Contractile effects and intracellular Ca\(^{2+}\) signalling induced by emodin in circular smooth muscle cells of rat colon

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**AIM:** To investigate whether emodin has any effects on circular smooth muscle cells of rat colon and to examine the mechanism underlying its effect.

**METHODS:** Smooth muscle cells were isolated from the circular muscle layer of Wistar rat colon and the cell length was measured by computerized image micrometry. Intracellular Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_i\)) signalling was studied in smooth muscle cells using Ca\(^{2+}\) indicator Fluo-3 AM on a laser-scanning confocal microscope.

**RESULTS:** Emodin dose-dependently induced smooth muscle cells contraction. The contractile responses induced by emodin were inhibited by preincubation of the cells with nifedipine, a voltage-gated Ca\(^{2+}\)-channel antagonist, and the sustained phase of the rise of [Ca\(^{2+}\)]\(_i\) was attenuated by extracellular Ca\(^{2+}\) removal with EGTA solution. Inhibiting Ca\(^{2+}\) release from ryanodine-sensitive intracellular stores by ryanodine reduced the peak increase in [Ca\(^{2+}\)]\(_i\). Using heparin, an antagonist of IP\(_3\)R, almost abolished the peak increase in [Ca\(^{2+}\)]\(_i\).

**CONCLUSION:** Emodin has a direct excitatory effect on circular smooth muscle cells in rat colon mediated via Ca\(^{2+}\)/CaM dependent pathways. Furthermore, emodin-induced peak [Ca\(^{2+}\)]\(_i\) increase may be attributable to the Ca\(^{2+}\) release from IP\(_3\) 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\(^{2+}\)]\(_i\).

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**INTRODUCTION**

Emodin (1, 3, 8-trihydroxy-6-methylanthaquinone) is an anthraquinone derivative isolated from *Rheum palmatum*\(^{[1]}\). Pharmaceutical preparations based on *Rheum palmatum* have been widely used in China for hundreds of years to treat gastrointestinal disorders\(^{[2-4]}\). The reported biological effects of emodin include antitumor, antibacterial and anti-inflammatory actions\(^{[5-7]}\). Emodin also possesses prokinetic effect on gastrointestinal smooth muscle. Stimulatory actions of emodin on gastrointestinal smooth muscle have been described in several studies, and emodin-induced contractions have been related to calcium ions\(^{[8-10]}\). However, the effects of emodin on the contractility of smooth muscle cells have not yet been explored. Thus, the present study was designed to determine whether emodin had any effects on circular smooth muscle cells of rat colon and to examine the mechanism underlying its effects.

**MATERIALS AND METHODS**

**Materials**

Fluo-3 AM (Molecular Probes, USA) was dissolved in DMSO (Sigma) and stored at -20 °C. Phloridzin F-127, collagenase type II, emodin, nifedipine, ryanodine, heparine, Egtazic acid (EGTA), trypsin inhibitor and HEPES were all purchased from Sigma Co. Ltd, USA. DMEM was purchased from GibCO USA, other chemicals were from LianXing BIO Co. Ltd (Beijing). Nifedipine, ryanodine and heparin were all dissolved in standard buffer and kept at 4 °C.

**Methods**

Preparation of dispersed smooth muscle cells  Smooth muscle cells were isolated from the circular muscle layer of Wistar rat colon as previously described with slight modifications\(^{[11]}\). Briefly, muscle strips were digested for 30 min at 31 °C in HEPES medium containing 0.1 % type II 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 500 μm Nitex filter and centrifuged at 350 g for 10 min, and the filtrate (cell suspension) was 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 muscle cell contraction  Contraction was measured in smooth muscle cells by computerized image micrometry as described previously\(^{[12]}\). An aliquot consisting of 1 x 10^4 cells in 0.25 ml of medium was added to 0.1 ml of a solution containing the test agents. The reaction was interrupted at 1 min by adding 0.1 ml of acrolein at a final concentration of 0.1 %. Individual cell length was measured by computerized image micrometry. The average length of cells in the control state or after adding test agents was obtained from 50 cells randomly. The contractile response was defined as the decrease in the average length of the 50 cells and expressed in percentage as compared with control length.

