Effects of Extracellular Ca^{2+} and Ca^{2+}-Antagonists on Endothelium-Dependent Relaxation in Rabbit Aorta

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(Received 19 April, 1990)

ABSTRACT

The effects of extracellular Ca^{2+} and various Ca^{2+} antagonists on endothelium-dependent relaxation to acetylcholine were studied in the isolated rabbit thoracic aorta in order to elucidate the control mechanism of endothelium derived relaxing factor (EDRF) release. Endothelium was removed from aortic strips by gentle rubbing with cotton ball. The effect of hemoglobin on basal tension was also observed with hemolysate. The results obtained were as follows:

1) Endothelium-dependent relaxation (EDR) to acetylcholine (ACh) showed biphasic pattern; the initial rapid relaxation phase and the late slow relaxation phase.

2) With the depletion of the extracellular Ca^{2+}, EDR was gradually suppressed, especially the late slow relaxation.

3) Verapamil, nifedipine, Mn^{2+} and Cd^{2+} had not any effect on EDR, while La^{3+} and Co^{2+} suppressed EDR completely.

4) The resting tension of the strips with rubbed endothelium was not altered by the addition of hemoglobin. That of the strips with intact endothelium, however, was enhanced and EDR to ACh was completely blocked.

From these results, we suggest that extracellular Ca^{2+} is necessary for ACh-induced slow relaxation while Ca^{2+} antagonists have not any effect on EDR.

Key Words: Endothelium-dependent relaxation, Ca^{2+}, Ca^{2+} antagonists

INTRODUCTION

Furchgott et al (1980a; 1980b) discovered that vascular endothelium plays essential role in the contractility of the vascular smooth muscle. Until this discovery, there was an unsolved controversy in the effect of acetylcholine (ACh) and other muscarinic agonists on the contractility of the vascular smooth muscle in vivo and in vitro; in vivo they produced vasodilation, but such vasodilation was observed in only a minority of the many vascular preparations tested in vitro. Furchgott reported that the vascular endothelium appears to be involved in the relaxation induced by ACh and other muscarinic agonists and the relaxation is mediated by endothelium-derived relaxing factor(s) (EDRF).

The vascular endothelium has many important physiological functions, which include transcapillary transport, regulation of plasma lipid metabolism, participation in the control of hemostasis, and modulation of the reactivity of vascular smooth muscle.

*This work was supported in part by the grant from Seoul National University Hospital, 1990.

The modulatory role is accomplished through several mechanisms; (a) the endothelium interposes a physical barrier between the vascular smooth muscle and some vasoactive substances as well as some hormones circulating in blood; (b) it extracts or metabolically degrades vasoactive substances such as norepinephrine (NE), serotonin, and kinins and thereby prevents or diminishes their activity in vascular smooth muscle; (c) it converts precursors (e.g. angiotensin I) into vasoactive products; (d) it secrets some vasoactive substances, primarily prostacyslin; and (e) it releases other still unidentified inhibitory and excitatory mediators in response to vasoactive stimuli (e.g. EDRF and EDCF) (Vanhoutte et al, 1986).

In vascular smooth muscle, EDRF induces the activation of soluble guanylate cyclase and the elevation of the level of cytosolic c-GMP (Forstermann et al, 1986; Ignarro et al, 1986). c-GMP causes inhibition both of calcium release from internal store and of calcium influx through the receptor-operated calcium channel (Griffith et al, 1988). And then the decrease of cytosolic calcium ion level causes vasorelaxation.

In vascular endothelium, the known EDRF agonists, thrombin and bradykinin, increases Ca^{2+} influx (Johns et al, 1987; Cannel and Sage, 1989), and there are reports suggesting that Ca^{2+} plays an important role in the release of EDRF and stimulates the release of EDRF from endothelial cells (Furchgott et al, 1983; Griffith et al, 1984). These works suggest that calcium ion plays an important role in the synthesis or release of EDRF and we think that making the role of calcium clear is the cornerstone of elucidating the mechanism and the nature of EDRF. Therefore we seek to investigate the effects of calcium and its antagonists on endothelium-dependent relaxation (EDR) and elucidate the role of calcium on EDR in this experiment.

METHODS

Rabbits of either sex, weighing about 2 kg, were killed by exanguination from the carotid artery under sodium pentothal anesthesia. The thoracic aorta was excised and immersed in the phosphate buffer Tyrode solution at room temperature, which was aerated with 100% O_2. After the removal of connective tissue, the aorta was cut into transverse strips (2 mm × 10 mm). Care was taken during dissection to avoid unnecessary stretching or contact of instruments with the luminal surface of the strips to ensure integrity of the endothelium. Endothelium was removed by gentle rubbing with cotton ball moistened with the solution, as described by Furchgott and Zawadzki (1980b). Scanning electronmicroscopic examination revealed that this method was sufficient to remove the endothelial cells (Fig. 1).

