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Full Length Research Paper

Epoxyaurapten inhibition of smooth muscle contraction and phosphorylation of myosin light chain by myosin light chain kinase

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Epoxyaurapten is a component of coumarin, which was isolated from Aurantii Fructus Immaturus. The objective of this study was to determine the inhibition of epoxyaurapten on smooth muscle *in vitro*. The experiment of smooth muscle contraction directly monitored the contractions of the isolated proximal duodenum by frequency and amplitude at different epoxyaurapten concentrations and under different incubation times. The results showed that epoxyaurapten inhibited contraction in intestinal muscles in a dose- and time-dependent manner. The effects of epoxyaurapten on myosin were measured in the presence of Ca^{2+}-calmodulin using the activities of 20 kDa myosin light chain (MLC_{20}) phosphorylation and myosin Mg^{2+}-ATPase. The results demonstrated that MLC_{20} phosphorylation by myosin light chain kinase (MLCK) decreased with increasing epoxyaurapten concentration. The myosin Mg^{2+}-ATPase activity also gradually deceased with increasing epoxyaurapten concentration. With prolonged incubation time, MLC_{20} phosphorylation by MLCK and myosin Mg^{2+}-ATPase activities furthered declined. The effect of epoxyaurapten on MLCK expression was measured by western blot, and the results showed that epoxyaurapten inhibited the expression of MLCK in a dose- and time-dependent manner. The findings demonstrated that epoxyaurapten could effectively attenuate the contractions of intestinal smooth muscle and therefore could be developed as a potential therapy in the future.

Key words: Epoxyaurapten, myosin light chain kinase (MLCK), 20 kDa regulating myosin light chain (MLC_{20}), myosin Mg^{2+}-ATPase, Aurantii Fructus Immaturus.

INTRODUCTION

Aurantii Fructus Immaturus belongs to the genus Citrus (Rutacae); the source of Aurantii Fructus Immaturus is a smaller, unripe fruit (Chuang et al., 2007a). Its major uses are in the treatment of gastroectasia, gastritis, functional gastrointestinal disorders, constipation, diarrhea, peptic ulcer and –ptoses, such as gastroptosis, metroptosis and archoptosis (Fang et al., 2009). The literature reports that Aurantii Fructus Immaturus contains various coumarins and glycosylated and nonglycosylated flavonoids (Chuang et al., 2007b). Various studies have revealed a variety of pharmacological activities for citrus coumarins, including anticancer (Hirata et al., 2009), anti-inflammatory (Fylaktakidou et al., 2004), anti-coagulant and anti-viral effects (Kostova et al., 2006), and several coumarin derivatives have been shown to be selective coronary vasodilators (Campos-Toimil et al., 2002). In this paper, we present the compound epoxyaurapten (Figure 1), which was isolation and structural determination of a coumarin from Aurantii Fructus Immaturus. The structure of epoxyaurapten was identified by comparison of spectroscopic data with values reported in Takase et al. (1994). From the literature, Aurantii Fructus Immaturus has a bilateral effect on gastrointestinal smooth muscle contraction (Fang et al., 2009) that is not yet clear. It is also unclear if epoxyaurapten is an active ingredient of Aurantii Fructus Immaturus, leading us to study the effect

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Abbreviations: SMCs, smooth muscle cells; **PAGE,** polyacrylamide gel electrophoresis; **MLC_{20},** 20 kDa regulating myosin light chain; **LC_{20},** unphosphorylated MLC_{20}; **p-LC_{20},** mono-phosphorylated MLC_{20}; **LC_{17},** 17 kDa myosin essential light chains.

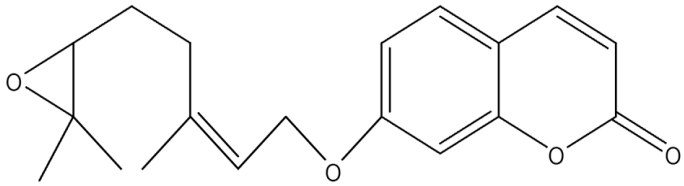

Figure 1. The chemical structure of epoxyaurapten.

of epoxyaurapten on intestinal smooth muscle. It had been accepted that the mechanism of regulation of smooth muscle contraction and relaxation is the reversible Ca^{2+}-calmodulin dependent phosphorylation of myosin light chains by myosin light chain kinase (MLCK) (Vale and Milligan, 2000; Yang et al., 2004; Zhang et al., 2006). Therefore, the modulation of myosin provides important indexes to evaluate the effects of modulators. In vertebrate tissues, MLCK is regulated by Ca^{2+}-calmodulin, and is rate-limiting for contraction of smooth muscle (Olson et al., 1990). In order to investigate epoxyaurapten function in smooth muscle, the experiments were chosen so as to inspect the contraction of intestinal smooth muscle, myosin phosphorylation levels and myosin Mg^{2+}-ATPase activity. Furthermore, in order to shed light on the mechanism of epoxyaurapten on smooth muscle cells, the expression of MLCK was examined using western blot analysis.

