RESEARCH ARTICLE

The effect of sphingosine-1-phosphate on colonic smooth muscle contractility: Modulation by TNBS-induced colitis

Aishah Al-Jarallah[1]*, Mabayoje Oriowo[2]

1 Department of Biochemistry, Faculty of Medicine, Health Sciences Center, Kuwait University, Jabreya, Kuwait, **2** Department of Pharmacology and Toxicology, Faculty of Medicine, Health Sciences Center, Kuwait University, Jabreya, Kuwait

* a.jarallah@hsc.edu.kw

Abstract

Aim

Increased levels of circulating sphingosine-1-phosphate (S1P) have been reported in ulcerative colitis. The objective of this study was to examine the effect of S1P on colonic smooth muscle contractility and how is it affected by colitis.

Methods

Colonic inflammation was induced by intrarectal administration of trinitrobenzene sulfonic acid. Five days later colon segments were isolated and used for contractility experiments and immunoblotting.

Results

S1P contracted control and inflamed colon segments and the contraction was significantly greater in inflamed colon segments. S1P-induced contraction was mediated by S1PR1 and S1PR2 in control and S1PR2 in inflamed colon segments. S1PR3 did not play a significant role in S1P-induced contractions in control or inflamed colon. S1PR1, S1PR2 and S1PR3 proteins were expressed in colon segments from both groups. The expression of S1PR1 and S1PR2 was significantly enhanced in control and inflamed colon segments, respectively. S1PR3 levels however were not significantly different between the two groups. Nifedipine significantly reduced S1P-induced contraction in control but not inflamed colon segments. Thapsigargin significantly reduced S1P-induced contraction of the inflamed colon. GF 109203X and Y-27632, alone abolished S1P-induced contraction of the control but not inflamed colon segments. Combination of GF 109203X, Y-27632 and thapsigargin abolished S1P-induced contraction of inflamed colon segments.

Conclusion

S1P contracted control colon via S1PR1 and S1PR2 and inflamed colon exclusively via S1PR2. Calcium influx (control) or release (inflamed) and calcium sensitization are involved in S1P-induced contraction. Exacerbated response to S1P in colitic colon segments may

explain altered colonic motility reported in patients and experimental models of inflammatory bowel disease.

Introduction

Inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease are characterized by chronic inflammation of unknown etiology. IBD involves a complex interaction between genetic and microbial factors and immune responses (recently reviewed in [1]). Abnormal colonic motility characterized by attenuated rhythmic phasic and tonic contractions and enhanced frequency of giant migrating contractions is an important feature of this condition [2–7]. Dysfunctional gastrointestinal motility has been demonstrated in vitro using patients' samples. For example, Snape and colleagues (1991) reported a decrease in contraction of colon circular muscle strips from patients with ulcerative colitis to electrical stimulation and bethanechol. Similarly, Al-Saffar and Hellstrom have also reported decreased reactivity of inflamed colon segments isolated from patients with IBD to tachykinins [8]. This has been confirmed by various studies in experimental models of colitis [9–17]. Several mechanisms have been proposed to explain the dysfunctional colonic contractility. These include impaired handling of intracellular calcium concentrations resulting from reduced calcium influx either through reduced expression [14, 16, 17] or enhanced nitrosylation [18] of L-type calcium channels, reduced sarco/endoplasmic reticulum ATPase-2 (SERCA2) [10], inhibition of myosin light chain (MLC) phosphorylation and reduced expression of CPI-17, an endogenous serine/threonine phosphatase in smooth muscle cells [16, 19] and impaired protein kinase C-dependent calcium sensitization [9]. Finally inflammation induced alterations in the enteric nervous system, specifically reduced nitric oxide synthase (NOS) immunoreactive neurons, were also implicated [11].The molecular mechanisms behind enhanced frequency of giant migrating contractions resulting in uncontrolled defecation, hemorrhage and thick mucus secretions associated with IBD [3, 5–7], are however less well defined.

Sphingosine-1-phosphate (S1P) is a bioactive sphingolipid that has been implicated in a variety of biological processes including cell proliferation, cell survival, cell migration and adaptive and immune responses [20]. Sphingosine is synthesized from ceramide, a metabolic product of sphingomyeline and is phosphorylated in vivo by sphingosine kinase (SK) 1 or 2 into the S1P [20]. S1P stimulated contractions in vascular smooth muscle [21–24] and non-vascular smooth muscle preparations [25–31]. S1P also induced relaxation of vascular smooth muscles [32, 33]. S1P-induced modulation of smooth muscle contractility has been implicated in some pathological conditions including hypertension [34–36], bladder overreactivity [28, 37] and asthma [26, 29, 38–42]. Even though elevated levels of S1P were reported in patients with IBD and confirmed in experimental models of ulcerative colitis [43], the effect of S1P on colonic smooth muscle in control and colitic rats has not been investigated.

The main objective of this project was to examine the effect of S1P on contraction of rat colon and how this is affected by experimentally induced colitis. The role of sphingosine-1-phosphate receptors (S1PRs) and potential downstream signal transduction pathways were also investigated.

Materials and methods

Animals

Rats were obtained from Charles River Research Models and Services. Seventy two male *Sprague-Dawley* rats weighing between 200–250 g were used in this study. The study was approved

by Health Science Center, Kuwait University Ethics Committee for animal use. The study was conducted according to the laboratory's animal care guidelines at Kuwait University, Kuwait in accordance with the international standards of animal care. Rats were maintained at 22˚C on 12-hr light/dark cycle (7 am–7 pm) and water and food were available ad libitum. Rats were anesthetized with ketamine (10 mg/Kg) and xylazine (20 mg/Kg). Experimental colitis was induced by intrarectal administration of 2,4,6-trinitrobenzenesulfonic acid (TNBS, 30 mg, Flucka, Gillingham, UK) dissolved in 0.5 ml of 50% ethanol via a catheter inserted approximately 8 cm from the anal margin and administered at a dose of 73.3 mg/Kg. Rats activity, type of stool and body weights were monitored daily post injection. Some animals died the day after TNBS injection. Dissecting the rats suggested perforation as a potential cause of death. About 5% of the animals died prior to the experimental endpoint. The animals were used five days after TNBS administration. In our experience maximal inflammatory response was obtained five days post TNBS treatment. Rats were sacrificed by concussion followed by exsanguination. Thereafter, the abdominal cavity was opened up and approximately 2 cm segments of the terminal colon was dissected and transferred into a petri-dish containing Krebs' solution at room temperature for physiological experiments or at 4˚C for immunoblotting experiments. The tissues were then cleaned and adherent tissues were removed. Colon segments were used immediately for physiological experiments or stored at -80˚C for immunoblotting studies. Smaller colon segments were preserved in phosphate buffered saline containing 10% formalin and processed for histological evaluation of colonic inflammation as previously described [9].

Physiological experiments

Preparation of colon segments. After removing all adhering tissues, colon segments were set up longitudinally in Krebs' solution at 37˚C contained in a 25ml organ bath for isometric tension recording. The composition of the Krebs' solution was as follows: NaCl, 119; KCl, 4.7; NaHCO$_3$, 25; KH$_2$PO$_4$, 1.2; MgSO$_4$, 1.2; CaCl$_2$, 2.5 and glucose, 11 mM. The solution was continuously gassed with a 5% CO$_2$/95% O$_2$ mixture. The preparations were allowed to equilibrate under a resting tension of 1.0 g for up to 60 min during which the bath fluid was changed at least once. Isometric contractions were recorded, through dynamometer UF1 transducers, on a Lectromed 4-channel polygraph (MultiTrace 4P). After the period of equilibration, KCl (80 mM) was added to the bath to test for tissue viability. The tissues were washed immediately after the peak contraction has been obtained. The tissues were washed repeatedly over the next 30 min period. The contraction to KCl was repeated every 30 min until two consecutive contractions did not differ by more than 5%. The last contraction to KCl was used as reference against which contractions to S1P were expressed. Thereafter, S1P (1, 10 or 40 μM) was added non-cumulatively to the bath and the contractions were recorded. Once the peak response to any concentration of S1P has been attained, the bath fluid was drained and replaced with fresh Krebs' solution. At the end of the experiments, the tissues were washed, weighed, frozen and stored at -80˚C until analyzed. S1P induced contraction was expressed as a percentage of KCl-induced contraction as mean KCl induced contraction was significantly not different between control and colitic colon segments (Control: 3.87 ± 0.51, Colitic: 3.93 ± 0.59). Similar experiments were performed using the selective S1PR1 receptor agonist, SEW2871. In experiments conducted with kinase inhibitors or receptor antagonists, the appropriate concentration of each antagonist was added to the organ baths and allowed to equilibrate with the tissues for 30 min before adding S1P. Only one antagonist was tested on any given preparation except when the antagonists were used in combination.