The transverse strips were mounted vertically in organ baths (50 and 100 cc). With the strips attached to a strain gauge force transducers (Grass FT-03 and Gould J 968), isometric tensions were recorded on pen recorders (Devices and Gould RS 3200). They were allowed to equilibrate for at least 2 hours before experimentation. During this period, they were stretched to approximately 0.2 g. Paired strips were studied in parallel in the same 50 cc chamber.

Preincubation with indomethacin (10^{-5} M) for 30 min did not change the magnitude of EDR induced by acetylcholine ($p<0.01$, $n=6$). So we executed this experiment without pretreatment of indomethacin.

Preparation of hemolysate

Human heparinized blood of 10~15 cc was centrifuged for 20 minutes at 190 g and the plasma supernatant was discarded. 2 cc of the remaining erythrocytes was hemolysated by the addition of 18 cc distilled water, as described by Bolton and Clapp (1986). The final concentration of hemoglobin was measured by Coulter counter.

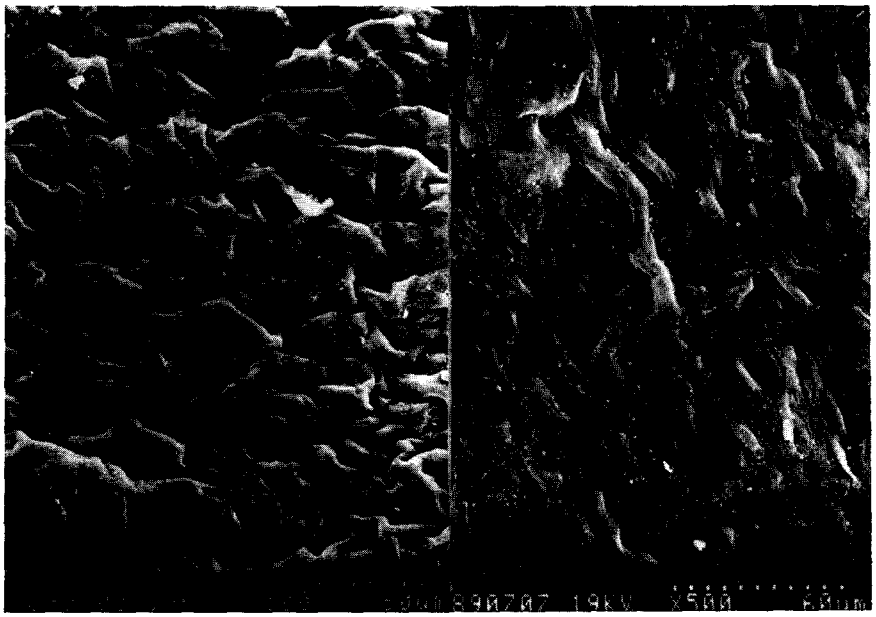

Fig. 1. Scanning electron micrographs of intimal surfaces of rabbit aortic strips with intact endothelium (left) and damaged endothelium (right). The strips were fixed and processed at the beginning of an experiment. In the strip with intact endothelium, it was observed that polygonal endothelial cells covered uniformly the intimal surface. In the strip with damaged endothelium, fibers of smooth muscles and connective tissues were observed.

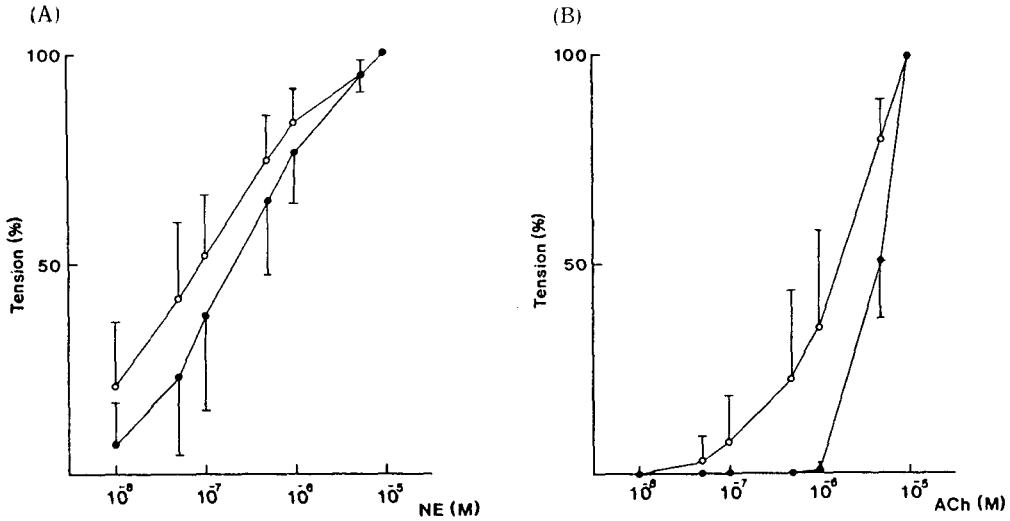

Fig. 2. (A) Concentration-response curves of norepinephrine (NE) in the rabbit aortic strips with intact endothelium (closed circle; n=12) or damaged endothelium (open circle; n=9). Tension was expressed as the percentage of the contraction induced by 10^{-5} M NE. Each point and vertical bar represents the mean±standard deviation (S.D.). (B) Concentration-response curves of ACh in the strips with intact endothelium (closed circle; n=5) or damaged endothelium (open circle; n=10). The tension was expressed as the percentage of the contraction induced by 10^{-5} M ACh.