Measurements of [Ca\(^{2+}\)]\(_i\) in smooth muscle cells  Changes in [Ca\(^{2+}\)]\(_i\) were estimated by fluorescence measurement using
Ca\(^{2+}\) indicator Fluo-3 AM as described elsewhere\(^{[13]}\), using a laser scanning confocal microscope (Radiance 2000; Bio-rad, Hertfordshire, UK).

Freshly dissociated smooth muscle cells were seeded onto glass coverslips and incubated with Fluo-3 working solution (Fluo-3 AM 5 \(\mu\)mol and Pluronic F-127 0.03 % 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 and incubated 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 stage of the microscope. The fluorescence in the cell was excited at 488 nm by an argon-ion laser, emission at wavelength between 515-545 nm was detected by a photomultiplier. Changes in the Fluo-3 fluorescence intensity indicating fluctuations in cytosolic Ca\(^{2+}\) were recorded using T-series acquisition. After stable baseline fluorescence intensity was measured, 10 \(\mu\)l of an agent was added to extracellular medium to yield a 1/100 concentration, and the fluorescence intensity was recorded. The ratio representing the intracellular calcium variations related to the basal level was calculated. Baseline and sustained phases of agonist induced [Ca\(^{2+}\)]\(_i\) were determined from the average of 5 data points. Peak [Ca\(^{2+}\)]\(_i\) was determined from the average of 3 data points including the absolute maximum of the response.

**Statistical analysis**

Data represented means ± standard error of the mean. Values of \(n\) were the numbers of cells. Student’s \(t\)-test was performed and a \(P\)-value of less than 0.05 was considered statistically significant.

**RESULTS**

**Effect of emodin on smooth muscle cell length**

In resting state, the average length of isolated smooth muscle cells was 81.27±6.29 \(\mu\)m. The application of emodin to freshly isolated smooth muscle cells induced a reduction in cell length. This reduction in cell length reflected contraction of the smooth muscle cells. Emodin at concentrations of 5 to 100 \(\mu\)mol/L induced a concentration-dependent contraction (Figure 1). Maximal contraction of 17.26±3.51 % was observed with 50 \(\mu\)mol/L of emodin. In order to determine the signal mechanism underlying emodin-induced contraction, the effect of ML-7, an inhibitor of MLCK, on the cell contraction induced by emodin was examined. 36.61±4.69 % of the contractile response induced by 50 \(\mu\)mol/L emodin was inhibited by preincubation of the cells with ML-7 (Figure 2).

**Role of calcium in emodin-induced responses**

The exposure of freshly isolated smooth muscle cells to emodin (50 \(\mu\)mol/L) induced an increase in [Ca\(^{2+}\)]\(_i\). The emodin-induced increase in [Ca\(^{2+}\)]\(_i\) was a biphasic rise, consisting of a transient peak followed by a decline to steady-state level that remained significantly above baseline during scanning (Figures 3 and 4).

**Figure 1** Contractile effect of emodin on isolated smooth muscle cell. Values were calculated as means ± SE from 3 experiments.

**Figure 2** Effect of ML-7 on emodin-induced contraction of smooth muscle cells. Values were means ±SE of 3 experiments. \(a P<0.05\) by Student’s \(t\)-test.

**Figure 3** Timecourse changes of [Ca\(^{2+}\)]\(_i\) induced by emodin in a circular colonic smooth muscle cells (analysed by BioRad Laserpix software).
To determine whether the emodin-induced increase in [Ca\(^{2+}\)]\(_i\) required calcium influx from extracellular medium, the effect of nifedipine, an antagonist of voltage-gated Ca\(^{2+}\) channel in response to emodin was investigated. Emodin was applied after 15 min-exposure to nifedipine. Exposing smooth muscle cells to nifedipine had no effect on the increase in [Ca\(^{2+}\)]\(_i\) induced by emodin (P>0.05). 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. Removal of extracellular Ca\(^{2+}\) by EGTA solution significantly reduced the sustained changes in [Ca\(^{2+}\)]\(_i\) (Figure 4, P<0.05) compared with the sustained increase in [Ca\(^{2+}\)]\(_i\) induced by emodin, and had no effect on peak changes in [Ca\(^{2+}\)]\(_i\) (Figure 4, P>0.05).