Determination of S1PR expression Levels by SDS-PAGE and immunoblotting

Total membranes were prepared to determine the expression levels of S1PRs. Colon samples were homogenized on ice for 3 minutes in 20 mM Tris-HCl, pH 7.5 containing 2 mM $MgCl_2$, 0.25 M sucrose, and 1x protease inhibitors (Sigma Aldrich, Munich, Germany). Homogenates were centrifuged at 3000 x g for 10 minutes at 4˚C and supernatants were subjected to another centrifugation step at 100,000 x g for 1 hour at 4˚C. The pellet was suspended in 50 mM Tris-HCl, pH 7.5 containing 1x protease inhibitors cocktail (Sigma Aldrich, Munich, Germany) and 0.1% sodium dodecyle sulfate. Protein concentration was measured using the BCA-protein determination kit (Themoscientific, Ottawa, Ontario, CA) and samples were stored at -80˚C for further analysis.

After boiling, samples from control and colitic colon segments (50 μg protein) were subjected to SDS-PAGE as previously described [44]. The membranes were immunoblotted with rabbit anti-S1PR1 (Cayman Chemical, MI, USA), rabbit anti-S1PR2 (Sigma Aldrich, Munich, Germany), rabbit anti-S1PR3 (Cayman Chemicals, MI, USA) or rabbit anti-actin (Abcam, MA, USA). HRP-conjugated donkey anti-rabbit antibody (Jackson ImmunoResearch, PA, USA) was used as secondary antibody. Bands were detected using Super Signal Western Pico chemiluminescence Substrate (Thermoscientific, Ottawa, Ontario, CA) and quantified using Image J software.

Drugs solutions

The following drugs were used in this study: S1P, SEW2871, W146, JTE-013 and CAY-10444, purchased from Cayman Chemical (MI, USA). Other drugs including nifedipine, thapsigargin, GF 109203X and Y-27632 were purchased from Tocris Bioscience (MN, USA). SEW2871, W146, JTE-013, nifedipine, CAY-10444 and GF 109203X were dissolved in dimethyl sulfoxide. Y-27632 was dissolved in water. S1P was dissolved in pre-warmed solution of bovine serum albumin (4 mg/ml) and incubated at 37˚C for 30 min with vortexing in between. This was followed by sonication for 10 sec in a water bath sonicator.

Statistical analysis

Data was analyzed using the Student's t-test (Microsoft Excel), represented as mean ± standard error of the mean (SEM) and was considered statistically significant when $P < 0.05$.

Results

Characterization of experimental colitis

Rats treated with TNBS weighed significantly less than control rats on day 5 post TNBS injection (223±3.84 g versus 240±2.58 g, $P < 0.05$). Colon segments from TNBS-treated rats were hypertrophic and weighted significantly ($P < 0.05$) more compared to colon segments from control rats (Fig 1A).

Macroscopically, colon segments from TNBS-treated rats were grayish thickened, ulcerated and filled with soft feces while colon segments from control rats were, on the other hand, pinkish and engorged with hard feces). Histologically, TNBS treatment resulted in mucosal ulcerations, goblet cells damage and depletion and inflammatory cell infiltration (Fig 1B).

S1P-induced contractions in control and inflamed colon segments

S1P-induced concentration-dependent contractions of colon segments isolated from control rats (Fig 2A).
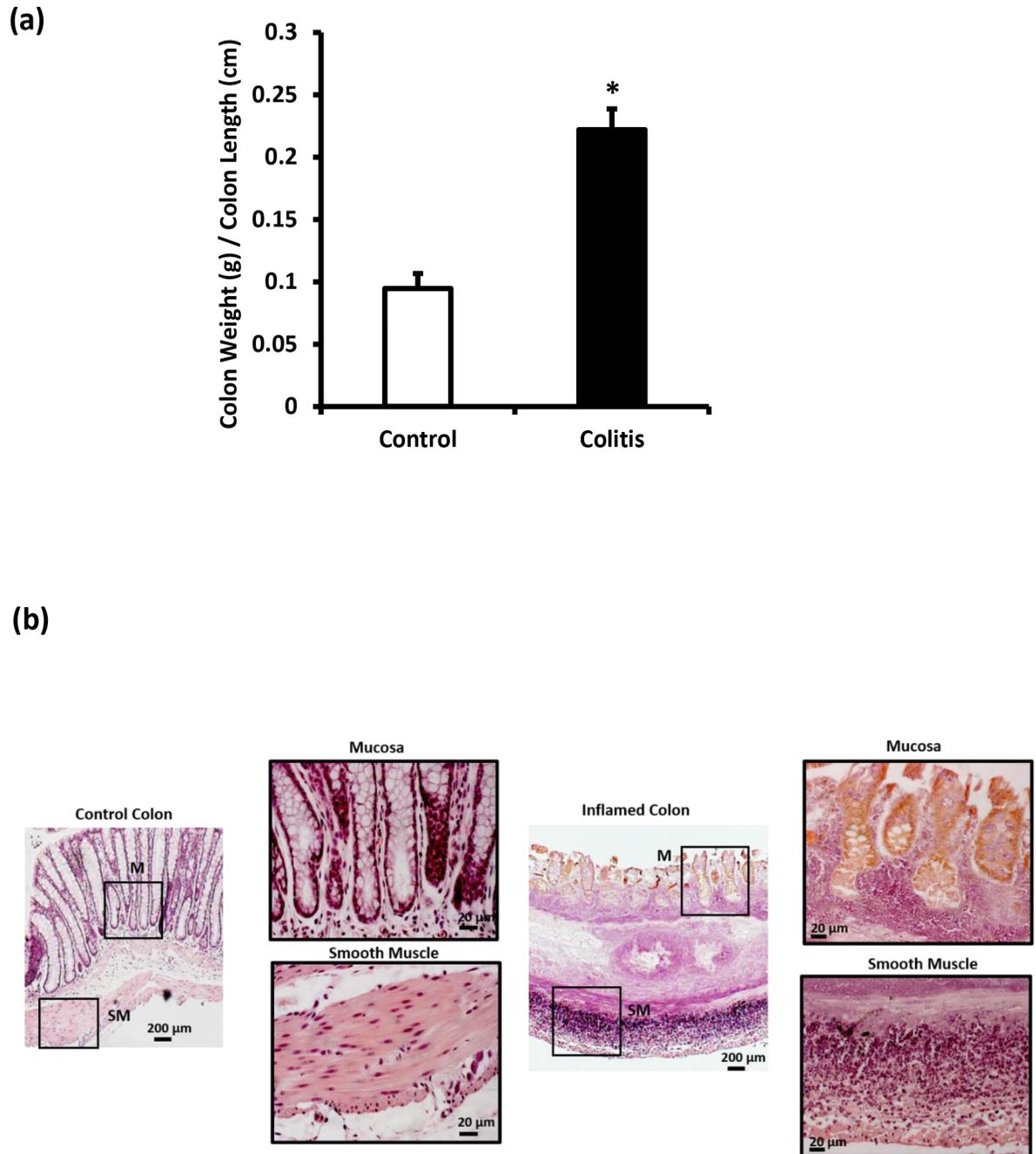
(a)

(b)

Fig 1. Characterization of ulcerative colitis. (A) Colon weight to length ratio in control and colitic rats. (B) Representative eosin and hematoxylin stained colon sections from control and TNBS-treated rats. Data is mean ± SEM, *P < 0.05.

