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Vasodilating Mechanism of Dibutyryl-cAMP and Forskolin in Rabbit Aorta[1]

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ABSTRACT

Dibutyryl-cyclic AMP (db-cAMP) and forskolin were used to investigate vasodilating mechanism of cAMP in rabbit aorta. Db-cAMP and forskolin inhibited the development of contractile tension induced by norepinephrine (NE) concentration-dependently. However, high K^+-induced contractile tension was inhibited less effectively by db-cAMP and forskolin. Db-cAMP and forskolin inhibited $^{45}Ca^{2+}$ uptake increased by NE. Forskolin seemed to inhibit $^{45}Ca^{2+}$ uptake increased by high K^+, but this inhibition was not significant statistically. Db-cAMP inhibited Ca^{2+}-transient contraction by NE in Ca^{2+}-free solution.

In conclusion, it seems that cAMP blocks Ca^{2+} influx through receptor operated Ca^{2+} channels (ROCs), but that the effect of cAMP on Ca^{2+} influx through voltage gated Ca^{2+} channels (VGCs) is not clear in this experiment. Furthermore, cAMP is likely to inhibit calcium release from the intracellular stores.

Key Words: Cyclic AMP, Ca^{2+} channel, Rabbit aorta

INTRODUCTION

Dibutyryl-cyclic AMP (db-cAMP) is a lipid-soluble derivative of cAMP which penetrates readily into the cellular membrane. Forskolin also increases cAMP in a cell following activation of adenylate cyclase. cAMP as a second messenger like cGMP, diacylglycerol plays an important role in physiological activation to various responses including vasodilatation (Hidaka *et al.*, 1985). Since smooth muscle contraction results from an increase of intracellular Ca^{2+}, which is caused by Ca^{2+} influx by neurotransmitter or membrane depolarization and/or Ca^{2+} release from cellular stores by inositol 1,4,5-trisphosphate (IP_3) or Ca^{2+} (Van Breemen and Saida, 1989), cAMP is expected to inhibit movement of Ca^{2+} resulting in vasodilatation. Furthermore, Ca^{2+}-calmodulin dependent phosphorylation of the 20,000-dalton myosin light chain by myosin light chain kinase (MLCK) is important regulatory mechanisms for smooth muscle contraction (Kamm and Stull, 1985; Marston, 1982; Sobieszek, 1985; Trybus and Lowey, 1985). Therefore, cAMP is also expected to affect above contractile processes.

Until now, it has been reported that cAMP inhibited Ca^{2+} influx via blocking voltage gated Ca^{2+} channels (VGCs) (Meisheri and Van Breemen, 1982; Hwang and Van Breemen, 1987). However, cAMP seemed not to inhibit VGCs but receptor operated Ca^{2+} channels (ROCs) (Ahn *et al.*, 1988; Abe and Karaki, 1988). On the other hands, cAMP has been known to decrease the affinity of myosin light chain kinase for Ca^{2+}/calmodulin (Adelstein and Eisenberg, 1980; deLanerolle *et al.*, 1984). However, Miller *et al.* (1983) reported that cAMP showed no evidence of a decreased kinase activity in the phosphorylation reaction in bovine tracheal smooth muscle. cAMP activates Ca^{2+}-ATPase of plasma membrane or sar-

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coplasmic reticulum resulting in decrease of intracellular Ca^{2+} (Eggermont *et al.*, 1988; Twort and Van Breemen, 1988).

Since effects of cAMP on Ca^{2+} channels are conflicting, we tried to examine the effect of cAMP on Ca^{2+} transport through calcium channels by using db-cAMP and forskolin.

MATERIALS AND METHODS

Preparation of aortic strip

Male New Zealand white rabbits weighing 2kg were killed by rapid infusion of sodium pentobarbital (50 mg/kg) and air into the ear vein. The thoracic aorta was rapidly removed and cut into spiral strips of 2-3mm width and 10-15mm long. The adventitial layer was removed from the media intimal layer (Karaki and Urakawa, 1977) in order to avoid the possible involvement of endogenous catecholamine (Karaki *et al.*, 1984_a). These aortic strips did not contain functionally intact endothelium.

