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Agonist-dependent difference in the relationship between cytosolic Ca^{2+} level and release of vascular relaxing factors in the endothelium of rabbit aortic valve

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Abstract

The correlation between changes in cytosolic Ca^{2+} levels ($[Ca^{2+}]_i$) and the release of vascular relaxing factor(s) was investigated in the endothelium of rabbit aortic valve. ATP, carbachol and thapsigargin increased endothelial $[Ca^{2+}]_i$ in rabbit aortic valve loaded with a leakage resistant, fluorescent Ca^{2+} indicator, fura-PE3. Release of relaxing factors was bioassayed using the 'sandwich' preparation in which contraction was measured in the endothelium-denuded rabbit aorta attached to the endothelial surface of the valve. Addition of ATP, carbachol and thapsigargin induced sustained relaxation of the phenylephrine-induced contraction of the aorta in the 'sandwich' preparation. N^G-monomethyl-L-arginine (L-NMMA) greatly attenuated the relaxation induced by carbachol, and combined treatment with tetra-*n*-butylammonium completely inhibited the relaxation. These results suggest that the endothelial relaxing factors released from aortic valve are nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF). When the increase in endothelial $[Ca^{2+}]_i$ was plotted against the relaxation, the carbachol-induced increase in $[Ca^{2+}]_i$ elicited greater relaxation than did ATP or thapsigargin at a given $[Ca^{2+}]_i$. This suggests that various agonists differently modulate the relationship between $[Ca^{2+}]_i$ and release of NO.

Keywords: ATP; Thapsigargin; Carbachol; Endothelium; EDRF (endothelium-derived relaxing factor); Ca^{2+} level; Cytosolic

1. Introduction

In the cardiovascular system, endothelial cells play important roles in the modulation of vascular contractility by releasing bioactive agents including nitric oxide (NO), endothelium-derived hyperpolarizing factor (EDHF) and prostaglandins. It has been reported that these agents are produced by Ca^{2+}-dependent mechanisms (Suttorp et al., 1985; Busse and Mülsch, 1990; Chen and Suzuki, 1990). Sato et al. (1990) showed that there is a positive correlation between the increase in cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) and release of NO (EDRF) in the rat aortic endothelium stimulated by carbachol, supporting the hypothesis that the activation of endothelial NO synthase is Ca^{2+}-dependent (Busse and Mülsch, 1990). Recently, however, it was shown that shear stress can increase NO production without an increase in $[Ca^{2+}]_i$ in bovine aortic endothelial cells (Corson et al., 1996). It has been shown that, in smooth muscle, the correlation between $[Ca^{2+}]_i$ and contraction varies depending on the type of contractile agonists because some agonists change the Ca^{2+} sensitivity of the contractile mechanism (Karaki et al., 1997). These results suggest the possibility that the coupling between $[Ca^{2+}]_i$ and NO release is different depending on the type of stimulation in endothelial cells. As the purpose of the present study was to examine this possibility, we measured the relationship between the changes in $[Ca^{2+}]_i$ and the release of vascular relaxing factors, using rabbit aortic valve stimulated by different types of endothelial agonists.

2. Methods

The experiments were carried out in accordance with the Guide for the Care and Use of Laboratory Animals as

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promulgated and adopted by the Graduate School of Agriculture and Life Sciences, The University of Tokyo.

2.1. Preparations and measurement of $[Ca^{2+}]_i$

Preparation of rabbit aortic valve and measurement of $[Ca^{2+}]_i$ in the endothelium have been described previously (Amano et al., 1994). Briefly, male Japanese white rabbits were killed by a sharp blow on the neck and exsanguination. Three aortic valves were removed from one rabbit heart and incubated with 5 μM acetoxymethyl ester of fura-PE3 (leakage resistant, Ca^{2+} fluorescent dye; Vorndran et al., 1995) and 0.02% cremophor EL for 4–6 h at room temperature.

An aortic valve loaded with fura-PE3 was held horizontally in a temperature-controlled organ bath filled with normal physiological salt solution (PSS) which contained NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.0, $NaHCO_3$ 23.8 and glucose 5.5 and aerated with 95% O_2–5% CO_2. EDTA 0.01 (mM) was also added to remove possibly contaminating heavy metals from the solution. Experiments were performed with a bio-fluorimeter (CAF-100, 102 or 110, Japan Spectroscopic, Tokyo, Japan), and the ratio of 500 nm fluorescence excited at 340 nm (F340) to that excited at 380 nm (F380) (R340/380) was used as indicator of $[Ca^{2+}]_i$. The resting $[Ca^{2+}]_i$ in normal PSS and the peak $[Ca^{2+}]_i$ in the presence of 10 μM ATP were taken as 0% and 100%, respectively. Experiments were performed at 37°C and 25°C. Since the relative effects of ATP and carbachol or thapsigargin were not affected by the change in temperature (see Section 3), since the results obtained at 25°C were more stable than those obtained at 37°C, and since leakage of dye was much less at the lower temperature, the results obtained at 25°C are presented unless otherwise stated.