Statistics

Results of the experiments are expressed as mean±SD. Students t-test was used for statistical analysis of the results and P value less than 0.05 was taken as significant.

Drugs and chemicals

The following drugs were used: Acetylcholine chloride, $CdCl_2$, $CoCl_2$, L-Arterenol bitartrate, $LaCl_3$, $MnCl_2$ (Sigma), Nifedipine (Bayer), Verapamil (Knoll AG). Inorganic calcium antagonists were applied 2 minutes before the addition of norepinephrine (NE), and organic calcium antagonists were applied 30 minutes before the NE treatment.

RESULTS

Effects of norepinephrine (NE) and acetylcholine (ACh) on strips with intact endothelium and with rubbed endothelium

With the removal of endothelium the contraction curve to NE was shifted to the left compared with that of strips with the intact endothelium (Fig. 2A). These data suggest that removal of endothelium may enhance the sensitivity to NE. ACh increased the tension of the strips with the rubbed endothelium in a dose-dependent manner (Fig. 2B). In contrast, the strips with intact endothelium did not contract apparently at up to 10^{-6} M of ACh, and the magnitude of contractions was abruptly increased at 5×10^{-6} M and 10^{-5} M of ACh. This finding is probably due to the dual effects of ACh on the vascular smooth muscle, namely direct contracting effect on muscle and indirect relaxing effect by stimulating EDRF release.

The concentration-dependent response to ACh was shown in Fig. 3 of the strips precontracted with 10^{-7} M of NE. In the strip with rubbed endothelium the magnitude of contractions was gradually increased with the addition of ACh. In the strips with intact endothelium the magnitude was gradually decreased by increasing the concentration of ACh and the maximal effect was shown at 10^{-6} M of ACh.

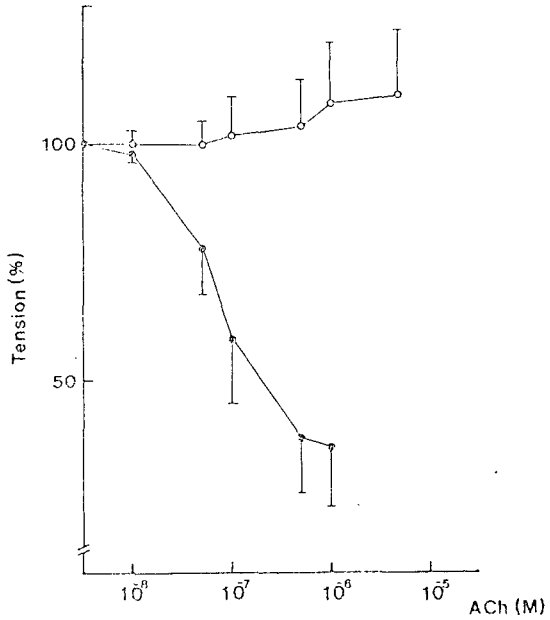

Fig. 3. Concentration-response curves of ACh in the precontracted strips with 10^{-7} M NE. The magnitudes of the responses were expressed as the percentage of the contraction induced 10^{-7} M NE. In the strips with damaged endothelium (open circle; n=9), the contractions were increased with the addition of ACh, while in the strips with intact endothelium (closed circle; n=9) the contractions were decreased in a dose-dependent manner.

Role of calcium on EDR

We examined the change in the magnitude of ACh (10^{-6} M)-induced relaxation by decreasing the extracellular calcium concentration on the strips, which were precontracted with 10^{-7} M of NE (Fig. 4). With the decrease of extracellular calcium ion level the magnitude of relaxation decreased significantly. As the extracellular Ca^{2+} level decreased, the magnitude of contractions of the strips with rubbed endothelium decreased, while that of the strips with intact endothelium did not decrease at 1 and 0.5 mM of

