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The Vasodilating Mechanism of Sodium Nitroprusside and Forskolin on Phorbol dibutyrate-Induced Contractions in Rat Aorta

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ABSTRACT

The objectives of this study is to compare the inhibitory mechanism of sodium nitroprusside and forskolin on the phorbol ester, activator of protein kinase C (PKC), -induced contractions in rat aorta.

0.1 μM phorbol dibutyrate (PDBu) induced sustained contractions and increased phosphorylations of myosin light chain (MLC) time-dependently. At 30 min, the contractions and phosphorylations of MLC by PDBu were augmented maximally and remained constant. Moreover, $^{45}Ca^{2+}$ uptake was increased 30 min after PDBu stimulation from resting values.

Sodium nitroprusside which activates guanylyl cyclase followed by increasing cGMP, inhibited the PDBu-induced contractions concentration-dependently. On the other hand, forskolin which activates adenylyl cyclase followed by increasing cAMP, also inhibited the PDBu-induced contractions concentration-dependently. However, sodium nitroprusside was more potent to inhibition of the PDBu-induced contractions than forskolin.

Sodium nitroprusside inhibited $^{45}Ca^{2-}$ uptake by PDBu stimulation. Forskolin also inhibited $^{45}Ca^{2+}$ uptake by PDBu stimulation.

Sodium nitroprusside and forskolin inhibited the phosphorylations of MLC by PDBu, respectively. However, sodium nitroprusside was more potent to inhibition of phosphorylations of MLC by PDBu than forskolin.

From these results, Sodium nitroprusside via cGMP or forskilin via cAMP may reduce myoplasmic Ca^{2-} followed by suppression of phosphorylations of MLC of PKC-mediated contractions, which results in vasodilation. However, cGMP may play a role more importantly than cAMP on the regulation of protein kinase C-mediated contraction in vascular smooth muscle.

Key words: Sodium nitroprusside, Forskolin, $^{45}Ca^{2+}$ uptake, Myosin light chain phosphorylations, Rat aorta

INTRODUCTION

Phorbol ester, activator of protein kinase C (PKC), induces slow developing and sustained contraction in vascular smooth muscle (Jiang and Morgan, 1987). Although Ca^{2+}-dependent 20-kDa myosin light chain (MLC) phosphorylations is essential for initiation of smooth muscle contraction, the maintenance of contraction may involve PKC-mediated regulation of contractile apparatus (Rasmussen *et al.*, 1987).

Phorbol duibutyrate (PDBu)-activated muscles phosphorylate MLC, which shows that two-thirds of the incorporated ^{32}P-phosphate was at-

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tributable to MLC kinase catalyzed phosphorylation and one-third was due to phosphorylation by protein kinase C (Barany *et al.*, 1992).

Besides MLC, PKC has been shown to phosphorylate calponin (Winder and Walsh, 1990; Rokolya and Moreland, 1993) and caldesmon (Adam *et al.*, 1989). PKC increases the myofilament Ca^{2+} sensitivity in rabbit mesenteric and rat pulmonary artery (Nishimura and van Breemen, 1989; Savineau *et al.*, 1993). Moreover, phorbol esters induce sustained contractions in the absence of extracellular Ca^{2+} without increases in intracellular free Ca^{2+} level measured with aequorin or fura 2 (Jiang and Morgan, 1987; Sato *et al.*, 1992; Itoh *et al.*, 1993) and the contractile mechanism remains to be determined. Recently, Ca^{2+}-independent PKC ε-isozyme was shown to translocate from cytosol to sarcolemma (Khalil *et al.*, 1992) and phosphorylate caldesmon through MAP-kinase pathway (Adam and Hathaway, 1993).

On the other hand, agents that elevate cAMP or cGMP such as sodium nitroprusside and forskolin, are known to relax vascular smooth muscle. The vasodilation by cyclic nucleotide-elevating agents may result from decrease $[Ca^{2+}]i$ or decrease in the $[Ca^{2+}]i$ sensitivity of MLC phosphorylation (Abe and Karaki, 1989; McDaniel *et al.*, 1991, 1992; Savineau *et al.*, 1993). However, how cyclic nucleotide-elevating agents regulate PDBu-induced contractions , Ca^{2+} uptake and MLC phosphorylations is not fully understood.

