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The Role of Ca^{2+}/Calmodulin-Dependent Protein Kinase II on the Norepinephrine and GTP-Increased Myosin Light Chain Phosphorylations in Rabbit Mesenteric α-toxin Permeabilized Artery

Hee Yul Ahn+, Hun Sik Kim* and Robert S. Moreland**

Department of Pharmacology, College of Medicine, Chungbuk National University, Cheongju 360-763, Korea
**National Health Institute of Safety Research, Seoul 122-020, Korea*
***Bockus Research Institute, Graduate Hospital, University of Pennsylvania, Philadelphia PA 19146, USA*

ABSTRACT

The role of Ca^{2+}/calmodulin-dependent protein kinase II in the increase of myofilament Ca^{2+} sensitivity by agonist and GTP was investigated in rabbit mesenteric α-toxin permeabilized artery.

0.3 μM Ca^{2+} increased myosin light chain phosphorylations monotonically. 10 μM norepinephrine and 10 μM GTP potentiated increase of myosin light chain phosphorylations by 0.3 μM Ca^{2+}, which reaches a peak at 5 min and gradually declines to the Ca^{2+} alone level at 20 min.

At the early phase (1 min), 10 μM KN 62, the inhibitor of Ca^{2+}/calmodulin-dependent protein kinase II, decreased myosin light chain phosphorylation levels by 10 μM norepinephrine and 10 μM GTP in the presence of 0.3 μM Ca^{2+}. However, 10 μM KN-62 did not affect the myosin light chain phosphorylations by 10 μM norepinephrine and 10 μM GTP in the presence of 0.3 μM Ca^{2+} at the peak (5 min) and plateau phases (20 min).

From these results, the role of Ca^{2+}/calmodulin-dependent protein kinase II may be different depending on time, which may play a role in increase of myofilamint Ca^{2+} sensitivity by norepinephrine and GTP resulting from increase of myosin light chain phosphorylations at the early phase. However, at plateau phase, Ca^{2+}/calmodulin-dependent protein kinase II may not be involved in the increase of myofilament Ca^{2+} sensitivity by norepinephrine and GTP in rabbit mesenteric α-toxin permeabilized artery.

Key Words: KN-62, Ca^{2+}-Calmodulin dependent protein kinase II, and Myosin light chain phosphorylations

INTRODUCTION

The phosphorylation of 20-kDa myosin light chain (MLC) seems to be necessary for the initiation of smooth muscle contraction (Hartshorne, 1987). However, the maintenance of smooth muscle contraction is not solely dependent on the phosphorylation of MLC. The dissociation between tension and MCL phosphorylations has been shown in various smooth muscles (Dillon *et al.*, 1981; Aksoy *et al.*, 1982). During the maintenance of smooth muscle contraction, MLC phosphorylation levels fall to suprabasal levels, which is termed latch bridges (non-cycling attached crossbridges) (Hai and Murphy, 1992). Recently, actin-binding thin filaments, calponin and/or caldesmon were suggested to play a role in the latch phenomenon (Winder *et al.*, 1991).

+To whom correspondence should be addressed

On the other hand, agonists were known to increase Ca^{2+} sensitivity to myofilaments of ferret portal vein (Morgan and Morgan, 1984). A variety of agonists, e.g. α-adrenergic (Nishimura *et al.*, 1988) and muscarinic (Kobayashi *et al.*, 1989) or eicosanoid (Himpens *et al.*, 1990) or endothelin (Sakata *et al.*, 1989) also increased the myofilamint Ca^{2+} sensitivity at a constant Ca^{2+}. After Nishimura et al. (1988) reported that increase of myofilament Ca^{2+} sensitivity by agonist was dependent on GTP in α-toxin permeabilized artery, G-protein seemed to play a role in increase of myofilament Ca^{2+} sensitivity in smooth muscle cell. Furthermore, it was suggested that increase of myofilament Ca^{2+} sensitivity was resulted from an increase in MLC phosphorylation following phosphatase inhibition by G-protein (Somlyo *et al.*, 1989; Kitazawa *et al.*, 1991). Phorbol esters, activator of protein kinase C, also increased myofilament Ca^{2+} sensitivity in smooth muscle (Rembold and Murphy, 1988: Nishimura *et al.*, 1990). Nishimura et al. (1992) and Ahn and Moreland (1993) suggested that activated protein kinase C by agonist and GTP may inhibit phosphatase directly or indirectly resulting in increase of myofilament Ca^{2+} sensitivity in rabbit mesenteric α-toxin permeabilized artery. However, the precise mechanism by which the increase of myofilament Ca^{2+} sensitivity by agonist and GTP still remains obscure.

