別紙様式１（修士申請者用）

# 修士学位論文

## 論　文　題　名

（注：学位論文題名が英語の場合は和訳をつけること。）

Caffeine inhibits smooth muscle actin-myosin interaction

カフェインは平滑筋ミオシンアクチン相互作用を抑制する

（西暦）　2017　年　01　月　06　日　提出

首都大学東京大学院
人間健康科学研究科 博士前期課程 人間健康科学専攻
フロンティアヘルスサイエンス学域
学修番号：15898601
氏　名：王志丹

（指導教員名：　渡辺　賢　）

別紙様式３（修士申請者用）

# （西暦）　~~2017~~ 2016　年度　博士前期課程学位論文要旨

学位論文題名（注：学位論文題名が英語の場合は和訳をつけること）

Caffeine inhibits smooth muscle actin-myosin interaction

カフェインは平滑筋ミオシンアクチン相互作用を抑制する

学位の種類：　修士（　健康科学　　学）
首都大学東京大学院
人間健康科学研究科　博士前期課程 人間健康科学専攻フロンティアヘルスサイエンス学域
学修番号:15898601
氏　名：王志丹
（指導教員名：渡辺 賢　　）

注：1ページあたり 1,000 字程度（英語の場合 300 ワード程度）で、本様式 1～2 ページ（A4 版）程度とする。

Caffeine is known to have inhibitory effects on $Ca^{2+}$ - induced contraction in smooth muscle, and possible mechanisms may contribute the inhibitory effects. Recently, it was found a caffeine effect on direct inhibition of myosin-actin interaction in smooth muscle. To clarify whether direct inhibition of myosin-actin interaction of caffeine to be dependent on the regulation of myosin light chain phosphorylation, in the present study, the author examined caffeine effects on skinned (cell membrane permeabilized) smooth muscle contraction. At 10 mM, caffeine significantly suppressed $Ca^{2+}$ induced contraction, which was dependent on myosin light chain phosphorylation. On the other hand, caffeine did not affect 15.5 mM $Mg^{2+}$ induced contraction, which was independent of myosin light chain phosphorylation. The present results suggest that caffeine inhibits actin-myosin interaction of smooth muscle through either inhibition of myosin light chain kinase activity or acceleration of myosin phosphatase activity.

Key words: caffeine, $Ca^{2+}$ - induced contraction, myosin-actin interaction, myosin light chain phosphorylation.

カフェインには平滑筋に Ca イオンの抑制があることが知られている。様々な機構がこの抑制に関与している。最近、カフェインは平滑筋のミオシンアクチン相互作用を直接的に抑制すること

が明らかになった。本研究では、細胞膜を破壊したスキンド盲腸紐標本を用いて、カフェインの平滑筋アクチン･ミオシン相互作用の抑制が、ミオシン軽鎖リン酸化の抑制によるものなのかを検討した。１０mMのカェインはミオシン軽鎖リン酸化に依存する Ca イオン活性化収縮張力を抑制したか。一方、カフェインはミオシン軽鎖リン酸化に依存しない１５．５mM Ｍｇイオンによる収縮張力を抑制しない。以上から、カフェインはミオシン軽鎖リン酸化酵素の活性阻害、もしくはミオシンホスファターゼの活性を促進することでミオシン軽鎖リン酸化レベルを低下させることで、平滑筋ミオシンアクチン相互作用を抑制し、平滑筋収縮を抑制することが示唆された。

キーワード：カフェイン、$Ca^{2+}$活性化収縮、ミオシン - アクチン相互作用、ミオシン軽鎖リン酸化

Abstract

Caffeine is known to have inhibitory effects on $Ca^{2+}$ - induced contraction in smooth muscle, and possible mechanisms may contribute the inhibitory effects. Recently, it was found a caffeine effect on direct inhibition of myosin-actin interaction in smooth muscle. To clarify whether direct inhibition of myosin-actin interaction of caffeine to be dependent on the regulation of myosin light chain phosphorylation, in the present study, the author examined caffeine effects on skinned (cell membrane permeabilized) smooth muscle contraction. At 10 mM, caffeine significantly suppressed $Ca^{2+}$ induced contraction, which was dependent on myosin light chain phosphorylation. On the other hand, caffeine did not affect 15.5 mM $Mg^{2+}$ induced contraction, which was independent of myosin light chain phosphorylation. The present results suggest that caffeine inhibits actin-myosin interaction of smooth muscle through either inhibition of myosin light chain kinase activity or acceleration of myosin phosphatase activity.