The objectives of this study is to investigate the mechanism how cyclic nucleotide-elevating agents affect protein kinase C-mediated contractions, Ca^{2+} uptake and MLC phosphorylations in rat thoracic aorta.

MATERIALS AND METHODS

Preparations

Adult male rats (Sprague-Dawley, 250~350 g) were killed by 100% CO_2 inhalation. Thoracic aorta was isolated and cleaned of fat and connective tissue. Endothelium was removed by gently rubbing the intimal space with a cotton swab. Segments (1.5 cm long were placed in organ chambers containing 10 ml of normal physiological salt solution (PSS) of the following composition (in mM): NaCl 136.9, KCl 5.4, glucose 5.5, $NaHCO_3$ 23.8, $CaCl_2$ 1.5, $MgCl_2$ 1.0 and ethylenediaminetetraacetic acid (EDTA) 0.01. Isosmotic 65.4 mM K^+ PSS was made by substituing 60 mM NaCl in the normal PSS with equimolar KCl.

Measurements of contractions

Muscle tension was recorded isometrically with force-displacement transducer connecting to the polygraph (Gould). Passive tension of 0.5 g was initially applied and muscle strips were allowed to equilibrate at 37℃ for 60 min and aerated with 95% O_2 and 5% CO_2. Drugs were cumulatively applied when 0.1 μM phorbol dibutyrate (PDBu)-induced tension reached a steady level.

Measurements of $^{45}Ca^{2+}$ uptake

After 90 min equilibration in normal PSS, the aortic rings were exposed to $^{45}Ca^{2+}$ labeled (2 μ Ci/ml) PSS or 0.1 μM PDBu-containing PSS for 30 min. Inhibitors were preincubated 30 min before addition of $^{45}Ca^{2+}$. Then the tissues were bathed in ice-cold Ca^{2+}-free PSS containing 2mM EGTA for 45 min in order to remove extracellular ^{45}Ca. The tissues were subsequently blotted, weighed and placed in 5 mM EDTA solution overnight. A scintillation cocktail was added and the tissues were counted (Meisheri *et al.*, 1981).

Measurements of phosphorylations of myosin light chain

Myosin light chain (MLC) phosphorylation levels were determined on long (~10 mm) strips of the thoracic aortas. After equilibration and drug pretreatment for 30 min, the tissues were 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 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 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. Visulations of the blotted proteins was performed by scanning densitometry of the nitrocellulose paper made by transparent by immersion in decalin. Values are reported as % by integration of the spot corresponding to the phosphorylated MLC as a % of the total of both the phosphorylated and unphosphorylated MLC.

Drugs

Forskolin (Sigma), Sodium nitroprusside (Sigma), Phorbol dibutyrate (Sigma), All other chemicals were of reagent grade purity.

Statistics

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

RESULTS

Time courses of phorbol dibutyrate (PDBu)-induced contractions and myosin light chain (MLC) phosphorylations in rat aorta

10^{-7} M PDBu induced a sustained contraction time dependently in rat aorta. The contraction reached a steady level at 30 min and the mag-

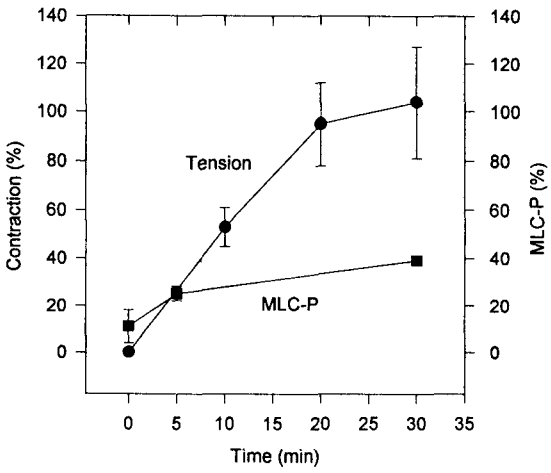

Fig. 1. Time-courses of 0.1 μM phorbol dibutyrate-induced contractions and phosphorylations of myosin light chain (MLC) in rat aorta. Data are expressed as means±S.E. (n=5~8). In left ordinate, 100% means the magnitude of isosmotic 65.4 mM KCl-induced contraction. The absolute magnitude of K-induced contraction was 1.0±0.1 g. In right ordinate, values are expressed as % by integration of the spot corresponding to the phosphorylated MLC as a % of the total of both the phosphorylated and unphosphorylated MLC.

