

Full Length Research Paper

Bi-directional regulation of ginsenoside Re on smooth muscle myosin of gizzard

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The ginsenoside Re is the major ingredient of ginsenosides, which was isolated from ginseng (genus *Panax*). The purpose of the study is to investigate into the effect of ginsenoside Re on Mg^{2+} -ATPase activity of smooth muscle myosin and phosphorylation of myosin light chain by myosin light chain kinase (MLCK), and try to find out the regular pattern resulting from ginsenoside Re on myosin function. Myosin and MLCK used in our study were purified from the chicken gizzard smooth muscle. Myosin phosphorylation was determined by glycerol polyacrylamide gel electrophoresis (PAGE). Myosin Mg^{2+} -ATPase activity was measured by Pi liberation method. The results showed that ginsenoside Re stimulates Mg^{2+} -ATPase activities, promotes phosphorylation of partially phosphorylated myosin, inhibits Mg^{2+} -ATPase activity and decrease phosphorylation of fully phosphorylated myosin in a dose dependent manner and in a time dependent manner. The findings demonstrated that the use of ginsenoside Re on various states of myosin phosphorylation produced different responses in a purified incubation system.

Key words: Ginsenoside Re, myosin phosphorylation, myosin Mg^{2+} -ATPase activity, bi-directional regulation.

INTRODUCTION

Ginseng (*Panax ginseng* C. A. Mey) has been widely used as a therapeutic herb in traditional Chinese medicine for thousands of years, and it has been increasingly used in the west as a complementary and alternative medicine (Hao et al., 2011). Ginseng shows a variety of pharmacological actions, including anti-diabetic effects (Reeds et al., 2011), vasorelaxing effects (Chen et al., 1984), protection against ischemia/reperfusion injury (Chen, 1996), neuroprotective effects (López et al., 2007), improvement of cognitive function (Geng et al., 2010), antitumor and anti-inflammatory activities (Park et al., 2004). The activity mechanisms of ginseng remain largely unclear. Nevertheless, the therapeutic effects of

ginseng have been attributed to its active ingredients, and ginsenosides, a class of steroid glycosides and triterpene saponins that are believed to account for most of the pharmacological activities of ginseng (Helms, 2004). Some pharmacological studies of the ginsenoside Re which is the major ingredient of ginsenosides have been reported. For example, ginsenoside Re showed antioxidant effects in cardiomyocytes from injury induced by both exogenous and endogenous oxidants (Xie et al., 2006); and it may improve diabetes through altering C-reactive protein by Re treatment (Cho et al., 2006); and it is useful for the management of tissue regeneration as a nonpeptide angiogenic agents with a superior stability (Yu et al., 2007), and so on. However, the effects of ginsenoside on smooth muscle myosin have not been evaluated to date. Therefore, we propose to study whether ginsenoside is related to the regulation of myosin function directly in a purified system.

It has been widely accepted that the mechanism of regulation of smooth muscle contraction and relaxation is the reversible Ca^{2+} -calmodulin dependent phosphorylation of myosin light chains (CDPM) by myosin light chain kinase (MLCK) (Vale and Milligan, 2000; Yang et al., 2004; Zhang et al., 2006). Thus, the

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Abbreviations: CDPM, Ca^{2+} -calmodulin dependent phosphorylation of myosin light chains; MLCK, myosin light chain kinase; PAGE, polyacrylamide gel electrophoresis; MLC₂₀, 20 kDa myosin regulating light chain; LC₂₀, unphosphorylated MLC₂₀; p-LC₂₀, mono-phosphorylated MLC₂₀; LC₁₇, 17 kDa myosin essential light chains.

modulation of myosin provides important indexes to evaluate the effects of modulators. The purpose of the study is to investigate into the effect of ginsenoside Re on Mg^{2+} -ATPase activity of smooth muscle myosin and phosphorylation of myosin light chain by MLCK, and try to find out the regular pattern resulting by ginsenoside Re on myosin function.