Key words: caffeine, $Ca^{2+}$ - induced contraction, myosin-actin interaction, myosin light chain phosphorylation.

## 要旨

カフェインには平滑筋に Ca イオンの抑制があることが知られている。様々な機構がこの抑制に関与している。最近、カフェインは平滑筋のミオシンアクチン相互作用を直接的に抑制することが明らかになった。本研究では、細胞膜を破壊したスキンド盲腸紐標本を用いて、カフェインの平滑筋アクチン･ミオシン相互作用の抑制が、ミオシン軽鎖リン酸化の抑制によるものなのかを検討した。１０mMのカェインはミオシン軽鎖リン酸化に依存する Ca イオン活性化収縮張力を抑制した。一方、カフェインはミオシン軽鎖リン酸化に依存しない１５．５mM Ｍｇイオンによる収縮張力を抑制しない。以上から、カフェインはミオシン軽鎖リン酸化酵素の活性阻害、もしくはミオシンホスファターゼの活性を促進することでミオシン軽鎖リン酸化レベルを低下させることで、平滑筋ミオシンアクチン相互作用を抑制し、平滑筋収縮を抑制することが示唆された。



キーワード：カフェイン、$Ca^{2+}$活性化収縮、ミオシン - アクチン相互作用、ミオシン軽鎖リン酸化

## Introduction

Smooth muscle contraction is dependent on phosphorylation of the regulatory light chain of myosin, which results in an increase in myosin $ATP_{ase}$ activity and cross-bridge cycling with actin[1].

Smooth muscle contraction is caused by the sliding of myosin and actin filaments. The energy for this to happen is provided by the hydrolysis of ATP. Movement of the filaments over each other happens when the globular heads protruding from myosin filaments attach and interact with actin filaments to form cross bridges. The process of interaction between myosin and actin is called cross bridge cycling. Unlike cardiac and skeletal muscle, smooth muscle contraction is initiated by a calcium-regulated phosphorylation of myosin light chain[1].

Considering the key role of free calcium ion ($Ca^{2+}$) in smooth muscle contraction, many factors are involved in concentration of $Ca^{2+}$ increase[1]. One of them is release of $Ca^{2+}$ from the sarcoplasmic reticulum. Therefore, an intracellular calcium channel called ryanodine receptor (RyR) is often applied. Whereas, caffeine, recognized as a kind of methylxanthine, at millimolar concentrations increase the $Ca^{2+}$ sensitivity of the RyR such that they become activated at basal $[Ca^{2+}]i$ in smooth muscle [2]. For this reason, caffeine is used to study excitation – contraction coupling in smooth muscle. Additionally, caffeine acts on the other several mechanisms involve in smooth muscle

contraction, such as adenosine receptor. Recently, Tazzeo [3] and colleagues reported that caffeine directly interfered actin myosin interaction resulting in inhibiting smooth muscle contraction. However, it is still unclear that whether the caffeine effects on actin-myosin interaction involve myosin light-chain phosphorylation. To clarify the mechanisms, the effects of caffeine on myosin light chain phosphorylation-dependent and- independent contraction were tested.

## Materials and Methods

All animal experiments were performed at Tokyo Metropolitan University Arakawa Campus. Animal experimental procedures conformed to the "Guidelines for Proper Conduct of Animal Experiments" approved by the Science Council of Japan, and were carried out under the rules and regulations of research ethics committee of Tokyo Metropolitan University. Hartley male guinea pigs weighing about 250 gm, were killed by intraperitoneal administration of pentobarbital sodium (Somnopentyl, K yoritsuseiyaku, Tokyo), then the taenia cecum were removed. The skinning (cell membrane permeabilization) procedure was described elsewhere [4)6)]. In short, the taenia cecum were treated with 100 microM beta-escin (Sigma,St. Loious, MO, USA) and 100 microM ionophore A23187 (Sigma) for 30 min. Next the skinned muscle preparations were kept in the relaxing solution (described below) with 50% glycerol at -15℃.

