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Effects of Emodin on Ca^{2+} Signal Transduction of Smooth Muscle Cells in Multiple Organ Dysfunction Syndrome[1]

Chen Zheyu,* Q. I. Qinghui,† Liu Lixin,* M. A. Tao,* Jian Xu,* Liang Zhang,‡ and Yan Lunan*,[2]

*Department of General Surgery of West China Hospital, Sichuan University, Chengdu, China; †First Affiliated Hospital, Medical University of DaLian, DaLian, China; ‡Division of Urologic Surgery, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, Massachusetts

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We have made several reports on the signal transduction mechanism that emodin enhance the calcium concentrations of smooth muscle cells (SMCs) in the physiological condition by inositol [1, 4, 5]-friphosphate (IP_3). The observation that IP3 concentrations in SMCs were decreased in multiple organ dysfunction syndrome (MODS) prompted us to ask whether emodin can activate SMCs to contract by way of elevating [Ca^{2+}] and thus modulating the critical Ca^{2+} signal transduction pathways involved in the contraction of the SMCs in the pathological setting of MODS. To test this hypothesis, we used the rat model of MODS to explore the potential roles of emodin in Ca^{2+} signal transduction in the SMCs of colon in rats. ML-7 [an inhibitor of myosin light-chain kinase (MLCK)] and Calphostin C [an inhibitor of protein kinase C (PKC)] were used to observe the influence of emodin on the muscle strips and SMCs in rats after MODS. Nifedipine (an antagonist of voltage-gated Ca^{2+} channel), EGTA (removal of extracellular Ca^{2+}), heparine (a specific IP3 receptor antagonist), and ryanodine were used to probe the potential mechanisms involved in emodin-mediated elevation of the global cytoplasmic Ca^{2+} in SMCs of colon in the rats after MODS. Our results show that emodin is capable of contract the smooth muscles of colon in rats after MODS by MLCK increasing [Ca^{2+}] of SMCs, and by PKC enhancing the calcium sensitivity of SMCs. The mechanism by which emodin triggers elevated [Ca^{2+}] of smooth muscles of colon in rats after MODS is likely to operate through IP_3 and RyR receptors in the sarcoplasm. It is hoped that deeper insights into how emodin modulates the critical calcium signaling in SMCs might lead to the potential development of emodin in the treatment of MODS.

Key Words: **calcium; SMC; MODS; emodin; signal transduction.**

INTRODUCTION

Pharmaceutical purgative preparations contained Rheum palmatum have been widely used in China for hundreds of years to treat gastrointestinal disorders [1, 2]. Emodin (1, 3, 8-trihydroxy-6-methylanthraquinone) is an anthraquinone derivative isolated from Rheum palmatum [3]. The reported biological effects of emodin include anti-tumor, antibacterial, and anti-inflammatory actions. Emodin also possesses prokinetic effects on gastrointestinal smooth muscles [4–7, 34, 35]. Stimulatory actions of emodin on gastrointestinal smooth muscles have been described in several studies, and emodin-induced contractions have been related to calcium ions [8, 9]. Recently, calcium has proved that it plays a key role in contraction of SMCs. The rise and fall in intracellular free Ca^{2+} are the principal mechanisms that initiate, respectively, contraction and relaxation in sooth muscles [10]. MA had demonstrated that Emodin has a direct excitatory effect on circular smooth muscle cells in colon of rats mediated via Ca^{2+}/CaM dependent pathways in physiological conditions. Furthermore, emodin-induced peak [Ca^{2+}]i increase may be attributable to the Ca^{2+} release from IP3 sensitive stores, which further promote Ca^{2+} release from ryanodine-sensitive stores through CICR mechanism. Additionally, Ca^{2+} influx from extracellular medium contributes to the sustained increase in [Ca^{2+}]i [11].

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[2] To whom correspondence and reprint requests should be addressed at Department of General Surgery, West China Hospital, Sichuan University, Chengdu, Sichuan 610000, China. E-mail: yanlunan688@yahoo.com.

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Our previous study showed that emodin can contract the muscle strips and cells of smooth muscles of colon in rats after MODS and the observed physiological effects of emodin on smooth muscles were shown to be related to calcium signaling [12]. It is critical to know whether the mechanisms of emodin-mediated calcium signaling pathway in the smooth muscle cells in rats after MODS is the same as used in physiological conditions. This study was conducted to test this idea and our experimental results in the rat model of MODS provide the rational for the potential development of emodin in the intervention of MODS.