Multifunctional Ca^{2+}/calmodulin-dependent protein kinase, also referred to as CaM kinase II, is a general protein kinase although the role in smooth muscle contraction is not known well (Schulman, 1993). In this study, we attempted to clarify the role of CaM kinase II in the increase of myofilament Ca^{2+} sensitivity by agonist and GTP, which results from increase of MLC phosphorylations, using KN-62, inhibitor of CaM kinase II, in rabbit mesenteric α-toxin permeabilized artery.

METHODS

Adult male New Zealand White rabbits (2~2.5 kg) were killed by 100% CO_2 inhalation. Second- and third-order branches of the mesenteric artery (~250 μm outer diameter) were isolated and cleaned of fat and connective tissue. Small helical strips ~5 mm in length were cut as close to perpendicular to the long axis of the vessel as possible. The strips used for the determination of MLC phosphorylation levels were equlibrated in a small beaker containing PSS previously described (Moreland and Moreland, 1987).

The vascular strips were permeabilized with *Staphylococcus aureus* α-toxin as described by Nishimura *et al.* (1988). After equilibration, the strips were stimulated with 10 mM ATP for 30 min which produced a phasic contraction and desensitization of purinergic receptors. All subsequent solutions contained a minimum of 1 mM ATP to maintain purinergic receptor desensitization. The strips were then contracted with 10 μM norepinephrine (NE) and 1 mM ATP in a calcium-free PSS containing 2 mM ethylene glycol-bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA) for 45 min to deplete cellular calcium. After this calcium depletion step, the smooth muscle cells were permeabilized by the addition of 2,500 hemolytic units/ml of α-toxin (GIBCO-BRL Life Sciences) for 60 min in a solution composed of (in mM) 2 EGTA, 100 K-methanesulfonate, 5.38 $MgCl_2$, 5.35 Na_2ATP, 10 creatine phosphate, and 20 tris (hydroxymethyl) aminomethane (Tris) maleate (pH 7.0). All Ca^{2+}-contracting solutions were composed of (in mM) 10 EGTA, 20 Tris maleate (pH 7.0), 10 creatine phosphate, 4 MgATP, 1 free Mg^{2+}, appropriate $CaCl_2$ to achieve the desired free [Ca^{2+}], and appropriate K-methanesulfonate to maintain ionic strength at 0.12 M.

MLC phosphorylation levels were determined on long (~10 mm) strips of the mesenteric arteries. After equilibration and permeabilization, the tissues were exposed to a Ca^{2+}-containing solution in the presence of 10 μM ionomycin for varying times and then rapidly frozen in a dry-ice acetone slurry containing 6% trichloroacetic acid. The tissues were slowly thawed to room temperature and then transferred to a vial containing 50 μl of a homogenization solution previously frozen in liquid nitrogen, composed of 1% sodium dodecyl sulfate, 10% glycerol, and 20 mM dithiothreitol. The vial containing tissue, solution, and a small stainless steel ball was cooled in liquid nitrogen and placed in a dental amalgamator (Wig-L-Bug, Crescent Dental) for homogenization of the tissue.

