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Investigating the mechanisms underlying U46619-induced contraction on porcine lower esophageal sphincter

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Number of text pages: 24
Number of tables: 0
Number of figures: 6
Number of references: 47
Number of words in the Abstract: 220
Number of words in the Introduction: 492
Number of words in the Discussion: 1030

Non-standard Abbreviations:
GERD: Gastroesophageal reflux disease; PPIs: proton pump inhibitors; LES: lower esophageal sphincter; TXA2 receptor: thromboxane A2 receptor; TTX: tetrodotoxin; IHC: immunohistochemistry; RT-PCR: reverse transcription polymerase chain reaction; GPCRs: G protein-coupled receptors; M receptor: muscarinic receptor; 5-HT: 5-hydroxytryptamine; CCK: cholecystokinin

Recommended Section Assignment:
Gastrointestinal, Hepatic, Pulmonary, and Renal
ABSTRACT

Gastroesophageal reflux disease (GERD) is associated with an incompetent lower esophageal sphincter (LES), resulting in the reflux of gastric contents into the esophagus. U46619, a thromboxane A2 (TXA2) receptor agonist, induces contractions in various smooth muscles. Therefore, this study aimed to investigate the effects and mechanisms of action of U46619 on the porcine LES. To achieve this, contractions of the clasp and sling strips of the porcine LES, induced by U46619 were measured using isometric transducers. Furthermore, the contractile mechanism of U46619 in the porcine LES was investigated by pretreating the strips with atropine (a muscarinic receptor antagonist), tetrodotoxin (a neuronal sodium channel blocker), nifedipine (a calcium channel blocker), and Ca\(^{2+}\)-free Krebs-Henseleit solution. Additionally, reverse transcription polymerase chain reaction and immunohistochemistry (IHC) were performed to determine the presence of the TXA2 receptor in porcine LES. The results of this study demonstrated that U46619 caused marked concentration-dependent contractions in both porcine sling and clasp strips. The mechanism of U46619-induced contraction of the porcine LES was found to be related to calcium channels. Furthermore, the reverse transcription PCR analysis and IHC revealed that the TXA2 receptor was expressed in the clasp and sling fibers of porcine LES. Consequently, this study suggests that U46619 mediates the contraction of porcine LES through calcium channels and has potential as a therapeutic approach for treating GERD.
Significance Statement: This study establishes that U46619 induces concentration-dependent contractions in porcine LES, primarily mediated by calcium channels. The presence of the TXA2 receptor in LES clasp and sling fibers is confirmed. These findings highlight U46619’s potential as a GERD therapeutic by targeting calcium channels for LES contraction modulation.
INTRODUCTION

Gastroesophageal reflux disease (GERD) is a common condition characterized by the reverse flow of gastric acid into the esophagus, causing symptoms such as heartburn, acid reflux, regurgitation, and chest discomfort. Additional manifestations include dysphagia, coughing, and vocal hoarseness. GERD can significantly impair an individual’s quality of life, disrupt sleep, and interfere with daily activities (Clarrett and Hachem, 2018; Vakil et al., 2006). The prevalence of GERD varies depending on geographic location and lifestyle factors, such as obesity, tobacco use, and dietary habits. Current research indicates a 10–20% prevalence in Europe and North America, while it remains below 10% in Asia and Africa. However, owing to lifestyle changes, the global prevalence of GERD is increasing (El-Serag et al., 2014).

The management of GERD involves lifestyle modifications and pharmacological interventions. Lifestyle adjustments included weight loss, avoiding trigger foods, and refraining from reclining after meals. Medications for GERD treatment include proton pump inhibitors (PPIs), histamine type 2 receptor antagonists, and antacids, with PPIs being the most effective. However, some patients respond poorly to these treatments, and surgeries that increase lower esophageal sphincter (LES) tension may offer benefits. Nevertheless, owing to surgical risks, these procedures are generally reserved for severely ill patients who are unresponsive to medications (Clarrett and Hachem, 2018). A limited number of drugs, such as baclofen, target LES enhancement and effectively alleviate GERD symptoms. However, due to the adverse effects of baclofen, such as drowsiness, dizziness, weakness, upset stomach, constipation, and trouble sleeping (Li et al., 2014; Warren and Davis, 2015), the development of alternative pharmaceutical agents targeting LES enhancement is essential.