MATERIALS AND METHODS

Ginsenoside Re was purchased from National Institutes for Food and Drug Control, Beijing, P. R. China (Batch No. 110754-201123, Grade: reference substance). Phenylmethylsulfonyl fluoride (PMSF) and dithiothreitol (DTT) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ethylene glycol bis(2-aminoethyl ether) tetraacetic acid (EGTA) was purchased from Wako (Osaka, Japan). All the other chemicals used were of the purest commercially available grade.

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). The purified myosin was unphosphorylated, as determined by 10% glycerol electrophoresis.

MLC₂₀ phosphorylation determination

Ca^{2+} -calmodulin dependent phosphorylation of myosin light chain by MLCK was carried out in a 20 mmol/L Tris-HCl (pH 7.4) buffer containing 1 mmol/L dithiothreitol (DTT), 5 mmol/L $MgCl_2$, 60 mmol/L KCl, 2 mmol/L ethylene glycol bis (beta-aminoethyl ether) N,N'-tetraacetic acid (EGTA), 4 μ mol/L myosin and 2 mmol/L ATP (Yang et al., 2003; Tang et al., 2010).

The high sensitivity and efficiency of CDPM by MLCK were known as those in the presence of Ca^{2+} and calmodulin. A very low concentration of MLCK was needed to phosphorylate myosin light chains (Tansey et al., 1994). In our previous study, myosin (4 μ mol/L) could be fully phosphorylated with 0.02 μ mol/L MLCK, and partially phosphorylated with 0.0002 μ mol/L MLCK in a purified system *in vitro* (Tang et al., 2005). Therefore, fully phosphorylated myosin and partially phosphorylated myosin were obtained by adding MLCK to the final concentrations of 0.02 and 0.0002 μ mol/L, respectively, in the assay. Various concentrations of MLCK, in different incubation time and incubation temperature for MLC₂₀ phosphorylation were described in detail in the corresponding figure legends.

After phosphorylation of MLC₂₀, solid urea and sample solution, which contained bromophenol blue (BPB) and glycerol, were added to the reaction mixture. Next, 10% glycerol polyacrylamide gel electrophoresis (Glycerol PAGE) was used to measure the extent of phosphorylation of MLC₂₀. Glycerol PAGE was made as follow: the separating gel containing 13.96% acrylamide, 0.372% bisacrylamide, 40% (v/v) glycerol, and 0.375 mol/L Tris (pH 8.7); and the stacking gel containing 5.72% acrylamide, 0.152% bisacrylamide, 10% (v/v) glycerol, and 0.125 mol/L Tris-HCl (pH 6.7). Myosin samples which contained 40% urea were added to the sample buffer containing 20% urea, 20% glycerol, 0.05 mol/L Tris (pH 6.7), 14 mmol/L 2-hydroxy-1-ethanethiol (β -ME), and a moderate amount of 0.01% BPB. The reaction mixture is loaded into the gel.

To analyze the percentage of MLC₂₀ phosphorylation, Scion

Image Software was applied to scan the density and size of phosphorylated MLC₂₀ and calculate the percentage of phosphorylation MLC₂₀ in total MLC₂₀. Mono-phosphorylation of the fully phosphorylated myosin control was chosen as the control and calculated as 100%.

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

The method of measuring Mg^{2+} -ATPase activity of myosin was performed as described previously (Zhang et al., 2006; Yang et al., 2004). In summary, the measurement 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 calmodulin, and 0.4 μ mol/L myosin at 25°C using the malachite green method.

Statistical analysis

The results are expressed as means \pm standard deviation ($\bar{x} \pm s$). Statistical analysis was performed with one-way ANOVA, using the SPSS software. Statistical significance was adopted at a level of $P < 0.05$.

Other procedures

Protein concentrations were determined by using the method of Bradford (1976). The graphs of phosphorylation of MLC₂₀ and myosin Mg^{2+} -ATPase activities were obtained with Microsoft Excel 2003.