**DISCUSSION**

The contractile effects of emodin on gastrointestinal smooth muscle have been described in several reports. In this study, we have attempted to investigate the effects of emodin on circular smooth muscle cells of rat colon and to examine the mechanisms underlying its effects.

The result of this study showed that emodin had a direct contractile effect on smooth muscle cells freshly isolated from rat colon, as application of emodin resulted in a decrease in cell length. It was also found that exposure of smooth muscle cells to emodin induced an increase in [Ca\(^{2+}\)]\(_i\), and the rising [Ca\(^{2+}\)]\(_i\) induced by emodin was a biphasic rise in [Ca\(^{2+}\)]\(_i\) consisting of a rapid, transient peak followed by a decline to sustained level that remained elevated than baseline. The involvement of calcium in emodin-induced contraction was in agreement with previous studies, reporting that emodin-induced contractions were related to calcium ion.

Calcium is believed to be the crucial signal for tension generation or shortening of smooth muscle cells. The increase in [Ca\(^{2+}\)]\(_i\) induced by contractile agonists in smooth muscle cells is accompanied by Ca\(^{2+}\)/CaM-dependent activation of myosin light chain (MLC) kinase, leading to activation of myosin ATPase and cell contraction. In these experiments, suppression of Ca\(^{2+}\)/CaM-dependent MLCK activity with ML-7 while maintaining Ca\(^{2+}\) mobilization partly inhibited emodin-induced cell contraction. This implies that Ca\(^{2+}\)/CaM-dependent MLCK signal pathway is involved in emodin-induced cell contraction.

**Figure 5** Average peak (top) and sustained (bottom) changes in [Ca\(^{2+}\)]\(_i\) in response to emodin under control conditions, in the presence of nifedipine, and EGTA solution. n=15. *P*<0.05 vs emodin group.

**Figure 6** Effect of ryanodine and heparin on the change in [Ca\(^{2+}\)]\(_i\) induced by emodin. n=15 *P*<0.05 vs emodin group.

Because emodin-induced peak increase in [Ca\(^{2+}\)]\(_i\) was not affected by pretreatment with EGTA and nifedipine, the contribution of calcium release from the intracellular stores to the changes in [Ca\(^{2+}\)]\(_i\) in response to emodin was examined. Smooth muscle cells were pretreated for 10 min with ryanodine (10^{-5}M) to inhibit Ca\(^{2+}\) release from ryanodine-sensitive intracellular stores. Ryanodine markedly attenuated the peak increase in [Ca\(^{2+}\)]\(_i\) in response to emodin (Figure 5, P<0.05).

In order to examine the role of IP\(_3\) in emodin-induced increases in [Ca\(^{2+}\)]\(_i\), the effect of low molecular weight heparin, a specific IP\(_3\) receptor antagonist was tested. Heparin did not diffuse across the plasma membrane, therefore, it was used in permeablized smooth muscle cells. In permeablized smooth muscle cells, the peak rise in [Ca\(^{2+}\)]\(_i\) induced by emodin was almost abolished by incubation with 10 µg/ml heparin (Figure 5).