MATERIALS AND METHODS

Animals, reagents and antibodies

Experiments were performed according to the rules of animals care and approved by the local animal protection committee (Liaoning University of Traditional Chinese Medicine). Male Sprague-Dawley rats (200 to 300 g) were used to study the effect of epoxyaurapten on the amplitude of smooth muscle contractions. BALB/C mice (18 to 22 g) were used for western blot analysis. Animals were obtained from the Experimental Animal Center of Dalian Medical University. Male Sprague-Dawley rats were housed five per cage and BALB/C mice were housed 10 per cage. Animals were maintained in a controlled environment at 25±2°C under a 12 h dark-light cycle (light on at 7:00 A.M.) and acclimatized for at least one week prior to use.

Phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT) and Anti-MLCK monoclonal antibody (mAb) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethylene glycol bis(2-aminoethyl ether)tetraacetic acid (EGTA) was purchased from Wako (Osaka, Japan). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS) and penicillin-streptomycin solution were purchased from Gibco (Carlsbad, CA, USA). Rabbit monoclonal anti-β-actin antibody was purchased from NanJing KeyGen Biotechnology Corporation (Nan Jing, China). All other chemicals used were of the purest commercially available grade.

Krebs's solution was created with the following proportions of constituents (in mmol/L): Sodium chloride 114.0, potassium chloride 4.7, magnesium chloride 1.2, calcium chloride 2.5, sodium dehydrogenate phosphate 1.8, glucose 11.5, sodium bicarbonate 18.0, and pH 7.4.

Preparation, perfusion of isolated duodenal segments and gastrointestinal motility

The abdomen was opened under urethane anesthesia. The duodenum between the pylorus and the Treitz ligament was removed and luminally perfused *ex vivo*, as described earlier (Park et al., 2010; Schumann and Hunder, 1996). Briefly, the perfusion conditions were as follows: Recirculation luminal perfusion with 30 ml of bicarbonate-buffered Tyrode solution (37°C, pH 7.2) equilibrated with 95% O_2 and 5% CO_2, 25 cm H_2O hydrostatic pressure, and 50 ml/min flow rate. The segments were kept in a moist chamber.

Contractions of the proximal duodenum were directly monitored by both frequency (contractions per minute) and amplitude (increase in pressure). In some cases, a motility index (frequency times mean amplitude) was calculated. Occasional movement artifacts were easily identified as spikes that appeared simultaneously in both recorded channels and were eliminated from data analysis. To determine the time at which tolerance was developed to the motility effects of epoxyaurapten, we compared the frequency of contractions in control recordings with the frequency of contractions after epoxyaurapten infusion in different groups of animals. Each animal served as its own control, and recording of intestinal motility was limited to 6 h.

Duodenum segments were also placed into a tissue chamber and allowed to equilibrate for 60 min. Isometric responses were measured with a force-displacement transducer. The effects of the duodenum on the isometric response included the relative value (the average of the contractile curve recorded over 5 min) compared to a normal control (Krebs' solution with 2.5 mmol/L $CaCl_2$), which was assigned a value of 100%

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Protein purification

Myosin and MLCK used in the assay were purified from fresh chicken gizzard smooth muscle using methods reported previously (Lin et al., 1994, 2000). The actin was purified from acetone powder of chicken breast muscle (Kohama, 1980). The purified myosin was unphosphorylated, as determined by 10% glycerol electrophoresis.

MLC_{20} phosphorylation determination

Ca^{2+}-calmodulin dependent phosphorylation of myosin light chain by MLCK was carried out according to the method of Yang et al. (2003). After phosphorylation of MLC_{20}, solid urea and sample solution, which contained bromophenol blue and glycerol, were added to the reaction mixture. Next, 10% glycerol polyacrylamide gel electrophoresis (PAGE) was used to measure the extent of phosphorylation of MLC_{20}. Scoin Image Software, densitometry software from Scion Co., Ltd., was applied to analyze the percentage of phosphorylated MLC_{20} in the total MLC_{20}. Mono-phosphorylation of the positive control was chosen as the control and calculated as 100%.