This procedure was repeated and then the vial was allowed to reach 4℃ and the contents were transferred to a storage tube. The vial was rinsed with 50 μl of homogenization solution, which was added to the storage tube, and then stored at -80° C. Within 1 wk of homogenization, the samples were thawed and subjected to two-dimensional electrophoresis as described by previously (Moreland and Moreland, 1987). After electrophoresis, the separated proteins were subjected to high-field-intensity Western blotting to nitrocellulose membrane. Visualization of the blotted proteins was performed using AuroDye forte gold protein stain (Amersham). Quantitation of the stained blots was performed by scanning densitometry of the nitrocellulose paper made by transparent by immersion in decalin. Values are reported as mol Pi per mol MLC by integration of the spot corresponding to the phosphorylated MLC as a percent of the total of both the phosphorylated and unphosphorylated MLC.

α-toxin (Gibco, BRL), KN-62 (Biomol, Pennsylvania USA), All other chemicals were of reagent grade purity.

Group mean values were compared using unpaired two-tailed Student's t test. A P value of $<$ 0.05 was taken as significant.

RESULTS

Effects of norepinephrine and GTP on the myosin light chain phosphorylation by Ca^{2+} alone

0.3 μM Ca^{2+} induces a gradual rise in the phosphorylation level of MLC, which reaches a peak at 5 min and stays at this level for at least 20 min in rabbit mesenteric α-toxin permeabilized artery (Fig. 1). The basal phosphorylation level is 0 mol Pi/mol MLC (n=3), and the plateau level (at 5 min) is 0.21 $\pm$ 0.01 mol Pi/mol MLC (n=3).

10 μM norepinephrine and 10 μM GTP potentiates MLC phosphorylation level by 0.3 μM Ca^{2+}, which reaches a peak at 5 min and gradually declines to the Ca^{2+} alone level at 20 min (Fig. 1). The basal phosphorylation level is 0.05 $\pm$ 0.05 mol Pi/mol MLC (n=4), and the peak level (at 5 min) is 0.37 $\pm$ 0.1 mol Pi/mol MLC (n=3) and the level at 20 min is 0.28 $\pm$ 0.06 mol Pi/mol MLC (n =4).

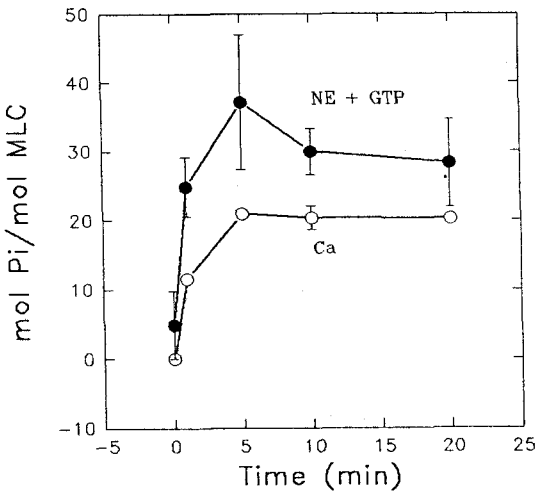

Fig. 1. Effect of 10 μM norepinephrine and 10 μM GTP on the 0.3 μM Ca^{2+}-induced myosin light chain phosphorylations for 20 minutes in rabbit mesenteric α-toxin permeabilized artery. Values are given as mean$\pm$standard errors of at least 3 determinations.