Because pretreatment with EGTA and nifedipine had no effect on emodin-induced peak increase in [Ca\(^{2+}\)]\(_i\), it seemed likely that the portion of peak emodin-induced Ca\(^{2+}\) transient mainly depended on the calcium release from intracellular stores. The sarcoplasmic reticulum (SR) is the physiological intracellular source and sink of activator Ca\(^{2+}\) in smooth muscle cells. The SR of smooth muscle is endowed with two different types of Ca\(^{2+}\) release channels, i.e. inositol 1, 4, 5-triphosphate receptors (IP\(_3\),Rs) and ryanodine receptors
Pretreating cells with ryanodine, a RyRs antagonist, attenuated but did not abolish the peak in \([Ca^{2+}]_i\). In contrast, heparin, which inhibits the IP3 binding to its receptor, almost abolished the peak component of the Ca\(^{2+}\) transient. These observations suggest that the residual Ca\(^{2+}\) peak observed in the presence of ryanodine is due to release of Ca\(^{2+}\) from IP3-sensitive stores, and Ca\(^{2+}\) release through RyR receptors is possibly linked with the Ca\(^{2+}\)-induced Ca\(^{2+}\)-release (CICR). CICR means a process that a rise in \([Ca^{2+}]_i\) results from extracellular Ca\(^{2+}\) influx or Ca\(^{2+}\) release from IP3-sensitive store triggers further calcium release from RYR in the SR [14,26-29]. Ryanodine receptors contain Ca\(^{2+}\) sensitive stores, which trigger Ca\(^{2+}\) release from ryanodine-sensitive stores through CICR mechanism. Additionally, Ca\(^{2+}\) influx from extracellular medium contributes to the sustained increase in \([Ca^{2+}]_i\) observed in response to emodin.