2.2. Bioassay to detect endothelial vascular relaxing factors

The thoracic aorta of rabbits, killed as in Section 2.1, was isolated and cut into approximately 2 mm wide and 8 mm long strips. The vascular endothelium was removed by gently rubbing with a finger moistened with normal PSS.

The strips were placed in normal PSS. Muscle tension was recorded isometrically with a force-displacement transducer (Orientec, Tokyo, Japan). Each muscle strip was attached to a holder under a resting tension of 10 mN and was equilibrated for about 120 min until the contractile response to 72.7 mM KCl, which was made by replacing NaCl with equimolar KCl in normal PSS, became stable. Then each preparation was contracted with phenylephrine (1 μM) and complete removal of endothelium was confirmed by the absence of a carbachol (1 μM)-induced relaxation.

Each strip was then removed from holders. The aortic valve was attached to the luminal surface of the strips, and the preparation was attached to the holders and suspended in an organ bath, again under a resting tension of 10 mN. Equilibration was for approximately 120 min until the contractile response to 72.7 mM KCl became stable. Then each preparation was contracted with phenylephrine (1 μM) and checked for carbachol (1 μM)-induced relaxation to verify the functional integrity of the endothelium of the aortic valve. The maximum relaxation thus obtained was taken as 100%, and the results of the experiments were expressed as percentages of this relaxation. Sixty minutes after wash-out of phenylephrine and carbachol, some preparations were again contracted with phenylephrine (1 μM) and a relaxant such as carbachol (1 μM), ATP (10 μM) or thapsigargin (1 μM) was added. Then, 100 μM N^G-monomethyl-L-arginine (L-NMMA) was added to determine if the relaxation induced by these agents had recovered. Fifty minutes after the wash-out, some preparations were treated with L-NMMA (100 μM) and/or tetra-*n*-butylammonium (1 mM) for 10 min and the relaxants were then added. All the experiments were performed at 37°C.

2.3. Chemicals

ATP was purchased from Yamasa Shoyu (Tokyo, Japan). Other chemicals used were L-NMMA (Wako, Osaka, Japan), carbamylcholine chloride (carbachol), phenylephrine, thapsigargin (Sigma, St. Louis, MO, USA), tetra-*n*-butylammonium chloride (Tokyo Kasei, Tokyo, Japan), cremophor EL (Nacalai Chemicals, Kyoto, Japan) and fura-PE3/AM (TEFLABS, Austin, TX, USA).

2.4. Statistics

The results of the experiments are expressed as means ± S.E. Analysis of variance (ANOVA; Scheffe's test) was used for analysis of the results.

3. Results

3.1. Effects of ATP, carbachol and thapsigargin on endothelial $[Ca^{2+}]_i$ of rabbit aortic valve

In rabbit aortic valves loaded with fura-PE3, ATP (10 μM) induced a large transient increase followed by a small sustained increase in $[Ca^{2+}]_i$ (32.0 ± 1.8% of peak $[Ca^{2+}]_i$, measured 10 min after the addition: $n = 16$) (Fig. 1A). Carbachol (1 μM) also induced a transient increase followed by a sustained increase in $[Ca^{2+}]_i$. Both changes were smaller than those induced by 10 μM ATP (39.0 ± 4.9% at peak level and 8.0 ± 2.7% at 10 min, $n = 4$) (Fig. 1B). Thapsigargin (1 μM) induced a slow increase in $[Ca^{2+}]_i$ (147 ± 28.1% at peak level and 130.6 ± 30.6% at 10 min, $n = 4$) (Fig. 1C). Phenylephrine (1 μM), an α_1-adrenoceptor agonist, induced a small transient increase in $[Ca^{2+}]_i$ (11.9 ± 2.0% at peak level, $n = 4$). The increases in $[Ca^{2+}]_i$ induced by ATP, carbachol, thapsigar-