The thromboxane A2 receptor (TXA2 receptor) is a G protein-coupled receptor that primarily modulates platelet aggregation and vascular tone. Yet, the expression of the TXA2 receptor is not limited to platelets and blood vessels; it can be found in a range of tissues, including the heart, lungs, kidneys, and brain (Raychowdhury et al., 1994; Smyth, 2010; Alqarni, 2023; Jude et al., 2023; Eskildsen et al., 2014; Liu et al., 2015; Lagier et al., 2019; Soper et al., 2012). In coronary arteries and cardiac myocytes, activation of the TXA2 receptor leads to vasoconstriction and platelet aggregation (Smyth, 2010). In the respiratory system, the TXA2 receptor is associated with bronchoconstriction and inflammation (Alqarni, 2023; Jude et al., 2023). Activation of the TXA2 receptor in the kidneys has been linked to vasoconstriction and a reduction in renal blood flow (Eskildsen et al., 2014; Liu et al., 2015). In the neurological context, TXA2 receptor activation correlates with increased blood–brain barrier permeability, neuroinflammation, and neurodegeneration (Lagier et al., 2019; Soper et al., 2012).
Current understanding of the TXA2 receptor’s expression and function in the LES remains sparse. This study sought to explore the presence of the TXA2 receptor in the LES and to assess the effects and underlying mechanisms of the TXA2 receptor agonist, U46619, on the porcine LES. Our findings hint at the potential of TXA2 receptor agonists as therapeutic targets for GERD.

MATERIAL AND METHODS

Preparation steps for in vitro LES contraction experiment
The standard Krebs-Henseleit solution used in this study contained 118 mM NaCl, 25 mM NaHCO₃, 4.7 mM KCl, 14 mM glucose, 1.2 mM NaH₂PO₄, 1.8 mM CaCl₂, and was maintained at 37°C with a pH value of 7.40 ± 0.05. The solution was continuously oxygenated using a gas mixture containing 95% O₂ and 5% CO₂. Fresh pig esophageal and stomach samples were obtained from the slaughterhouse, stored in Krebs-Henseleit solution, and transported to the laboratory within 30 min. The stomach was cut longitudinally along the greater and lesser curvatures, and the gastric mucosa was removed. The muscle layer located between the greater curvature and esophagus, which appears as a semicircular thickening, was identified as the sling muscle. The muscle layer located between the sling muscles near the lesser curvature was identified as the clasping muscle. The sling and clasp muscles of the LES were excised, as described by Farré et al. (2007). These muscles were then trimmed into strips approximately 1.5 cm in length and 0.5 cm in width. One end of the LES strips was fixed to a clamp, and the other end was tied with a silk thread and attached to a force transducer. Each bath contained 5 ml of the Krebs-Henseleit solution, supplied with 95% oxygen and 5% carbon dioxide. A tension of 1 mg was applied, and after 30 min, when the tension curve reached a steady state, 1 μM carbachol was added to induce LES contraction to test its activity. The LES tension curve is allowed to reach equilibrium, and then U46619 at concentrations of 10 nM, 30 nM, 100 nM, 300 nM, and 1 μM is added individually to test whether it can cause LES contraction (Chen and Huang, 2011; Tsai et al., 2015). Contraction curves were recorded, and the amplitude of contraction induced by 1 μM carbachol was considered to be 100%. The percentage amplitude induced by U46619 was recorded at each concentration tested. The study was exempt from review by the Institutional Animal Care and Use Committee of the E-DA Hospital, as the esophagus and stomach of the pigs were classified as food products rather than live animal parts, in accordance with the applicable regulations.

Using TXA2 receptor antagonists to block U46619-induced contraction of porcine LES
In this study, the effectiveness of TXA2 receptor antagonists, namely seratrodast and GR32191,
in inhibiting U46619-induced contraction of porcine LES was investigated. The experimental protocol closely followed a previously described method. Initially, a concentration of 1 μM carbachol was employed to elicit contraction of the LES, and its activity was subsequently assessed. The tissue was then rinsed three times with standard Krebs-Henseleit solution and allowed to rest for 30 minutes. Subsequently, 1 μM seratrodast or 10 μM GR32191 was administered, followed by the addition of 100 nM U46619 after a 6-minute interval. The resulting contraction curve was then recorded (Sharma et al., 2016; Jin et al., 2016; Yan et al., 2019).

