Korean J. of Pharmacology
Vol. 27, No. 2, pp. 125-133, 1991

Release of a Stable Endothelium-derived Relaxing Factor by A23187 from the Rabbit Aortic Endothelium

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ABSTRACT

In the isolated rabbit mesenteric artery denuded of endothelium, we characterized the identity of the A23187-induced endothelium-dependent relaxing factor (EDRF) released from the endothelium of rabbit aorta, which is distinct from that of acetylcholine-induced relaxing factor.

In the normal physiological salt solution (PSS), the dose-response curves to A23187 and acetylcholine were overlapped together. Their effects were also inhibited by methylene blue. Upon application of hypoxanthine and xanthine oxidase into the bath, the phenylephrine-induced precontraction was transiently increased followed by the sustained relaxation. During the burst of hypoxanthine-xanthine oxidase reaction, the Ca^{++} ionophore, A23187 but not acetylcholine was able to cause an immediate relaxation. However, A23187-induced relaxation was not manifested when precontracted by 50 mM K^{+}-PSS. Nevertheless, in the presence of superoxide dismutase, A23187 could produce an immediate relaxation without accompanying the transient contraction as acetylcholine did during the hypoxanthine-xanthine oxidase reaction.

On the other hand, acetylcholine-induced relaxation was more sensitively inhibited by phorbol 12-myristate 13-acetate (PMA) than A23187-induced relaxation. Endothelium-independent relaxation to sodium nitroprusside was not affected by PMA.

Based on these results it is suggested that both A23187 and acetylcholine cause the methylene blue-inhibitable endothelium-dependent relaxation, and in addition, A23187 may release a stable EDRF which is resistant to superoxide anion and PMA.

Key Words: Endothelium-derived relaxing factor, A23187, Endothelium, Vasorelaxation, Superoxide anion, Acetylcholine

INTRODUCTION

It is widely reported that the relaxation of the vascular smooth muscle in response to acetylcholine and A23187 is mediated by the release of a labile relaxing factor deried from endothelium (Furchgott, 1983; Vanhoutte *et al.*, 1986).

A23187, an ionophorous antibiotic agent, increases the permeability to divalent cation (Ca^{++}) of the biological membrane with an apparent selectivity and causes contraction of skeletal, cardiac and smooth muscles (Pressman, 1973; Reed and Lardy, 1972). Relaxation induced by A23187 in the rabbit aorta was reported to be mediated by a vascular relaxing factor released from the endothelial cells (Furchgott, 1983) and associated with the membrane hyperpolarization of the vascular smooth muscle in an endothelium-dependent manner (Chen and Suzuki, 1990).

Otherwise, phorbol 12-myristate 13-acetate (PMA), the activator of protein kinase C (Castagna *et al.*, 1982) has been reported to suppress

the muscarinic receptor mediated cyclic GMP synthesis and inhibit the endothelium-dependent responses evoked by acetylcholine (Weinheimer *et al.*, 1986; Lewis and Henderson, 1987; Rubanyi *et al.*, 1989).

Recently, we have observed that there exists a difference between acetylcholine and A23187 in the endothelium-dependent relaxation of the rabbit mesenteric artery. Thus, this study was designed to characterize the relaxing factor released from endothelium in response to A23187, which is resistant to the superoxide anion and PMA in comparison with that to acetylcholine.

METHODS

New Zealand white rabbits (1.5~2.5 kg) of either sex were killed by stunning of back head and rapidly exsanguinated. In the present study, both donor and detector muscles were mounted in the same bath together.

To supply sufficient amounts of endothelium, the rabbit thoracic aortic segment with endothelium was cut into rings of 10 mm in length, and opened along its longitudinal axis with care taken not to hurt the endothelial lining. This segment was suspended in the bath under 1 g of resting tension for the donor muscle. For the detector muscle, the ring segment (about 3 mm in length) of rabbit mesenteric artery denuded of endothelium was mounted in the same bath. To remove the endothelium, the intimal surface of rabbit mesenteric artery was mechanically rubbed with a moistened cotton bud under stereoscepe (Furchgott and Zawadzki, 1980) and thereafter, the tissue was immersed shaking in the physiological salt solution (PSS) containing CHAPS (0.3%) for 15 sec, and mounted in the bath with fresh PSS. The removal of the intimal surface was verified by a loss of relaxation in response to acetylcholine on the precontracted tissue. The mesenteric arterial ring segments were mounted in the muscle chamber, aerated with 95% O_2-5% CO_2 at 37°C. The upper part was connected to the isometric transducer (Myograph, Grass instruments) and the tissues were equilibrated for 2 hrs under resting tension of 2 g. The composition of the PSS was (in mM): 130 NaCl, 4.7 KCl, 1.18 $NaH_2PO_4 \cdot 2H_2O$, 1.17 $MgSO_4 \cdot 7H_2O$, 1.6 $CaCl_2 \cdot 2H_2O$, 14.9 $NaHCO_3$, and 5.5 dextrose. In the case of K^+-PSS, an equimolar KCl was substituted for NaCl in the PSS. Propranolol (0.2 μM) and indomethacin (5 μM) were included in the PSS to rule out the involvement of adrenergic component and the production of prostaglandins.