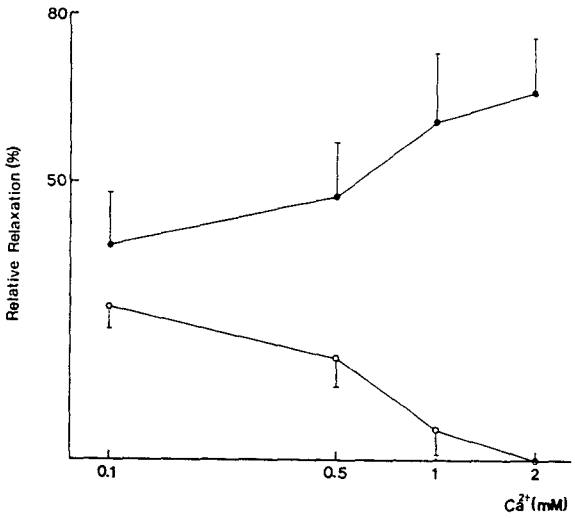

Fig. 4. Effect of extracellular Ca^{2+} on ACh (10^{-6} M)-induced relaxation in the presence of endothelium. The magnitude of relaxation was expressed as the percentage of the contraction induced by NE (10^{-7} M). With the decrease of extracellular Ca^{2+}, the magnitude of relaxation decreased significantly (closed circle; $p<0.05$, $n=9$). Open circles represent the differences between the magnitude of relaxation in extracellular Ca^{2+} concentration, 2 mM and those in the Ca^{2+} concentration, 1, 0.5 and 0.1 mM.

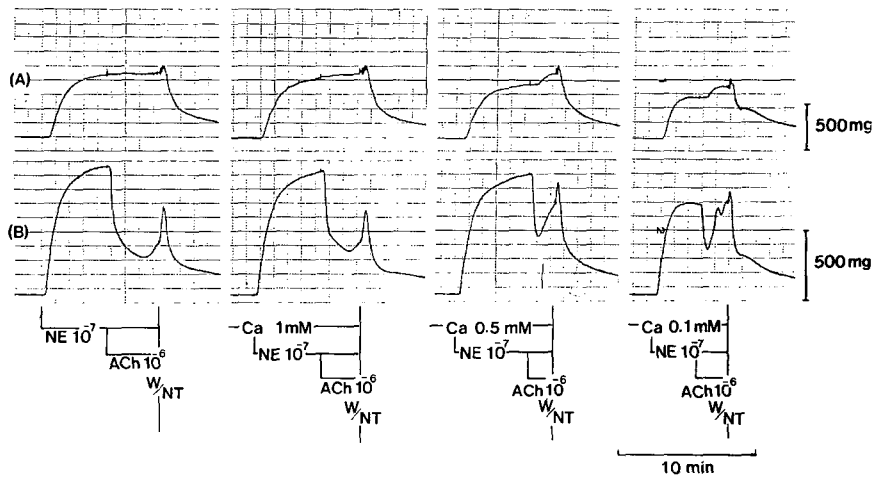

Fig. 5. Effects of low Ca^{2+} concentration on NE-induced contraction and ACh-induced relaxation. The contraction of the strip with damaged endothelium (A) deceased with the decrease of the Ca^{2+} concentration, while the contraction of the strip with intact endothelium (B) did not decrease in 1 and 0.5 mM Ca^{2+} and decreased in 0.1 mM Ca^{2+}. The relaxation induced by ACh showed biphasic pattern; initial rapid relaxation phase followed by subsequent slow relaxation phase. At the concentration of extracellular Ca^{2+}, 0.5 and 0.1 mM, the late slow relaxation disappeared, but the early rapid relaxation was not decreased.

calcium ion concentration (Fig. 5). The relaxation showed biphasic pattern; initially rapid relaxation and subsequently late slow relaxation. At 0.5 and 0.1 mM calcium ion level, the late relaxation phase disappeared, but the early one did not decrease.

The effects of La^{3+} and Co^{2+} on the responses of the strips to ACh and NE were similar to those of the decrease of extracellular Ca^{2+} concentration (Fig. 6, 7). In the presence of La^{3+} or Co^{2+}, however, ACh-induced relaxation was greatly reduced; the late phase was abolised and the early phase decreased to various degrees according to the concentration of