**Measurements of mechanical responses using skinned preparation**

In each experiment, the smooth muscle preparations were used from a small muscle layer strip (0.2-0.3 mm wide and 3-4 mm long) by cutting off the taenia cecum. Then the preparation was attached to a pair of tungsten wires with silk thread monofilaments, and connected to a force transducer (ULA-10GR, Minebea, Tokyo) to convert mechanical stress into electrical signal. After the signal amplified (AM32AZ, Uniplus, Tokyo), the signal was digitalized with an analog digital converter (PowerLab2/26; AD Instruments Japan, Tokyo) and recorded the continuous change in a computer software (Labchart v.7, AD Instruments Japan, Tokyo). In order to change the solution quickly, we choose a bubble plate system with six wells (0.135 ml each) [5)8)]. Experimental temperature was kept at 30 ℃ to prevent deterioration of the preparation[7)].

The skinned preparation was firstly fixed in relaxing solution. After the passive tension reached a steady level (resting tension, ~10 μN), the preparation was immersed in $10^{-5.0}$ M $Ca^{2+}$ to reach the maximal $Ca^{2+}$ -induced contraction (control group). When the active tension maintained in a steady level, the preparation was removed to the relaxing solution again. Subsequently the muscle strip was reactivated in various concentrations of $10^{-5.0}$ M $Ca^{2+}$ or 15.5 mM $Mg^{2+}$ in the absence or presence of caffeine (test groups).

**Solution and chemical**

Artificial intracellular solutions for skinned preparations were prepared according to the method of Horiuti [8]. The relaxing solution was made with 115 mM K (methanesulfonate), 1.2 mM Mg (methanesulphonate)$_2$, 1.35 mM $Na_2ATP$ (Sigma), 20 mM creatine phosphate (Nacalai Tesque), and 10 mM ethylene glycolbis-(2-aminoethyl)-tetraacetic acid(EGTA) (Wako Pure Chemicals, Osaka). $10^{-5.0}$ M $Ca^{2+}$ solutions were prepared by mixing the relaxing solution that contained 10 mM EGTA and 9.64 mM Ca (methanesufonate)$_2$ (Tokyo Kasei). And when elicit the maximal contraction, 1 μM calmodulin (Wako Pure Chemicals) was also needed. All solutions, contained 0.85 mM free $Mg^{2+}$, 1.0 mM MgATP (8, 40). $Mg^{2+}$ and MgATP concentrations were chosen based on those measured in the intact-taenia cecum [9]. To keep the MgATP supply sufficiently, concentrations of creatine phosphate (20 mM) were chosen about 10 times higher than that in the intact taenia cecum preparations. The apparent dissociation constant of $Ca^{2+}$-EGTA was assumed to be $10^{-6.4}$M. 15.5 mM $Mg^{2+}$ MgCTP solutions, which were used for myosin light chain phosphorylaion-indepenent contraction, contained 15.5 mM, 1.0 mM MgCTP (1.4 mM total $Na_2CTP$; Sigma), 20 mM creatine phosphate, and 10 mM EGTA, since MgCTP is a poor substrate for several kinase reactions including myosin light chain phosphorylation but strongly activates myosin $ATP_{ase}$ [10)11)]. Caffeine was purchased from Wako. All other chemicals were

reagent grade.

**Data analysis of the mechanical properties**

The developed tension levels of the test contraction of skinned preparations were expressed as: relative tension= (an observed tension of the test contraction – the basal tension)/ (the Maximal of the control contraction – the basal tension)

To estimate caffeine concentration for half maximal effect of the active tension ($ED_{50}$), data were fitted to a modified Hill equation with the program kaleida Graph (v.4.aJ, Hulinks, Tokyo) using the Levenberg-Marquardt algorithm [5]:

Relative tension = $F_{min} + (F_0 - F_{min}) \times [caffeine]^n / ([caffeine]_{50}^n + [caffeine]^n)$, where $F_0$, $F_{min}$ and $[caffeine]_{50}$ denote a relative tension level of the test contraction in the absence of caffeine, the maximally suppressed-relative tension level of the test contraction by caffeine, and caffeine concentration for half maximal inhibition of the relative tension, respectively. The Hill coefficient (n) is a measure of the slope.