RESULTS

Ginsenoside Re inhibiting the phosphorylation of fully phosphorylated myosin and enhancing the phosphorylation of partially phosphorylated myosin by MLCK in different ginsenoside Re concentrations

Inhibitory effects of ginsenoside Re on the phosphorylation extent of myosin of fully phosphorylated myosin are showed in Table 1 and Figure 1A. It can be seen that the phosphorylation extent of fully phosphorylated myosin is decreased along with the increasing concentration of ginsenoside Re. As the concentrations increased to 80 and 160 μ mol/L, the phosphorylation extents of myosin in fully phosphorylated state drop to $72.00 \pm 8.25\%$ and $56.17 \pm 7.88\%$ respectively, while for the corresponding negative control without ginsenoside Re, the extent is $97.50 \pm 8.45\%$ ($**P < 0.01$).

Figure 1B shows concentrations of 0.0002 μ mol/L MLCK and 4 μ mol/L myosin for partial phosphorylation of MLC₂₀. As listed in Table 1, the phosphorylation extent of the partially phosphorylated myosin increases with the increasing concentration of ginsenoside Re. Especially, when the concentrations of ginsenoside Re are 80 and 160 μ mol/L, the phosphorylation extents of MLC₂₀ of partially phosphorylated myosin rise up to $43.33 \pm 5.32\%$, $67.33 \pm 7.66\%$ respectively ($**P < 0.01$ vs negative control).

Table 1. Ginsenoside Re inhibited the phosphorylation of fully phosphorylated myosin and enhanced the phosphorylation of partially phosphorylated myosin by MLCK in different ginsenoside Re concentrations ($\bar{X} \pm s$, $n = 6$).

Concentration of Ginsenoside Re ($\mu\text{mol/L}$)	Phosphorylation extent of MLC_{20} (%)	
	Fully phosphorylated myosin (Figure 1A)	Partially phosphorylated myosin (Figure 1B)
0 (Negative control)	97.50 \pm 8.45	13.83 \pm 2.63
40	96.33 \pm 7.81	14.50 \pm 3.61
80	72.00 \pm 8.25**	43.33 \pm 5.32**
160	56.17 \pm 7.88**	67.33 \pm 7.66**

The data are statistical results for myosin phosphorylation after treatment with different ginsenoside Re concentrations, analyzed using Scion Image Software. Fully mono-phosphorylation of MLC_{20} was chosen as the control and calculated as 100%. The data are $\bar{X} \pm s$ of six experiments and asterisks (**) denote significant difference ($P < 0.01$) from the corresponding negative control group.