However, we were not able to attain the maximum response as this would involve a lot of S1P. We therefore chose to use 40 µM, a concentration that gave 30% contraction relative to KCl, for subsequent experiments. As shown in (Fig 2B and 2C), S1P (40 µM) induced contractions that were significantly (P < 0.05) greater in colon segments from TNBS-treated rats compared to similar preparations from control rats (Fig 2B). S1PR1 selective agonist, SEW2871, induced contractions in colon segments from control rats (Fig 3A).
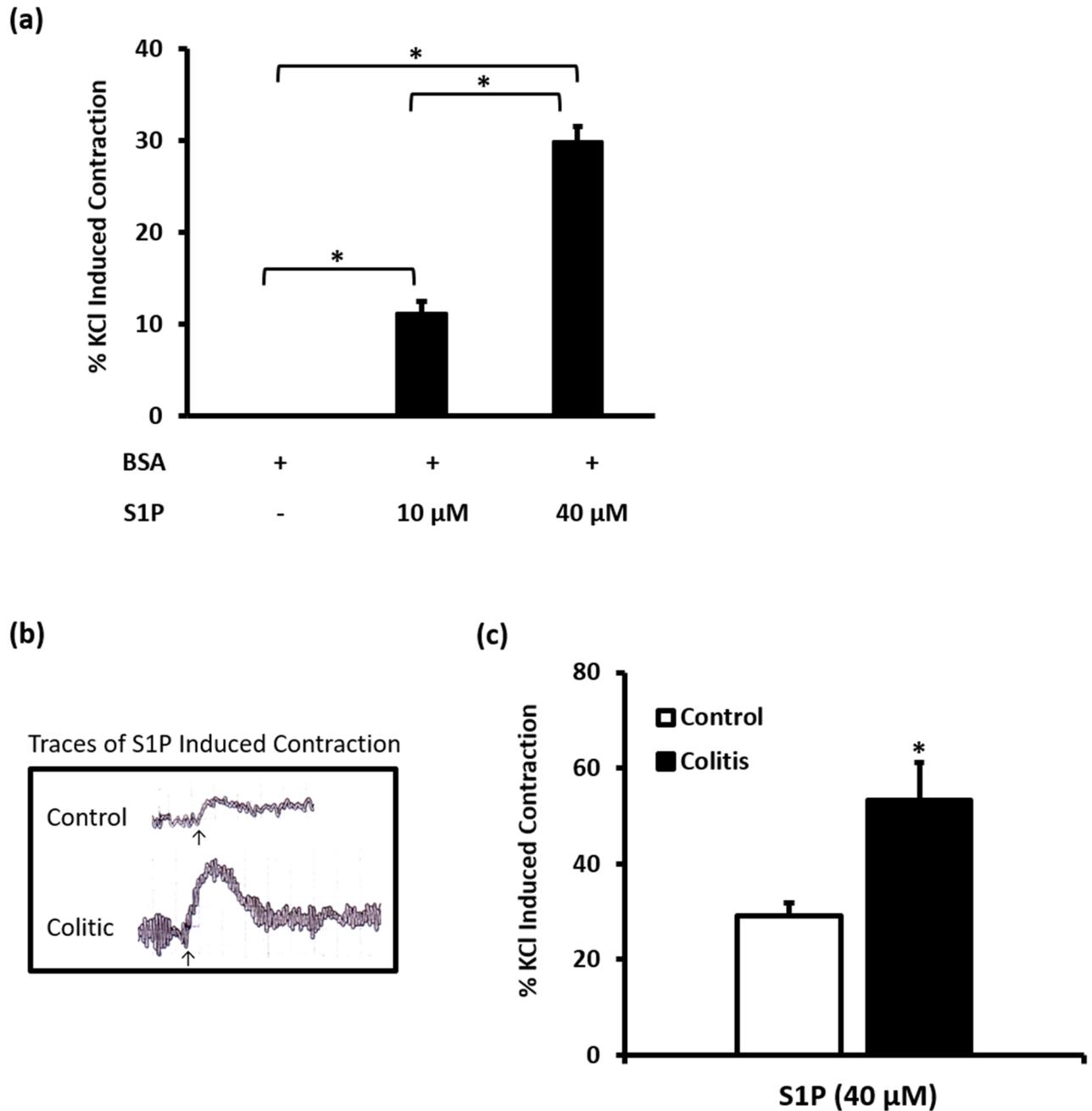
Fig 2. Effect of S1P on KCl-induced contractions in control and colitic colon segments. After equilibration colonic segments from control rats were contracted twice with KCl (80m M) and washed immediately after reaching a peak contraction. Then colonic segments were incubated with BSA (40 µM) or S1P (10 or 40 µM) and allowed to reach a maximal response (A). Traces of S1P (40µM) induced contraction in control and colitic colon segments are shown in (B), arrows indicate S1P addition. Mean S1P (40µM) induced contraction in control and colitic colon segments is shown in (C). Data is mean ± SEM of S1P induced contraction expressed relative to the second KCl-induced contraction, * $P < 0.05$.

SEW2871 however failed to induce a contraction in colon segments from TNBS-treated rats (Fig 3B). Consistent with this finding, the selective S1PR1 antagonist, W146, significantly ($P < 0.05$) reduced S1P induced contractions in colon segments from control but not TNBS-treated rats (Fig 3A and 3B). In contrast the selective S1PR2 receptor antagonist, JTE-013,
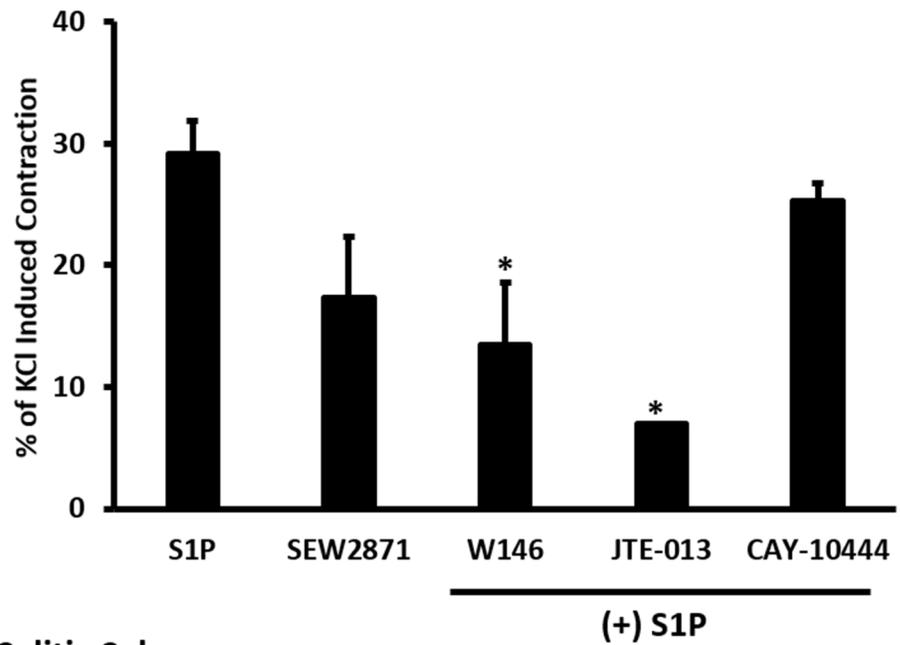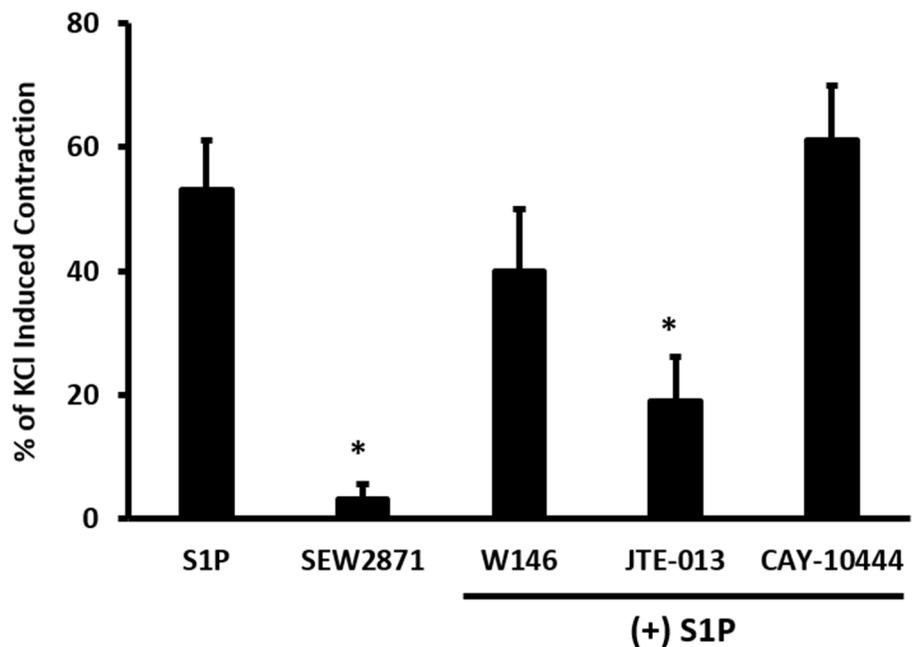
Fig 3. The Role of S1PR in S1P induced contraction in control and colitic colon. Colonic segments from control (A) and colitic (B) rats were equilibrated in Krebs solution for 30 min and contracted twice with KCL (80mM) with consecutive washes after the maximal contraction was reached. Tissues were then preincubated with S1PR1 agonist (SEW2871, 1μM) S1PR1 antagonist (W146, 10μM), S1PR2 antagonist (JTE-013, 1μM) or with S1PR3 antagonist (CAY-10444, 10μM) for 30 min before the addition of S1P (40μM). Data is mean ± SEM of S1P induced contraction is expressed relative to the second KCl-induced contraction, * P < 0.05 relative to S1P induced contraction.

https://doi.org/10.1371/journal.pone.0170792.g003

significantly (P < 0.05) reduced S1P induced contraction in colon segments from control and TNBS-treated rats by 75% and 60% respectively (Fig 3B). CAY-10444, a selective S1PR3 receptor antagonist had no significant effect on S1P-induced contractions in colon segments from control or TNBS-treated rats (Fig 3A and 3B).

Immunoblotting experiments

As shown in (Fig 4), S1PR1, S1PR2 and S1PR3 receptor proteins are expressed in colon segments from control and TNBS-treated rats.

S1PR1 was expressed at significantly (P < 0.05) higher levels in colon segments from control rats relative to colon segments from TNBS-treated rats (Fig 4A). S1PR2 protein levels were on the other hand significantly (P < 0.05) higher in colon segments from TNBS-treated rats (Fig 4B) whereas S1PR3 expression levels were similar in colon segments from control and TNBS-treated rats (Fig 4C).