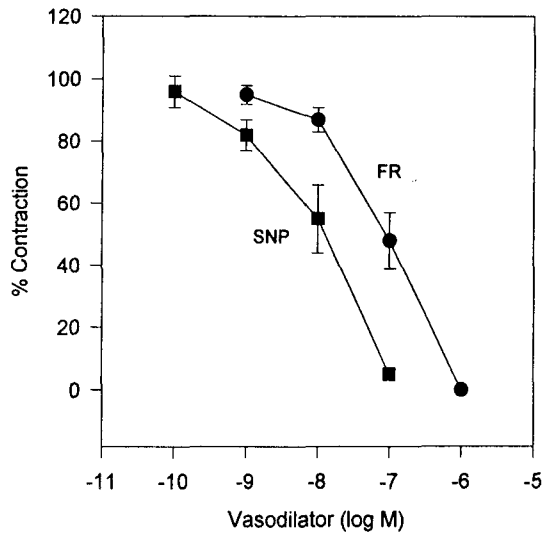

Fig. 2. Concentration-response curves for sodium nitroprusside (SNP) and forskolin (FR) in vessels that had been made to contract with 0.1 (M phorbol dibutyrate in rat aorta. 100% means the magnitude of 0.1 μM phorbol dibutyrate-induced contraction which corresponds to the 120.0±12.8% in the case of SNP (n=6), 145.7±15.7% in the case of FR (n=6) of high K contraction (0.7±0.1 g, 0.6±0.1 g, respectively). Data are expressed as means±S.E.

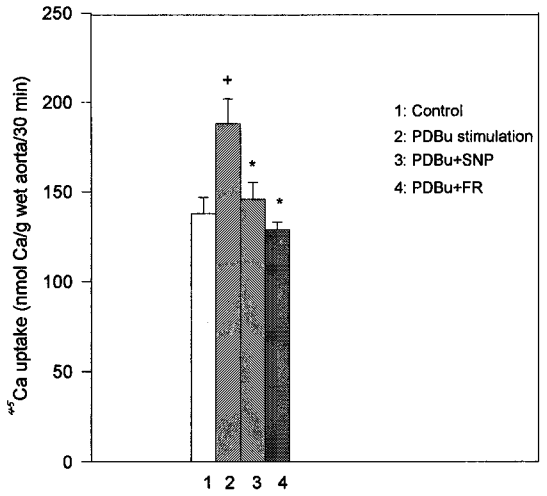

Fig. 3. Bar graph showing that 0.1 μM sodium nitroprusside (SNP) or 1 μM forskolin (FR) decreased ^{45}Ca uptake induced by 0.1 μM phorbol dibutyrate (PDBu) in rat aorta. SNP or FR was preincubated 30 min before application of PDBu, respectively. +means $P<0.05$ vs control, *means $P<0.05$ vs PDBu stiumlation.

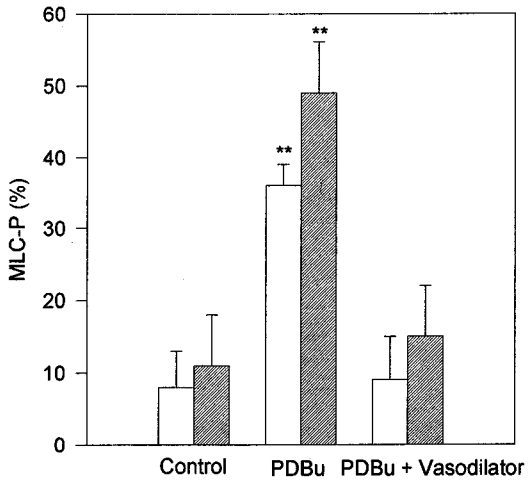

Fig. 4. Bar graph showing that sodium nitroprusside (open column) and forskolin (closed column) decreased the phosphorylations of myosin light chain (MLC) by 0.1 μM phorbol dibutyrate at 30 min. 0.1 μM sodium nitroprusside and 1 μM forskolin were treated 30 min bedore the application of phorbol dibutyrate, respectively. Data are expressed as mean±S. E. (n=4~5). Values are expressed as % by integration of the spot corresponding to the phosphorylated MLC as a % of the total of both the phosphorylated and unphosphorylated MLC. ** $P<0.01$ vs. values of control

nitude of contraction was almost same with that of high K-induced contraction.