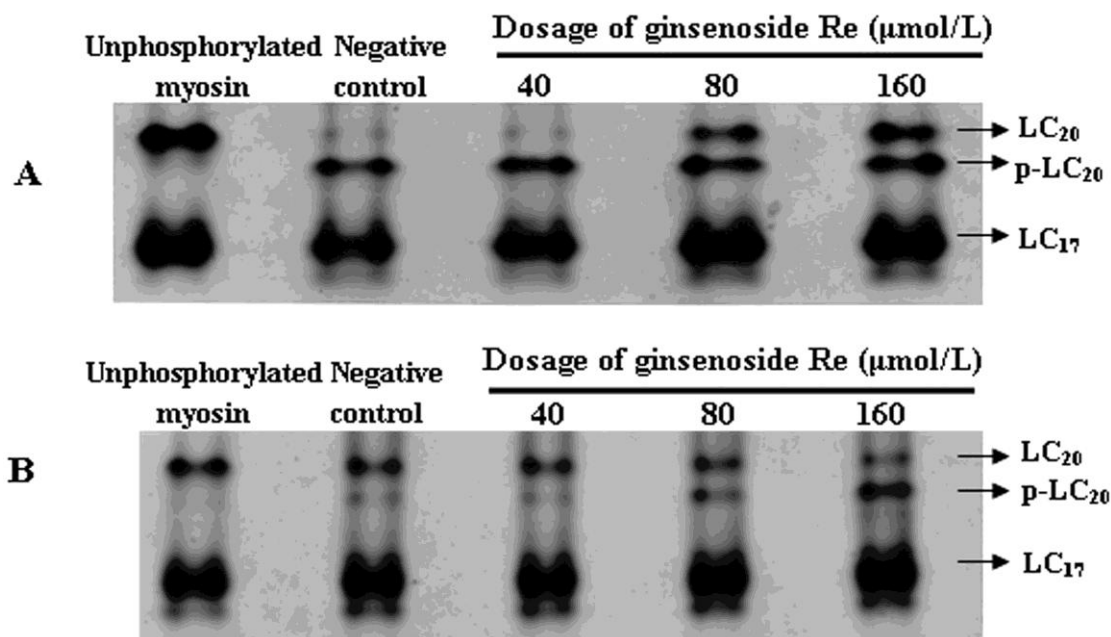


Figure 1. Ginsenoside Re inhibited the phosphorylation of fully phosphorylated myosin and enhanced the phosphorylation of partially phosphorylated myosin by MLCK in different ginsenoside Re concentrations ($\bar{X} \pm s$, $n = 6$). A) 0.02 $\mu\text{mol/L}$ MLCK and 4 $\mu\text{mol/L}$ aliquot of myosin ($1.76 \text{ mg}\cdot\text{ml}^{-1}$) were used at 25°C for 20 min in the assay. B) 0.0002 $\mu\text{mol/L}$ MLCK and 4 $\mu\text{mol/L}$ aliquot of myosin ($1.76 \text{ mg}\cdot\text{ml}^{-1}$) were used at 25°C for 20 min in the assay. The glycerol electrophoresis results for MLC_{20} phosphorylation by MLCK after treatment with different ginsenoside Re concentrations. The lane marked with unphosphorylated myosin is the blank control without incubation. The lane marked with negative control is the model control without ginsenoside Re. The other lanes represent 40, 80, 160 $\mu\text{mol/L}$ ginsenoside Re at the same incubation time, respectively. LC_{20} , p-LC_{20} and LC_{17} were separated into 3 isolated bands in glycerol PAGE. Their mobilities were in the following order: $\text{LC}_{20} < \text{p-LC}_{20} < \text{LC}_{17}$. LC_{20} , unphosphorylated MLC_{20} (20 kDa regulatory myosin light chain); p-LC_{20} , mono-phosphorylated MLC_{20} ; LC_{17} , 17kDa myosin essential light chains.

(Table 1).

Ginsenoside Re inhibiting the phosphorylation of fully phosphorylated myosin and enhancing the phosphorylation of partially phosphorylated myosin by MLCK in different incubation time

To investigate the influence of incubation time on the

extent of MLC_{20} phosphorylation by MLCK with the presence of ginsenoside Re, three different incubation times (that is, 5, 10, 20 min) were selected while the concentration of ginsenoside Re was fixed at 80 $\mu\text{mol/L}$. Furthermore, we also chose 0.02 $\mu\text{mol/L}$ MLCK and 4 $\mu\text{mol/L}$ myosin for full phosphorylation of MLC_{20} , and 0.0002 $\mu\text{mol/L}$ MLCK and 4 $\mu\text{mol/L}$ myosin for partial phosphorylation of MLC_{20} , as mentioned earlier.

The extents of MLC_{20} phosphorylation with different

Table 2. Ginsenoside Re inhibited the phosphorylation of fully phosphorylated myosin and enhanced the phosphorylation of partially phosphorylated myosin by MLCK in different incubation time ($\bar{X} \pm s$, $n = 6$).