MATERIALS AND METHODS

Materals

Male Wistar rats weighing 200 to 250 g were supplied by the Center of Experimental Animal in Sichuan University. Animals were bred in a controlled environment with a 12-h light/dark cycle. A Biopac system MP150 Physiometer (Biopac, Goleta, CA) was used for measuring the contractility of circular muscle strips of colon. Laser scanning confocal microscopy (Radiance 2000; Bio-Rad, Hertfordshire, United Kingdom) was used for determining the concentrations of calcium in SMCs. An image analysis system (Cimas2000, Beijing, China) was used for observing the changes in length of SMCs. Nifedipine, ryanodine, heparin, EGTA, Calphostin C, ML-7, emodin, collagenase type 2 (Sigma Co., St. Louis, MO)'Fluo-3 AM, F-127 (Molecular Probes Co., Eugene, OR) were used in this study. Nifedipine, ryanodine, and heparin were all dissolved in standard buffer and kept at 4°C.

Establishment of the Model with MODS

This study was approved for animal use by the Sichuan University and conformed to the National Institutes of Health guidelines for the care and use of laboratory animals. Forty male Wistar rats were divided randomly into two groups of $n = 20$. The model of MODS was established according to Chen [12]. Briefly, 1 ml suspension of 8×10^8cfu/ml of *Escherichia coli* strain O127 H6 (Sigma), which contained 10% $BaSO_4$, was injected under sterile conditions into the abdominal cavity of the rats in the MODS group. The control group was injected 1 ml of normal saline.

Preparation and Measurement the Smooth Muscle Strip

Twenty-four hours after injection, the rats were humanely killed. The abdomen was opened, the distal colon was removed quickly, and placed in cold pre-oxygenated Krebs-Ringer buffer (KRB, Sigma). Standard KRB contained (mM): Na^+, 137.4; K^+, 5.9; Ca^{2+}, 2.5; Mg^{2+}, 1.2; Cl^-, 134; HCO_3^-, 15.5; $H_2PO_4^-$, 1.2; and glucose, 11.5. This physiological solution was gassed with 97% $O_2$to 3% CO_2 to establish a pH of 7.4. The mucosa of colon was curetted to make a 15 × 5 mm circular muscle strip. The strips were fixed in the physiometer and contractility was measured after incubating the strips in Krebs fluid for 1 h at 37°C. The tension and contraction wave was used as criterion of contractility of the circular muscle strip of colon. The average amplitude of the tension and contraction was taken as the base value. Recording was initiated after the tension and contraction waves stabilized. After the administration of medicine, the tension and contraction were recorded for 5 min. The average values were calculated for treatment with each agent. The changes in percentage = (effect value − base value) ÷ base value × 100%.

Preparation of Dispersed Smooth Muscle Cells

Smooth muscle cells were isolated from the circular muscle layer of the colon in the rats as described previously with slight modifications [13]. Briefly, muscle strips were digested for 30 min at 31°C in HEPES buffer [M:pH 7.4; HEPES 24.6; NaCL 115; KCL 5.7; KH_2PO_4 2.0; $Cacl_2$ 1.9; $MgCL_2$ 0.6; glucose 5.6; 0.184% (wt/vol) DWEM] containing 0.1% type 2 collagenase and 0.01% trypsin inhibitor. The partly digested strips were washed with PBS, and muscle cells were allowed to disperse spontaneously for 30 min. The cells were harvested by filtration through a 500-μm Nitex filter and centrifuged at 350 × g for 10 min, and the filtrates (cell suspension) were equilibrated for 20 min before the experiment. For some experiments, cells were permeabilized with a brief exposure to saponin (75 μg/ml for 4 min) and equilibrated in a cytosolic buffer.

Measurement of SMCs Contraction

The contractions of SMCs of the colon were measured using computerized image micrometry as described previously [11]. Briefly, the cell suspensions of 0.25-ml consisting of 1×10^4 cells were added to 0.1 ml of the solutions containing the test agents. The reaction was interrupted after 1 min by adding 0.1 ml of acrolein at a final concentration of 0.1%. The length of individual cell was measured by computerized image micrometry and the average length of cells before and after adding test agents was calculated from 50 cells randomly. The contractile response after each agent was defined as the decrease in the average length of the 50 cells and expressed as a percentage compared to the length before administration [14].