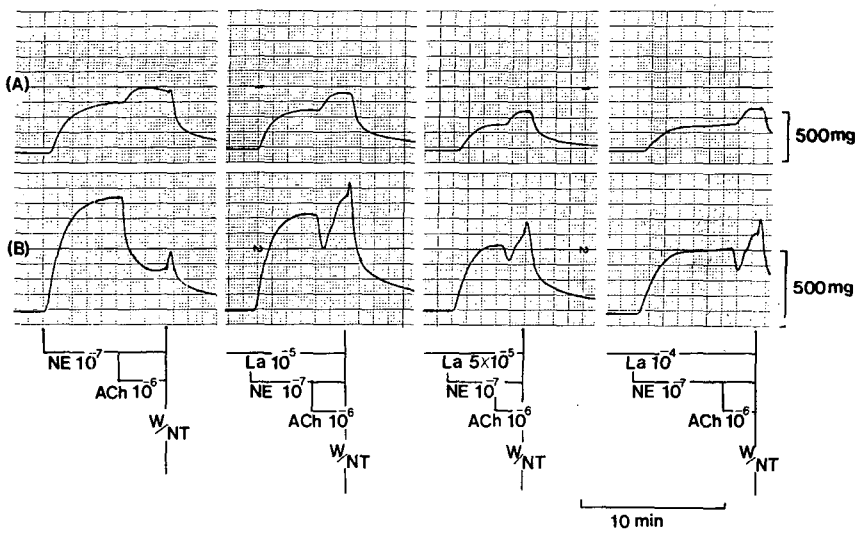

Fig. 6. Effect of La^{3+} on the contraction by NE (10^{-7} M) and relaxation by ACh (10^{-6} M) in the absence (A) or presence of endothelial cells (B). NE-induced contractions of both strips were decreased significantly in the presence of La^{3+} ($p<0.05$, $n=4$). In the presence La^{3+}, ACh-induced relaxations were greatly reduced; the late slow relaxations were abolished and the early rapid relaxations were decreased.

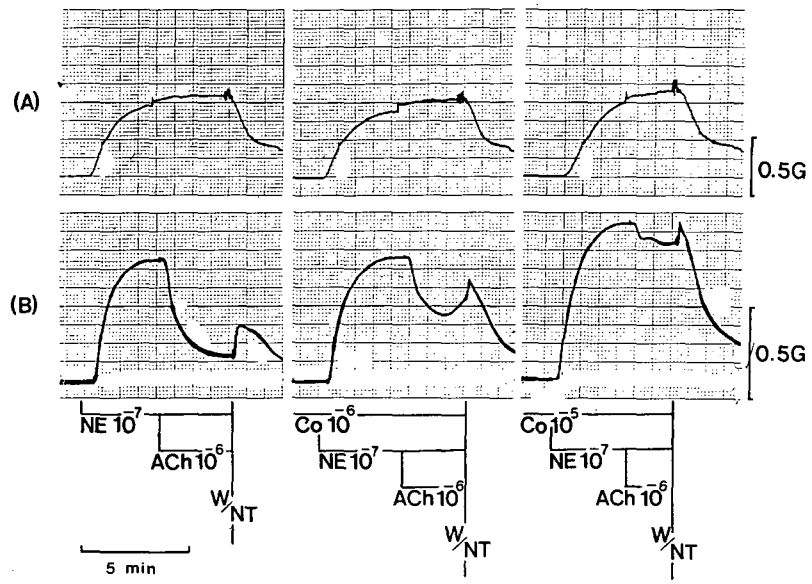

Fig. 7. Effects of Co^{2+} on NE-induced contractions and ACh-induced responses in the absence (A) or presence of endothelial cells (B). In the strips with damaged endothelium, NE-induced contractions were not altered by application of Co^{2+}. In the strips with intact endothelium, however, the magnitudes of the contractions were gradually increased with the increase of Co^{2+} concentration and ACh-induced relaxations were markedly reduced.

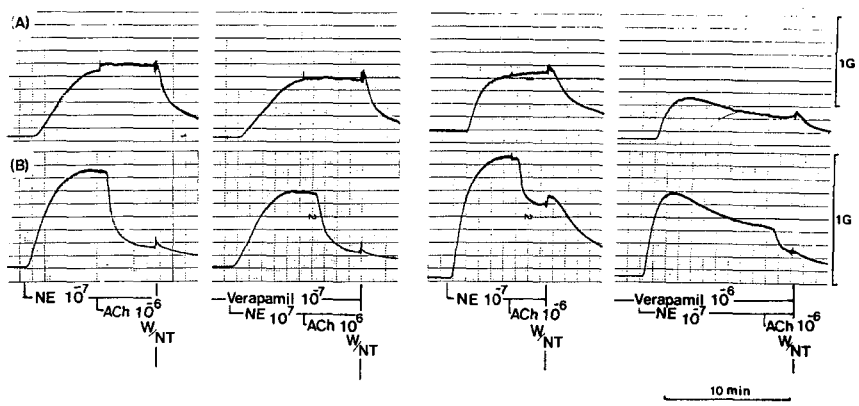

Fig. 8. Effect of verapamil on NE-induced contractions and ACh-induced responses in the absence (A) or presence of endothelial cells (B). In both groups, verapamil suppressed NE-induced contractions. ACh-induced relaxations in the strip with intact endothelium, however, were not changed by verapamil.