Moreover, 10^{-7} M PDBu increased MLC phosphorylations time dependently in rat aorta. At 30 min, the MLC phosphorylations were increased from 11.4±7.0% to 38.7±1.9% significantly ($P<0.01$, n=5) (Fig. 1).

Effects of sodium nitroprusside and forskolin on the phorbol dibutyrate-induced contractions

Sodium nitroprusside (10^{-10}-10^{-7} M) and forskolin (10^{-9}-10^{-6} M) depressed the 10^{-7} M PDBu-induced contractions concentration-dependently (Fig. 2). IC_{50} of sodium nitroprusside and forskolin on the PDBu-induced contractions were 1.2×10^{-8} M and 9.8×10^{-8} M, respectively.

Effects of sodium nitroprusside and forskolin on the phorbol dibutyrate-increased $^{45}Ca^{2+}$ uptake

10^{-7} M PDBu increased ^{45}Ca uptake significantly 30 min after stimulation from resting values, 137.8±9.1 nmol Ca/g wet weight to 188± 14 nmol Ca/g wet weight ($P<0.05$, n=4). 30 min pretreatment of 10^{-7} M sodium nitroprusside inhibited PDBu-induced $^{45}Ca^{2+}$ uptake to 145.8± 9.5 nmol Ca/g wet weight (n=4) significantly. Moreover, 30 min pretreatment of 10^{-6} M forskolin inhibited PDBu-induced $^{45}Ca^{2+}$ uptake to 129±4.1 nmol Ca/g wet weight (n=4) significantly (Fig. 3).

Effects of sodium nitroprusside and forskolin on the phorbol dibutyrate-increased myosin light chain phosphorylations

10^{-7} M PDBu increased MLC phosphorylations significantly from 8.1±5.1% , resting values to 35.5±3.3% at 30 min after application ($P<0.01$, n=4). 30 min pretreatment of 10^{-7} M sodium nitroprusside decreased PDBu-induced MLC phosphorylations significantly to 9.4±5.5% ($P<0.01$, n=4). On the other hand, 30 min pre-

treatment of 10^{-6} M forskolin decreased PDBu-induced MLC phosphorylations significantly from 48.8±7.0% to 14.7±6.9% ($P<0.05$, $n=4$) (Fig. 4).

DISCUSSION

The goal of this study was to compare the vasodilating mechanism of cAMP with that of cGMP on protein kinase C (PKC)-mediated contractions in rat thoracic aorta. Phorbol dibutyrate (PDBu) directly binds to and activates PKC (Kikkawa *et al.*, 1983). Forskolin, activator of adenylyl cyclase, and sodium nitroprusside , activator of guanylyl cyclase, are known to increase in cAMP and cGMP in smooth muscle, respectively (Seamon *et al.*, 1981; Nambi *et al.*, 1986; McDaniel *et al.*, 1992). So, we investigated the vasodilating effects of forskolin and sodium nitroprusside on PDBu-induced contractions.

The contraction of smooth muscle tissues is associated with an increase in cytosolic Ca^{2+} and the Ca^{2+}-dependent phosphorylation of the 20-kDa myosin light chain (MLC) (Dillon *et al.*, 1981). On the other hand, phorbol ester develops sustained contraction with increases of $[Ca^{2+}]i$ and 20-kDa MLC phosphorylations in vascular and tracheal smooth muscle (Sato *et al.*, 1992; Gunst *et al.*, 1994).

In this experiment, PDBu induced a sustained contraction and increased phosphorylations of MLC time-dependently (Fig. 1). Moreover, PDBu increased Ca^{2+} uptake 30 min after stimulation. Ahn and Moreland (1993) suggested that MLC phosphatase activity may be down-regulated by G-protein dependent activation of protein kinase C. Itoh *et al.* (1993) also suggested that PDBu may cause a decrease in MLC phosphatase activity through activation of protein kinase C. Thus the mechanism(s) by which PDBu develops contractions in smooth muscle may involve Ca^{2+} dependent activation of MLCK and inhibition of phosphatase followed by increase of MLC phosphorylations.