Determination of Signal Transduction of Emodin on Smooth Muscle Contraction

Calcium ions directly regulate the contraction of smooth muscle through the activation of Ca^{2+}/calmodulin-dependent myosin light-chain kinase (MLCK) [38]. In this study, smooth muscle strips and SMCs pre-treated with Ml-7, an inhibitor of MLCK [36], were incubated with emodin. It is predicted that inhibition of emodin-induced contraction will indicate a key role of MICK in the calcium signaling in the SMCs initiated by emodin. Otherwise, MLCK is not associated with the emodin-activated contraction of SMCs.

Protein kinase C (PKC) may regulate the contractility of SMCs in a variety of ways, including the phosphorylation of actin binding proteins and ion channels and increased Ca^{2+} sensitivity of contractile proteins [38]. As with ML-7 treatment, muscle strips and SMCs pre-treated with Calphostin C, an inhibitor of PKC [37], were incubated with emodin. Inhibition of emodin-mediated activation of SMCs implicates a role of PKC in this process. Conversely, PKC is predicted not to be involved in the contraction of SMCs activated by emodin.

Measurements of $[Ca^{2+}]i$ in Smooth Muscle Cells

Calcium concentrations in the SMC***s*** were estimated by fluorescence measurement using Ca^{2+} indicator Fluo-3 AM. with a laser scanning confocal microscopy as described Jacques [15]. Briefly, freshly dissociated smooth muscle cells were seeded onto glass coverslips and incubated in Fluo-3 working solution (Fluo-3 AM 7.5 μmol and Pluronic F-127 0.02% dissolved in standard buffer) at 37°C under an atmosphere of 5% CO_2. After a loading period of 30 min, the cells were washed with PBS to remove extracellular Fluo-3 AM followed by incubation for an additional 20 min to allow complete desterilization of the cytosolic Fluo-3 AM. Coverslips mounted on the chamber slide (Molecular Probe) were placed on the plate of the confocal microscope. The fluorescence in the cell was excited at 488 nm by an argon-ion laser, emitted at wavelength between 515 to 545 nm, and was detected by a photomultiplier. The amount of Fluo-3 fluorescence indicating the cytosolic Ca^{2+} concentration was recorded.

In SMCs, voltage-gated Ca^{2+} channel is the main channel of extracellular calcium influx. To determine whether it was related to the emodin-induced increase in $[Ca^{2+}]i$, the effects of nifedipine, an antagonist of voltage-gated Ca^{2+} channel in response to emodin were investigated. Emodin was applied after 15 min-exposure to nifedipine, if the observed level of increase in $[Ca^{2+}]i$ is not significantly different ($P < 0.05$) from that treated by emodin alone, then voltage-gated Ca^{2+} channel is predicted not to be associated with the process of $[Ca^{2+}]i$ increases induced by emodin. Otherwise, the level of increase in $[Ca^{2+}]i$ is expected to be inhibited if voltage-gated Ca^{2+} channel is involved in the emodin-mediated calcium signaling. To further evaluate the role of extracellular Ca^{2+} influx to the emodin-induced rise in $[Ca^{2+}]i$, emodin was applied to cells incubation with Ca^{2+}-free extracellular solution. Extracellular Ca^{2+} was removed by EGTA solution. Significantly lower levels ($P < 0.05$) of increase in $[Ca^{2+}]i$ induced by treatment with EGTA and emodin together than that induced by emodin alone will indicate the involvement of extracellular Ca^{2+} influx in the emodin-mediated increase in $[Ca^{2+}]i$. Conversely, no significant difference ($P < 0.05$) in the observed increase of $[Ca^{2+}]i$ between the two treatments will rule out the contribution of extracellular Ca^{2+} influx in calcium signaling triggered by emodin.