Generation of superoxide anion

To produce a flux of superoxide anion, an enzyme substrate system consisting of hypoxanthine (HX) and xanthine oxidase (XO) was used at pH 7.4. Xanthine oxidase was dialyzed in the one liter of distilled water for 4 hrs to preclude direct effect of ammonium sulfate. Superoxide anion produced by the HX (100 μM)-XO (0.02 unit/ml) reaction was measured by monitoring the superoxide dismutase (SOD)-inhibitable reduction of cytochrome c at 550 nm by spectrophotometer (Giford 2600).

^{86}Rb efflux

In this experiment, ^{86}Rb was used as a marker for K^+ as Rb^+ behaves similarly to K^+ (Imaizumi and Watanabe, 1981). Rubbed aortic strip or mesenteric segment impaled on a wire was immersed in the test tube containing 5 ml PSS at 37°C bubbled with 95% O_2-5% CO_2. After 30 min equilibration in PSS, the segment was loaded with ^{86}RbCl, 1 μCi/ml for 90 min. After washing out the excess radioactivity, the ^{86}Rb was allowed to efflux from the tissues by transferring them to the tubes containing 2 ml PSS for every 5 min period. At the end of the experiment, the ^{86}Rb content of the solution were counted for radioactivity. Each artery was blotted, weighed and digested overnight in 2 ml of 1 N NaOH. Then 0.15 ml of 6 N HCl was added to the resulting solution and the mixture was counted for radioactivity by liquid scinitllation counter (Packard instrument 300C). The efflux data were expressed in terms of the rate coefficient (functional loss of ^{86}Rb from the tissue, expressed as percent per min).

Drugs

All chemicals used were a reagent grade. Hypoxanthine, xanthine oxidase (Grade 1), superoxide dismutase, catalase, indomethacin, phenylephrine, sodium nitroprusside, propranolol, acetylcholine chloride and phorbol 12-myris-

tate 13-acetate were purchased from Sigma Chemical Co. A23187 was donated through the courtesy of Nelson Research and Developmental Company. The initial stock solution of PMA was dissolved in dimethylsulfoxide (DM-SO). Subsequent dilutions were in PSS. Final bath concentrations of DMSO were less than 0.1 % v/v, which did not alter the contraction or relaxation responses. ^{86}Rb was purchased from New England Nuclear Co.

Statistics

Data are expressed as mean±S.E.M. Statistical analysis was carried out by the Student's t-test and one-way ANOVA test. When $p<0.05$, the differences were considered to be statistically significant.

RESULTS

Assessment of superoxide radicals

HX (100 μM) was previously added into the cuvette cells without and with SOD (20 and 100 units/ml) for 5 min at 37°C, and then the reaction was initiated by addition of XO (0.02 unit/ml). Fig. 1 shows the representative time course of superoxide anion production by HX-XO reaction. In the absence of SOD, the average production rate of superoxide anion was 28.6 nmoles/min. This amount of product was completely negated by addition of 100 units/ml of SOD and partially reduced by 20 units/ml of SOD.

Release of EDRF

The dose-dependent relaxant effects of A23187 and acetylcholine were evaluated in the normal PSS in comparison with those of soduium nitroprusside, nitrosovasodilator. Cumulative dose-response relateionships for acetylcholine-, A23187- and sodium nitroprusside-stimulated reaxation of the rabbit mesenteric artery which had been precontracted with 10^{-7} M phenylephrine are shown in Fig. 2. The ED_{50} values for the acetylcholine- and A23187-stimulated relxation were 1.6×10^{-8} and 1.8×10^{-8} M, respectively. Acetylcholine and A23187 are more potent than sodium nitroprusside ($ED_{50}= 7.7\times10^{-8}$ M) in vasodilatation (Table 1).