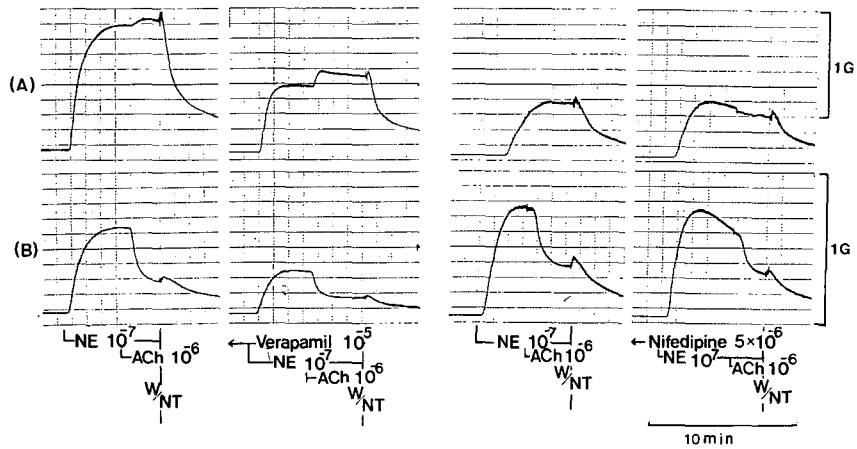

Fig. 9. Effects of verapamil and nifedipine on NE-induced contractions and ACh-induced relaxations in the absence (A) or presence of endothelial cells (B). Nifedipine (5×10^{-6} M) did not reduce NE-induced phasic contraction, but suppressed the tonic contraction. ACh-induced relaxations were not changed by nifedipine.

La^{3+} or Co^{2+}. Moreover, NE-induced contractions of the strips with rubbed endothelium were not altered and those of the strips with intact endothelium increased greatly in the presence of Co^{2+}. In contrast, ACh-induced relaxation was markedly reduced in the same conditions.

Verapamil, nifedipine, Mn^{2+} and Cd^{2+} suppressed NE-induced contractions regardless the existence of endothelium, but ACh-induced relaxations were not influenced by these Ca^{2+}-antagonists (Fig. 8, 9, 10 and 11).

Release of EDRF on resting state

We observed the effect of hemoglobin, which is known to inhibit EDRF, on relaxation elicited by ACh (Fig. 12). Hemoglobin (10^{-5} M), pretreated 5

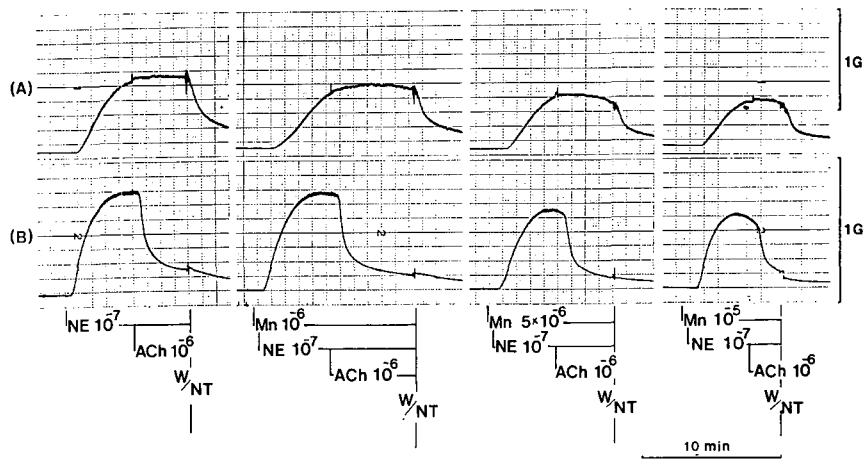

Fig. 10. Effect of Mn^{2+} on NE-induced contractions and ACh-induced relaxations in the absence (A) or presence of endothelial cells (B). The effect of Mn^{2+} was similiar to that of verapamil.

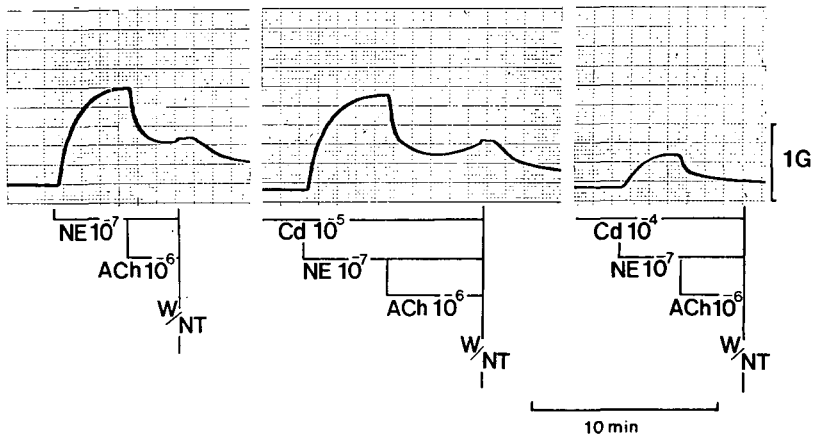

Fig. 11. Effect of Cd^{2+} on NE-induced contractions and ACh-induced relaxations in the strips with intact endothelium. The effect was similiar to that of verapamil.

minutes before the application of NE, induced an increase of basal tension (mean±S.D.=9.25±5.91%, n=4; the magnitude of basal tension increase was expressed as the percentage of the contraction induced by 10^{-7} M NE) and suppressed the ACh-induced relaxation completely. In contrast, hemoglobin had no effects on basal tension of the strips with rubbed endothelium and NE-induced contractions of the strips with intact and rubbed endothelium.