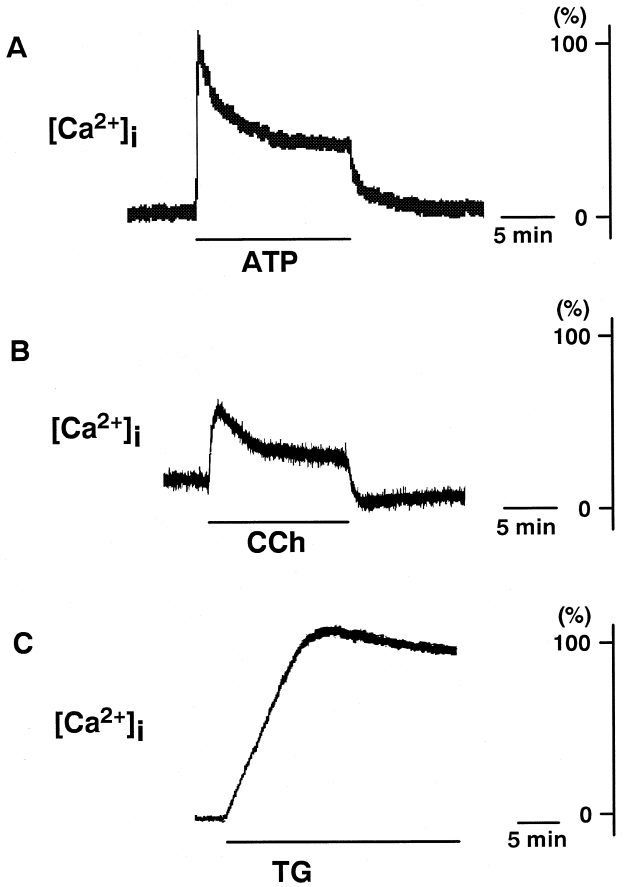

Fig. 1. Typical recordings of the increase in endothelial $[Ca^{2+}]_i$ of rabbit aortic valve induced by 10 μM ATP, 1 μM carbachol (CCh) and 1 μM thapsigargin (TG). Changes in $[Ca^{2+}]_i$ were monitored by measuring the fura-PE3 fluorescence ratio (R340/380). The maximum response to 10 μM ATP, obtained before starting the experiment, was taken as 100% and the resting $[Ca^{2+}]_i$ level as 0%.

gin or phenylephrine were abolished when the surface of the valve was rubbed with cotton swabs to remove the endothelium ($n = 2$, each). To find if the change in temperature affects the effects of agonists, we also measured the effects at 37°C. The increase in $[Ca^{2+}]_i$ induced by 1 μM carbachol at 37°C was 42.6 ± 11.2% at peak level and 17.7 ± 6.6% at 10 min of the maximum increase in $[Ca^{2+}]_i$ induced by 10 μM ATP ($n = 3$). The increase in $[Ca^{2+}]_i$ induced by 1 μM thapsigargin was 104.7 ± 22.1% at peak level and 88.6 ± 13.3% at 10 min of the maximum increase induced by 10 μM ATP ($n = 3$). These values were not significantly different from those obtained at 25°C, indicating that the relative effects of ATP, carbachol and thapsigargin were not affected by the change in temperature.

3.2. Release of endothelium-derived relaxing factors induced by ATP, carbachol and thapsigargin

Rabbit aorta without endothelium was attached to an aortic valve and precontracted with phenylephrine (1 μM). Addition of ATP (10 μM) induced a sustained relaxation of the aorta (44.8 ± 0.7%, measured 10 min after addition, $n = 4$) (Fig. 2A). When an aortic valve was not attached to the aorta, ATP (10 μM) had no effect (13 in 26 preparations) or induced only a small sustained decrease of the phenylephrine-induced contraction (12.3 ± 2.1% in 13 out of 26 preparations).

In the aorta attached to an aortic valve and precontracted with phenylephrine (1 μM), carbachol (1 μM) induced a sustained relaxation (96.2 ± 2.3%, measured 10 min after addition, $n = 4$) (Fig. 2B). When an aortic valve was not attached to the aorta, carbachol (1 μM) had no