Elucidating the mechanism underlying U46619-induced contraction of the porcine LES

As shown in Fig. 1, to investigate the mechanisms underlying U46619-induced porcine LES contraction, we employed four experimental protocols following the preparation steps described previously. Firstly, to determine the involvement of neuronal pathways, we pretreated the LES muscle strips with 1 μM tetrodotoxin, a selective voltage-gated sodium channel blocker, for 15 minutes before stimulating them with 100 nM U46619. Second, to assess muscarinic receptor involvement, we pretreated the strips with 1 μM atropine as a non-selective muscarinic receptor antagonist for 6 minutes prior to U46619 stimulation. Third, we examined the role of L-type voltage-gated calcium channels by pretreating the strips with 1 μM nifedipine for 20 minutes before U46619 introduction. Finally, to evaluate the necessity of extracellular calcium, we conducted U46619-induced contractions in calcium-free Krebs-Henseleit solution containing 0.5 mM EGTA as a calcium chelator. Contraction recordings were obtained with a computer following U46619 stimulation in the presence of these pharmacological inhibitors.

Using immunohistochemistry (IHC) to verify the expression of TXA2 receptor in the porcine LES

To ascertain the expression of TXA2 receptors in the porcine LES, we employed standard techniques to prepare formalin-fixed and paraffin-embedded samples of porcine sling and clasp muscle fibers for immunohistochemical (IHC) staining. The staining process was conducted using the BOND-MAX Automated Staining System (Leica Microsystems, Germany).
retrieval, heat-induced epitope retrieval was carried out with bond epitope retrieval solution 2 (EDTA, pH 9.0; Leica Microsystems). Subsequently, the sections were incubated with a polyclonal antibody against TXA2 receptors (Elabscience, Houston, TX, USA) at a dilution of 1:100 for 30 minutes at room temperature. This was followed by a 10-minute incubation with an anti-rabbit horseradish peroxidase polymer. To visualize the staining, we applied 3,3′-diaminobenzidine tetrahydrochloride as the chromogen for 10 minutes at room temperature, and counterstaining was achieved with hematoxylin for 5 minutes. As part of our quality control, we included a negative control by staining sections of the sling and clasp muscles with normal rabbit IgG at equimolar concentrations.

Using reverse transcription polymerase chain reaction (RT-PCR) to verify the expression of TXA2 receptor in the porcine LES

In brief, for the extraction of RNA from porcine tissue, we harvested porcine LES muscle fibers and immersed them in RNAlater solution (Applied Biosystems Inc., Foster City, CA, USA) at 4°C for a period of two days to facilitate tissue penetration. Afterward, we carefully removed any excess solution and stored the tissue at -80°C until further processing.

RNA extraction was carried out using the GeneJET RNA Purification Kit (Thermo Fisher Scientific Inc., Waltham, USA) following the manufacturer’s protocol, which employs the guanidine isothiocyanate method. To assess the purity and quality of the isolated RNA, we utilized an ultraviolet-visible spectrophotometer (DU800; Beckman Coulter, CA, USA).

To synthesize cDNA from the isolated RNA, a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Inc.) was used. Following the manufacturer’s instructions, 1 μg of RNA was used for cDNA synthesis. The cDNA samples were then diluted to 100 μL for RT-PCR analysis.

The primers used to amplify the β-actin sequence were TCGGTTGGATGGACATCCCC (forward) and GGGGAGGAGGGACTTCTGTAA (reverse). The primers used to amplify the TXA2 receptor sequence were TTCATGACCAGACAGTTCT (forward) and GATCGACGGCGCTCGAAAC (reverse). The PCR was performed using an All-in-One PCR Mix (Protech, Taipei, Taiwan) in a thermal cycler (Thermo PX2, MA, USA). The program included annealing at 58.8 °C, 40 cycles of amplification, and a final extension at 72 °C. β-actin was used as an internal control.