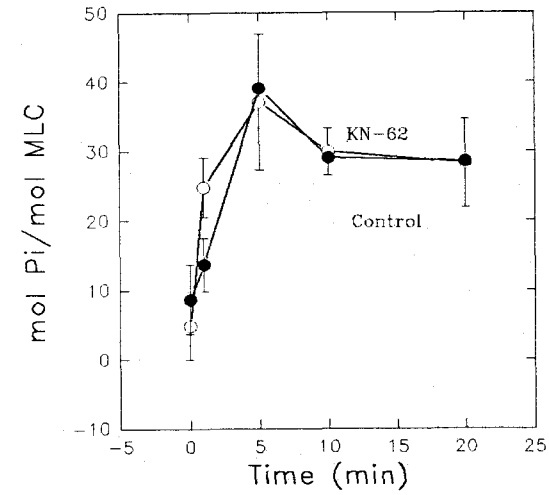

Fig. 2. Effects of Ca^{2+}/calmodulin-dependent protein kinase II inhibitor, KN 62 on the norepinephrine and GTP-increased myosin light chain phosphorylations in rabbit mesenteric α-toxin permeabilized artery. 10 μM KN-62 was treated 15 minute before addition of 10 μM norepinephrine and 10 μM GTP in the presence of 0.3 μM Ca^{2+} in the muscle strips. Values are given as mean$\pm$standard errors of at least 3 determinations.

Effects of Ca^{2+}/calmodulin-dependent protein kinase II inhibitor, KN-62, on the myosin light chain phosphorylations by norepinephrine and GTP

15 min pretreatment of 10 μM KN-62 did not modify the phosphorylation level by 10 μM norepinephrine and 10 μM GTP during 20 min except the early phase (Fig. 2). At 1 min, the phosphorylation level of MLC by 10 μM norepinephrine and 10 μM GTP is 0.25 $\pm$ 0.04 mol Pi/mol MLC (n=5). 10 μM KN-62 decreased the phosphorylation level by 10 μM norepinephrine and 10 μM GTP to 0.14 $\pm$ 0.04 mol Pi/mol MLC (n=3).

DISCUSSION

In this study, 10 μM norepinephrine (NE) and 10 μM GTP increased myosin light chain (MLC) phosphorylations by 0.3 μM Ca^{2+} transiently. With continued NE and GTP stimulation, MLC phosphorylation levels fell to levels similar to those of 0.3 μM Ca^{2+} alone. These results are consistent with previous results by Moreland *et al.* (1992). They showed that increase in myofilament Ca^{2+} sensitivity by NE and GTP results from increase of MLC phosphorylations in rabbit mesenteric α-toxin permeabilized artery and the pattern of increase of MLC phosphorylations by NE and GTP was transient. The mechanism of increase of myofilament Ca^{2+} sensitivity by agonist and GTP may be resulted from inhibition of MLC phosphatase (Somlyo *et al.*, 1989; Kitazawa *et al.*, 1991; kubota *et al.*, 1992) or inhibition of MLC phosphatase following protein kinase C activation (Nishimura *et al.*, 1992; Ahn and Moreland, 1993), which increases MLC phosphorylation resulting in development of contraction in smooth muscle.

However, during the maintenance of the receptor and G protein-dependent increase in myofilament Ca^{2+} sensitivity, MLC phosphorylation levels fall to Ca^{2+} alone level although contractile tension by agonist and GTP persists larger than that of Ca^{2+} alone (Moreland *et al.*, 1992 and Fig. 1). Therefore, another contractile regulatory mechanism(s) should be considered. The thin filament-associated proteins, caldesmon and calponin, have both been implicated in the regulation of smooth muscle contraction, and both are phosphorylated in vitro by PKC resulting in disinhibition of actin-myosin interaction (Sobue and Sellers, 1991; Winder *et al.*, 1991). Thus, the role of calponin and/or caldesmon on the sustained phase of increase in myofilament Ca^{2+} sensitivity by agonist and GTP should be further investigated in rabbit mesenteric α-toxin permeabilized artery.

Recently, Ca^{2+}/calmodulin-dependent protein kinase II, CaM kinase II is known to phosphorylate myosin light chain kinase (MLCK) followed by inhibition of MLCK activity, which results in inhibition of smooth muscle contraction (Tansey and Stull, 1992). However, CaM kinase II can phosphorylate serine-19 residue of 20-kDa MLC, which is prerequisite for initiation of smooth muscle contraction (Edelman *et al.*, 1990). So, the role of CaM kinase II in smooth muscle contraction seems to be complicated with unknown effects.