Incubation time (min)	Phosphorylation extent of MLC ₂₀ (%)	
	Fully phosphorylated myosin (Figure 2A)	Partially phosphorylated myosin (Figure 2B)
20 (Negative control)	97.17 \pm 5.49	19.67 \pm 7.12
5	90.00 \pm 4.00*	40.83 \pm 8.08**
10	80.17 \pm 4.96**	45.50 \pm 9.73**
20	70.17 \pm 5.84**	49.67 \pm 7.34**

The data are statistical results for myosin phosphorylation after different incubation time with the same ginsenoside Re concentration (80 μ mol/L), analyzed using Scion Image Software. Fully mono-phosphorylation of MLC₂₀ was chosen as the control and calculated as 100%. The data are expressed as s.e. means of calculated value from 6 experiments including the representative records in Figure 2A or 2B. $p^* < 0.05$, $p^{**} < 0.01$ vs the corresponding negative control group.

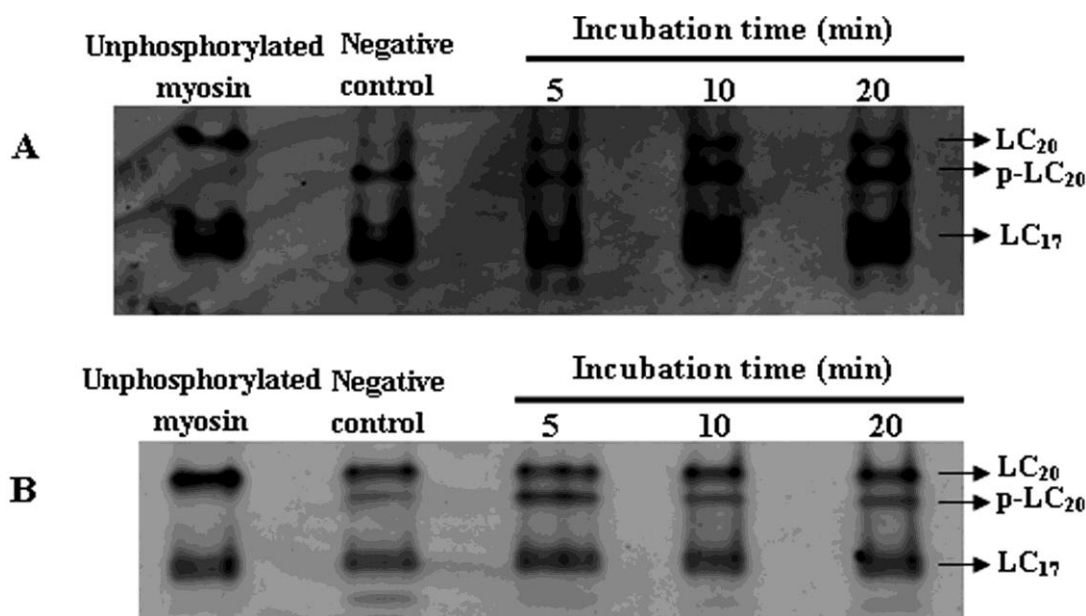


Figure 2. Ginsenoside Re inhibited the phosphorylation of fully phosphorylated myosin and enhanced the phosphorylation of partially phosphorylated myosin by MLCK in different incubation time ($\bar{X} \pm s$, $n = 6$). A) 0.02 μ mol/L MLCK and 4 μ mol/L aliquot of myosin (1.76 $\text{mg} \cdot \text{ml}^{-1}$) were used at 25°C in the assay. B) 0.0002 μ mol/L MLCK and 4 μ mol/L aliquot of myosin (1.76 $\text{mg} \cdot \text{ml}^{-1}$) were used at 25°C in the assay. The glycerol electrophoresis results of MLC₂₀ phosphorylation by MLCK after different incubation time with the same ginsenoside Re concentration (80 μ mol/L). Lanes in Figure 2 show the unphosphorylated myosin control (blank control without incubation), negative control (without ginsenoside Re), 5, 10 and 20 min incubation time groups, respectively.

incubation time are listed in Table 2. The results indicate that the extent of MLC₂₀ phosphorylation of fully phosphorylated myosin decreases with the increasing incubation time. Significant difference can be observed between group 5 min and the corresponding negative control ($* P < 0.05$). As the incubation time increases to 20 min, the difference becomes more evident with the extent of MLC₂₀ phosphorylation, which is 70.17 \pm 5.84%, about 30% lower than that of negative control without ginsenoside Re ($**P < 0.01$).