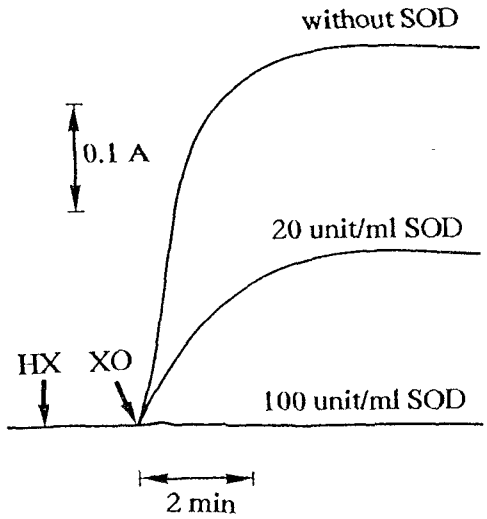

Fig. 1. Time course production of superoxide anion by HX(100 μM)-XO(0.02 unit/ml) reaction was measured. The figure shows superimposed tracings from spectrophotometer chart recordings. Extinction coefficients was 2.1×10 M/cm.

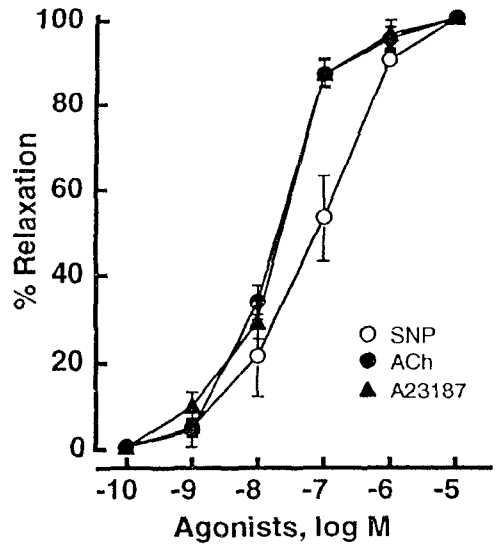

Fig. 2. Dose-response relaxation curves of acetylcholine, A23187 and sodium nitroprusside(SNP) in the precontracted strip of the mesenteric artery denuded of endothelium. The intact endothelium of rabbit aorta was used for donor of relaxing factor. Each point represents mean ±S.E.M. of 5-7 experiments.

Table 1. Effect of phobol 12-myristate 13-acetate(PMA) on ED_{50} for acetylcholine, A23187 and sodium nitroprusside

	Acetylcholine	A23187	Sodium nitroprusside
None	$1.6\pm0.2\times10^{-8}$	$1.8\pm0.3\times10^{-8}$	$7.7\pm3.2\times10^{-8}$
PMA, 1 μM	$5.1\pm1.7\times10^{-7}$*	$4.1\pm1.4\times10^{-8}$	–
PMA, 3 μM	$4.8\pm1.5\times10^{-5}$**	$6.6\pm1.1\times10^{-8}$**	$7.6\pm3.3\times10^{-8}$

*, $p<0.01$; **, $p<0.001$ vs. None

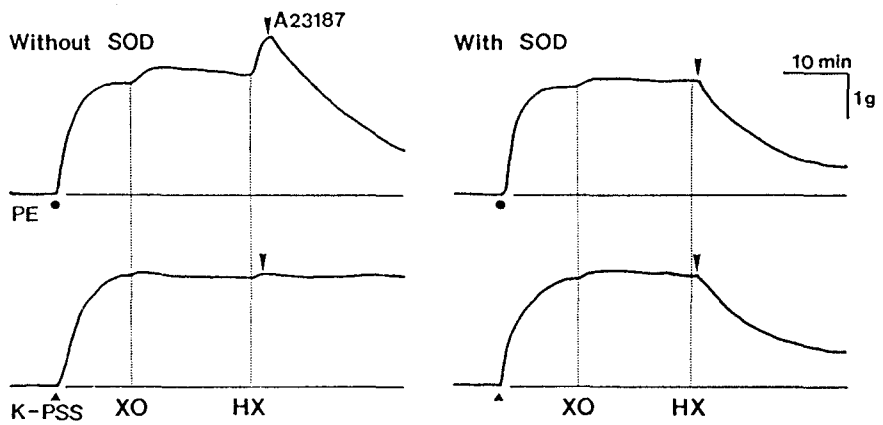

Fig. 3. A23187(0.05 μM)-induced relaxation(▼) during HX(100 μM)-XO(0.02 unit/ml) reaction in the absence (left) and presence (right) of SOD (100 unit/ml). ●, Contraction to 1 μM phenylephrine; ▲ Contraction by K^+(50 mM)-PSS.