Measurement of myosin Mg^{2+}-ATPase activity

The method for measuring Mg^{2+}-ATPase activity of myosin was performed as described previously (Yang et al., 2003, 2004; Zhang et al., 2006).

Briefly, the measuring of Mg^{2+}-ATPase activity was carried out in a 20 mmol/L Tris-HCl (PH 7.4) buffer containing 60 mmol/L KCl, 5 mmol/L $MgCl_2$, 1 mmol/L DTT, 0.5 mmol/L ATP, 0.1 mmol/L $CaCl_2$, 0.6 mmol/L CaM, and 0.4 µmol/L myosin at 25°C using the malachite green method.

Cell culture

The primary smooth muscle cells (SMCs) were obtained from BALB/C mice colons using a method previously reported (Wells et al., 2003; Zulian et al., 2010) with slight modification. The colon was pinned to ice-cold PBS, and the mucosa and submucosa were removed by sharp dissection. The remaining muscle sheet was cut into strips that were then placed into trypsin-EDTA solution. Next, the strips were centrifuged at 1500 g for 10 min. The operation was repeated twice. The strips were mixed in cell medium and the solution was filtered through a 70-μm cell strainer and centrifuged at 1500 g for 10 min. Last, the precipitate was suspended in cell medium (DMEM with 10% FBS and 1% penicillin-streptomycin solution) to produce primary SMCs. The SMCs were then plated in 6-well plates at a density of 1.5×10^6 cells/ml (2 ml/well). The SMCs of passages 3 to 4 were used in the experiments.

Western blot analysis

In accordance with the experimental design, the smooth muscle cells were exposed to epoxyaurapten in a complete medium (DMEM with 10% FBS and 1% penicillin-streptomycin solution). After incubation, the cells were harvested and pelleted by centrifugation at 500 g for 5 min. Cell pellets were lysed with a cell lysate kit (KeyGen). The total protein concentration of each sample was measured using a MicroBCA Protein Assay Reagent Kit (KyeGen). The cell lysates containing 20 μg of protein were electrophoresed in 12% SDS-polyacrylamide gel and electroblotted onto a polyvinylidene difluoride membrane (Lin et al., 2010). The membrane was then blocked with 5% fat-free milk in PBS with 0.1% Tween 20 for 1 h at room temperature. Anti-MLCK mAb (1:100 dilution) was incubated overnight at 4°C, followed by incubation with 1:5000 diluted HRP-conjugated goat antibody against rabbit IgG and stained with an enhanced chemiluminescence kit (KyeGen). Six independent experiments were performed, and the results were reproducible. The resulting western blot images were analyzed with Scoin Image Software to determine the integrated density value of each protein band normalized to the integrated density value of β-actin.

Statistical analysis

The results are expressed as means ± standard deviation ($\overline{X}$ ±s). Statistical analysis was performed with a Student's t-test. Statistical significance was accepted at a level of $P<0.05$.

RESULTS

Effect of epoxyaurapten on the amplitude of smooth muscle contraction at different concentrations

The dose-response curve for epoxyaurapten-induced decreased duodenum contraction after epoxyaurapten incubation for 5 min in rats is shown in Figure 2. Epoxyaurapten (20, 40, 80, 160, 320 μmol/L, respectively) resulted in an inhibition of the amplitude of contraction (from 100±6.3 to 91±7.6, 69±5.9, 62±4.4, 48±6.1 and 29±3.9%) in the duodenum in a dose-dependent manner.

There was significant difference between the amplitudes of the different concentration groups (40, 80, 160 and 320 μmol/L) and the corresponding negative control (**p<0.01).

Effect of epoxyaurapten on the amplitude of smooth muscle contraction under different incubation times

The side effects of epoxyaurapten are not clear. Figure 2 illustrates that 80 μmol/L epoxyaurapten had a significant effect. In order to avoid potentially side effects for high concentrations of epoxyaurapten, we chose 80 μmol/L epoxyaurapten to incubate duodenum. In Figure 3, administration of epoxyaurapten (80 μmol/L) for 4, 8, 12, 16 and 20 min resulted in a decrease in the amplitude of contraction in a time-dependent manner. Furthermore, there was a significant difference between the amplitudes of the timed groups and the corresponding negative control (**p<0.01).