The normal physiological salt solution (PSS) containing NaCl 136.9, KCl 5.4, glucose 5.5, $NaHCO_3$ 23.8, $CaCl_2$ 1.5, $MgCl_2$ 1.0 and ethylenediamine tetraaceticacid (EDTA) 0.01 (mM) was used as a standard solution. Isosmotic 65.4mM K^+ solution was made by substituting 60mM NaCl in the normal PSS with equimolar KCl. Ca^{2+}-free solution was made by omitting $CaCl_2$ and adding 1mM ethyleneglycol bis (β-aminoethylether)-N,N,N′,N′-tetraacetic acid (EGTA). The solutions were aerated with a mixture of 95% O_2 and 5% CO_2 at 37°C (pH 7.4)

Measurement of contractile tension

Muscle strips were attached to holders and suspended in baths containing 20ml PSS. Muscle tension was recorded isometrically with a force-displacement transducer connected to a polygraph (Gould, USA). Passive tension of 1g was initially applied and tissues were allowed to equilibrate for 60 min before beginning the experiment. Isosmotic 65.4mM KCl solution was applied for 15 min followed by PSS wash-out for 15 minutes. This procedure was repeated until the amplitude of high K^+-contraction was maximally developed. Thereafter, 1μM norepinephrine or isosmotic 65.4mM KCl solution was applied to muscle strips. Dibutyryl-cyclic AMP (db-cAMP) or forskolin was cumulatively applied when the contractile tension induced by stimulants reached a steady level. The concentration of db-cAMP or forskolin required to induce a 50% inhibition (IC_{50}) was calculated from the cumulative concentration-inhibition curves.

Norepinephrine-induced transient contraction in aorta was obtained. After exposure of the muscle strips to Ca^{2+}-free solution for 10 min, 1μM norepinephrine was added for 5 min to induce a transient contraction. Following a 10 min wash with Ca^{2+}-free solution, 1.5mM Ca^{2+} was added for 15 min to load Ca^{2+} store in the muscle. Muscle strips were then rinsed with Ca^{2+}-free solution for 10 min followed by the application of 1μM norepinephrine. This procedure was repeated until steady transient contractions were obtained. Db-cAMP was added 10 min before the application of norepinephrine.

Measurement of Ca^{2+} influx

Ca^{2+} influx was measured as described by Karaki and Weiss (1979). Muscle strips were allowed to equilibrate in normal PSS for 3 h and then incubated with $^{45}Ca^{2+}$ (1.6 μCi/ml) for 5 min. Db-cAMP or forskolin was added 30 min before the $^{45}Ca^{2+}$ exposure. Agonists were added simultaneously with $^{45}Ca^{2+}$. Tissues were then washed for 30 min with an ice-cold lanthanum-solution containing $LaCl_3$ 73.8mM, glucose 5.5mM and tris (hydroxymethyl) aminomethane (Tris) 24.0mM. This soluton was adjusted to pH 6.8-6.9 at 0.5°C with 1N maleic acid. After the La^{3+}-wash period, muscle strips were removed from the holders, blotted, and placed in scintillation vials and $^{45}Ca^{2+}$ was extracted overnight with 1ml of 20mM EGTA solution. Scintillation mixture (Beckman Ready-Solv, HP) was added to each vial and radioactivity was counted with a liquid scintillation counter (Beckman, USA).

Statistics

Results of the experiments are expressed as Mean ± S.E. of Mean. Values were considered to be significantly different when P value was less than 0.05 by use of Student's t-test.

Drugs and chemicals

Dibutyryl-cyclic AMP (Yamasa, Japan), Forskolin (Sigma), Norepinephrine bitartrate (Sigma), EGTA (Sigma), TRIS (Sigma), $LaCl_3$ (Nacali Tesque, Japan), EDTA (Junsei chemicals, Japan).

RESULTS

Effects of db-cAMP and forskolin on sustained contraction

Db-cAMP inhibited norepinephrine (NE, 1μM)-induced contractions concentration dependently. When added during the high K^+-induced sustained contractions of aorta, however, db-cAMP did not inhibit the muscle contraction. Concentration-inhibition curves for db-cAMP on high K^+- and NE-induced contractions are shown in Figure 1. IC_{50} values for db-cAMP on NE-induced contraction were 4.3×10^{-4}M. Forskolin also inhibited NE-induced contractions more effectively than high K^+-induced contractions. Concentration-inhibition curves for forskolin on high K^+- and NE-induced contractions are shown in Figure 2. IC_{50} values for forskolin on high K^+-induced contractions and NE-induced contractions were 8.4×10^{-6} and 3.9×10^{-7}M, respectively.