Inositol triphosphate receptors (IP_3R) and ryanodine receptors (RyR) were localized to the sarcoplasmic reticulum (SR), show to act as ion channels and play key roles in Ca^{2+} release [10]. Heparine (a specific IP3 receptor antagonist) and ryanodine (inhibit Ca^{2+} release from ryanodine-sensitive intracellular stores) were used to determine whether IP_3R and RyR were involved in the increase of $[Ca^{2+}]i$ induced by emodin with the same principles as nifedipine and EGTA.

Statistical Analysis

Unless otherwise indicated, data are presented as mean ± SEM. Comparisons of means were performed using ANOVA followed by comparisons of individual pairs of means using Student's t-test. χ^2 test was used to evaluate the differences in mortality between groups. These tests are included in the SPSS v. 10.1 statistical analysis program (SPSS Inc., Chicago, IL). When P was less than 0.05, the difference was considered statistically significant.

RESULTS

The Pathobiology of MODS in the Rat Model

Twenty-four hours after injection, eleven rats remained in the MODS group (45% mortality), compared to the control group where all 20 rats remained (no mortality) ($P < 0.01$, compared to the MODS group). Blood was sampled from the caudal vein and the serum ALT and BUN were measured to determine liver and kidney function using an automatic biochemistry analyzer (HITACHI-7110A, Japan). The levels of serum ALT and BUN in the control group ($N = 20$) were 49.51 ± 10.64 IU/L and 4.79 ± 1.14 M, respectively. However, the serum ALT and BUN levels in the MODS group ($N = 11$) were three times higher than the upper limit observed in the control (173.15 ± 30.97 IU/L and 16.79 ± 1.83 M, respectively). This difference was statistically significant (Fig. 1) and suggested that the liver and kidney function of the MODS group had been severely damaged. This model of MODS based on the establishment of bacteria peritonitis, as observed commonly in clinical cases, which resulted in a high rate of mortality. These characteristic makes the model well suit for observing pathological changes and the effects of treatment.

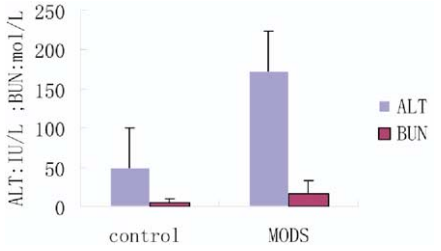

FIG. 1. The levels of serum ALT and BUN in the control group ($N = 20$) were 49.1 ± 10.64 IU/L and 4.79 ± 1.14 M, respectively, and 173.15 ± 30.97 IU/L and 16.79 ± 1.83 M in the MODS group ($N = 11$). The levels of ALT and BUN in the MODS group were three times higher than the upper limit of the control ($P < 0.05$).

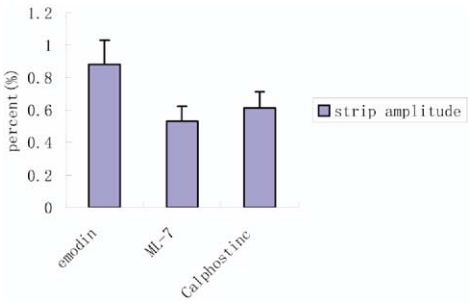

FIG. 2. ML-7 and Calphostin C could significantly decrease the contraction amplitudes of the circular muscle strips in the rats after MODS, respectively ($P < 0.05$).

Effects of ML-7 and Calphostin C on Muscle Strip

After 100μ mol/L emodin effecting on the circular muscle strips in the rats after MODS, the average amplitudes were elevated about 0.73 ± 0.29%. When the circular muscle strips were pretreated by 10 μmol/L ML-7 and 5 μmol/L Calphostin C (an inhibitor of PKC) respectively, significant decrease were found in this two conditions compared to 100 μmol/L emodin used alone ($P < 0.05$) (Fig. 2).

Effects of ML-7 and Calphostinc on Smooth Muscle Cells

The length of colon smooth muscle cells was about 113.2 ± 30.4 μM in the rats after MODS. When 100-μmol/L emodin was used, the length of colon smooth muscle cells was about 71.2 ± 21.9 μM, which decreased significantly compared with the former ($P < 0.05$). When smooth muscle cells pretreated with 10 μmol/L ML-7 and 5 μmol/L Calphostin C respectively were incubated with emodin, their lengths were 91.1 ± 23.8 μM and 85.3 ± 25.9 μM (Fig. 3). The effects of emodin on cellular calcium contraction of smooth muscle cells in MODS rats are shown in Fig. 4. The effects of nifedipine, EGTA, heparine, and ryanodine on the changes of $[Ca^{2+}]$ induced by emodin are shown in Fig. 5.