Effect of epoxyaurapten on MLC_{20} phosphorylation by MLCK at different epoxyaurapten concentrations

As the literature reports, the high sensitivity of MLC_{20} phosphorylation by MLCK means only low concentrations of MLCK are needed in the presence of Ca^{2+} and CaM (Tang et al., 2010; Tansey et al., 1994), so 4 μmol/L myosin and 0.02 μmo/L MLCK were added to the assay for MLC_{20} phosphorylation. Figure 4 indicates that epoxyaurapten at concentrations from 20 to 320 μmol/L induced a dose-dependent inhibition of MLC_{20} phosphorylation by MLCK. With increases in epoxyaurapten concentration, MLC_{20} phosphorylation by MLCK significantly decreased (**P<0.01).

Effect of epoxyaurapten on MLC_{20} phosphorylation by MLCK after different incubation times

In Figure 5, to investigate MLC_{20} phosphorylation by MLCK after incubation with epoxyaurapten for different times, three different incubation times (i.e., 5, 10 and 20 min) are displayed. As can be seen, the extent of MLC_{20} phosphorylation in lanes 2′, 3′ and 4′ decreased along incubation time. Furthermore, there was a significant difference between the time groups (5, 10 and 20 min) and the corresponding positive control group (** P < 0.01).

Effect of epoxyaurapten on myosin Mg^{2+}-ATPase activity

The results in Figure 6 indicate that at different incubation times, epoxyaurapten (40, 80, 160 and 320 μmol/L) induced a dose-dependent inhibition of Mg^{2+}-ATPase activity.

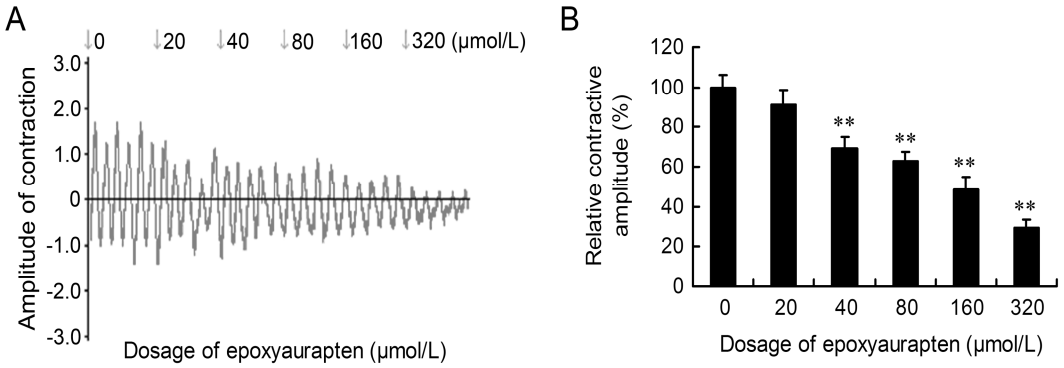

Figure 2. Effect of epoxyaurapten on the amplitude of smooth muscle contraction at different concentrations ($\overline{X}$ ±s, n = 6). (A) The diagram of scope curves of smooth muscle contraction. The arrow shows that the contraction scope changed at 0, 20, 40, 80, 160 and 320 μmol/L epoxyaurapten. (B) Histogram of statistical results for different epoxyaurapten concentrations. The abscissa is different epoxyaurapten concentrations and the ordinate is the relative extent of contraction amplitude. Data are means±SD of six experiments and asterisks (**) denote significant difference (P < 0.01) from the negative control group.

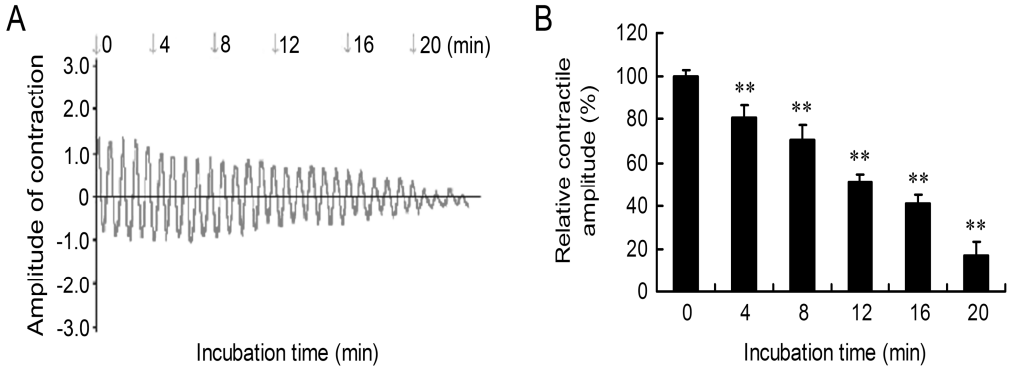

Figure 3. Effect of epoxyaurapten on amplitude of smooth muscle contraction after different incubation times ($\overline{X}$ ±s, n=6). A) The scope curves of smooth muscle contraction. The arrow shows that the contraction scope changed for 80 μmol/L epoxyaurapten after different incubation times (4, 8, 12, 16 and 20 min). B) Histogram for statistical results of the amplitude of smooth muscle contraction after different incubation times with 80 μmol/L epoxyaurapten. The abscissa is different incubation times and the ordinate is the relative extent of contraction amplitude. Data are means±SD of six experiments and asterisks (**) denote significant difference (P < 0.01) from the negative control group.