If CaM kinase II works in increase of myofilament Ca^{2+} sensitivity during the sustained phase of the contraction by agonist and GTP, which shows no increase MLC phosphorylation levels compared with those of Ca^{2+} alone in rabbit mesenteric α-toxin permeabilized artery, the inhibitor of CaM kinase II, KN-62 may affect the MLC phosphorylation levels by NE and GTP at sustained phase. To test this hypothesis, we investigated the effect of 10 μM KN-62 on the MLC phosphorylation level by 10 μM NE and 10 μM GTP. Unexpectedly, KN-62 decreased the phosphorylation levels of MLC by NE and GTP at 1 min. However, KN-62 did not modify the plateau levels of MLC phosphorylation by NE and GTP at 20 min as well as peak levels of MLC phosphorylation at 5 min by NE and GTP.

Accordingly, CaM kinase II may not involved in increase of myofilament Ca^{2+} sensitivity during the maintenance of contraction by NE and GTP in the presence of low Ca^{2+}, which may result from another mechanism(s). However, during the early phase in increase of myofilamint Ca^{2+} sensitivity by NE and GTP in the presence of low Ca^{2+}, CaM kinase II may inhibit phosphatase and/or activate phosphatase inhibitor followed by increase of MLC phosphorylations in rabbit mesenteric α-toxin permeabilized artery.

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=국문초록=

α-독으로 처리한 토끼장간막동맥에서 Norepinephrine과 GTP에 의한 마이오신 인산화의 증가에 대한 Ca^{2+}/calmodulin-dependent protein kinase II의 역할

충북대학교 의과대학 약리학교실
*국립보건안전연구원
**펜실바니아 대학 Graduate 병원 Bockus 연구소

안희열 · 김헌식* · Robert S. Moreland**

수용체작동약물과 GTP에 의한 수축단백질의 칼슘이온의 감수성의 증가에 대하여 Ca^{2+}/calmodulin-dependent protein kinase II의 역할을 α-독으로 처리한 토끼장간막동맥에서 조사하였다.

0.3 μM의 칼슘이온은 마이오신의 인산화를 시간의존적으로 증가시켰고 5분 이후부터 평형에 도달하였다. 한편, 10 μM의 norepinephine과 10 μM의 GTP는 칼슘이온 존재하에 칼슘이온 단독에 의한 것 보다 더 마이오신의 인산화를 증가시켰는데, 5분 후에 최대에 달하였고 그 후는 감소하기 시작하여 20분 후에는 칼슘이온 단독에 의한 마이오신 인산화의 증가와 거의 차이가 없었다.

Ca^{2+}/calmodulin-dependent protein kinase II 억제제인 KN-62를 전처치하여 norepinephrine과 GTP에 의한 마이오신 인산화 증가의 변화를 경시적으로 관찰하였다. 10 μM KN-62는 1분에서 10 μM norepinephrine과 10 μM GTP에 의한 마이오신의 인산화의 증가를 억제하였다. 그러나 5분에서 관찰되는 norepinephrine과 GTP에 의한 마이오신 인산화의 증가의 최대치에는 영향이 없였고 그 이후에도 KN-62는 아무 영향을 끼치지 못하였다.

이상과 같은 결과로 볼때 norepinephrine과 GTP에 의하여 일어나는 평활근 수축단백의 칼슘이온의 감수성의 증가는 이미 알려진 바와 같이 마이오신 인산화의 증가에 기인하며 이 증가는 일과성임을 확인하였다. 이때 Ca^{2+}/calmodulin-dependent protein kinase II의 역할은 시간의존적으로 norepinephrine과 GTP의 자극 초기에 관여되는 것으로 생각되며 그 이후에는 관여가 없는 것으로 사료된다.