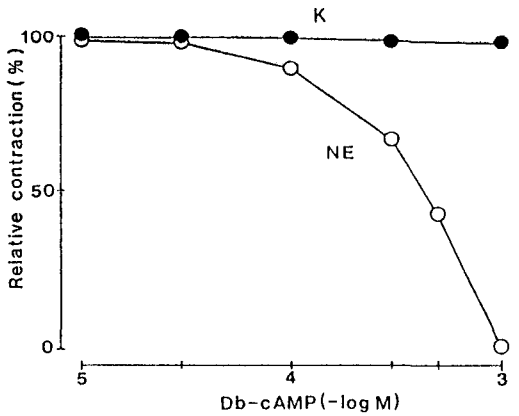

Fig. 1. Inhibitory effect of db-cAMP on norepinephrine- and KCl-induced contraction in rabbit aorta. Aortic strips were precontracted either by 1μM of NE or by 65.4mM of KCl. 100% represents the level of the developed tension before the addition of db-cAMP. Actual contractile tension induced by high K^+ or NE was 1.28±0.41g, 1.29±0.06g, respectively. Means of 6 experiments are shown.

Effects of db-cAMP and forskolin on $^{45}Ca^{2+}$ influx

The resting $^{45}Ca^{2+}$ influx in aorta was 105.9±5.3 nmol g^{-1} wet weight 5 min^{-1}. NE (1μM) increased the $^{45}Ca^{2+}$ influx to 160.8±7.5 nmol g^{-1} wet weight 5 min^{-1}. The addition of 1mM db-cAMP significantly decreased the $^{45}Ca^{2+}$ influx induced by NE (Fig. 3). Forskolin (10μM) decreased NE-induced $^{45}Ca^{2+}$ influx significantly from 181.2±8.4 nmol g^{-1} wet weight 5 min^{-1} to 110.3±7.4 nmol g^{-1} wet weight 5 min^{-1}. Furthermore, forskolin decreased high K^+-induced $^{45}Ca^{2+}$ influx from 263.8±28.7 nmol g^{-1} wet weight 5 min^{-1} to 192.6±36.8 nmol g^{-1} wet weight 5 min^{-1} but this decrease was not significantly different (Fig. 4).

Effects of db-cAMP on transient contraction

In Ca^{2+}-free solution, 1μM NE induced a transient contraction in rabbit aorta. Db-cAMP inhibited a transient contraction induced by NE. Concentration-inhibition curves for db-cAMP on NE-induced tran-

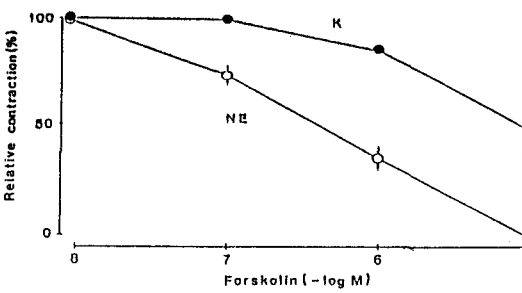

Fig. 2. Inhibitory effect of forskolin on norepinephrine- and KCl-induced contraction in rabbit aorta. 100% represents the level of the developed tension before the addition of forskolin. Actual contractile tension induced by high K^+ or NE was 0.76±0.22g, 0.89±0.21g, respectively. Means of 3-4 experiments are shown.

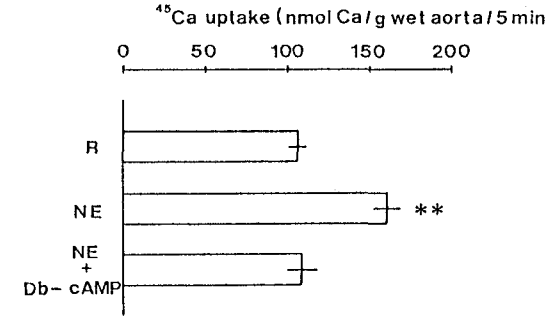

Fig. 3. Inhibitory effect of db-cAMP (1mM) on $^{45}Ca^{2+}$ uptake induced by norepinephrine (1μM). Means of 5-6 experiments are shown. ** indicates values of norepinephrine are significantly different from those of norepinephrine + db-cAMP ($p<0.01$).
R; means the resting $^{45}Ca^{2+}$ influx.