Effect of epoxyaurapten on MLCK expression at different epoxyaurapten concentrations

In Figure 7, epoxyaurapten at concentrations ranging from 40 to 320 μmol/L induced a dose-dependent expression of MLCK. The expression of MLCK decreased with increasing epoxyaurapten concentration.

Effect of epoxyaurapten on MLCK expression after different incubation times

The results in Figure 8 demonstrate that for over five different incubation times (that is, 12, 24, 48, 72 and 96 h), the expression of MLCK was decreased gradually with increasing incubation time.

DISCUSSION

Smooth muscle contraction is activated primarily via Ca^{2+}-calmodulin (CaM)-dependent phosphorylation of MLC_{20} by MLCK. The phosphorylation can be simply described as interaction of Ca^{2+} with CaM inducing a conformational change of MLCK that activates MLCK. The activated MLCK then catalyzes phosphorylation of

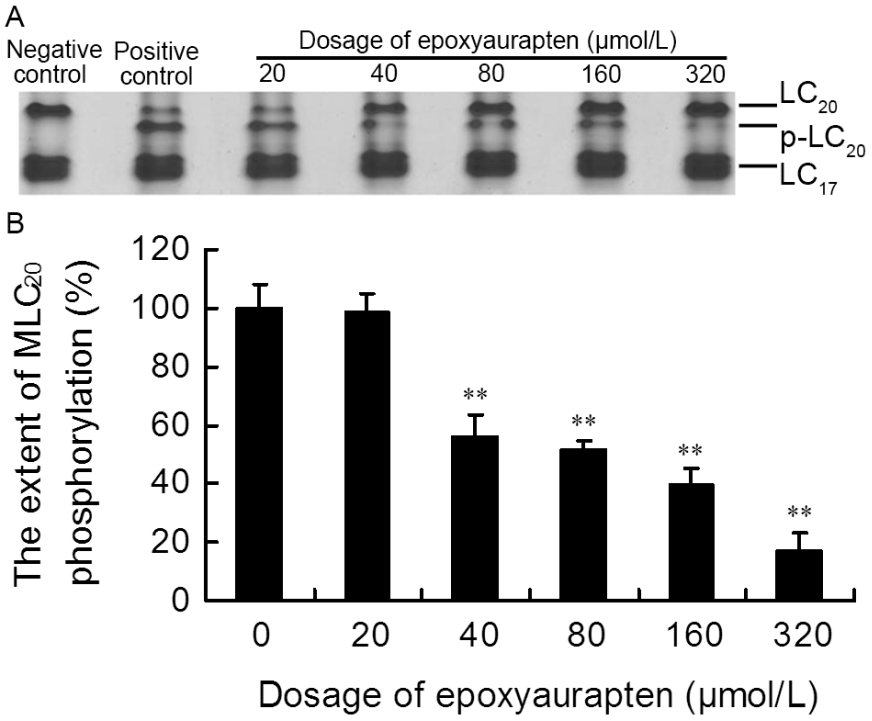

Figure 4. Effect of MLC_{20} phosphorylation by MLCK at different epoxyaurapten concentrations ($\overline{X}$ ±s, n = 6). (A) The glycerol electrophoresis results for MLC_{20} phosphorylation by MLCK after treatment with different epoxyaurapten concentrations. Lane 0 is the negative control (blank control without incubation); lane 1 is the positive control (without epoxyaurapten); lanes 2 to 6 represent 20, 40, 80, 160, 320 μmol/L epoxyaurapten at the same incubation time, respectively. A 4 μmol/L aliquot of myosin (1.76 mg·ml^{-1}) was used at 25°C for 20 min in the incubation. LC_{20}, p-LC_{20} and LC_{17} were separated into 3 isolated bands in glycerol polyacrylamide gel electrophoresis (PAGE). Their mobilities were in the following order: LC_{20} < p-L20 < L_{17}. (B) Histogram of statistical results for myosin phosphorylation after treatment with different epoxyaurapten concentrations, analyzed using Scoin Image Software. The abscissa is different epoxyaurapten concentrations and the ordinate is the relative extent of MLC_{20} phosphorylation. Mono-phosphorylation of positive control was chosen as the control and calculated as 100%. Data are means±SD of six experiments and asterisks (**) denote significant difference (P < 0.01) from the positive control group.