In contrast, the results in Figure 2B and Table 2 show that for the partially phosphorylated myosin. The extent of

MLC₂₀ phosphorylation is increasing significantly along with increasing incubation time, which is 5, 10 and 20 min respectively ($**P < 0.01$ vs negative control without ginsenoside Re).

Ginsenoside Re inhibiting Mg^{2+} -ATPase activity of fully phosphorylated myosin and stimulating Mg^{2+} -ATPase activities of partially phosphorylated myosin by different dosage at different time respectively

Different incubation times that is, 5, 10, and 20 min

Table 3. Ginsenoside Re inhibited Mg^{2+} -ATPase activity of fully phosphorylated myosin by different dosage at different time respectively ($\bar{X} \pm s$, n = 6).

Concentration of ginsenoside Re ($\mu\text{mol/L}$)	Relative Mg^{2+} -ATPase activity at different incubation time (%)		
	5 (min)	10 (min)	20 (min)
0 (Negative control)	268.83 \pm 37.60	434.17 \pm 44.66	525.67 \pm 62.27
40	243.33 \pm 25.74	390.67 \pm 39.81	475.00 \pm 26.94
80	207.83 \pm 39.58*	335.67 \pm 33.81** [#]	402.17 \pm 22.09** ^{##}
160	157.50 \pm 20.68*** ^{##} \$	254.17 \pm 23.35*** ^{##} \$	289.00 \pm 26.77*** ^{##} \$

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 control without ginsenoside Re respectively, # $P < 0.05$, ## $P < 0.01$ vs group with 40 $\mu\text{mol/L}$ ginsenoside Re, \$ $P < 0.05$, \$\$\$ $P < 0.01$ vs group with 80 $\mu\text{mol/L}$ ginsenoside Re.

Table 4. Ginsenoside Re stimulated Mg^{2+} -ATPase activities of partially phosphorylated myosin by different dosage at different time respectively ($\bar{X} \pm s$, n = 6).

Concentration of ginsenoside Re ($\mu\text{mol/L}$)	Relative Mg^{2+} -ATPase activity at different incubation time (%)		
	5 (min)	10 (min)	20 (min)
0 (Negative control)	109.33 \pm 10.31	161.00 \pm 23.30	201.17 \pm 17.55
40	121.67 \pm 18.18	189.33 \pm 16.60*	227.67 \pm 20.92*
80	140.50 \pm 13.26**	221.83 \pm 16.10** [#]	265.00 \pm 22.25** ^{##}
160	163.83 \pm 21.76*** ^{##}	255.33 \pm 18.94*** ^{##} \$	310.00 \pm 24.53*** ^{##} \$

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 control without ginsenoside Re respectively, # $P < 0.05$, ## $P < 0.01$ vs group with 40 $\mu\text{mol/L}$ ginsenoside Re, \$ $P < 0.01$ vs group with 80 $\mu\text{mol/L}$ ginsenoside Re.

respectively were chosen to examine the myosin Mg^{2+} -ATPase activities in different states of smooth muscle myosin at three ginsenoside Re concentrations.