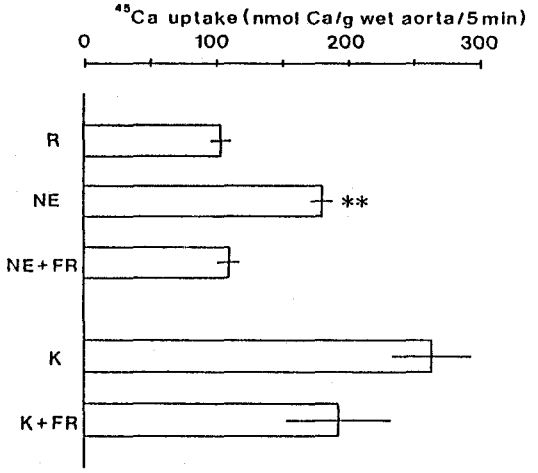

Fig. 4. Inhibitory effect of forskolin (FR, 10μM) on $^{45}Ca^{2+}$ uptake induced by norepinephrine (1μM) and KCl (65.4mM). Means of 6 experiments are shown. ** indicates values of norepinephrine are significantly different from those of norepinephrine + forskolin ($p<0.01$). However, values of KCl are not significantly different from those of KCl + forskolin. R; means the resting $^{45}Ca^{2+}$ influx.

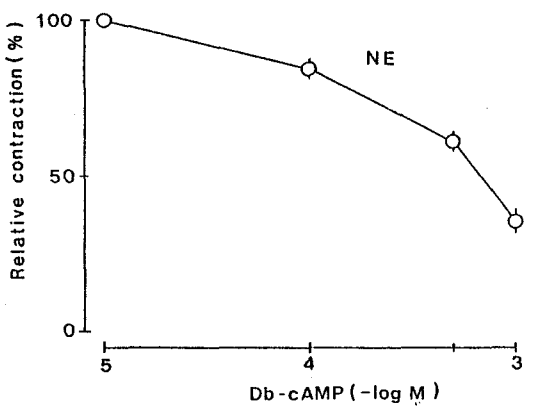

Fig. 5. Inhibitory effect of db-cAMP on norepinephrine (NE, 1μM)-induced contraction in Ca^{2+}-free solution. 100% represents the tension level before the addition of db-cAMP. Actual contractile tension induced by NE was 0.41 ± 0.02g. Means of 4 experiments are shown.

sient contraction are shown in Figure 5. IC_{50} values for db-cAMP were 6.8×10^{-4}M.

DISCUSSION

Db-cAMP, a permeable analogue of cAMP, inhibited sustained contractions induced by 1μM norepinephrine (NE) concentration-dependently in rabbit aorta. However, high K^+-induced contractions were not nearly inhibited by db-cAMP (Fig. 1). Forskolin, a diterpene activator of adenylate cyclase, also inhibited sustained contractions induced by 1μM NE more effectively than by high K^+ (Fig. 2). Since forskolin as well as db-cAMP is known to increase cAMP in the cell (Lincoln and Fisher-Simpson, 1983; Vegesna and Diamond, 1983, 1984; Marshall and Fain, 1985; Abe and Karaki, 1988), cAMP seems to be more effective in the inhibition of NE-induced contractions than that of high-K^+-induced contractions in rabbit aorta. In rat aorta, Lincoln and Fisher-Simpson (1983) reported that forskolin relaxed the NE-induced contractile tension more effectively than the KCl-induced contractile tension. Abe and Karaki (1988, 1989) also reported that forskolin relaxed the NE-induced contractile tension more effectively than the KCl-induced contractile tension in rabbit aorta. Therefore, our data were consistent with previous results.

Ca^{2+} entry into cytoplasm is regulated by various Ca^{2+} channels. Membrane depolarization has been demonstrated to open voltage gated Ca^{2+} channels (VGC_s) and NE has been shown to increase Ca^{2+} entry through receptor operated Ca^{2+} channels (ROC_s) (Bolton, 1979; Van Breemen *et al.*, 1979). Since db-cAMP and forskolin relaxed sustained contractions induced by NE or high K^+ in rabbit aorta, we investigated the effect of db-cAMP and forskolin on Ca^{2+} entry. Db-cAMP inhibited $^{45}Ca^{2+}$ uptake increased by NE (Fig. 3). Forskolin also inhibited $^{45}Ca^{2+}$ uptake increased by NE (Fig. 4). Therefore, cAMP seems to inhibit NE-contracted aorta by blocking Ca^{2+} entry through ROC_s. However, it is uncertain that cAMP affects on Ca^{2+} entry through VGCs in rabbit aorta because the decrease of high K^+-induced $^{45}Ca^{2+}$ uptake by forskolin was not significant (Fig. 4).