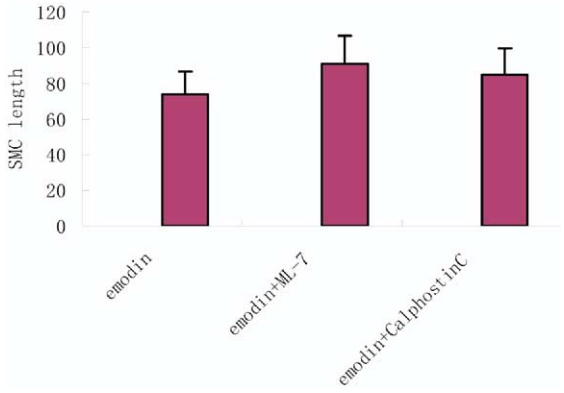

FIG. 3. After SMCs were pre-treated with 10 μmol/L ML-7 and 5 μmol/L Calphostin C, respectively, the actions of emodin-induced contraction were significantly inhibited and compared to the SMCs incubated with emodin alone ($P < 0.05$).

DISCUSSION

Calcium, of both intracellular and extracellular sources, is believed to be an important second messenger for regulating the contractions of smooth muscle cells [16–19]. Extracellular calcium influx in SMCs through voltage-gated Ca^{2+} channel of the membrane, intracellular calcium stored in SR is mainly released by two ion channels: IP_3R and RyR. IP_3R are present in the central as well as in the peripheral SR, which can bind IP_3 to open ion channel and induce Ca^{2+} release [20–26]. The ryanodine receptors contain Ca^{2+} binding sites, allowing increased Ca^{2+} to initiate release from intracellular calcium stores. RyR can be activated through calcium-induced calcium release (CICR), which is a process that a rise in $[Ca^{2+}]$ resulted from extracellular Ca^{2+} influx or Ca^{2+} release from IP3-sensitive store triggers further calcium release from RyR in the SR [27–31].

Ma had proved that exposure of smooth muscle cells to emodin induced an increase in $[Ca^{2+}]$i, and the resultant rising in $[Ca^{2+}]$i was a biphasic rise in $[Ca^{2+}]$ consisting of a rapid, transient peak followed by a decline to sustained level that remained elevated above baseline [11]. It is considered the main mechanism for emodin to elevate Ca^{2+} in SMCs in the physiological conditions via IP_3 route. It is expected that contractions of SMCs will be weakened under the physiological conditions of decreasing IP_3 and increasing NO levels owing to the fall of $[Ca^{2+}]$i. The relevant question to ask is whether emodin can contract the smooth muscles of colon in MODS, which displays altered physiological conditions. The results of this study address this issue by showing that emodin induced an increase in $[Ca^{2+}]$ in the setting of MODS. The mechanisms of $[Ca^{2+}]$ rise in MODS were similar as physiological conditions. Therefore, one of the important mechanisms of emodin regulating the contraction of gut in MODS is to elevate $[Ca^{2+}]$.

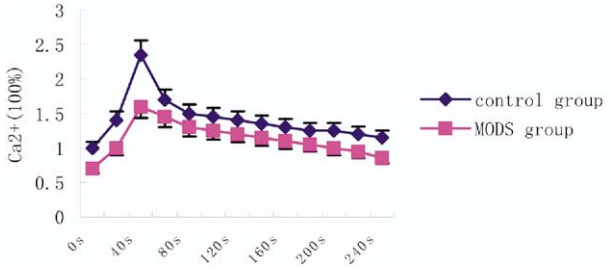

FIG. 4. If the average concentrations of calcium ions in resting state were set as 1 ± 0.07 in SMCs of the control group (the number of SMC was 30), and 0.7 ± 01 (the number of SMC was 31) in the MODS group. The maximal concentrations of $[Ca^{2+}]$ induced by emodin in MODS could reach 1.6 ± 0.29, and $[Ca^{2+}]$ could attain the peak in 30 to 50 s quickly, then falling gradually. The concentrations of $[Ca^{2+}]$I in the two groups were significantly different ($P < 0.05$).