Role of calcium and calcium-sensitization pathways in S1P-induced contractions

Nifedipine (1 µM), a concentration that abolished KCl-induced contraction [45], significantly (P < 0.05) reduced but did not abolish S1P induced contraction of colon segments from control rats (Fig 5A).

The same concentration of nifedipine did not however affect S1P-induced contractions of colon segments from TNBS-treated rats (Fig 5A). Thapsigargin (10 µM) abolished S1P-induced contraction of colon segments from control rats and significantly (P < 0.05) reduced S1P-induced contraction (approximately 60% reduction) of colon segments from TNBS-treated rats (Fig 5A). GF 102903X and Y-27632 were used to investigate the role of PKC-dependent calcium sensitization pathways in S1P-induced contractions of the colon segments. PKC inhibitor, GF 102903X (1 µM) and Rho-kinase inhibitor (Y-27632, 10 µM) abolished S1P-induced contractions of colon segments from control rats (Fig 5B). However, the same concentrations of these inhibitors reduced S1P-induced contractions of colon segments from TNBS-treated rats by 56% and 68%, respectively when used individually. A combination of GF 109203X (1 µM) and Y-27632 (10 µM) reduced S1P-induced contraction by approximately 85% in colon segments from TNBS-treated rats (Fig 5B). The addition of thapsigargin to GF 102903X and Y-27632 however completely abolished S1P-induced contraction of colon segments from TNBS-treated rats.

Discussion

The objective of this study was to test the effects of S1P on colon contractility and test if these effects are modulated by colonic inflammation. The major findings of the study are: 1) S1P induced contractions in control and inflamed colon segments and these contractions were greater in inflamed colon segments. 2) S1P stimulated contractions are mediated by distinct S1PR isoforms in control and inflamed colon segments. 3) Calcium influx through L-type Calcium channels is required for S1P induced contraction in control but not inflamed colon segments. 4) Calcium release from the sarcoplasmic reticulum and calcium sensitization pathways all contributed to S1P induced contractions in control and inflamed colon segments however their contribution differed (Fig 6).

S1P produces its effects by activating G-protein coupled S1P receptors (S1PRs). Of the five S1PRs that have been identified, S1PR1, S1PR2 and S1PR3 receptors are widely expressed in smooth muscles. One or more of these receptors have been reported to mediate the contractile effect of S1P in a variety of smooth muscle preparations. It has been reported that S1P-induced
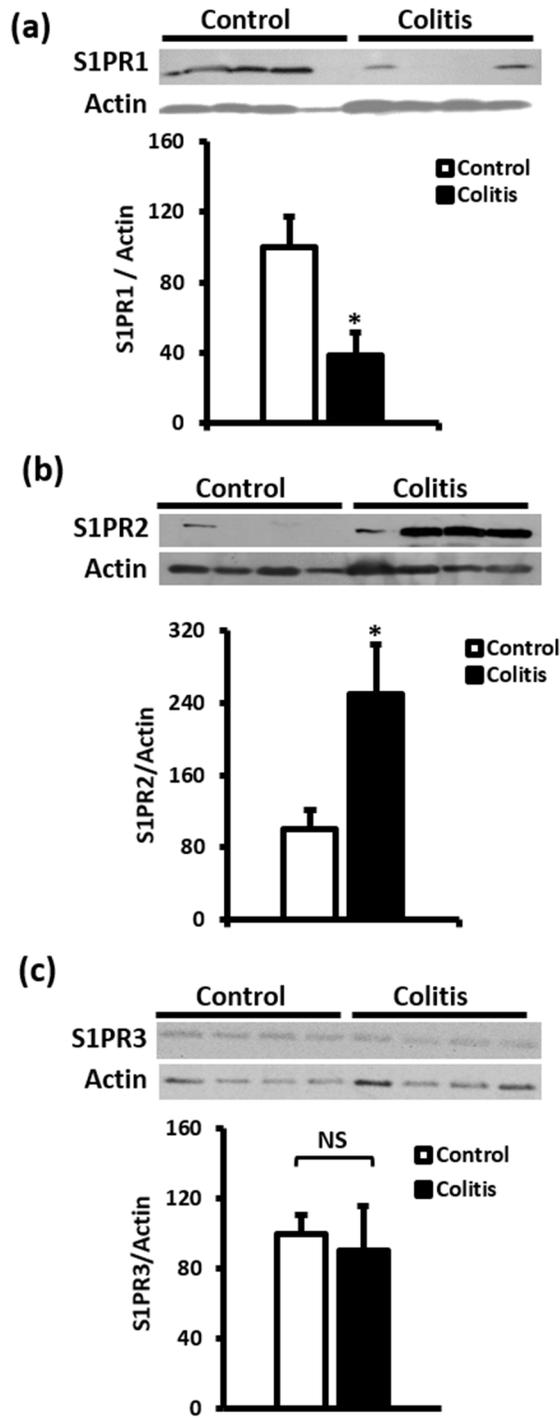
Fig 4. Immunoblotting of S1PR expression in control and colitic colon segments. Representative images of total membrane preps (50 μg protein) from control and colitic colon tissues, from 8–12 independent rats, that were probed with S1PR1 (a), S1PR2 (b) and S1PR3 (c) antibodies. Band intensities were normalized to actin and expressed relative to the controls. Data is mean ± SEM, * P < 0.05 and NS indicates no significance.

https://doi.org/10.1371/journal.pone.0170792.g004

(a)

The role of calcium influx and intracellular calcium release

(b)

The role of calcium sensitization

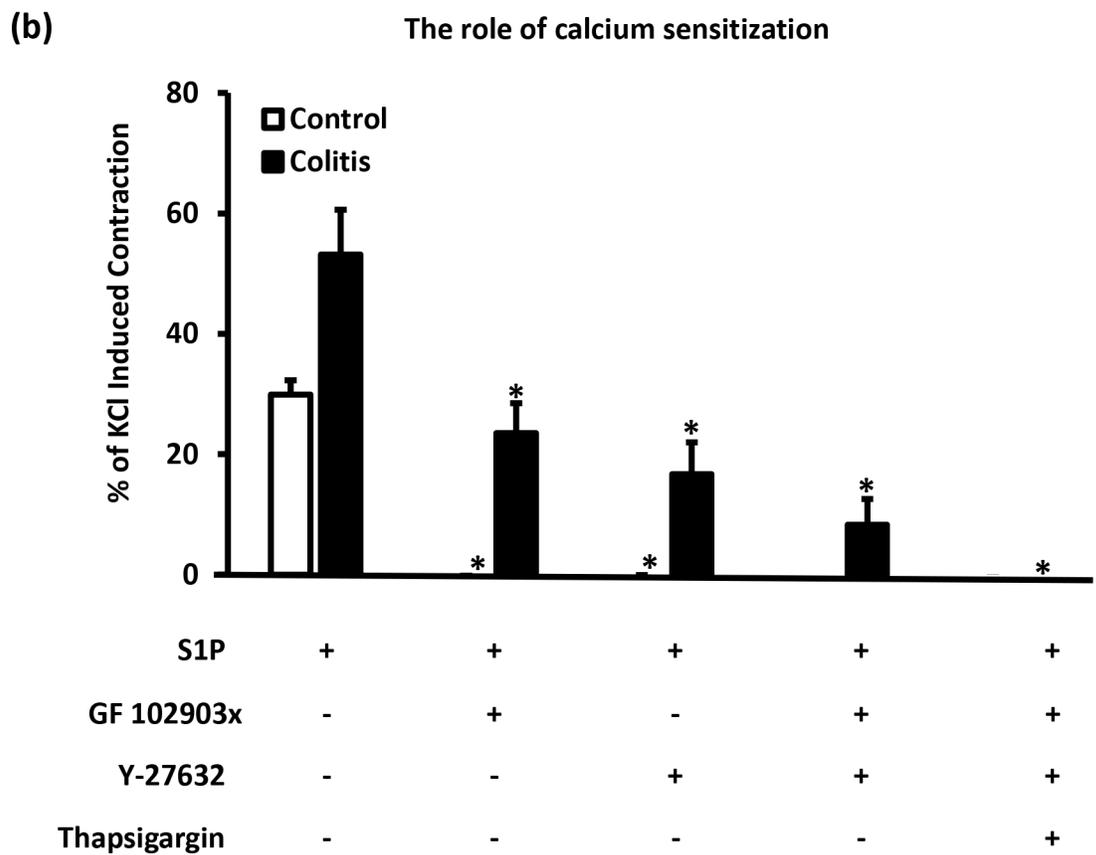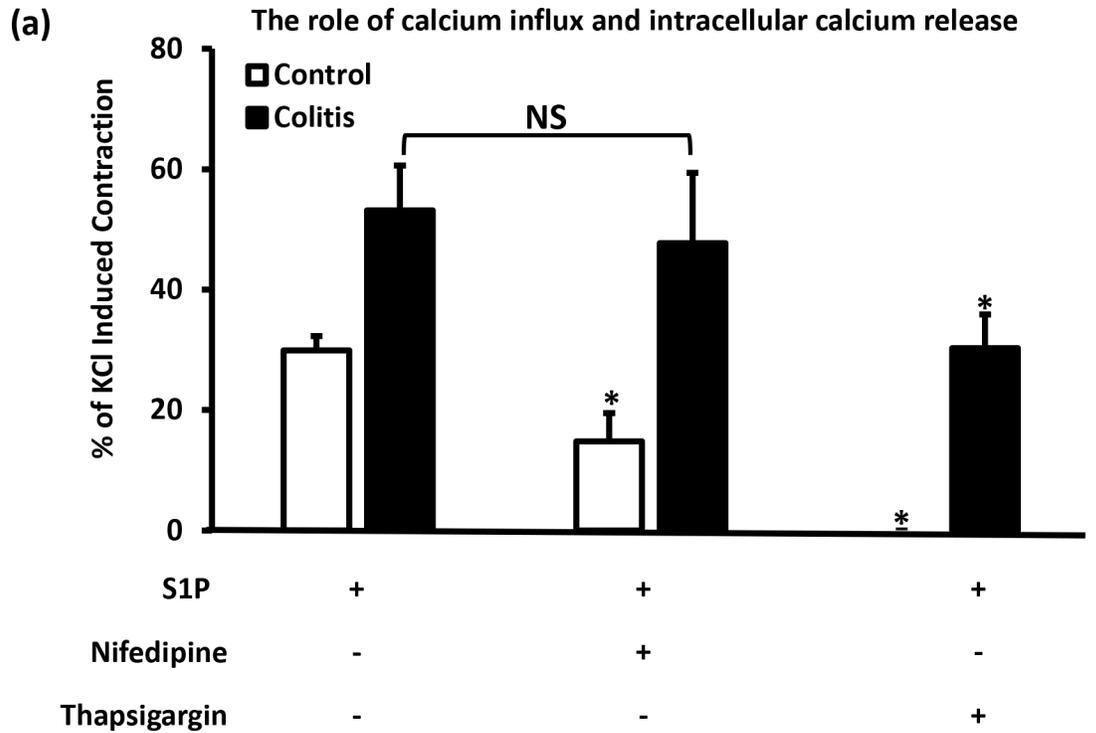**Fig 5. The Role of calcium and calcium sensitization pathways in S1P induced contraction in control and colitic colon.** Colonic segments from control (n = 3–6) and colitic (n = 3–7) rats were equilibrated in Krebs solution for 30 min and contracted twice with KCL with consecutive washes after the maximal contraction was reached. Tissues were then preincubated with L-type calcium channel blocker (nifedipine, 1μM) or SERCA antagonist (thapsigargin, 10μM) (A) or with PKC antagonist (GF 102903x, 1μM), ROCK inhibitor (Y-27632, 10μM) or inhibitor cocktails (B) for 30 min before the addition of S1P (40μM). Data is mean ± SEM of S1P induced contraction expressed relative to the second KCl-induced contraction, * P < 0.05 versus S1P induced contraction in corresponding group.