A 10 minute exposure to the solution with depleted Ca^{2+} changed the magnitude of NE-induced contractions. Compared with responses obtained in the same strips under control condition (2 mM of Ca^{2+}, Fig. 13), the magnitudes of contractions decreased significantly in the strips with rubbed endothelium. In the strips with intact endothelium the magnitudes were not changed significantly at 1 and 0.5 mM of Ca^{2+} level and decreased significantly at 0.1 mM of Ca^{2+}

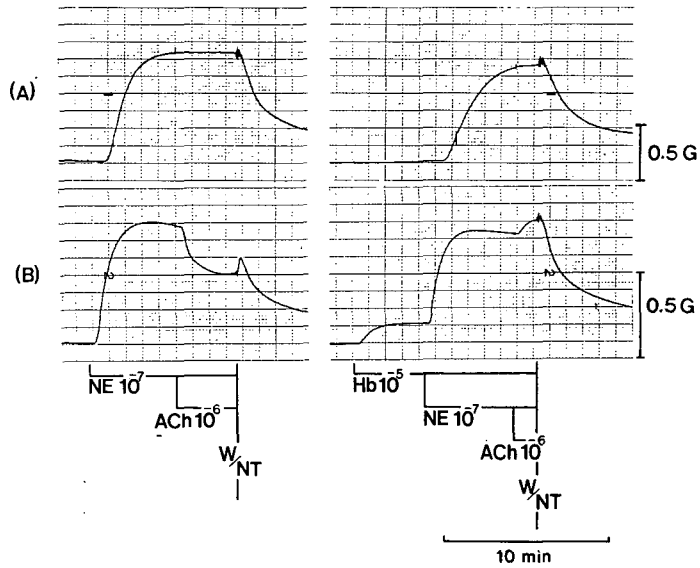

Fig. 12. Effect of hemoglobin (Hb) on basal tension and ACh-induced response in the absence (A) or presence of endothelial cells (B). In the strips with intact endothelium, the basal tensions were increased (mean±S.D.=9.25±5.91%, n=4; The magnitude of basal tension increase was expressed as the percentage of the contraction inducecd by 10^{-7} M NE)

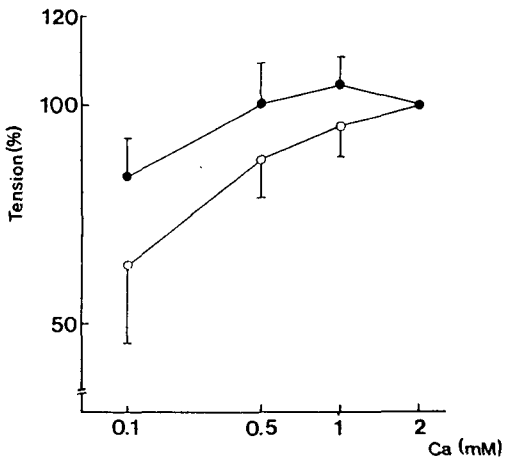

Fig. 13. Effect of extracellular Ca^{2+} on NE-induced contractions in the presence (closed circle) or absence of endothelial cells (open circle). The magnitudes of NE (10^{-7} M)-induced contractions at 0.1, 0.5 and 1 mM Ca^{2+} were expressed as the percentage of the contraction at 2 mM Ca^{2+}.

($p<0.05$, n=9). In addition there was significant difference between the two curves ($p<0.05$, n=9). We suppose that the difference may be due to compromise of the decrease of contraction with reduced EDRF release at low Ca^{2+} condition in the strips with intact endothelium.

DISCUSSION

The contraction induced by stimulation of α-receptors is divided into initial phasic contraction and late tonic contraction in rabbit aorta; intracellular Ca^{2+} appears to be utilized mainly in phasic contraction and extracellular Ca^{2+} seems to be utilized mainly in tonic contraction (Cony et al, 1984). With gradual depletion of extracellular Ca^{2+} and by application of Ca^{2+}-antagonists diminished not only NE-induced phasic contraction but also tonic one (Fig. 5). This indicates that extracellular Ca^{2+} would be involved in both phasic and tonic contractions.