Difference in the pattern of relaxation between A23187 and acetylcholine

In the present experiment, one bath system was used as an ordinary procedure. The apparent changes in muscle tension of rabbit mesenteric arterial segments were diretly observed upon release of the relaxant substances from the donor vessels.

As shown in Fig. 3, XO and HX were introduced into the organ bath separately with the lag time of 10 min. In the PSS, upon introduction of HX into the PSS of the bath containing XO, the strip precontracted by phenylephrine still exerted a transient contraction followed by relaxation. The contraction was sustained for approximately 5 min and the average maximum height was 1.4±0.2 g. When the level of relaxation was measured after 20 min elapse from the point where HX was applied, it was reduced by 50~60 %. When A23187 (5×10^{-8}) was added into the bath at 30 sec after application of HX, the relaxation started immediately. However, in the case of acetylcholine (5×10^{-8} M), the transient contraction was sustained up to over 7 min as shown in the control (Fig. 4).

Depression of A23187-induced relaxation in the K^+-PSS

In this study, the A23187-induced relaxation which was occurred in the phenylephrine-induced precontraction during HX-XO reaction was not observed in the high K^+ (50 mM)-induced contraction (Fig. 4). However, in the presence of SOD (100 units/ml), a rapid relaxation to A23187 of the mesenteric artery was reproducible in the high K^+ (50 mM)-induced contraction as well as in the phenylephrine-induced contraction without showing the transient contraction.

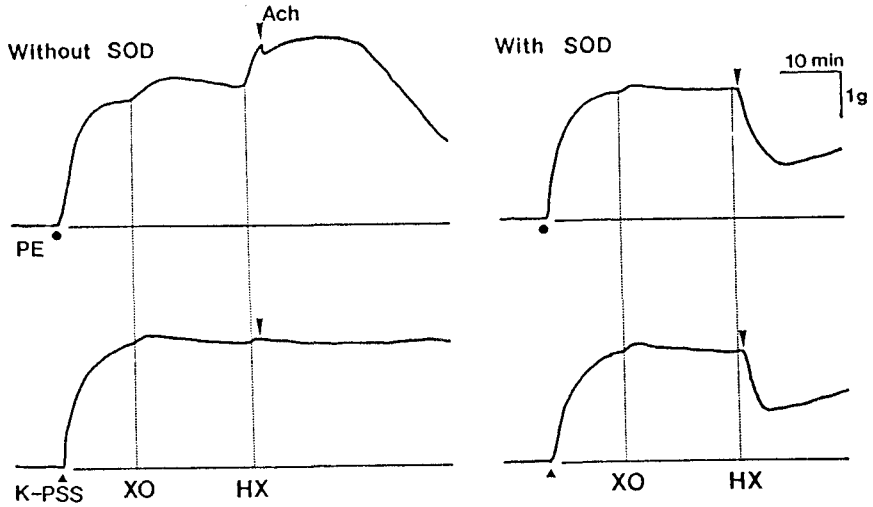

Fig. 4. Effect of acetylcholine (0.05 μM) after HX-XO reaction in the absence (left) and presence (right) of SOD. In the absence of SOD, the rapid relaxation to acetylcholine was not manifested in the normal PSS. The delayed relaxation was exhibited independent of actylcholine. ●, phenylephrine;▲, K^+(50 mM)-PSS.