Data analysis

Data are presented as mean ± standard error of the mean. GraphPad Prism 5 was used to calculate EC50 values. To analyze the results, statistical tests such as Student’s t-tests or one-way ANOVA
followed by Dunnett’s post hoc test were utilized. $P < 0.05$ was considered significant for all the comparisons made.

RESULTS

The effects of the TXA2 receptor agonist on porcine LES

In this study, the effects of U46619 on the contraction of LES sling and clasp muscle strips were investigated, and the results indicated that U46619 caused detectable contractions in both muscle types (Fig. 2A and 2B). Specifically, in the LES sling muscle strips, U46619 induced tension values of 15.3±3.2%, 28.7±3.8%, 52.4±5.3%, 72.2±5.6%, and 81.4±5.4% of the tension induced by 1 μM carbachol at concentrations of 10 nM, 30 nM, 100 nM, 300 nM, and 1 μM, respectively (n≥4, all $P < 0.05$; tensions induced by other concentrations compared with tension induced by 10 nM (*), 30 nM (#) and 100 nM (¶), as shown in Fig. 3A). The EC$_{50}$ was 79.3 nM. Similarly, in the LES clasp muscle strips, U46619 caused detectable contractions. At concentrations of 10 nM, 30 nM, 100 nM, 300 nM, and 1 μM, U46619 induced tension values of 14±3.8%, 26.8±3.1%, 49.2±7.8%, 62.3±9.3%, and 72.5±4.6%, respectively, compared to the tension induced by 1 μM carbachol (n≥4, all $P < 0.05$; tensions induced by other concentrations compared with tension induced by 10 nM (*)) and 30 nM (#), as shown in Fig. 3B). The EC$_{50}$ was 67.1 nM. These findings suggest that U46619 causes significant dose-dependent contractions in both types of muscle strips.

The inhibitory effects of TXA2 receptor antagonists on U46619-induced contraction in porcine LES

A concentration of 100 nM was selected to test the effects of TXA2 receptor antagonists on U46619-induced contraction in the LES sling muscle strip based on the reference EC$_{50}$ concentration. As shown in Fig. 4A, both 1 μM seratrodast and 10 μM GR32191 significantly inhibited the contraction induced by 100 nM U46619 in the LES sling muscle strip (both $P < 0.05$). These findings suggest that the contractions elicited by U46619 are mediated by the TXA2 receptor.

The effects of TTX, atropine, nifedipine, and Ca$_{2+}$-free Krebs-Henseleit solution on U46619-induced contractions in the porcine LES

Muscle strips were treated with TTX, atropine, nifedipine, or a Ca$_{2+}$-free Krebs-Henseleit solution prior to U46619 stimulation. As depicted in Fig. 4B, TTX did not produce a significant effect
on U46619-induced contractions ($P > 0.05$, compared with U46619 alone, $n = 4$). Similarly, atropine did not exhibit a notable effect on U46619-induced contractions (both $P > 0.05$, compared to U46619 alone, $n = 4$). In contrast, the L-type Ca$^{2+}$ channel blocker, nifedipine, at a concentration of 1 μM, as well as the Ca$^{2+}$-free Krebs-Henseleit solution, significantly inhibited the U46619-induced contraction of LES sling muscle fibers (both $P < 0.05$, compared with U46619 alone, $n = 4$). These findings suggest that the U46619-induced contraction of the LES sling muscle is primarily mediated by calcium influx through L-type calcium channels.

Analysis of TXA2 receptor transcript levels in the LES using RT-PCR

With the utilization of primers specific to the TXA2 receptor, we successfully amplified a PCR product measuring 159 bp from both sling and clasp muscle fibers. In parallel, the internal control β-actin PCR product demonstrated a size of 148 bp ($n = 3$; Fig. 5). These findings unequivocally confirm the presence of the TXA2 receptor in both porcine sling and clasp muscle fibers.

IHC Analysis of TXA2 Receptor in Porcine LES

The IHC results demonstrated that the expression of the TXA2 receptor was widespread across the sling (Fig. 6A) and clasp (Fig. 6B) muscle fibers of the LES ($n = 3$). These findings suggested that the TXA2 receptor was present in the fibers of both porcine sling and clasp muscles.