MLC_{20}. Next, MLC_{20} phosphorylation triggers cycling of myosin cross-bridges along actin filaments, resulting in motive force (Kamm and Stull, 1985; Tang et al., 2010). Since the changes in myosin phosphorylation and Mg^{2+}-ATPase activity reflect the regulation of myosin function, in these experiments, we focused on these changes as important indexes to determine the effects of epoxyaurapten on myosin. From the results, the functional features of epoxyaurapten on the contraction of intestinal smooth muscle and myosin were discovered. In order to reveal the internal mechanism of the effect of epoxyaurapten on smooth muscle, the MLCK expression was quantified. From the experiments results, we found that epoxyaurapten could inhibit the contraction of isolated proximal duodenum under normal conditions. In addition, epoxyaurapten inhibited the contraction of intestinal muscle in a dose- and time-dependent manner. The results further showed that epoxyaurapten could act directly on intestinal smooth muscle.

We then used glycerol PAGE to inspect the effect of epoxyaurapten on MLC_{20} phosphorylation by MLCK. The activity of myosin phosphorylation by MLCK was observed via the expression of mono-phosphorylation on glycerol PAGE (Tang et al., 2010). The results demonstrated that epoxyaurapten could inhibit the extent of phosphorylation of myosin in a dose- and time-dependent manner. Furthermore, since MLC_{20} can be phosphorylated and the phosphorylation of smooth muscle myosin is associated with an increase in actin-activated Mg^{2+}-ATPase activity (Hong et al., 2009; Kamm and Stull, 1985), this studied investigated the effect of epoxyaurapten on myosin Mg^{2+}-ATPase activity. From the results, we could see that epoxyaurapten also decreased the extent of Mg^{2+}-ATPase activity in a dose-dependent