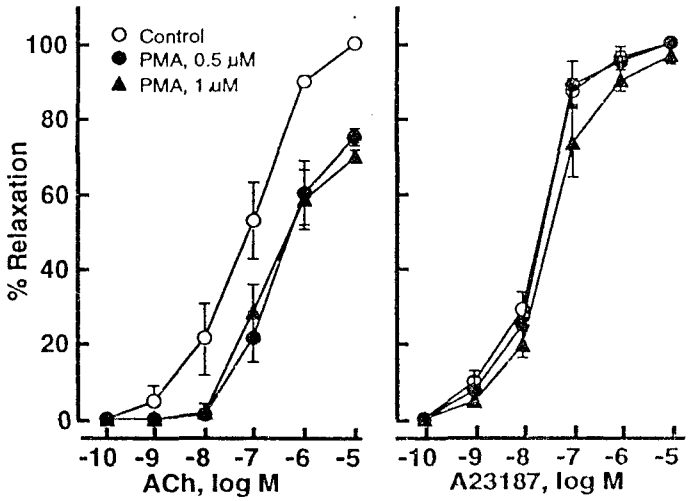

Fig. 5. Effect of phorbol 12-myristate 13-acetate(PMA; 0.5-1 μM) on the endothelium-dependent relaxations evoked by acetylcholine (left) and A23187 (right) in the rabbit mesenteric artery. The intact endothelium of rabbit aorta was used for donor of relaxing factor.

These findings were similarly manifest in the case of acetylcholine (Fig. 3 and 4).

Effect of PMA on the relaxation

Effect of PMA on the relaxation induced by acetylcholine, A23187 and sodium nitroprusside were assessed (Fig. 5 and 6). The phenylephrine-induced contraction was little affected by the PMA within the concentration range (0.5~3 μM). The basal tone of the vascular rings was also not affected by PMA up to 1 μM. Dose-response curves to acetylcholine were markedly shifted to the right by pretreatment with PMA (0.5 and 3 μM) with increased ED_{50} values, whereas those to either A23187 or sodium nitroprusside were affected little (Table 1).

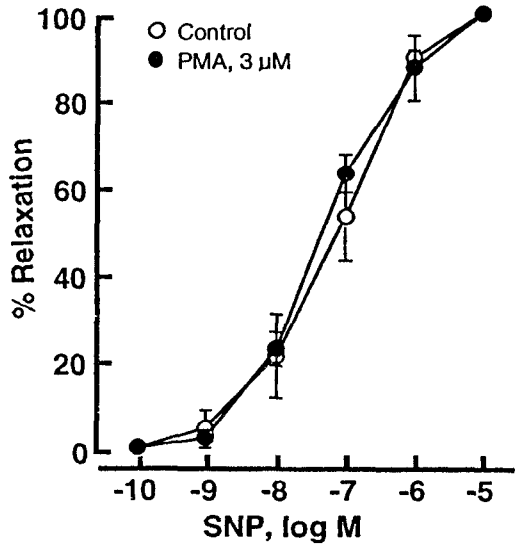

Fig. 6. Effect of phorbol 12-myristate 13-acetate (PMA; 3 μM) on the endothelium-independent relaxation evoked by sodium nitroprusside (SNP, 10^{-10} to 10^{-5} M) in rabbit mesenteric artery denuded of endothelium. Arterial rings were contracted with phenylephrine and responses are expressed as a percentage of relaxation of this contraction.

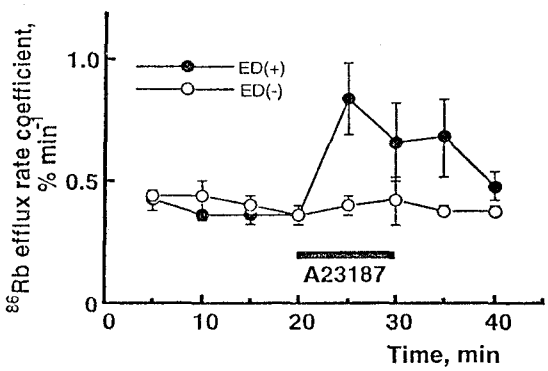

Fig. 7. Effect of A23187 on the ^{86}Rb efflux rate from the rabbit thoracic aorta with and without endothelium preloaded with 1 μM Ci / ml of ^{86}RbCl. ^{86}Rb efflux is expressed as the rate constant coefficient, % min^{-1}. Points represent the measn ± S.E.M. of 4 experiments.

Effect of A23187 on the ^{86}Rb efflux

This study shows endothelium-dependent increase in A23187-induced ^{86}Rb efflux from the rabbit aorta in the normal PSS. In untreated, endothelium-intact ring segments of the rabbit aorta, the basal efflux rate of ^{86}Rb was 0.36±0.04 % min^{-1}. Exposure to A23187 (1 μM) produced a rapid increase in ^{86}Rb efflux (0.84±0.22 % min^{-1}), which was sustained over 10 min (Fig. 7). However, A23187 did not increase the ^{86}Rb efflux rate from the aorta without endothelium.