DISCUSSION

The tonicity of the LES is crucial for regulating food flow and preventing gastric acid reflux. G protein-coupled receptors (GPCRs), located on the cell surface, play a pivotal role in recognizing extracellular molecules and initiating cellular responses across various physiological functions (Sutkeviciute and Vilardaga, 2020). Numerous studies have investigated the impact of GPCRs on LES contraction and revealed important findings. For instance, extracts from *Arecaej pericarpium* have been shown to induce contractions in the porcine LES, which are potentially mediated by muscarinic (M) receptors (Tey et al., 2021). Activation of bombesin receptor subtypes 2 and 3 has been identified as responsible for mediating tonic contraction of the porcine LES following bombesin stimulation (Tsai et al., 2015). LES contraction primarily involves M3 receptors and relies on pertussis toxin-insensitive G9-G11 protein (Sohn et al., 1993; Farré and Sifrim, 2008). Additionally, evidence suggests the presence of endothelin B receptors in guinea pig LES that play a significant role in its
contraction (Huang, 2005). Furthermore, tachykinins stimulate circular muscle contraction in the human LES through neurokinin 2 receptor receptor activation (Farré and Sifrim, 2008, Huber et al., 1993). In rats, fenoldopam, a selective dopamine-1 agonist, increased LES pressure (Sigala et al., 1994). Activation of GABA(B) receptor leads to increased basal LES pressure (Chen et al., 2011). Moreover, canine LES smooth muscle contracts in response to 5-hydroxytryptamine (5-HT) and SK&F 103829 by stimulating 5-HT2 receptors (Barnette et al., 1992). Sling fibers in the LES demonstrated stronger contractions upon exposure to cholecystokinin (CCK) and gastrin than clasp fibers. CCK-A receptor activation primarily governs the contractile response of sling fibers, while both CCK-A and CCK-B receptors contribute to the regulatory role in clasp fiber function (Liu et al., 2008). These studies showed that GPCRs have a significant impact on LES contraction.

TXA2 plays a central role in the gastrointestinal tract by influencing secretion, motility, inflammation, and neoplasia. A remarkable aspect of TXA2 in the colon is its involvement in calcium ion regulation. Studies have shown that 9,11-epithio-11,12-methano-TXA2 stimulation results in increased intracellular calcium concentrations in colonic crypt cells (Ikari et al., 1999). Furthermore, thromboxanes influence chloride transport in the colonic epithelium, which is mediated through the calcium and cAMP pathways (Diener and Rummel, 1991; Horikawa et al., 2005). In the context of inflammatory bowel disease, TXA2 receptor, when antagonized by ONO-NT-126, shows promise in ameliorating colonic damage (Taniguchi et al., 1997). Equally noteworthy is the role of platelet-derived TXA2 in promoting colitis and fibrosis following intestinal injury (Sacco et al., 2019). Interestingly, TXA2 agonists increase intracellular calcium concentrations and induce contractions in the intestinal smooth muscle (Frings et al., 2000; Sametz et al., 2000; Amstutz and Diener, 1997; Diener and Gabato, 1994). Additionally, TXA2 modulates intestinal motility through the orchestration of pacemaker activities in the interstitial cells of Cajal, a process that bypasses G protein- and protein kinase C-dependent pathways and involves the modulation of both extracellular and intracellular calcium (Kim et al., 2008). In the context of aging, the downregulation of TXA2 and angiotensin II type 1 receptors has been associated with a reduction in internal anal sphincter tone (Mohanty et al., 2019). From a neoplastic perspective, the cAMP pathway, mediated by the TXA2 receptor, appears to enhance Kv7.1 channels, thereby promoting colon cancer cell proliferation (Shimizu et al., 2014). Hence, the diverse effects of TXA2 highlight its potential therapeutic significance in gastrointestinal disorders.