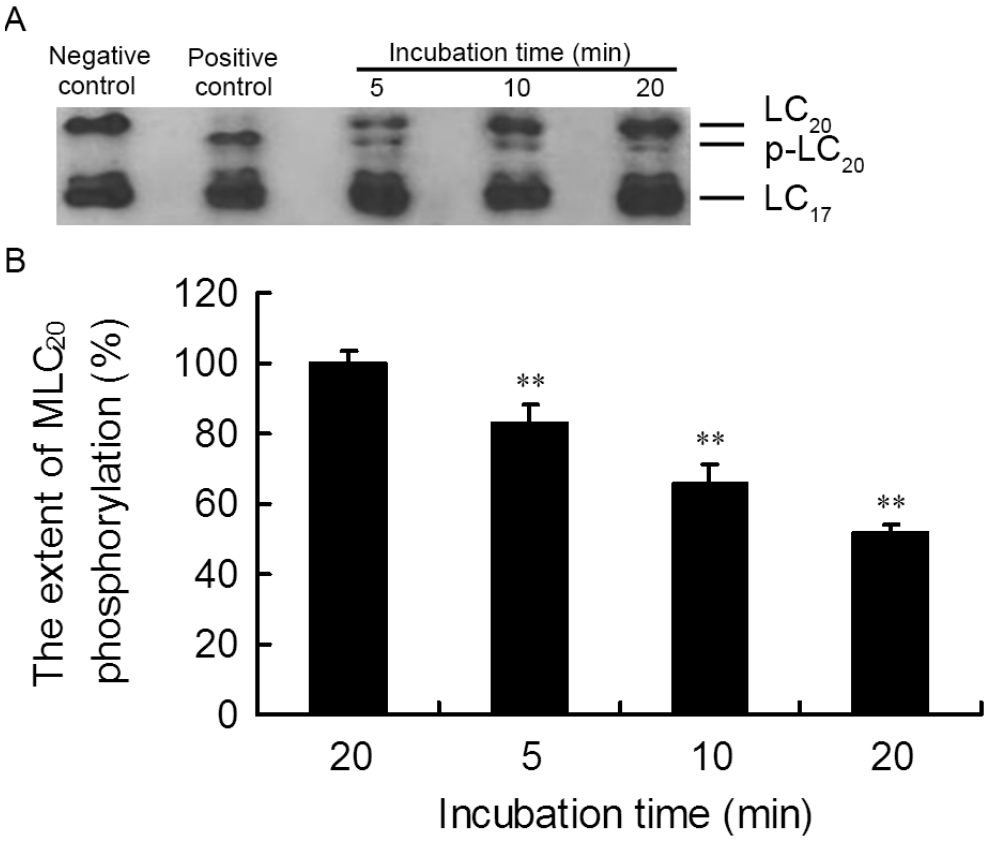

Figure 5. Effect of epoxyaurapten on MLC_{20} phosphorylation by MLCK after different incubation times ($\bar{x}$ ±s, n = 6). (A) The glycerol electrophoresis results of MLC_{20} phosphorylation by MLCK after different incubation times with the same epoxyaurapten concentration. Lanes 0' to 4' show the negative control (blank control without incubation), positive control (without epoxyaurapten), 5, 10 and 20 min incubation time groups, respectively. A 4 μmol/L aliquot of myosin (1.76 mg ml^{-1}) was used at 25°C in the incubation. LC_{20}, p-LC_{20} and LC_{17} were separated into 3 isolated bands on a glycerol PAGE. Their mobilities were in the following order: LC_{20} < p-L20 < L_{17}. (B) Histogram of statistical results for myosin phosphorylation after different incubation times with the same epoxyaurapten concentration was (80 μmol/L), analyzed using Scoin Image Software. The abscissa is different incubation times and the ordinate is the relative extent of MLC_{20} phosphorylation. Mono-phosphorylation of positive control was chosen as the control and calculated as 100%. Data are means±SD of six experiments and asterisks (**) denote significant difference (P < 0.01) from the positive control group.

manner. These results indicated that epoxyaurapten could inhibit the function of smooth muscle myosin, thereby inhibiting the contraction of intestinal smooth muscle.

MLCK expression can directly affect the amount of MLC_{20} phosphorylation and the level of myosin Mg^{2+}-ATPase activity (Lin et al., 2010). By western blot analysis, increased concentrations of epoxyaurapten and extended incubation times both decreased the expression of MLCK. These results indicated that the mechanism of smooth muscle contraction inhibition of epoxyaurapten was related to the expression of MLCK. Herein, we report for the first time that epoxyaurapten inhibits the contraction of intestinal smooth muscle. The experiment results suggested that epoxyaurapten might have a future therapeutic role in the treatment of the pathological state of smooth muscle disorders. It has also been reported that Aurantii Fructus Immaturus can antagonize acetylcholine-induced contraction of intestines (Fang et al., 2009), so epoxyaurapten might also be responsible for this observed inhibition of smooth muscle contraction. One possible explanation for this phenol-menon is that epoxyaurapten may be an active ingredient of Aurantii Fructus Immaturus for the inhibition of smooth muscle contraction. The experiment results also provided a pharmacological basis for the regulation of Aurantii Fructus Immaturus in the treatment of gastrointestinal disorders. Moreover, these results provide important information for further research into the mechanism and future clinical applications of epoxyaurapten.

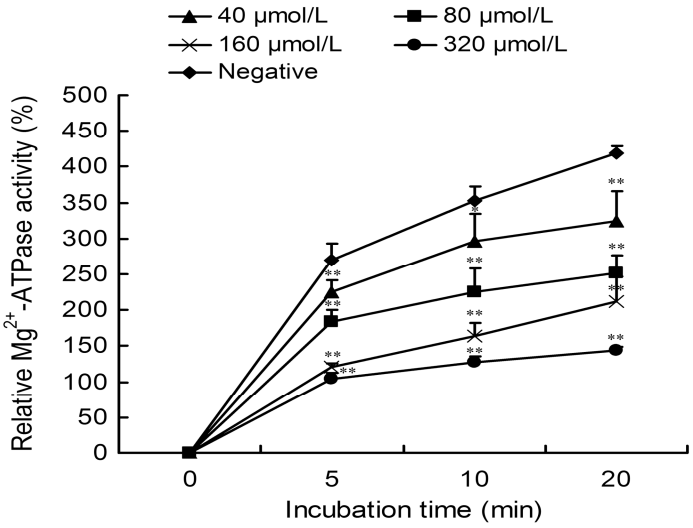

Figure 6. Effect of different concentrations epoxyaurapten at different incubation times on myosin Mg^{2+}-ATPase activity. In the graphic, ▲, ■, ×, ● and ◆ represent the Mg^{2+}-ATPase activity with 40, 80, 160 and 320 μmol/L epoxyaurapten and the negative control group. The Mg^{2+}-ATPase activity of unphosphorylated myosin was 100%. The other data are relative values compared to the Mg^{2+}-ATPase activity of unphosphorylated myosin. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. negative group without epoxyaurapten control.