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REFERENCES

1. Liang JW, Hsu SL, Wu PP, Chao PD. Emodin pharmacokinetics in rabbits. Planta Med 1995; 61: 406-408
2. Xia Q, Jiang JM, Gong X, Chen GY, Li L, Huang ZW. Experimental study of Tong Xia purgative method in ameliorating lung injury in acute necrotizing pancreatitis. World J Gastroenterol 2000; 6: 115-118
3. Chen H, Wu X, Guan F. Protective effects of tongo gongxia herbs on gut barrier in rat with multiple organ dysfunction syndrome. Zhongguo Zhongxiyi Jiehe Zazhi 1995; 61: 429-431
4. You SY, Wu XZ, Liu ML. Effects of dachengqi decoction on gut hormones and intestinal movement after cholecystectomy. Zhongguo Zhongxiyi Jiehe Zazhi 1994; 4: 522-524
5. Lee HZ. Effects and mechanisms of emodin on cell death in human lung squamous cd1 carcinoma. Br J Pharmacol 2001; 134: 11-20
6. Lee HZ. Protein kinase C involvement in aloe-emodin- and emodin-induced apoptosis in lung carcinoma cell. Br J Pharmacol 2001; 134: 1093-1103
7. Kuo YC, Meng HC, Tsai WJ. Regulation of cell proliferation, inflammatory cytokine production and calcium mobilization in primary human T lymphocytes by emodin from Polygonum hypoleucum Ohwii. Inflamm Res 2001; 50: 73-82
8. Jin ZH, Ma DL, Lin XZ. Study on effect of emodin on the isolated intestinal smooth muscle of guinea pigs. Zhongguo Zhongxiyi Jiehe Zazhi 1994; 14: 429-431
9. Li JY, Yang WX, Hu WW, Wang J, Jin ZG, Wang XY, Xu WS. Effects of emodin on the activity of K channel in guinea pig taenia coli smooth muscle cells. Xiyue Xuebao 1998; 33: 321-325
10. Yang WX, Wang J, Li JY, Yu Y, Xu WS. Characteristics of emodin evoked \([Ca^{2+}]_i\) and inhibition of GDP in guinea pig taenia coli cells. Shengwu Wuli Xuebao 2001; 17: 165-169
11. Wang P, Bitar KN. RhoA regulates sustained smooth muscle contraction through cytoskeletal reorganization of HSP27. Am J Physiol 1998; 275(GPT 1): 1454-1462
12. Yamada H, Strahler J, Weish J, Bitar KN. Activation of MAP kinase and translocation with HSP27 in bombesin-induced contraction of rectosigmoid smooth muscle. Am J Physiol 1995; 269 (SPT 1): 683-691
13. Jacques D, Sader S, El-Bizni N, Choufani S, Hassan G, Shbaklo H. Neuroepitope-Y induced increase of cytosolic and nuclear Ca\(^{2+}\) in heart and vascular smooth muscle cells. Can J Physiol Pharmacol 2000; 78: 162-172
14. Sanders KM. Invited review: mechanisms of calcium handling in smooth muscles. J Appl Physiol 2001; 91: 1438-1449
15. Bolton TB, Prestwich SA, Zhols A, Gordienko DV. Excitation-contracting coupling in gastrointestinal and other smooth muscles. Annu Rev Physiol 1999; 61: 85-115
16. Makhlouf GM, Murthy KS. Signal transduction in gastrointestinal smooth muscle. Cell Signal 1997; 9: 269-276
17. Sayeski PP, Ali MS, Bernstein KE. The role of Ca\(^{2+}\) mobilization and heterotrimERIC Ca\(^{2+}\) activation in mediating tyrosine phosphorylation signaling patterns in vascular smooth muscle cells. Mol Cel Biochem 2000; 212: 91-98
18. Fan J, Byron KL. Ca\(^{2+}\) signalling in rat vascular smooth muscle cells: a role for protein kinase C at physiological vasoconstrictor concentrations of vasopressin. J Physiol 2001; 524(Pt 3): 821-831
19. White C, Mc Geown JG. Regulation of basal intracellular calcium concentration by the sarcoplasmic reticulum in myocytes from the rat gastric antrum. J Physiol 2000; 529(Pt 2): 395-404
20. Carl A, Lee HK, Sanders KM. Regulation of ion channels in smooth muscles by calcium. Am J Physiol 1996; 271(Pt 1): 9-34
21. McCarron JG, Flynn ER, Bradley KN, Muir TC. Two Ca\(^{2+}\) entry pathways mediate InsP\(_3\)-sensitive store refilling in guinea-pig smooth muscle. J Physiol 2000; 525(Pt 1): 113-124
22. Takeuchi M, Watanabe J, Horiochi S, Karibe A, Katoh H, Baba S, Shinozaki T, Miura M, Fukuchi M, Kagaya Y, Shirato K. Interaction between L-type Ca\(^{2+}\) channels and sarcoplasmic reticulum in the regulation of vascular tone in isolated rat small arteries. J Cardiovasc Pharmacol 2000; 36: 548-554
23. van Helden DF, Imtiaz MS, Nurgaliyeva K, Von der Weid P, Dosen PJ. Role of calcium stores and membrane voltage in the generation of slow wave action potentials in guinea-pig gastric pylorus. J Physiol 2000; 524(Pt 1): 245-265
24. Flynn ER, Bradley KN, Muir TC, McCarron JG. Functionally separate intracellular Ca\(^{2+}\) stores in smooth muscle. J Biol Chem 2001; 276: 36411-36418
25. Kotlikoff ML, Wang YX, Xin HB, Ji G. Calcium release by ryanodine receptors in smooth muscle. N ovaris Found Sym 2002; 246: 109-119
26. Ji G, Barsotti RJ, Feldman ME, Kotlikoff MI. Stress-induced calcium release in smooth muscle. J Gen Physiol 2002; 119: 533-544
27. Collier ML, Ji G, Wang Y, Kotlikoff MI. Calcium-induced calcium release in smooth muscle: loose coupling between the action potential and calcium release. J Gen Physiol 2000; 115: 653-662
28. Morgan JM, Gillespie JI. The modulation and characterisation of the Ca\(^{2+}\)-induced Ca\(^{2+}\) release mechanism in cultured human myometrial smooth muscle cells. FEBS Lett 1995; 369: 295-300
29. Henkel CC, Asubn J, Cebabalos G, del Carmen Castillo M, Castillo EF. Relationship between extra and intracellular sources of calcium and the contractile effect of thiopental in rat aorta. Can J Physiol Pharmacol 2001; 79: 407-414
30. Somlyo AP, Somlyo AV. The sarcoplasmic reticulum: then and now. N ovaris Found Sym 2002; 246: 258-268
31. Chambers P, Neal DE, Gillespie JI. Ryanodine receptors in human bladder smooth muscle. Exp Physiol 1999; 84: 41-46

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