Smooth muscle contraction, including contraction of the LES, is a multifactorial process governed by neural factors, muscarinic receptors, and calcium channels (Fig. 1) (Hafen et al., 2022).
The LES, possessing inherent myogenic properties, exhibits a sustained closure state regulated by a balance of inhibitory and excitatory neurons (Goyal and Chaudhury, 2008). Activation of postganglionic excitatory neurons results in acetylcholine release, which, upon binding to muscarinic receptors on the LES, triggers intracellular pathways, culminating in contractions (Goyal and Chaudhury, 2008). Upon activation, these muscarinic receptors, which are G protein-coupled entities, stimulate the Gq/11 protein, leading to phospholipase C activation and the subsequent generation of inositol triphosphate (IP3) and diacylglycerol. IP3, in turn, prompts the release of calcium from the sarcoplasmic reticulum into the cytoplasm. Additionally, calcium influx into smooth muscle cells is facilitated by two types of channels: voltage-gated calcium channels, which are activated by membrane depolarization (Catterall, 2011), and receptor-operated calcium channels, which are responsive to neurotransmitters, hormones, and mechanical stimuli (McFadzean and Gibson, 2002). An increase in intracellular calcium, which interacts with calmodulin, activates myosin light chain kinase, ultimately driving smooth muscle contraction (Hafen et al., 2022, Ehlert et al., 1997; Hafen and Burns, 2022).

In this study, U46619 induced dose-dependent contractions in the LES muscle. Moreover, the inhibitory effects of the TXA2 receptor antagonists seratrodast and GR32191 on U46619-induced LES contractions were examined, providing evidence for a direct association between U46619-induced LES contractions and TXA2 receptor activity.

Furthermore, the effects of specific blockers were assessed to elucidate the involvement of neuronal and muscarinic receptor pathways in U46619-induced LES contraction. Notably, the selective voltage-gated sodium channel blocker TTX and the muscarinic antagonist atropine were ineffective in inhibiting U46619-induced contractions in porcine LES. These findings suggest that U46619 exerts its contractile effects on the LES through mechanisms independent of neuronal signaling and muscarinic receptor activation. In contrast, the application of calcium channel blockers such as nifedipine and a calcium-free solution containing 0.5 mM EGTA significantly inhibited U46619-induced contractions in porcine LES. These results strongly implicate the involvement of voltage-dependent calcium channels and subsequent calcium influx in U46619-induced LES contraction. Furthermore, the expression of the TXA2 receptor in the porcine LES was confirmed using RT-PCR and immunohistological staining, supporting its functional relevance in mediating the contractile response to U46619.
Taken together, U46619 elicited dose-dependent contractions in the porcine LES mediated via TXA2 receptor activation and calcium channels. While U46619 shows promise for GERD management, our study has limitations. Our porcine model, though anatomically similar to humans, may not fully capture the complex nature of GERD, necessitating caution in extrapolation. The intricate interplay of mechanical, neural, hormonal, and inflammatory factors in human GERD may not be fully replicated in our model. Although the ex vivo approach isolates tissue responses, systemic influences are absent. To apply our findings to humans, further validation in in vivo animal models resembling GERD’s milieu and subsequent human studies are essential. Additionally, we didn’t explore potential toxicity at high U46619 doses, which can induce adverse effects. Comprehensive dose-ranging toxicity studies, encompassing cardiopulmonary and esophageal effects, are crucial to establish a safe therapeutic window before considering human trials. Future research should prioritize assessing U46619’s safety profile across various organ systems.

CONCLUSION

In conclusion, this study highlights the dose-dependent contractions induced by U46619 in the porcine LES, which are mediated by the activation of TXA2 receptor and involve voltage-dependent calcium channels and calcium influx. Although these results suggest the potential of U46619 as a novel therapeutic approach for GERD, additional studies are necessary to comprehensively evaluate its safety profile and efficacy in humans.
ACKNOWLEDGEMENTS

None
DATA AVAILABILITY

The authors declare that all the data supporting the findings of this study are contained within the paper.
AUTHOR CONTRIBUTIONS

Participated in research design: Su Y. and Tsai C.

Conducted experiments: Kek H., Su Y., Lin K., and Tsai C.

Performed data analysis: Kek H., Su Y., Lin K., Chang L., Yang M., Yang Y. and Tsai C.

