM K⁺ solution-induced contraction (*n* = 5). **P* < 0.05 compared with control (in the absence of fasudil).

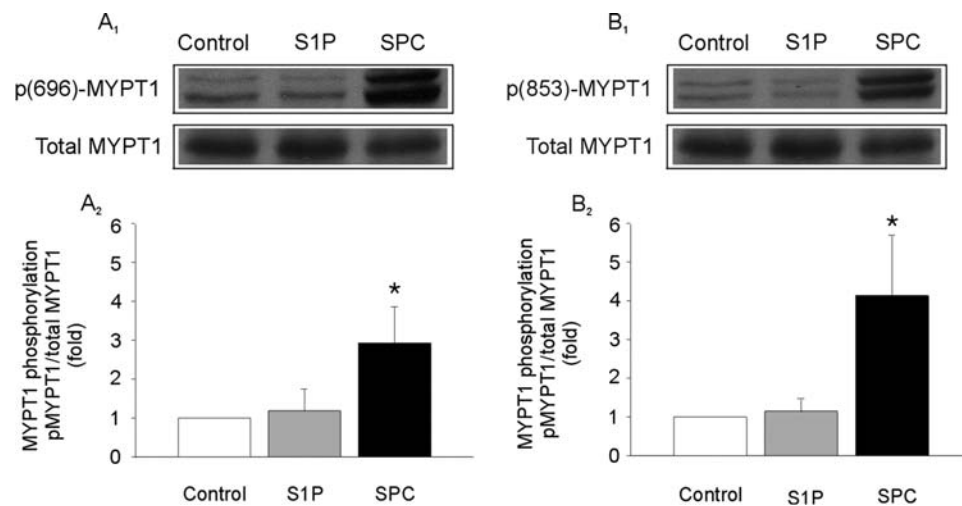


Figure 7 Effects of sphingosylphosphorylcholine (SPC) and sphingosine-1-phosphate (S1P) on phosphorylation of MYPT1. (A₁ and A₂) sphingosylphosphorylcholine and sphingosine-1-phosphate induced Thr⁶⁹⁶ MYPT1 phosphorylation. (B₁ and B₂) sphingosylphosphorylcholine and sphingosine-1-phosphate induced Thr⁸⁵³ MYPT1 phosphorylation. Thr⁶⁹⁶ phosphorylation and Thr⁸⁵³ phosphorylation of MYPT1 were analysed in resting and sphingosylphosphorylcholine (or sphingosine-1-phosphate)-treated strips by western blotting using phosphorylation site-specific anti-phospho-MYPT1 antibodies. Results are representatives of immunoblots from three independent preparations. **P* < 0.05 compared with control.

4. Discussion

4.1 Comparison of contractile responses to SPC and S1P

In this study, we have shown that SPC induced a concentration-dependent and sustained contraction, whereas S1P evoked a concentration-dependent rapid and transient rise in force which was followed by a secondary sustained contraction of lower magnitude. These results are consistent with previous reports concerning SPC- or

S1P-induced contractions.^{12,14,15,21,27,34} However, the patterns of S1P-induced contraction between the present study and previous results show some differences.^{12,14,27} In canine basilar artery, S1P also induced biphasic contraction,²⁷ similar to our results with coronary arteries, but most other previous results reported that S1P induced a sustained contraction.^{12,14} These discrepancies are unlikely to be related to the presence of an endothelium—present in others experiments²⁷ but absent in our experiments—but may be related to vessel-specific and/or cell-specific

receptor expressions/coupling. Although there are many reports concerned with SPC- and S1P-induced contractions, the present study is the first to compare directly the patterns of SPC- and S1P-induced contractions in the same coronary arterial bed.

4.2 Comparison of signalling mechanisms involved in SPC- and S1P-induced contractions

It has been shown that SPC- and S1P-induced vasoconstrictions are mediated by combination of Ca^{2+} mobilization from IP_3 -sensitive store,^{16,35} Ca^{2+} influx through L-type Ca^{2+} channel³⁶ and other Ca^{2+} entry mechanisms,³⁴ and Ca^{2+} sensitization of contractile protein via RhoA/ROCK pathway.^{13,15,16,21–23,37–39} In the present study, to evaluate whether increases in $[\text{Ca}^{2+}]_i$ contribute to the SPC- and S1P-induced contractions, we first measured the effect of the removal of extracellular Ca^{2+} and CPA on the SPC- and S1P-induced contractions in rabbit coronary arterial rings. SPC-induced contraction was only slightly decreased by the absence of extracellular Ca^{2+} , but S1P-induced contraction was significantly suppressed in the absence of extracellular Ca^{2+} . Pre-treatment with CPA did not affect the SPC-induced sustained contraction but significantly decreased the initial transient force induced by S1P. Furthermore, S1P, but not SPC, significantly increased $[\text{Ca}^{2+}]_i$ in isolated coronary artery smooth muscle cells. In the absence of extracellular Ca^{2+} , the S1P-induced increase in $[\text{Ca}^{2+}]_i$ was significantly inhibited, and combined treatment with CPA completely abolished S1P-induced increase in $[\text{Ca}^{2+}]_i$. These results suggest that S1P-induced increase in $[\text{Ca}^{2+}]_i$ might be responsible for the S1P-induced contraction consistent with the previous findings.^{16,20,36} However, in the present study, the discrepancy between SPC-induced contraction in intact strips and changes in $[\text{Ca}^{2+}]_i$ in isolated single cells still exists. In spite of SPC barely elevating $[\text{Ca}^{2+}]_i$ in isolated cells, SPC-induced contraction was slightly decreased by the absence of extracellular Ca^{2+} . Therefore, a minor involvement of Ca^{2+} influx on the SPC-induced contraction still remains a possibility to be fully explored.