contractions were mediated via S1PR2 receptors in the urinary bladder [28, 31, 37], myometrium [25], airway smooth muscle [26], esophageal [27] and gastric smooth muscle [29, 46]. S1P-induced contraction of gastric smooth muscle is also mediated in part by S1PR1 receptors [29, 46]. There are some reports of S1PR3 receptors mediating S1P-induced contractions in vascular smooth muscle preparations [21, 22, 47]. We therefore sought to determine the receptor(s) mediating the contractile effect of S1P in rat colon. The results showed that in colon segments from control rats, the selective S1PR1 receptor agonist SEW2871 produced a contraction that was not significantly different from S1P suggesting a role for S1PR1 receptor isoform in mediating S1P-induced contraction of the rat colon. This was confirmed by the fact that the contraction was antagonized by W146, a selective S1PR1 receptor antagonist. In addition, S1P-induced contraction of control rat colon was significantly reduced by selective
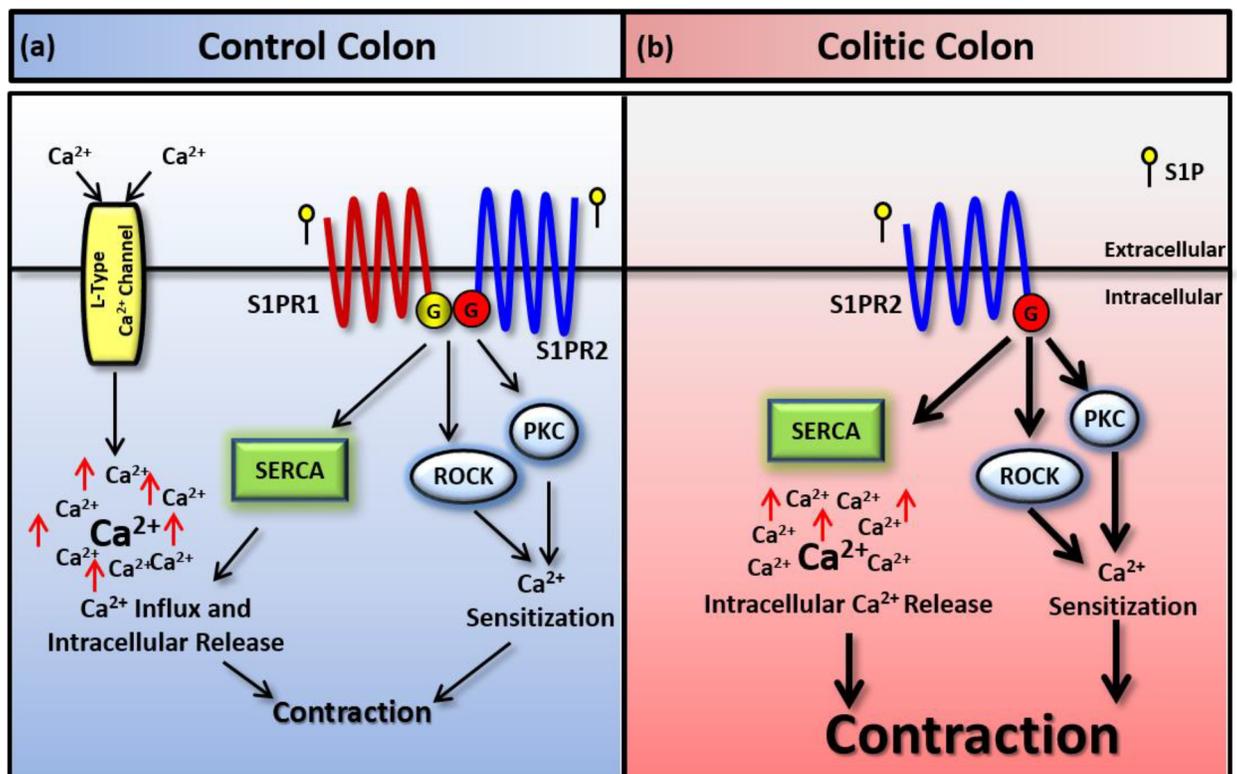

Fig 6. Proposed mechanism of S1P induced contraction in control and colitic colon. In control colon S1P activates S1PR1 and S1PR2 and induce contractions that are dependent on calcium influx via L-type calcium channels, calcium release from the sarcoplasmic reticulum and calcium sensitization pathways including PKC and Rho kinase (A). In colitic colon however S1P induces larger contractions via S1PR2 that are independent of calcium influx through L-type calcium channels yet it involves intracellular calcium release and calcium sensitization pathways (B). The contribution of these pathways however appears to be different in control and inflamed colon segments. Whereas the blockade of a single pathway completely abolishes the response to S1P in control segments, S1P induced contractions appear to be salvaged by other pathways in inflamed colon segments. Eradication of S1P induced contraction in inflamed colon require simultaneous blockade of intracellular calcium release (SERCA) and calcium sensitization pathways (PKC and Rho kinase).