Agents that elevate cAMP levels are known to relax arterial smooth muscle by decreasing $[Ca^{2+}]i$ or decreasing in the $[Ca^{2+}]i$ sensitivity of phosphorylation without change in $[Ca^{2+}]i$ (Abe and Karaki, 1989; Conti and Adelatein, 1981; Mcdaniel *et al.*, 1991; Savineau *et al.*, 1993) or activation of cGMP-dependent protein kinase (Lincoln and Corbin, 1983). However, isoproterenol-induced Ca^{2+}-desensitization of myosin light chain phosphorylation and force development in KCl-contracted smooth muscle is not mediated via myosin light chain kinase phosphorylation (Tang *et al.*, 1992). On the other hand, nitrovasodilators induce relaxation by both decreasing $[Ca^{2+}]$ and uncoupling force from myosin phosphorylation (McDaniel *et al.*, 1992; Karaki *et al.*, 1988). cGMP-dependent protein kinase decreases the sensitivity of the contractile machinery in vascular smooth muscle (Jiang and Morgan, 1987; Nishimura and van Breemen, 1989; Savinaeu *et al.*, 1993). However, Nishikawa *et al.* (1984) demonstrated that phosphorylation of myosin light chain kinase by G-kinase did not affect its function. Therefore, the mechanism for cyclic nucleotide dependent smooth muscle relaxation appears to be complex.

In this experiment, forskolin depressed the PDBu-induced contractions and phosphorylations of MLC (Fig. 2, 4). Sodium nitroprusside also depressed the PDBu-induced contractions and phosphorylations of MLC (Fig. 2, 4). However, sodium nitroprusside was more potent to inhibition of contractions and phosphorylations of MLC by PDBu. Furthermore, sodium nitroprusside or forskolin inhibited Ca^{2+} uptake by PDBu (Fig. 3).

Thus, sodium nitroprusside or forskolin may block Ca^{2+} influx followed by depressing phosphorylations of MLC, which results in inhibition of PDBu-induced contractions.

In conclusion, cGMP or cAMP may reduce myoplasmic Ca^{2+} followed by suppression of MLC phosphorylations of PKC-mediated contractions, which may result in vasodilation in rat aorta. However, cGMP may play a role more importantly than cAMP on the regulation of protein kinase c-mediated contraction in vascular smooth muscle.

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=국문초록=

Sodium nitroprusside와 Forskolin의 Phorbol ester 수축에 대한 혈관이완작용의 기전

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안 희 열

본 연구의 목적은 protein kinase C의 활성물질인 phorbol ester의 수축에 대한 cGMP 및 cAMP의 조절기전을 명확히 하기 위하여 흰쥐의 대동맥을 재료로 실험을 수행하였다. Sodium nitroprusside는 guanylyl cyclase를 활성화시켜 cGMP를, forskolin은 adenylyl cyclase를 활성화시켜서 cAMP를 증가시키는 것으로 보고되어 있으므로 위의 두 약물을 선택하였다. Phorbol ester는 시간경과와 함께 지속적인 수축을 발생하였으며 30분경 안정상태에 도달하였다. 동시에 20-kDa myosin light chain (MLC)의 인산화도 증가하였으며 30분경 최대치를 나타내었다. Sodium nitroprusside와 forskolin은 phorbol ester에 의한 수축을 농도의존적으로 억제하였으나 sodium nitroprusside가 forskolin보다 더욱 민감하게 억제하였다. Phorbol ester는 $^{45}Ca^{2+}$의 유입을 증가시켰고 sodium nitroprusside와 forskolin은 이 증가된 $^{45}Ca^{2+}$을 유의하게 억제하였다. Phorbol ester에 의하여 증가된 MLC의 인산화는 sodium nitroprusside 및 forskolin 각각의 최대농도로 억제되었다.

이상과 같은 결과로 볼때 아마도 cGMP와 cAMP는 phorbol ester에 의한 수축을 Ca^{2+} 유입억제에 이은 MLC 인산화 억제에 의하여 이완작용을 나타내는 것으로 추측되며 cGMP가 cAMP보다 protein kinase C 매개의 수축조절에 더 중요하게 작용하리라 추측된다.