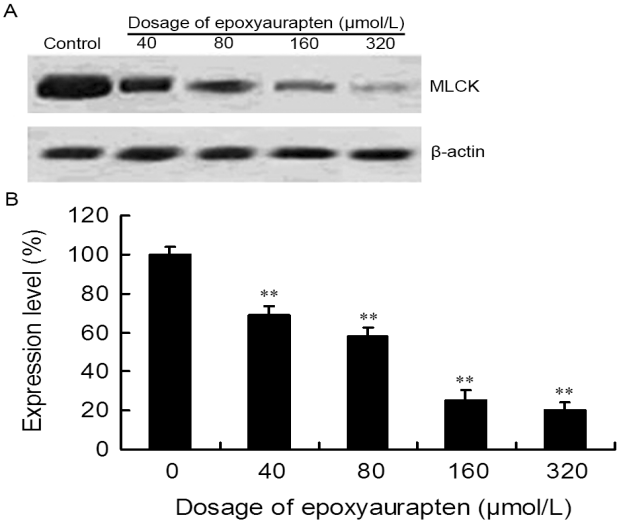

Figure 7. Representative western blots depicting the effect of different epoxyaurapten concentrations on MLCK expression in smooth muscle cells ($\bar{x}$±s, n = 6). (A) Representative gel showing the MLCK and β-actin bands of the positive control and samples for four different concentrations ofepoxyaurapten after 48 h incubation in complete medium. Lanes 1 to 5 were control (without epoxyaurapten), 40, 80, 160 and 320 μmol/L epoxyaurapten, respectively. (B) Histogram of statistical results for expression of MLCK after treatment with different epoxyaurapten concentrations, analyzed using Scoin Image Software. All data were obtained from six independent experiments. Error bars represent the means±SD significantly different from the corresponding control ($^{**}P < 0.01$ vs. control group).

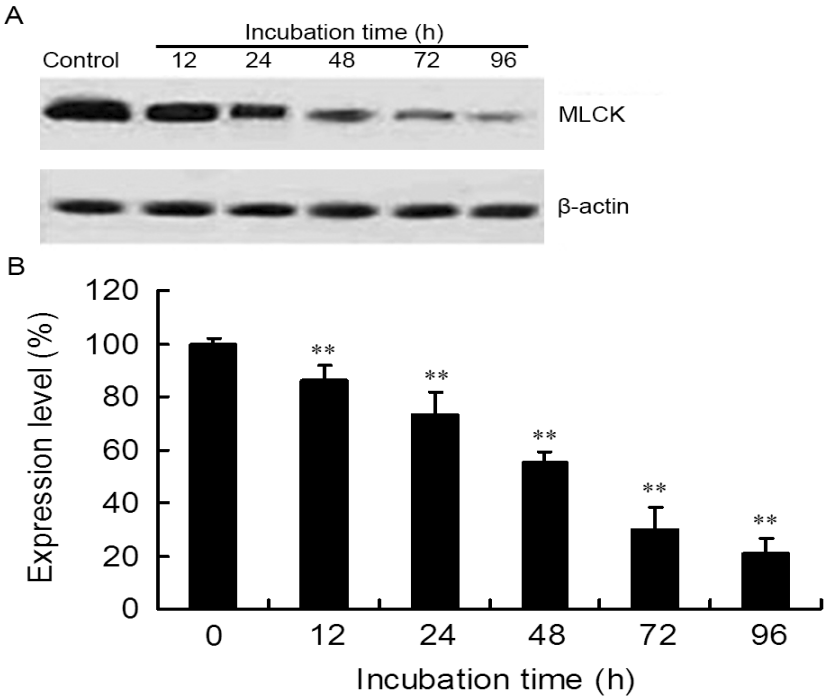

Figure 8. Representative western blots depicting the effect of 80 μmol/L epoxyaurapten on MLCK expression after different incubation times ($\overline{X}$ ±s, n = 6). (A) Representative gel showing the MLCK and β-actin bands of the positive control and five different incubation times in complete medium. Lanes 1 to 6 were control (without epoxyaurapten) 12, 24, 48, 72 and 96 h incubation times with epoxyaurapten (80 μmol/L) in complete medium, respectively. (B) Histogram of statistical results for expression of MLCK after different incubation times with the same epoxyaurapten concentration (80 μmol/L), analyzed using Scoin Image Software. All data were obtained from six independent experiments. Error bars represent the means±SD significantly different from the corresponding control (**P* < 0.01 vs. control group).

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