Wrote or contributed to the writing of the manuscript: Kek H. and Tsai C.
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Footnotes

This study was supported by intramural funding, provided by E-Da Hospital (112-EDN0005, EDAHS112030, and EDAHI112002). No author has an actual or perceived conflict of interest concerning the content of this article.
FIGURE LEGENDS

Figure 1. Schematic overview of the mechanisms governing smooth muscle contraction, including neural stimulation, muscarinic receptor activation, and calcium influx. Acetylcholine (ACH) release from excitatory neurons binds to muscarinic receptors (M) on the smooth muscle cell surface. This triggers activation of the Gq/11 protein, which then stimulates phospholipase C (PLC). PLC cleaves phosphatidylinositol 4,5-bisphosphate (PIP2) into inositol triphosphate (IP3) and diacylglycerol (DAG). IP3 causes calcium release from the sarcoplasmic reticulum (SR) into the cytoplasm. This cytoplasmic calcium increase, along with calcium influx through voltage-gated and receptor-operated calcium channels (VDCC and ROC), interacts with calmodulin to activate myosin light chain kinase (MLCK). MLCK phosphorylation of myosin leads to crossbridge cycling and smooth muscle contraction. Additionally, myosin light-chain phosphatase (MLCP) is also involved in this intricate regulatory pathway.

Figure 2. Characteristic contraction profiles of both porcine lower esophageal sphincter sling (A) and clasp (B) muscle strips, each demonstratively influenced by escalating concentrations of U46619 at 30, 100, and 300 nM.

Figure 3. Contractile response of porcine lower esophageal sphincter sling (A) and clasp (B) muscle strips to different concentrations of U46619. The elicited contractions are expressed as a percentage of the contraction caused by a standard 1 µM carbachol stimulation. The displayed results are derived from a minimum of four separate experiments, with vertical bars denoting the standard error of the mean. The asterisk (*) indicates a statistically significant difference compared to 10 nM, the hash symbol (#) to 30 nM, and the paragraph symbol (¶) to 100 nM U46619-induced contractions, all at \( P < 0.05 \).
Figure 4. Responses of porcine lower esophageal sphincter (LES) to U46619 and various inhibitors.
(A) Effects of thromboxane A2 receptor antagonists on U46619-induced contractions in LES sling muscle strips. When strips are treated with either 1 μM seratrodast or 10 μM GR32191 and then exposed to 100 nM U46619, both seratrodast and GR32191 significantly inhibit contractions. The asterisk indicates contractions that are statistically different from those induced by 100 nM U46619 alone ($P < 0.05$).

(B) Effects of various inhibitors on U46619-induced contractions in LES sling muscle. The LES strips are treated with 1 µM tetrodotoxin (TTX), 1 µM atropine, 1 µM nifedipine, or calcium-free Kreb’s solution before exposure to 100 nM U46619. Only nifedipine and the calcium-free Kreb’s solution significantly inhibit contractions. The asterisk indicates contractions that are statistically different from those triggered by 100 nM U46619 alone ($P < 0.05$).

Figure 5. Amplification of total RNA is shown after reverse transcription with thromboxane A2 (TXA2) receptor-specific primers. The amplified products were analyzed via electrophoresis on an agarose gel, stained with ethidium bromide, and visualized under UV light. The results displayed, representative of three separate experiments, confirm the presence of TXA2 receptor PCR products in both the sling and clasp muscles (lane TXA2R). As an internal control, the β-actin specific PCR product of 148 bp is presented (lane ACTB).

Figure 6. Immunohistochemical examination of paraffin-embedded sling and clasp muscle fibers, stained with thromboxane A2 receptor-specific antibodies. Notably, immunostaining for the thromboxane A2 receptor is observed within the smooth muscle (SM) of both lower esophageal sphincter sling (A) and clasp (B) muscle fibers (magnified 400x). As a control, sections stained with equimolar concentrations of normal rabbit IgG, serving as a negative control, are also provided for both the sling (C) and clasp (D) muscles (magnified 400x).
Figure 1
Figure 2

**A**

Sling

- 300 nM U46619
- 100 nM U46619
- 30 nM U46619

**B**

Clasp

- 300 nM U46619
- 100 nM U46619
- 30 nM U46619
Figure 3

A: Sling

B: Clasp

CONTRACTION (% 1 μM Carbachol-induced)

U46619 CONCENTRATION (Log M)
Figure 4
Figure 5