Table 3 shows that the Mg^{2+} -ATPase activities are all enhanced with prolonging the incubation time, when the fully phosphorylated myosin was used. The results also indicated that at all incubation times, the highest Mg^{2+} -ATPase activity is observed when studied in the negative group, and the enzyme activity of myosin is decreased progressively with increasing concentration of ginsenoside Re. The differences between Mg^{2+} -ATPase activities in different groups become more obvious with the extension of incubation time. It was significant between negative group and group (80 $\mu\text{mol/L}$), group (160 $\mu\text{mol/L}$) at each incubation time (* $P < 0.05$, ** $P < 0.01$).

Using partially phosphorylated myosin (Table 4), we have observed the following characteristics. (a) The Mg^{2+} -ATPase activities of the three conditions were all enhanced with prolonging the incubation time. (b) At different incubation time, the highest Mg^{2+} -ATPase activity was observed when myosin was in the group with 160 $\mu\text{mol/L}$ ginsenoside Re; the second one was the group with 80 $\mu\text{mol/L}$ ginsenoside Re; the third one was the group with 40 $\mu\text{mol/L}$ ginsenoside Re and the lowest was the negative group without ginsenoside Re. (c) The difference between Mg^{2+} -ATPase activities in different

groups also became more and more obvious as incubation time increasing. The detailed results are listed in Table 4. So if partially phosphorylated myosin is used, the higher the concentration of ginsenoside Re used and the longer incubation time, the higher is the Mg^{2+} -ATPase activity of partially phosphorylated myosin.

DISCUSSION

Smooth muscle contraction is activated primarily via CDPM by MLCK. The phosphorylation can be simply described as interaction of Ca^{2+} with calmodulin inducing a conformational change of MLCK that activates it. The activated MLCK then catalyzes phosphorylation of MLC_{20} of myosin. 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 ginsenoside Re on myosin.

Our results demonstrate some interesting characteristics of the regulation of ginsenoside Re on gizzard smooth muscle myosin. In summary, ginsenoside Re yields a bi-directional regulation on both Mg^{2+} -ATPase

activities of CDPM and the phosphorylation of myosin. Stimulatory effects are observed in partially phosphorylated myosin with 0.0002 $\mu\text{mol/L}$ MLCK, and inhibitory effects are obtained for fully phosphorylated myosin using 0.02 $\mu\text{mol/L}$ MLCK. The bi-directional regulatory effects from ginsenoside Re imply that gastrointestinal smooth muscle should not be over contracted or over relaxed.

From an alternative viewpoint, we may notice that bi-directional regulation is a common characterization of these modulators in regulating the activities of smooth muscle myosin. For example, (1) Protein kinase C (PKC) showed bi-directional regulation on myosin activity, that is, PKC could inhibited the activity of myosin by phosphorylating MLCK, but stimulated the activity of myosin by phosphorylating myosin phosphatase (Nishikawa et al., 1985; Weber et al., 1999; Pfizer, 2001). (2) Calponin stimulates phosphorylated myosin activity in the absence of actin by direct interaction with myosin, and inhibits the activity in the presence of actin (Winder and Walsh, 1990; Lin et al., 1993).

We aforementioned the two types of bi-directional regulation rather than illustrated all the bi-regulation in regulating myosin activities of smooth muscle. We observed that the use of ginsenoside Re on various states of myosin phosphorylation produced different responses. These results provide some important information for us to further evaluate the possible mechanism and future clinical application of ginsenoside Re before we offer any conclusion.

We supposed that, when MLC_{20} of myosin was partially phosphorylated as the low concentration of MLCK (0.0002 $\mu\text{mol/L}$), ginsenoside Re showed to stimulate directly the activity of phosphorylated myosin, because it was more likely to bind with phosphorylated myosin and interact with phosphorylated myosin. When MLC_{20} of myosin was fully phosphorylated by MLCK of high concentration (0.02 $\mu\text{mol/L}$), ginsenoside Re was easier to bind to MLCK, and showed the inhibitory effects by inhibiting the kinase activity of MLCK. So, further study need to reveal the exact mechanism of the bi-directional regulation of ginsenoside Re on the activity of smooth muscle myosin.

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