DISCUSSION

EDRF is recognized to be exceedingly labile in the biological media with an extremely short half life (6~50 sec; Griffith *et al.*, 1984; Angus and Cocks, 1987) and most readily degraded by superoxide anion (Gryglewski et al., 1986; Moncada *et al.*, 1986). Recently, some reports have proposed that EDRF is the NO (Furchgott and Khan, 1986; Palmer *et al.*, 1987). A number of indirect lines of evidence have shown a high similarity and a few dissimilarity between EDRF and NO. The similarties of both substances are based on the facts: (1) Easy degradation of the their activities in the buffers. (2) Prolongation of the activites by SOD. (3) Inactivation by oxyhemoglobin and methylene blue. (4) The formation of cyclic GMP formation preceding the relaxation of the muscle (Long and Berkowitz, 1989). Nevertheless, some dissimilarties between EDRF and NO have been noted (Shikano *et al.*, 1987).

Recently, we have reported that a stable EDRF which is distinct from prostaglandins and methylene blue-inhibitable is produced upon exposure of the endothelial cells to the superoxide anion generated enzymatically or electrolysis (Hong *et al.*, 1989a & b), suggesting that there are more than one EDRF (Vanhoutte, 1987).

In this experiment, two tissue strips were mounted: one is a donor vessel (rabbit thoraic aorta with endothelium) and the other is the detector muscle (rabbit mesenteric arterial ring segment denuded of endothelium). A dose-dependent relaxation either to acetylcholine or to A23187 of the mesenteric artery was overlapped

each other and the vasorelaxation by these drugs was abolished by pretreatment with methylene blue (1 μM, data not shown), indicating that the EDRF released from the rabbit aorta by A23187 must be identical to that produced by acetylcholine.

However, upon application of A23187 to the mesenteric artery during the burst of HX-XO reaction, the relaxation was immediately evident in contrast to that of acetylcholine. The precontraction by phenylephrine showed another transient increase in the tone followed by sutained relaxation in response to the HX-XO reaction in the muscle bath. Consistent with the report of Katusic and Vanhoutte (1989), the transient contraction in response to HX-XO reaction was ascribed to the superoxide anion generated because it was not exhibited in the absence of endothelium or following pretreatment with SOD. In the relaxation by A23187, an involvement of prostacyclin and/or other oxygen free radicas than SOD could be precluced because 150 units/ml catalase and 5 μM indomethacin were included in the PSS throughout the experiment (McCord, 1974).

Based on the fact that the NO-like EDRF is excedingly labile in biological media (Angus and Cocks, 1987; Griffith *et al.*, 1984) and readily destroyed by superoxide anion (Gryglewski *et al.*, 1984), and further, in the presence of SOD, the relaxation was consistently exhibited in response to acetylcholine as was to A23187 even in the midst of HX-XO reaction, it is indicated that, in the normal PSS, both relaxation to A23187 and acetylcholine were mediated by SOD-inhibitable NO-like EDRF. Thus it is clear that this relaxing factor is different from the aleady known NO-like EDRF. Rather, it is likely that some pharmacological actions of this relaxing factor are closely similar to the action of superoxide-dependent endothelium-dependent relaxing factor reported by Hong *et al.* (1989a & b), in that exposure of endothelial cells to enzymatically generated superoxide anion causes a production of stable secondary EDRF (approximately 4.5 min).

Here, a question arises as to how A23187, the Ca^{++} ionophore, can elicit the vasorelaxation in the burst of HX-XO reaction.

As Ca^{++} ionophore, A23187 reportedly has the property to potentiate the Ca^{++} movement at the outer membrane by ionophoretic action and also increase the intracellular release of Ca^{++}, thereby leading to an increase in intracellular Ca^{++} of endothelium (Desmedt and Hainaut, 1976). In this study, an increase in ^{86}Rb efflux by A23187 from the aortic segment with endothelium was observed. The increase in ^{86}Rb efflux in association with concentration-dependent hyperpolarization of the smooth muscle membrane has been demonstrated either by A23187 (Bolton and Clapp, 1984; Chen and Suzuki, 1990) or by acetylcholine (Bolton *et al.*, 1984; Chen *et al.*, 1988; Feletou and Vanhoutte, 1988). However, it is not known whether acetylcholine has the property to release the similar relaxing factor as does A23187.