To investigate the role of Ca^{2+} -sensitization mechanisms in the SPC- and S1P-induced contractions, we investigated the effect of SPC and S1P on contractility at constant $[\text{Ca}^{2+}]_i$. We showed that SPC induced significant augmentation of the pCa 6.3-induced force in β -escin permeabilized strips, but S1P had little or no effect. These results suggest that Ca^{2+} -sensitization mechanism may play a key role in SPC-induced contraction, but not in S1P-induced contraction and are also consistent with previous findings that a Ca^{2+} -sensitization mechanism is involved in SPC-induced vasoconstriction.^{13,15,21}

It is known that vascular contractility depends on the 20 kDa myosin light chain (MLC_{20}) phosphorylation, which is regulated by myosin light chain kinase (MLCK) and MLCP activities. Phosphorylation of MLC_{20} is a key event in the contraction of vascular smooth muscle and reflects the relative activities of the Ca^{2+} /calmodulin-dependent MLCK and the Ca^{2+} -independent MLCP. MLCP is composed of three subunits: a catalytic subunit of type 1 phosphatase, PP1c; a targeting subunit, termed myosin phosphatase target subunit, MYPT1; and a smaller subunit, M20, of unknown function. Phosphorylation of inhibitory sites on MYPT1, Thr⁶⁹⁶, and

Thr⁸⁵³ results in inhibition of PP1c activity.^{38,39} It is well established that RhoA activates ROCK, which can phosphorylate MYPT1 and inhibit its activity, thus increasing MLC_{20} phosphorylation.³⁷

In the present study, to investigate whether RhoA/ROCK pathway is involved in Ca^{2+} -sensitization mechanism, we examined the effect of fasudil hydrochloride, ROCK inhibitor, on SPC- and S1P-induced vasoconstrictions in intact and permeabilized tissues. We showed that fasudil hydrochloride significantly inhibited SPC-induced contraction in intact and permeabilized tissues. These results suggest that the RhoA/ROCK pathway-mediated Ca^{2+} -sensitization mechanism plays a major role in SPC-induced contraction. These results are consistent with the previous findings that RhoA/ROCK pathway is involved in SPC-induced vasoconstriction.^{13,15,21} To further confirm the involvement of RhoA/ROCK pathway-mediated Ca^{2+} sensitization in SPC- and S1P-induced vasoconstrictions, we examined the effect of SPC and S1P on the MYPT1 phosphorylation level. SPC induced a several-fold increase in Thr⁶⁹⁶ and Thr⁸⁵³ phosphorylation of MYPT1 in rabbit coronary artery, but S1P did not significantly affect phosphorylation of MYPT1 either at Thr⁶⁹⁶ or at Thr⁸⁵³. Taken together, our results suggest that RhoA/ROCK pathway is activated by SPC, resulting in the inhibition of MLCP by phosphorylation of MYPT1.

Our results are compatible with the hypothesis that SPC and S1P may be spasmogenic substances in coronary arteries. However, SPC may be a better candidate than S1P because SPC-induced contraction is evoked by the activation of RhoA/ROCK pathway, which is known as a major mechanism in vasospasm. In the present study, we used second branches of left descending coronary artery. The size of this artery is larger than that of resistance artery. Therefore, the comparison of SPC- and S1P-induced contractions in resistance coronary arteries also requires elucidation.

S1P receptors have been identified.⁴⁰ These receptors were initially called endothelial differentiation gene (EDG) receptors and have been renamed as S1P receptors. They are S1P₁ (EDG-1), S1P₂ (EDG-5), S1P₃ (EDG-3), S1P₄ (EDG-6), and S1P₅ (EDG-8). Because we did not elucidate which receptor is involved in SPC- or S1P-induced contraction in the present study, the nature of the receptor(s) involved in the SPC or S1P response still remains elucidated. SPC can bind to EDG receptors with a potency about two orders of magnitude lower than S1P.

In summary, our results suggest that SPC-induced contraction in rabbit descending coronary arteries shows gradual and sustained patterns but S1P-induced contraction shows an initial transient and then a secondary sustained pattern. Furthermore, activation of RhoA/ROCK pathway and phosphorylation of MYPT1 may be key mechanisms for SPC-induced contraction, whereas elevation of $[\text{Ca}^{2+}]_i$ may be a key mechanism for S1P-induced contraction.

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