S1PR2 receptor antagonist (JTE-013) but not by the selective S1PR3 receptor antagonist (CAY-10444). Together this suggests that S1P-induced contraction of the control rat colon was mediated via S1PR1 and S1PR2 but not S1PR3 receptor isoforms. This is consistent with the reported involvement of S1PR1 and S1PR2 in mediating S1P-induced contraction of gastric smooth muscle [29, 46]. This result would possibly suggest regional differences in S1PR isoform mediating S1P-induced contraction along the gastrointestinal tract such that S1PR2 receptor mediates contraction in esophageal smooth muscle [27], S1PR1 and S1PR2 receptors in gastric smooth muscle [29, 46] and now in the colon. Interestingly, S1P-induced greater contractions of colon segments from TNBS-treated rats and these contractions were not inhibited by the selective S1PR1 receptor antagonist, W146, at the same concentration that inhibited S1P-induced contraction of colon segments from control rats. This would suggest that S1PR1 receptor did not mediate S1P-induced contraction of inflamed colon segments. In addition, S1P-induced contractions were significantly inhibited by JTE-013 (S1PR2 antagonist) but not CAY-10444 (S1PR3 antagonist). Collectively this data suggests that S1P-induced contraction of inflamed colon segments was mediated by entirely by S1PR2 isoform. These findings were further confirmed by data from immunoblotting experiments. Our results showed that S1PR1, S1PR2 and S1PR3 receptors are expressed in colon segments from control and TNBS-treated rats. S1PR1 protein levels were significantly reduced in colon segments from TNBS-treated rats consistent with lack of involvement of S1PR1 in S1P-induced contraction of inflamed colon segments. On the other hand, S1PR2 protein expression was significantly upregulated in inflamed colon segments which is also consistent with the involvement of S1PR2 in mediating S1P-induced contraction of inflamed colon. Low levels of S1PR3 were detected in control and colitic colon segments however they were not significantly different between the two groups. These finding demonstrate a shift from S1PR1 and S1PR2 in mediating S1P-induced contraction of control colon segments to entirely S1PR2 in mediating S1P-induced contraction of inflamed colon segments. Alterations in the expression levels of S1PR isoforms have been demonstrated in other pathological states for instance upregulation of S1PR1 and S1PR3 and downregulation of S1PR2 were reported in multiple sclerosis lesions [48] and in experimental asthma [49], respectively. The reason behind enhanced response to S1P in inflamed colon segments however remains unclear. S1PR2 was reported to interact with $G_{i/o}$, $G_{12/13}$ and G_q [50]. S1PR1 on the other hand is solely coupled to $G_{i/o}$ [50]. S1PR2, via multiple G-proteins, could thus simultaneously activate multiple downstream signaling pathways. Enhanced expression of S1PR2 observed in colitis may therefore result in enhanced activation of downstream signaling pathways and exacerbated response to S1P.

S1P-induced contractions are associated with increased intracellular concentration of calcium resulting from influx of extracellular calcium and/or release from intracellular stores. The contribution of extracellular or intracellular calcium varies between preparations and with the receptor type mediating the S1P response. S1P-induced contraction of porcine retinal arterioles involved influx of extracellular calcium through L-type voltage operated channels [51] with no role for intracellular calcium. On the other hand, S1P-induced contraction of rat cerebellar artery by releasing calcium from intracellular stores [52]. Rosenfeldt and colleagues have shown that S1P-induced contractions of human airway smooth muscle cells is dependent on influx of extracellular calcium [26]. This has been confirmed by [38]. We therefore determined the role of intra- and extracellular calcium in S1P-induced contraction of the rat colon. The results showed that nifedipine, an inhibitor of L-type voltage-gated calcium channels significantly reduced S1P-induced contraction but did not abolish it indicating that S1P-induced contraction of the rat colon was partly mediated via influx of extracellular calcium in agreement with previous reports in the literature [26, 38, 51]. Thapsigargin abolished S1P-induced contraction indicating contribution of intracellular calcium in S1P-induced contractions.

Thus both extra- and intracellular calcium are involved in S1P-induced contractions of the rat colon. However, influx of extracellular calcium did not appear to be involved in S1P-induced contraction of inflamed colon segments. This was based on our observation that nifedipine at the same concentration that significantly reduced S1P-induced contraction of colon segments from control rats, had no effect on S1P-induced contraction of the inflamed colon. Previous studies have shown that inflammation impaired influx of extracellular calcium [53–55] possibly due to downregulation of L-type calcium channels in the inflamed tissues [14, 54]. Our results are in agreement with these observations. Thapsigargin significantly reduced S1P-induced contraction of the inflamed colon indicating a role for intracellular calcium in S1P-induced contraction of the inflamed colon.

In addition to increasing intracellular calcium concentration, contractile agonists also sensitize the contractile machinery to calcium. This process of sensitization involves PKC and RhoA/Rho-kinase pathways. According to Chiba et al (2010), S1P-induced contraction of murine bronchial smooth muscle was abolished by Y-27632, an inhibitor of Rho kinase indicating a role for Rho-kinase pathway of calcium sensitization in S1P-induced contraction of the bronchial smooth muscle. The Rho kinase pathway of calcium sensitization has also been implicated in S1P-induced contraction of guinea-pig trachea [38], urinary bladder [31, 37], human airway smooth muscle [26] and porcine retinal arterioles [51]. Kim et al have reported a role for the PKC pathway of calcium sensitization in S1P-induced contraction of feline esophageal smooth muscle cells [56]. Other studies have shown that both PKC and Rho-kinase pathways of calcium-sensitization are involved in S1P-induced contractions of the myometrium [25] and porcine renal arterioles [51]. Our results show that Y-27632 (Rho kinase inhibitor) and GF 109203X (PKC inhibitor) abolished S1P-induced contraction of colon segments from control rats. This would suggest that both PKC and Rho-kinase pathways of calcium-sensitization are involved in S1P-induced contraction of the rat colon. The fact that both inhibitors individually abolished S1P-induced contractions would possibly reflect a cross-talk between the pathways as suggested by Kamiya et al in porcine renal arterioles [51]. S1P could sequentially activate PKC and Rho-kinase pathways of calcium sensitization. Accumulating evidence in the literature suggests the sequential activation of PKC and Rho-kinase pathways of calcium sensitization in smooth muscle cells [57–60]. In inflamed colon however, Y-27632 and GF 109203X significantly reduced S1P-induced contraction, yet did not abolish it. This may suggest reduced contribution of PKC and Rho kinase to S1P induced contraction in inflamed colon. We have previously reported reduced PKC mediated calcium sensitization in response to carbachol in inflamed colon segments [9]. A combination of Y-27632 and GF 109203X did not abolish S1P-induced contraction and left a residual contraction of about 15%. The residual contraction however was completely abolished in the presence of thapsigargin.

It was therefore concluded that S1P-induced contractions of the rat colon via S1PR1 and S1PR2 in segments from control rats but exclusively via S1PR2 in inflamed segments from TNBS-treated rats. The contractions involved multiple signaling pathways including influx of extracellular calcium through nifedipine-sensitive L-type voltage-gated channels, calcium release from intracellular stores and calcium sensitization through PKC and Rho-kinase pathways. Enhanced contractile response to S1P in inflamed colon accompanied with the reported increase in S1P levels may possibly contribute to the reported increase in colonic motility in patients and experimental models of inflammatory bowel disease.

Supporting information

S1 File. Raw data underlying the results.
(XLSX)

Acknowledgments

Author Contributions

Conceptualization: AA MO.

Formal analysis: AA.

Funding acquisition: AA MO.

Investigation: AA MO.

Methodology: AA MO.

Project administration: AA.

Resources: AA.

Software: AA MO.

Supervision: AA MO.

Validation: AA MO.

Visualization: AA MO.

Writing – original draft: AA.

Writing – review & editing: MO.

References

1. Zhang YZ, Li YY. Inflammatory bowel disease: pathogenesis. World J Gastroenterol. 2014; 20(1):91–9. PubMed Central PMCID: PMC3886036. https://doi.org/10.3748/wjg.v20.i1.91 PMID: 24415861

2. Coulie B, Camilleri M, Bharucha AE, Sandborn WJ, Burton D. Colonic motility in chronic ulcerative proctosigmoiditis and the effects of nicotine on colonic motility in patients and healthy subjects. Aliment Pharmacol Ther. 2001; 15(5):653–63. PMID: 11328259

3. Sethi AK, Sarna SK. Colonic motor activity in acute colitis in conscious dogs. Gastroenterology. 1991; 100(4):954–63. PMID: 2001831

4. Snape WJ Jr. The role of a colonic motility disturbance in ulcerative colitis. The Keio journal of medicine. 1991; 40(1):6–8. PMID: 2046212

5. Bassotti G, de Roberto G, Chistolini F, Sietchiping-Nzepa F, Morelli O, Morelli A. Twenty-four-hour manometric study of colonic propulsive activity in patients with diarrhea due to inflammatory (ulcerative colitis) and non-inflammatory (irritable bowel syndrome) conditions. Int J Colorectal Dis. 2004; 19(5):493–7. https://doi.org/10.1007/s00384-004-0604-6 PMID: 15083326

6. Loening-Baucke V, Metcalf AM, Shirazi S. Anorectal manometry in active and quiescent ulcerative colitis. Am J Gastroenterol. 1989; 84(8):892–7. PMID: 2756980

7. Jouet P, Sarna SK, Singaram C, Ryan RP, Hillard CJ, Telford GL, et al. Immunocytes and abnormal gastrointestinal motor activity during ileitis in dogs. Am J Physiol. 1995; 269(6 Pt 1):G913–24.