**Statistical Analysis**

Results are presented as the mean ± standard error (SEM). Statistical hypotheses on the differences between means were tested with Student's t-test for paired samples unless noted otherwise. The null hypotheses were rejected when P was less than 0.05.

## Results

Effects of caffeine on the $Ca^{2+}$ - induced contraction of skinned preparations. Figure

1 represents typical tension traces of the ß-escin taenia cecum skinned preparations from guinea pig. When a preparation was activated with $10^{-5}$ M $Ca^{2+}$ and 1 μM calmodulin, active tension gradually increased and reached a sustained level around 5 min. In the presence of 10 mM caffeine, the developed tension level was clearly lower than that in the absence of caffeine. Figure 2 shows the caffeine effects on the relationship between $Ca^{2+}$ concentration and the active tension. At 10 mM concentration of caffeine partially and significantly reduced the $Ca^{2+}$ - induced tension development. The estimated $ED_{50}$ value for inhibition of $F_{max}$ was 5.12 ± 0.78 mM.

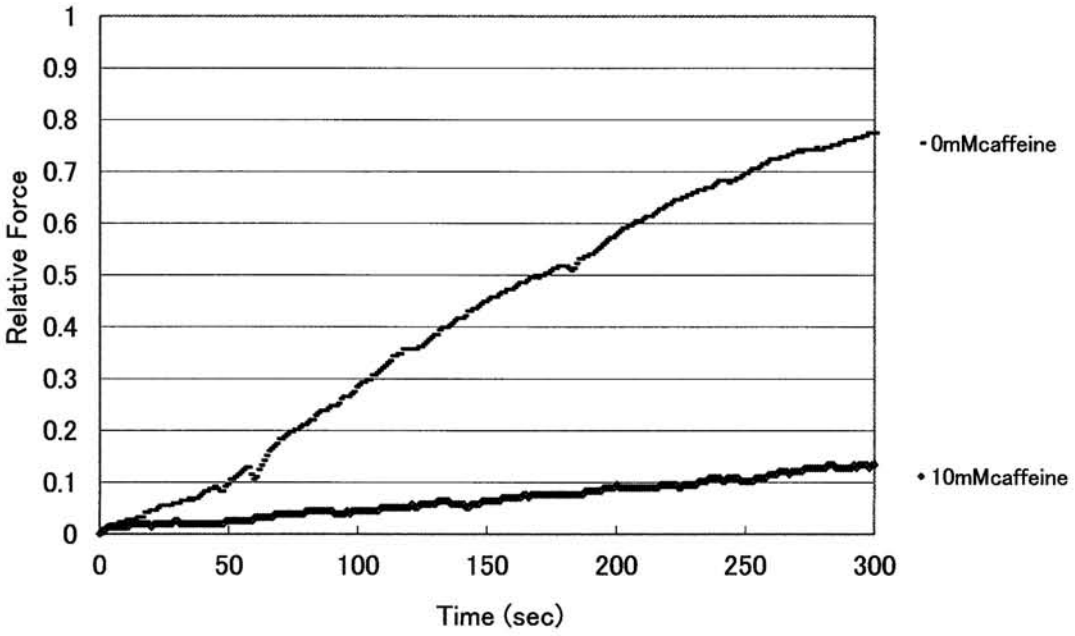


Figure 1: typical tension traces of $Ca^{2+}$ -induced contraction of skinned taenia cecum from guinea pig. Caffeine at 10 μM suppressed the contraction (30.0 ± 1.0℃).