Alternatively, endothelium-dependent relaxation caused by acetylcholine was significantly inhibited by PMA, whereas that by A23187 was not affected. Histamine-induced endothelium-dependent relaxation was inhibited by the phorbolester, phorbol 12, 13-dibyutyrate in the guinea pig pulmonary artery (Weinheimer *et al.*, 1986) and acetylcholine-induced relaxation in the canine femoral and coronary artery (Rubanyi *et al.*, 1989), whereas A23187-induced relaxation was not affected by the phorbol ester. In this regard, there are several considerations which may explain the different findings between acetylcholine- and A23187-induced endothelium-dependent relaxation is that: the former is due to receptor-stimulated response and the latter is due to non-receptor-stimulated relaxation. At present, there is an indication that A23187 releases different forms of EDRF, which is resistant to superoxide anion and PMA. If PMA interferes with endothelium-dependent relaxation to acetylcholine in vascular smooth muscle by activation of protein kinase C which inactivate the GTP-binding protein by phosphrylation it will block the acetylcholine-induced signal transduction as indicated with histamine (Weinheimer *et al.*, 1986). Lai and El-Fakahany (1987) have reported that, in mouse neuroblastoma cells, PMA suppressed muscarinic and histaminic receptor-mediated cyclic GMP formation without affecting the effect by A23187. The inability of PMA to inhibit cyclic GMP formation mediated by A23187 in contrast to acetylcholine may be explained that the phobol ester can not exert its effect when the cyclic GMP formation is mediated through non-

receptor signal transduction pathway or bypassing the cell surface receptors in the case of A23187. A chemical interaction of PMA with EDRF or nonspecific effects of PMA are unlikely. Recently, Vidal *et al.* (1991) have demonstrated that, in canine femoral veins, endothelium-derived factors released in response to A23187 produce a relaxation of the smooth muscle by a mechanism distinct from changes in cyclic GMP.

On the basis of these results, it is likely that the possible release of the stable endothelium-derived factor(s) other than NO-like EDRF may mediate the vasorelaxation under certain circumstances.

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= 국문요약 =

토끼 대동맥 내피에서 A23187에 의하여 유리되는 혈관이완물질의 특성에 관한 연구

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내피세포가 제거된 토끼의 적출 장간막 동맥에서 토끼의 대동맥 내피세포로 부터 A23187과 acetylcholine은 NO와 유사한 혈관 이완성 물질 (EDRF)을 유리한다. 이에 첨가하여 A23187은 acetylcholine과는 달리 superoxide anion에 의하여 파괴되지 않는 EDRF도 유리시킴을 확인하고 이의 특성에 대하여 연구하였다.

정상적인 생리영양액에서는 A23187과 acetylcholine의 용량-반응 곡선은 내피세포에 의존하지 않는 sodium nitroprusside의 곡선과 유사하였다. 이들은 methylene blue에 의하여 억제되었다. Hypoxanthine (HX)과 xanthine oxidase (XO)를 bath내로 투여시 phenylephrine에 의한 수축이 일과성으로 증가한 후 지속적으로 이완되었다. HX-XO 반응중에는 A23187은 장간막 동맥을 즉각적으로 이완시켰으나 acetylcholine의 이완작용은 소실되었다. A23187에 의하여 야기되는 장간막동맥의 이완은 50 mM K^+-PSS로 수축을 야기시켰을 때에는 나타나지 아니하였다. Superoxide dismutase를 전처치하였을 때는 HX-XO 반응중에도 acetylcholine 뿐만아니라 A23187에 의한 장간막 동맥의 수축은 이완되었다.

한편, acetylcholine에 의하여 야기되는 장간막 동맥의 이완은 A23187에 의하여 야기되는 이완에 비하여 phorbol 12-myristate 13-acetate (PMA)에 훨씬 더 민감하게 억제되었다. 내피세포의 기능과는 무관한 sodium nitroprusside에 의하여 야기되는 이완은 PMA에 의하여 영향을 받지 아니하였다.

이상의 결과로 미루어 볼때, A23187과 acetylcholine은 methylene blue에 의하여 억제되는 내피세포 의존성 이완을 야기시키고, 첨가하여 A23187은 어떤 병적 환경 아래서는 superoxide anion과 PMA에 저항성을 지닌 혈관이완성 물질을 유리하는 것으로 사료된다. 앞으로 A23187에 의하여 유리되는 혈관 이완성 물질이 superoxide anion에 의존하여 생성된 것인지에 대하여는 추후의 연구과제이다.