8. Al-Saffar A, Hellstrom PM. Contractile responses to natural tachykinins and selective tachykinin analogs in normal and inflamed ileal and colonic muscle. Scand J Gastroenterol. 2001; 36(5):485–93. Epub 2001/05/11. PMID: 11346201

9. Al-Jarallah A, Khan I, Oriowo MA. Role of Ca2+-sensitization in attenuated carbachol-induced contraction of the colon in a rat model of colitis. Eur J Pharmacol. 2008; 579(1–3):365–73. Epub 2007/11/27. https://doi.org/10.1016/j.ejphar.2007.10.069 PMID: 18037403

10. Al-Jarallah A, Oriowo MA, Khan I. Mechanism of reduced colonic contractility in experimental colitis: role of sarcoplasmic reticulum pump isoform-2. Mol Cell Biochem. 2007; 298(1–2):169–78. Epub 2006/11/30. https://doi.org/10.1007/s11010-006-9363-8 PMID: 17131044

11. Auli M, Nasser Y, Ho W, Burgueno JF, Keenan CM, Romero C, et al. Neuromuscular changes in a rat model of colitis. Auton Neurosci. 2008; 141(1–2):10–21. Epub 2008/06/07. https://doi.org/10.1016/j.autneu.2008.04.005 PMID: 18534920

12. Cao W, Vrees MD, Kirber MT, Fiocchi C, Pricolo VE. Hydrogen peroxide contributes to motor dysfunction in ulcerative colitis. Am J Physiol Gastrointest Liver Physiol. 2004; 286(5):G833–43. Epub 2003/12/13. https://doi.org/10.1152/ajpgi.00414.2003 PMID: 14670823

13. Cheon GJ, Cui Y, Yeon DS, Kwon SC, Park BG. Mechanisms of motility change on trinitrobenzenesulfonic Acid-induced colonic inflammation in mice. The Korean journal of physiology & pharmacology: official journal of the Korean Physiological Society and the Korean Society of Pharmacology. 2012; 16 (6):437–46. PubMed Central PMCID: PMC3526749.

14. Khan I. Molecular basis of altered contractility in experimental colitis: expression of L-type calcium channel. Dig Dis Sci. 1999; 44(8):1525–30. Epub 1999/09/24. PMID: 10492128

15. Lundberg S, Holst M, Hellstrom PM. Expression of iNOS mRNA associated with suppression of colonic contraction in rat colitis. Acta Physiol (Oxf). 2006; 187(4):489–94. Epub 2006/07/27.

16. Ohama T, Hori M, Fujisawa M, Kiyosue M, Hashimoto M, Ikenoue Y, et al. Downregulation of CPI-17 contributes to dysfunctional motility in chronic intestinal inflammation model mice and ulcerative colitis patients. J Gastroenterol. 2008; 43(11):858–65. Epub 2008/11/18. https://doi.org/10.1007/s00535-008-2241-2 PMID: 19012039

17. Ohama T, Hori M, Ozaki H. Mechanism of abnormal intestinal motility in inflammatory bowel disease: how smooth muscle contraction is reduced? J Smooth Muscle Res. 2007; 43(2):43–54. Epub 2007/06/30. PMID: 17598957

18. Ross GR, Kang M, Shirwany N, Malykhina AP, Drozd M, Akbarali HI. Nitrotyrosylation of Ca2+ channels prevents c-Src kinase regulation of colonic smooth muscle contractility in experimental colitis. J Pharmacol Exp Ther. 2007; 322(3):948–56. https://doi.org/10.1124/jpet.107.123075 PMID: 17551092

19. Ohama T, Hori M, Momotani E, Iwakura Y, Guo F, Kishi H, et al. Intestinal inflammation downregulates smooth muscle CPI-17 through induction of TNF-alpha and causes motility disorders. Am J Physiol Gastrointest Liver Physiol. 2007; 292(5):G1429–38. Epub 2007/02/20. https://doi.org/10.1152/ajpgi.00315.2006 PMID: 17307724

20. Maceyka M, Harikumar KB, Milstien S, Spiegel S. Sphingosine-1-phosphate signaling and its role in disease. Trends Cell Biol. 2012; 22(1):50–60. Epub 2011/10/18. PubMed Central PMCID: PMC3253987. https://doi.org/10.1016/j.tcb.2011.09.003 PMID: 22001186

21. Choi SK, Ahn DS, Lee YH. Comparison of contractile mechanisms of sphingosylphosphorylcholine and sphingosine-1-phosphate in rabbit coronary artery. Cardiovasc Res. 2009; 82(2):324–32. Epub 2009/02/17. https://doi.org/10.1093/cvr/cvp054 PMID: 19218288

22. Murakami A, Takasugi H, Ohnuma S, Koide Y, Sakurai A, Takeda S, et al. Sphingosine 1-phosphate (S1P) regulates vascular contraction via S1P3 receptor: investigation based on a new S1P3 receptor antagonist. Mol Pharmacol. 2010; 77(4):704–13. Epub 2010/01/26. https://doi.org/10.1124/mol.109.061481 PMID: 20097776

23. Salomone S, Soydan G, Ip PC, Hopson KM, Waeber C. Vessel-specific role of sphingosine kinase 1 in the vasoconstriction of isolated basilar arteries. Pharmacol Res. 2010; 62(6):465–74. Epub 2010/09/21. PubMed Central PMCID: PMC2974794. https://doi.org/10.1016/j.phrs.2010.09.002 PMID: 20850539

24. Salomone S, Yoshimura S, Reuter U, Foley M, Thomas SS, Moskowitz MA, et al. S1P3 receptors mediate the potent constriction of cerebral arteries by sphingosine-1-phosphate. Eur J Pharmacol. 2003; 469(1–3):125–34. Epub 2003/06/05. PMID: 12782194

25. Leiber D, Banno Y, Tanfin Z. Exogenous sphingosine 1-phosphate and sphingosine kinase activated by endothelin-1 induced myometrial contraction through differential mechanisms. Am J Physiol Cell Physiol. 2007; 292(1):C240–50. Epub 2006/09/08. https://doi.org/10.1152/ajpcell.00023.2006 PMID: 16956968

26. Rosenfeldt HM, Amrani Y, Watterson KR, Murthy KS, Panettieri RA Jr., Spiegel S. Sphingosine-1-phosphate stimulates contraction of human airway smooth muscle cells. FASEB J. 2003; 17(13):1789–99. Epub 2003/10/02. https://doi.org/10.1096/fj.02-0836com PMID: 14519658

27. Song HJ, Choi TS, Chung FY, Park SY, Ryu JS, Woo JG, et al. Sphingosine 1-phosphate-induced signal transduction in cat esophagus smooth muscle cells. Mol Cells. 2006; 21(1):42–51. Epub 2006/03/03. PMID: 16511346

28. Watterson KR, Berg KM, Kapitonov D, Payne SG, Miner AS, Bittman R, et al. Sphingosine-1-phosphate and the immunosuppressant, FTY720-phosphate, regulate detrusor muscle tone. FASEB J. 2007; 21 (11):2818–28. Epub 2007/04/24. https://doi.org/10.1096/fj.06-7326com PMID: 17449719

29. Zhou H, Murthy KS. Distinctive G protein-dependent signaling in smooth muscle by sphingosine 1-phosphate receptors S1P1 and S1P2. Am J Physiol Cell Physiol. 2004; 286(5):C1130–8. Epub 2004/04/13. https://doi.org/10.1152/ajpcell.00429.2003 PMID: 15075212

30. Hu W, Huang J, Mahavadi S, Li F, Murthy KS. Lentiviral siRNA silencing of sphingosine-1-phosphate receptors S1P1 and S1P2 in smooth muscle. Biochem Biophys Res Commun. 2006; 343(4):1038–44. Epub 2006/04/01. https://doi.org/10.1016/j.bbrc.2006.03.079 PMID: 16574065

31. Kendig DM, Matsumoto AK, Moreland RS. Sphingosine-1-phosphate induced contraction of bladder smooth muscle. Eur J Pharmacol. 2013; 720(1–3):355–62. PubMed Central PMCID: PMC3849345. https://doi.org/10.1016/j.ejphar.2013.10.004 PMID: 24120660

32. di Villa Bianca R, Sorrentino R, Imbimbo C, Palmieri A, Fusco F, Maggi M, et al. Sphingosine 1-phosphate induces endothelial nitric-oxide synthase activation through phosphorylation in human corpus cavernosum. J Pharmacol Exp Ther. 2006; 316(2):703–8. Epub 2005/10/20. https://doi.org/10.1124/jpet.105.093419 PMID: 16234413

33. Mair KM, Robinson E, Kane KA, Pyne S, Brett RR, Pyne NJ, et al. Interaction between anandamide and sphingosine-1-phosphate in mediating vasorelaxation in rat coronary artery. Br J Pharmacol. 2010; 161 (1):176–92. Epub 2010/08/20. PubMed Central PMCID: PMC2962826. https://doi.org/10.1111/j.1476-5381.2010.00878.x PMID: 20718749

34. Spijkers LJ, van den Akker RF, Janssen BJ, Debets JJ, De Mey JG, Stroes ES, et al. Hypertension is associated with marked alterations in sphingolipid biology: a potential role for ceramide. PLoS One. 2011; 6(7):e21817. Epub 2011/08/06. PubMed Central PMCID: PMC3139577. https://doi.org/10.1371/journal.pone.0021817 PMID: 21818267

35. Ikeda H, Nagashima K, Yanase M, Tomiya T, Arai M, Inoue Y, et al. Sphingosine 1-phosphate enhances portal pressure in isolated perfused liver via S1P2 with Rho activation. Biochem Biophys Res Commun. 2004; 320(3):754–9. Epub 2004/07/09. https://doi.org/10.1016/j.bbrc.2004.04.207 PMID: 15240112

36. Kegeyama Y, Ikeda H, Watanabe N, Nagamine M, Kusumoto Y, Yashiro M, et al. Antagonism of sphingosine 1-phosphate receptor 2 causes a selective reduction of portal vein pressure in bile duct-ligated rats. Hepatology. 2012. Epub 2012/04/17.