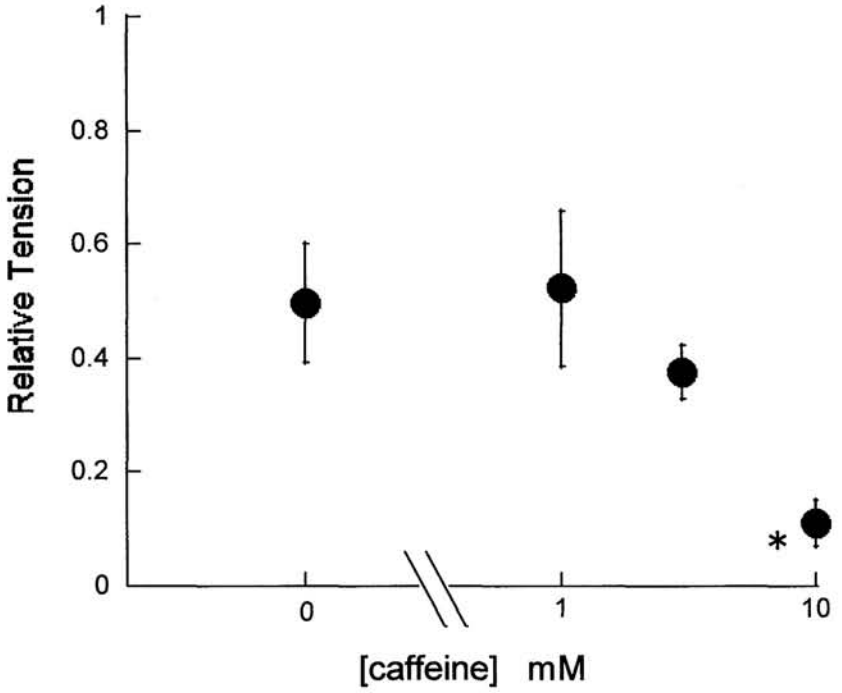


Figure 2: effects of caffeine on the 10 $\mu$M $Ca^{2+}$ induced contraction. Data were fitted to the modified Hill equation (straight line). Values are means ± SEM of 6-7 experiments. Asterisk indicates the significant difference of the active force compared with that of control, where P values are less than 0.05. 30.0 ± 1.0℃.

***Effects of caffeine on the high $Mg^{2+}$ - induced contraction of skinned preparations***

Figure 3 presents typical tension traces of high $Mg^{2+}$ - induced contraction of skinned taenia cecum from guinea pig. Pharmacological concentrations of $Mg^{2+}$ are known to induce force development without changing the phosphorylation level of myosin light chain. 15.5 mM $Mg^{2+}$ initiated an increase in the tension in the short time-course [7]. Figure 4 shows the effects of caffeine on the $Mg^{2+}$ concentration-relative tension relationship. Caffeine at 10 mM or lower did not significantly affect the 15 mM $Mg^{2+}$ induced contractile force.