In rabbit aorta, db-cAMP and forskolin reduced $^{45}Ca^{2+}$ influx stimulated by high K^+ (Hwang and Van Breemen, 1987). However, Abe and Karaki (1988, 1989) reported that forskolin did not reduce $^{45}Ca^{2+}$ influx stimulated by high K^+ in rabbit aorta but reduced $[Ca^{2+}]_{cyt}$ induced by high K^+ using fura-2-Ca^{2+} fluorescence in rat aorta. The reason of this discrepancy is uncertain at this time. Species or methodological differences may explain above incon-

sistency. Moreover, intracellularly perfused cyclic AMP had no effect on voltage-dependent calcium current in smooth muscle cells (Ohya *et al.*, 1987).

Besides the effect of cAMP on Ca^{2+} entry, cAMP has been shown to affect contractile machinery or cellular enzymes. The increased cAMP following activation of adenylate cyclase by forskolin decreases the affinity of myosin light chain kinase for Ca^{2+}/calmodulin (Adelstein and Eisenberg, 1980; deLanerolle *et al.*, 1984) and/or activate Ca^{2+}-ATPase of plasma membrane or sarcoplasmic reticulum (Mueller and Van Breemen, 1979; Saida and Van Breemen, 1984; Hertog *et al.*, 1985; Eggermont *et al.*, 1988; Twort and Van Breemen, 1988). However, opposite results were also reported. For example, db-cAMP and forskolin were ineffective in relaxing precontracted skinned rat aortic rings (McMahon *et al.*, 1986).

Db-cAMP was not effective in relaxing high K^+-contracted aorta but forskolin in our data. This inconsistency may reflext cAMP is not solely responsible for the relaxation of high K^+-contracted aorta caused by these agents.

Db-cAMP inhibited NE-induced Ca^{2+}-transient contraction in Ca^{2+}-free solution (Fig. 5). Ca^{2+} release from sarcoplasmic reticulum (SR) has been postulated to proceed through the inositol-1,4,5-trisphosphate (IP_3) or Ca^{2+} (Endo, 1970; Streb *et al.*, 1983; Suematsu *et al.*, 1984). Therefore, db-cAMP may reduce Ca^{2+} release activated by NE through inhibition of phosphatidylinositol turnover.

In conclusion, cAMP may block Ca^{2+} influx through receptor operated Ca^{2+} channels (ROC_s), but the effect of cAMP on Ca^{2+} influx through voltage gated Ca^{2+} channels (VGC_s) is uncertain at this time. Furthermore, cAMP inhibits NE-induced transient contraction in rabbit aorta.

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= 국문초록 =

Dibutyryl-cyclic AMP와 Forskolin의 혈관평활근 이완작용

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세포막을 투과하는 cyclic AMP의 유도체인 Dibutyryl-cyclic AMP(db-cAMP)와 ad-enylate cyclase를 활성화시킴으로써 세포내에 cAMP를 증가시키는 Forskolin을 이용하여 토끼 대동맥평활근 이완작용의 기전을 검토하여 다음과 같은 결과를 얻었다.

1. Db-cAMP는 1μM norepinephrine에 의한 지속성 수축을 농도의존적으로 억제하였으나 고농도의 K에 의한 수축은 억제하지 못하였다.

2. Forskolin은 1μM norepinephrine에 의한 지속성 수축을 농도의존적으로 억제하였으며, 고통도의 K에 의한 수축보다 더 효과적으로 억제하였다.

3. Db-cAMP는 1μM norepinephrine에 의한 ^{45}Ca 유입증가를 억제하였다.

4. Forskolin은 1μM norepinephrine에 의한 ^{45}Ca 유입증가를 억제하였으며, 고농도의 K에 의한 ^{45}Ca 유입증가도 억제하였으나 유의차는 없었다.

5. Db-cAMP는 칼슘이온 제거용액에서 1μM norepinephrine에 의한 일과성 수축을 농도의존적으로 억제하였다.

이상의 결과에서 cAMP는 수용체작동성 칼슘채널(ROCs)을 통한 칼슘이온의 유입을 억제함으로써 norepinephrine에 의한 수축을 억제하며, 고농도의 K수축 억제가 전위의존성칼슘채널(VGCs)을 통한 칼슘이온의 유입의 억제에 의한 것인지는 확실치 않다.

또한 cAMP는 norepinephrine에 의한 세포내 칼슘이온의 유리에 의한 일과성 수축도 억제한다.