EDR was divided into two phases; initial rapid relaxation phase followed by late slow relaxation one. The late relaxation phase is greatly impaired with extracellular Ca^{2+} depletion, while the initial phase is not or slightly impaired (Fig. 5). This fact indicates that extracellular Ca^{2+} would be required for EDR, especially in the late phase. From this result we suggest that extracellular Ca^{2+} may be necessary for the continuous production of EDRF. There was a slightly different finding from that by extracellular Ca^{2+} depletion (Fig. 6, 7). It has been known that La^{3+} inhibits not only Ca^{2+} influx but also the efflux (van Breeman et al, 1972), and that the sequence of blocking effectiveness in barnacle muscle is $La^{3+} > Co^{2+} > Mn^{2+}$ (Hille, 1984). In present study the sequence of effectiveness to inhibit EDR is $Co^{2+} > La^{3+}$ > extracellular Ca^{2+} depletion, and Mn^{2+} has no effect on EDR. Moreover, La^{3+} did not suppress NE-induced contraction and Co^{2+} increased the contraction. The mechanisms by which these results occurred are not clear at present. We only suppose that La^{3+} and Co^{2+} may inhibit EDR by some other mechanisms except Ca^{2+} entry blocking effect. However, there are reports that the greater affinity for phosphate and carboxyl groups of La^{3+} than that of Ca^{2+} is ideal for the displacement of extracellular bound Ca^{2+} (van Breeman et al, 1972) and that EDRF is a compound with carbonyl group (Griffith et al, 1984). If these reports were valid, La^{3+} reacts with EDRF and then inactivate it. Rees et al (1989) reported L-arginine is a source of EDRF. From these reports and data, we suppose that Co^{2+} and La^{3+} inactivate EDRF by reacting with amino group and carboxyl group and that EDRF is a compound with amino and carboxyl group, probably amino acid. We think that further study is necessary to confirm this suggestion.

Ca^{2+}-antagonists had no effect on EDR (Fig. 8, 9 10 and 11). Peach et al. reported that preincubation for sufficient time (more than 30 min) was necessary to suppress EDR with Ca^{2+}-antagonists. But we failed to suppress EDR induced by acetylcholine in spite of the sufficient preincubation time (30 min) with Ca^{2+}-antagonists. Our result is consistent with those of Jayakody et al. (1987) and Vanhoutte (1988).

In the present experiments, hemoglobin elevates basal tension of the strips with intact endothelium without modifying the contracting response to NE, while it does not change that of the strips with rubbed endothelium. As hemoglobin inhibits EDR by binding to EDRF (Martin et al, 1985), we suppose such an effect would be due to inactivation of EDRF released spontaneously at rest by binding to hemoglobin. We also suppose that the significant difference between the contraction curve to NE of the strip with intact endothelium and that of the strip with rubbed endothelium may be due to compromise of the decrease of contraction to NE with the reduced EDRF release at rest in reduced extracellular Ca^{2+} concentration in the strips with intact endothelium.

Removal of endothelial cells shifted the NE concentration-responce curve to the left, compared with that of the strips with intact endothelium. Cocks and Angus (1988) suggested that this finding was due to releasing EDRF from endothelial cells by NE. However, Furchgott (1988) reported that removal of endothelial cells did not interfere with the sensitivity of preparations of rabbit aorta to contracting agents (e.g. NE) if care was taken not to overstretch the smooth muscle during the rubbing procedure. In addition, EDRF can interact with NE (Rubanyi et al, 1985).

Through this experiment we conclude that extracellular Ca^{2+} would be necessary for ACh-induced slow relaxation but Ca^{2+}-antagonists would have no effect on the EDR.

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국문초록 =

토끼 대동맥 평활근의 내피세포 의존성 이완에 미치는 Ca^{2+} 및 Ca^{2+} 길항제의 효과

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토끼 흉부 대동맥을 이용하여 내피세포 의존성 혈관이완에 대한 세포외 Ca^{2+}과 여러가지 Ca^{2+} 길항제의 효과를 분석하여 EDRF의 작용기전을 밝혀 보고자 하였다. 대동맥 횡단 절편의 등장성 수축은 10^{-7} M 노에피네프린으로 유발시켰으며, 10^{-6} M 사세틸콜린으로 내피세포 의존성 혈관이완을 일으켰다. 내피세포는 작은 솜뭉치로 부드럽게 문질러서 제거하였으며, hemolysate를 사용하여 EDRF에 대한 헤모글로빈의 효과를 관찰하였다.

결과를 종합하면 다음과 같다.

1) 아세틸콜린에 의한 내피세포 의존성 혈관이완은 두 시기, 즉 초기급속이완기와 후기완만이완기로 나타났다.

2) 세포외 Ca^{2+}을 낮추면, 아세틸콜린에 의한 내피세포 의존성 혈관이완이 감소하였으며, 특히 후기완만이완기가 감소하였다.

3) Verapamil, nifedipine, Mn^{2+} 및 Cd^{2+}은 내피세포 의존성 혈관이완에 영향이 없었던 반면 La^{3+}와 Co^{2+}는 억제시켰다.

4) 헤모글로빈을 투여하면 내피세포가 없는 절편에서는 기초긴장도의 변화가 없었으나 내피세포가 있는 절편에서는 기초긴장도가 증가하였고 아세틸콜린에 의한 내피세포 의존성 혈관이완도 완전히 억제되었다.

이상의 결과로부터 세포외 Ca^{2+}은 주로 후기완만이완기에 작용하며 이때 사용되는 Ca^{2+} 유입통로는 Ca^{2+} 길항제로 억제되지 않는 것으로 결론지을 수 있다.