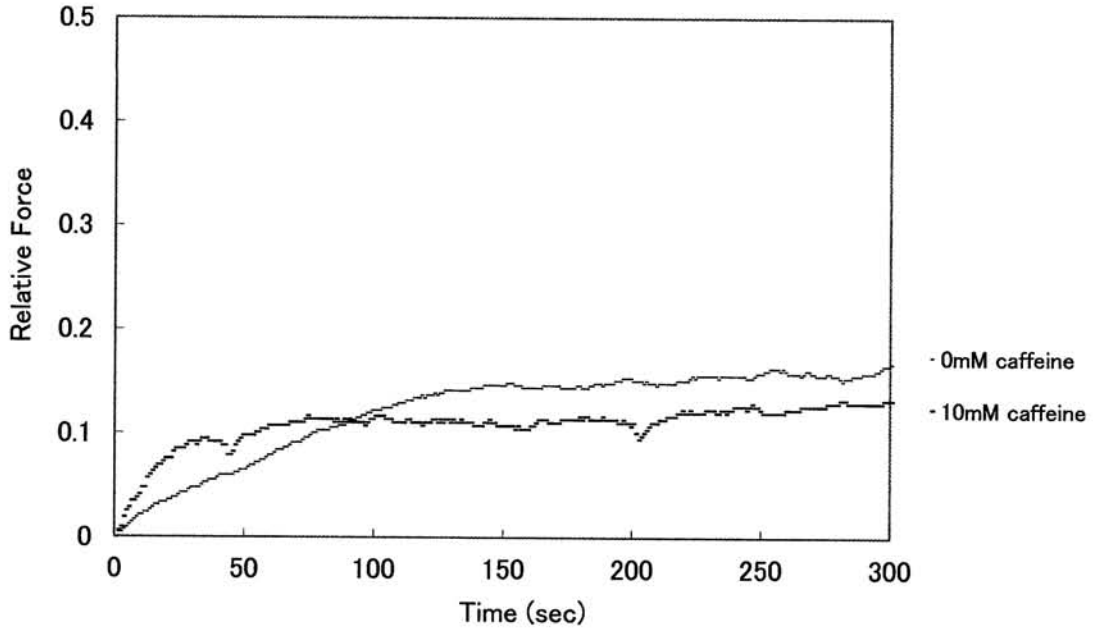


Figure 3: typical tension traces of high $Mg^{2+}$ - induced contraction of skinned taenia cecum from guinea pig. The preparations were immersed in 15.5 mM $Mg^{2+}$ with 0 mM and 10 mM caffeine.

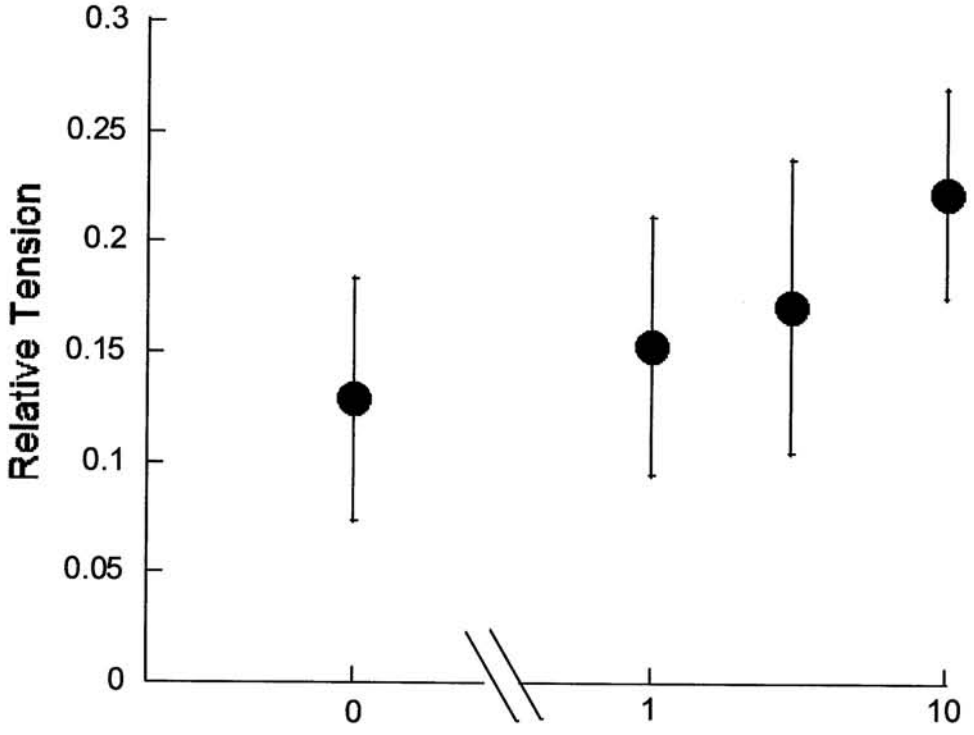


Figure 4: Effects of caffeine on the 15.5 mM $Mg^{2+}$ induced contraction. Values are

means ± SEM of 6-7 experiments. 30.0 ± 1.0℃

## Discussion

In the present study, the author found that in ß-escin skinned tracheal smooth muscle preparations from guinea pig, caffeine at 10 mM significantly suppressed the $Ca^{2+}$-calmodulin induced active tension development. However, when 15.5 mM $Mg^{2+}$ was applied, resulting in tension development independent of myosin light chain phosphorylation, caffeine did not affect the tension development. These results suggest that caffeine inhibits myosin-actin interaction in smooth muscle through decrease in myosin light chain phosphorylation level. In a previous study, Tazzeo et al[3] shows that caffeine directly induced actin filament depolimerization and disorganization causing inhibition of smooth muscle contraction. If so, 15.5 mM $Mg^{2+}$ - induced, myosin light chain phosphorylation independent, contraction should be inhibited. Therefore, in taenia cecum, this mechanism seems not to contribute caffeine induced myosin-actin interaction.