37. Aydin M, Downing K, Villegas G, Zhang X, Chua R, Melman A, et al. The sphingosine-1-phosphate pathway is upregulated in response to partial urethral obstruction in male rats and activates RhoA/Rho-kinase signalling. BJU Int. 2010; 106(4):562–71. Epub 2010/02/05. https://doi.org/10.1111/j.1464-410X.2009.09156.x PMID: 20128782

38. Kume H, Takeda N, Oguma T, Ito S, Kondo M, Ito Y, et al. Sphingosine 1-phosphate causes airway hyper-reactivity by rho-mediated myosin phosphatase inactivation. J Pharmacol Exp Ther. 2007; 320 (2):766–73. Epub 2006/11/16. https://doi.org/10.1124/jpet.106.110718 PMID: 17105828

39. Makino Y, Kume H, Oguma T, Sugishita M, Shiraki A, Hasegawa Y, et al. Role of Sphingosine-1-Phosphate inbeta-adrenoceptor Desensitization via Ca2+ Sensitization in Airway Smooth Muscle. Allergol Int. 2012; 61(2):311–22. Epub 2012/03/24. https://doi.org/10.2332/allergolint.11-OA-0350 PMID: 22441633

40. Pfaff M, Powaga N, Akinci S, Schutz W, Banno Y, Wiegand S, et al. Activation of the SPHK/S1P signalling pathway is coupled to muscarinic receptor-dependent regulation of peripheral airways. Respir Res. 2005; 6:48. Epub 2005/06/02. PubMed Central PMCID: PMC1156956. https://doi.org/10.1186/1465-9921-6-48 PMID: 15927078

41. Roviezzo F, Di Lorenzo A, Bucci M, Brancaleone V, Vellecco V, De Nardo M, et al. Sphingosine-1-phosphate/sphingosine kinase pathway is involved in mouse airway hyperresponsiveness. Am J Respir Cell Mol Biol. 2007; 36(6):757–62. Epub 2007/02/27. https://doi.org/10.1165/rcmb.2006-0383OC PMID: 17322125

42. Jolly PS, Rosenfeldt HM, Milstien S, Spiegel S. The roles of sphingosine-1-phosphate in asthma. Mol Immunol. 2002; 38(16–18):1239–45. Epub 2002/09/10. PMID: 12217390

43. Snider AJ, Kawamori T, Bradshaw SG, Orr KA, Gilkeson GS, Hannun YA, et al. A role for sphingosine kinase 1 in dextran sulfate sodium-induced colitis. FASEB J. 2009; 23(1):143–52. Epub 2008/09/26. PubMed Central PMCID: PMC2626622. https://doi.org/10.1096/fj.08-118109 PMID: 18815359

44. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature. 1970; 227(5259):680–5. Epub 1970/08/15. PMID: 5432063

45. Oriowo MA. Chloride channels and alpha1-adrenoceptor-mediated pulmonary artery smooth muscle contraction: effect of pulmonary hypertension. Eur J Pharmacol. 2004; 506(2):157–63. https://doi.org/10.1016/j.ejphar.2004.11.008 PMID: 15588736

46. Hu W, Mahavadi S, Huang J, Li F, Murthy KS. Characterization of S1P1 and S1P2 receptor function in smooth muscle by receptor silencing and receptor protection. Am J Physiol Gastrointest Liver Physiol. 2006; 291(4):G605–10. Epub 2006/05/06. https://doi.org/10.1152/ajpgi.00147.2006 PMID: 16675745

47. Salomone S, Potts EM, Tyndall S, Ip PC, Chun J, Brinkmann V, et al. Analysis of sphingosine 1-phosphate receptors involved in constriction of isolated cerebral arteries with receptor null mice and pharmacological tools. Br J Pharmacol. 2008; 153(1):140–7. PubMed Central PMCID: PMC2199385. https://doi.org/10.1038/sj.bjp.0707581 PMID: 18026125

48. Van Doorn R, Van Horssen J, Verzijl D, Witte M, Ronken E, Van Het Hof B, et al. Sphingosine 1-phosphate receptor 1 and 3 are upregulated in multiple sclerosis lesions. Glia. 2010; 58(12):1465–76. https://doi.org/10.1002/glia.21021 PMID: 20648639

49. Chiba Y, Suzuki K, Uechi M, Kurihara E, Goto K, Sakai H, et al. Downregulation of sphingosine-1-phosphate receptors in bronchial smooth muscle of mouse experimental asthma. Pharmacol Res. 2010; 62 (4):357–63. Epub 2010/06/18. https://doi.org/10.1016/j.phrs.2010.05.005 PMID: 20554039

50. Rosen H, Gonzalez-Cabrera PJ, Sanna MG, Brown S. Sphingosine 1-phosphate receptor signaling. Annual review of biochemistry. 2009; 78:743–68. https://doi.org/10.1146/annurev.biochem.78.072407.103733 PMID: 19231986

51. Kamiya T, Nagaoka T, Omae T, Yoshioka T, Ono S, Tanano I, et al. Role of Ca2+ -dependent and Ca2+ -sensitive mechanisms in sphingosine 1-phosphate-induced constriction of isolated porcine retinal arterioles in vitro. Experimental eye research. 2014; 121:94–101. https://doi.org/10.1016/j.exer.2014.01.011 PMID: 24486793

52. Coussin F, Scott RH, Wise A, Nixon GF. Comparison of sphingosine 1-phosphate-induced intracellular signaling pathways in vascular smooth muscles: differential role in vasoconstriction. Circ Res. 2002; 91 (2):151–7. Epub 2002/07/27. PMID: 12142348

53. Kinoshita K, Sato K, Hori M, Ozaki H, Karaki H. Decrease in activity of smooth muscle L-type Ca2+ channels and its reversal by NF-kappaB inhibitors in Crohn's colitis model. Am J Physiol Gastrointest Liver Physiol. 2003; 285(3):G483–93. Epub 2003/03/21. https://doi.org/10.1152/ajpgi.00038.2003 PMID: 12646416

54. Liu X, Rusch NJ, Striessnig J, Sarna SK. Down-regulation of L-type calcium channels in inflamed circular smooth muscle cells of the canine colon. Gastroenterology. 2001; 120(2):480–9. PMID: 11159888

55. Shi XZ, Sarna SK. Impairment of Ca(2+) mobilization in circular muscle cells of the inflamed colon. Am J Physiol Gastrointest Liver Physiol. 2000; 278(2):G234–42. Epub 2000/02/09. PMID: 10666047

56. Kim YS, Song HJ, Park SY, Min YS, Im BO, Ko SK, et al. The signaling mechanism of the sphingosylphosphorylcholine-induced contraction in cat esophageal smooth muscle cells. Arch Pharm Res. 2007; 30(12):1608–18. Epub 2008/02/08. PMID: 18254249

57. Wang T, Kendig DM, Smolock EM, Moreland RS. Carbachol-induced rabbit bladder smooth muscle contraction: roles of protein kinase C and Rho kinase. American journal of physiology Renal physiology. 2009; 297(6):F1534–42. PubMed Central PMCID: PMC2801330. https://doi.org/10.1152/ajprenal.00095.2009 PMID: 19794111

58. Wang T, Kendig DM, Trappanese DM, Smolock EM, Moreland RS. Phorbol 12,13-dibutyrate-induced, protein kinase C-mediated contraction of rabbit bladder smooth muscle. Frontiers in pharmacology. 2012; 2:83. PubMed Central PMCID: PMC3249380. https://doi.org/10.3389/fphar.2011.00083 PMID: 22232602

59. Nakayama K, Obara K, Tanabe Y, Saito M, Ishikawa T, Nishizawa S. Interactive role of tyrosine kinase, protein kinase C, and Rho/Rho kinase systems in the mechanotransduction of vascular smooth muscles. Biorheology. 2003; 40(1–3):307–14. PMID: 12454420

60. Kandabashi T, Shimokawa H, Miyata K, Kunihiro I, Eto Y, Morishige K, et al. Evidence for protein kinase C-mediated activation of Rho-kinase in a porcine model of coronary artery spasm. Arteriosclerosis, thrombosis, and vascular biology. 2003; 23(12):2209–14. https://doi.org/10.1161/01.ATV.0000104010.87348.26 PMID: 14592852