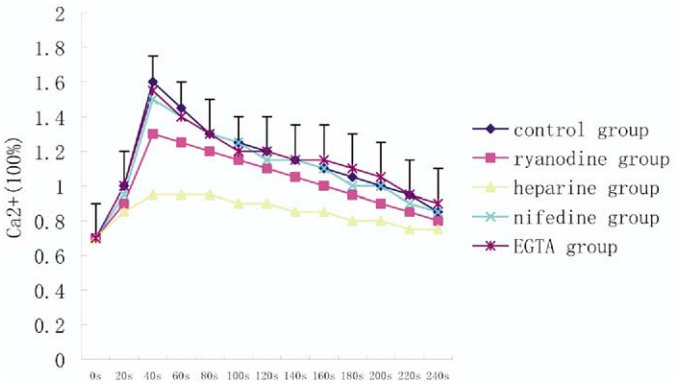

FIG. 5. When SMCs were pre-treated by the solutions of 10^{-5} mol/L nifedipine (an antagonist of voltage-gated Ca^{2+} channel) and 2 mmol/L EGTA (removal of extracellular Ca^{2+}), respectively, their concentrations of Ca^{2+} weren't significantly decreased and suggested that they had no significant effects on the increase of $[Ca^{2+}]$ of SMCs induced by emodin ($P > 0.05$). When SMCs were pre-treated by 100 u/L heparine solutions, the concentrations of Ca^{2+} were only 0.27 ± 0.11 of the control group, and were significantly inhibited ($P < 0.05$). The concentrations of $[Ca^{2+}]$ in SMCs after using ryanodine were 0.72 ± 0.29 of the control group, and ryanodine could significantly decrease the increase of $[Ca^{2+}]$ induced by emodin ($P < 0.05$).

In this study, EGTA, nifedipineh, heparine, and ryanodine were respectively used to determine the mechanism of emodin induced an increase in $[Ca^{2+}]$ of SMCs after MODS as Ma described in the physiological conditions [11]. The data suggested that treatment with heparin, which inhibits the IP3 binding to its receptor, almost abolished the peak component of the Ca^{2+} transient. This adds support to the notion that IP_3 induced Ca^{2+} release is the major mechanism of emodin elevating Ca^{2+} of SMCs in the rats after MODS. The peak of $[Ca^{2+}]$ in SMCs pretreated with ryanodine, a RyR antagonist, was attenuated but not abolished in the rats after MODS. This result suggested that RyR also took part in the process of the increase of $[Ca^{2+}]$ induced by emodin in MODS. EGTA and nifedipine had no detectable effect on emodin-induced peak increase of $[Ca^{2+}]$ in MODS, suggesting that the sources of increasing

$[Ca^{2+}]$ of SMCs in MODS induced by emodin is independent of extracellular Ca^{2+} influx through voltage-gated Ca^{2+} channel and came from intracellular calcium stored in SR through IP_3 and RyR. It needs to be further addressed whether calcium spark plays a role in elevating $[Ca^{2+}]$ induced by emodin.

In smooth muscles, Ca^{2+}/CaM-dependent activation of MLCK leads to the activation of myosin ATPase and cells contractions [32]. It is important to know whether emodin-induced $[Ca^{2+}]$ increase contract smooth muscles is regulated by Ca^{2+}/CaM-dependent MLCK in MODS. In this experiment, ML-7 partly inhibited emodin-induced muscle strips and cells contractions. This implied that Ca^{2+}/CaM-dependent MLCK signal pathway was likely to be involved in emodin-induced smooth muscle contractions in the setting of MODS in rats.

PKC also can activate the contractions of smooth muscles [33]. Calphostin C was used to inhibit the activity of PKC in this study. Our results suggested that PKC could play a certain role in emodin-induced smooth muscle contractions after MODS.

In conclusion, the present study shows that emodin is capable of directly contracting the colonic smooth muscle in the MODS model of rat by both the signal path of MLCK to increase the concentration of calcium ion and the PKC path to enhance calcium sensibility. The mechanism underlying emodin-induced increases in calcium ion is mainly through IP_3 and RyR receptors in the sarcoplasm. These results suggest the potential clinical application of emodin in the treatment of MODS.

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