The significant inhibitory effect of caffeine on $Ca^{2+}$-induced contraction smooth muscle has highlighted the importance of myosin light chain phosphorylation. When the light chains are phosphorylated, myosin becomes active and will allow contraction to occur. The enzyme that phosphorylates the light chain is called myosin light chain kinase, which only works when the muscle is stimulated to contract. Electronic or /and

pharmacological stimulation will increase the intracellular concentration of calcium ions which bind to a molecule called calmodulin, then Ca-calmodulin complex activates myosin light chain kinase[1)2)]. On the other hand, phosphorylated myosin is dephosphorylated with myosin phosphatase, a protein phosphatase 1, and inducing dissociation of myosin-actin[1)]. In the present study, $Ca^{2+}$-concentration in the preparation was well kept and sarcoplasmic reticulum, a Ca store in smooth muscle, was destroyed with A23187[4)], caffeine effects on $Ca^{2+}$ regulation must be neglected. Also cell membrane of the preparations was destroyed with β-escin[4)], cell membrane receptor coupled mechanisms such as caffeine effects on adenosine receptor should also be eliminated. Caffeine is known to act phosphodiesterase[12)], and may inhibit smooth muscle contraction via myosin phosphatase activation through inhibition of phosphodiesterase activity[3)]. Therefore, in the present knowledge, caffeine effects on inhibition of $Ca^{2+}$ induced contraction in the skinned taenia cecum in the present experimental condition seems to be due to the acceleration of myosin phosphatase activity. Further studies are necessary to clarify the effects of caffeine on myosin phosphatase activity on skinned teaenia cecum.

References

1). Somlyo AP, Somolyo AV. Signal transduction and regulation in smooth muscle. Nature. 372:231-6, 1994.

2). Karaki H, Weiss GB. Calcium release in smooth muscle. Life Sci. 42, 111-22, 1998.

3). Tazzeo T, Bates G, Roman HN, Lauzon AM, Khasnis MD, Eto M, et al. Caffeine relaxes smooth muscle through actin depolymerization. Am J Physiol Lung Cell Mol Physiol, 303(4): L334–L342, 2012.

4). Watanabe M, Takano-Ohmuro H. Extensive skinning of cell membrane diminishes the force-inhibiting effect of okadaic acid on smooth muscles of Guinea pig hepatic portal vein. Jpn J Physiol, 52(2): 141-7, 2002.

5). Yumoto M, Watanabe M: Blebbistatin, a myosin II inhibitor, suppresses $Ca^{2+}$-induced and "sensitized" – contraction of skinned tracheal muscles from guinea pig. J Smooth Muscle Res, 49: 89-98, 2013.

6). Sakurai W, Watanabe M, Yamashina A, Konishi M. Effects of hydrogen peroxide on contraction of skinned aorta from guinea pigs. Jpn J Physiol. 53: 471-4, 2002.

7). Watanabe M, Yumoto M, Tanaka H, Wang HH, Katayama T, Yoshiyama S, Black J, et al.: Blebbistatin a myosin II inhibitor, suppresses contraction and disrupts contractile filaments organization of skinned taenia cecum from guinea pig. Am J Physiol Cell Physiol, 298(5): C118-1126, 2010.

8). Horiuti K.: Mechanism of contracture on cooling of caffeine-treated frog skeletal muscle fibres. J Physiol Lond, 398: 131-148, 1988.

9). Nakayama S and Clark JF. Smooth muscle and NMR review: an overview of smooth muscle metabolism. Mol Cell Biochem: 17-30, 2003.

10). Cassidy P, and Kerrick WG. Superprecipitation of gizzard actomyosin, and tension in gizzard muscle skinned fibers in the presence of nucleotides other than ATP. Biochim Biophys Acta 705: 63-69, 1982.

11). Flockhart DA, Freist W, Hoppe J, Lincoln TM, and Corbin JD.: ATP analog specificity of cAMP-dependent protein kinase, cGMP-dependent protein kinase, and phosphorylase kinase. Eur J Biochem 140: 289-295, 1984.

12). Wells JN, Kramer GL. Phosphodiesterase inhibitors as tools in cyclic nucleotide research: a precautionary comment. Mol Cell Endocrinol 23: 1–9.