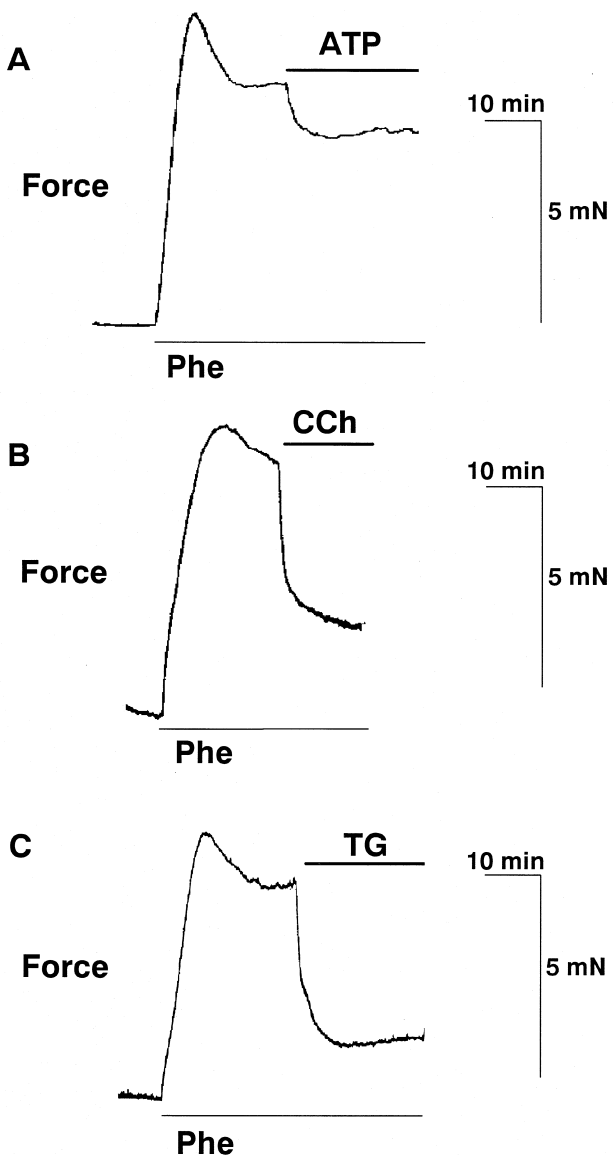

Fig. 2. Typical recordings of the relaxations induced by 10 μM ATP (A), 1 μM carbachol (B) and 1 μM thapsigargin(C) in endothelium-denuded aorta attached to the valve as a donor of vascular relaxing factor(s). Rabbit aorta had been precontracted with 1 μM phenylephrine (Phe) before the addition of ATP, carbachol or thapsigargin.

effect (91 in 114 preparations) or induced only a small sustained increase of the phenylephrine-induced contraction (6.3 ± 0.7% in 23 out of 114 preparations).

In the aorta attached to an aortic valve and precontracted with phenylephrine (1 μM), thapsigargin (1 μM) induced a sustained relaxation (80.4 ± 15.2%, measured 10 min after addition, $n = 4$) (Fig. 2C). In an aorta which was not attached to an aortic valve, thapsigargin (1 μM) had no effect (13 in 16 preparations) or induced a small decrease of the phenylephrine-induced contraction (13.5 ± 4.2% in 3 out of 16 preparations).

After relaxation had been induced with carbachol, ATP or thapsigargin, the addition of L-NMMA (100 μM), an inhibitor of NO synthase, increased muscle tension to a level higher than that before the addition of the relaxant (carbachol: 9.3 ± 2.0%;ATP: 11.2 ± 1.3%; thapsigargin: 20.8 ± 6.6% of 1 μM phenylephrine-induced contraction; $n = 5–10$). A higher concentration of L-NMMA (200 μM) induced no additive effect ($n = 3$ each). In the absence of an aortic valve, addition of L-NMMA (100 μM) had no effect. In the aorta attached to an aortic valve and precontracted with phenylephrine (1 μM), L-NMMA (100 μM) induced a small increase in muscle tension (about 19% of 1 μM phenylephrine-induced contraction, $n = 2$). In the absence of aortic valve, in contrast, L-NMMA (100 μM) had no effect ($n = 3$).

To examine whether the presence of ATP, carbachol or thapsigargin affects the NO-mediated vascular relaxation, we compared the concentration–response relationship for the inhibitory effect of sodium nitroprusside. It was found that ATP, carbachol and thapsigargin had no effect on muscle relaxation induced by sodium nitroprusside in rabbit aorta precontracted with 1 μM phenylephrine ($n = 3–4$, data not shown).

3.3. Relationship between the increase in endothelial $[Ca^{2+}]_i$ and relaxation of aorta

Fig. 3 shows the time courses of the increase in endothelial $[Ca^{2+}]_i$ of aortic valve and relaxation of aorta induced by ATP (10 μM), carbachol (1 μM) or thapsigargin (1 μM). The increase in endothelial $[Ca^{2+}]_i$ induced by ATP and carbachol reached a maximum within 1 min, followed by a gradual decrease to the respective sustained levels. In response to the increase in endothelial $[Ca^{2+}]_i$, muscle force stimulated by phenylephrine was decreased in a time-dependent manner and reached its maximum after about 10 min (see also Fig. 2). The maximum decrease in muscle force induced by carbachol was much greater (about 220%) than that induced by ATP, although the increase in $[Ca^{2+}]_i$ induced by carbachol was only about 40% of that induced by ATP (Fig. 3A,B). The time course of the increase in endothelial $[Ca^{2+}]_i$ induced by thapsigargin, in contrast, correlated well with that of the decrease in muscle force (Fig. 3C). L-NMMA (100 μM) and phenylephrine (1 μM) had no effect on the carbachol-, ATP- and thapsigargin-induced increase in $[Ca^{2+}]_i$.

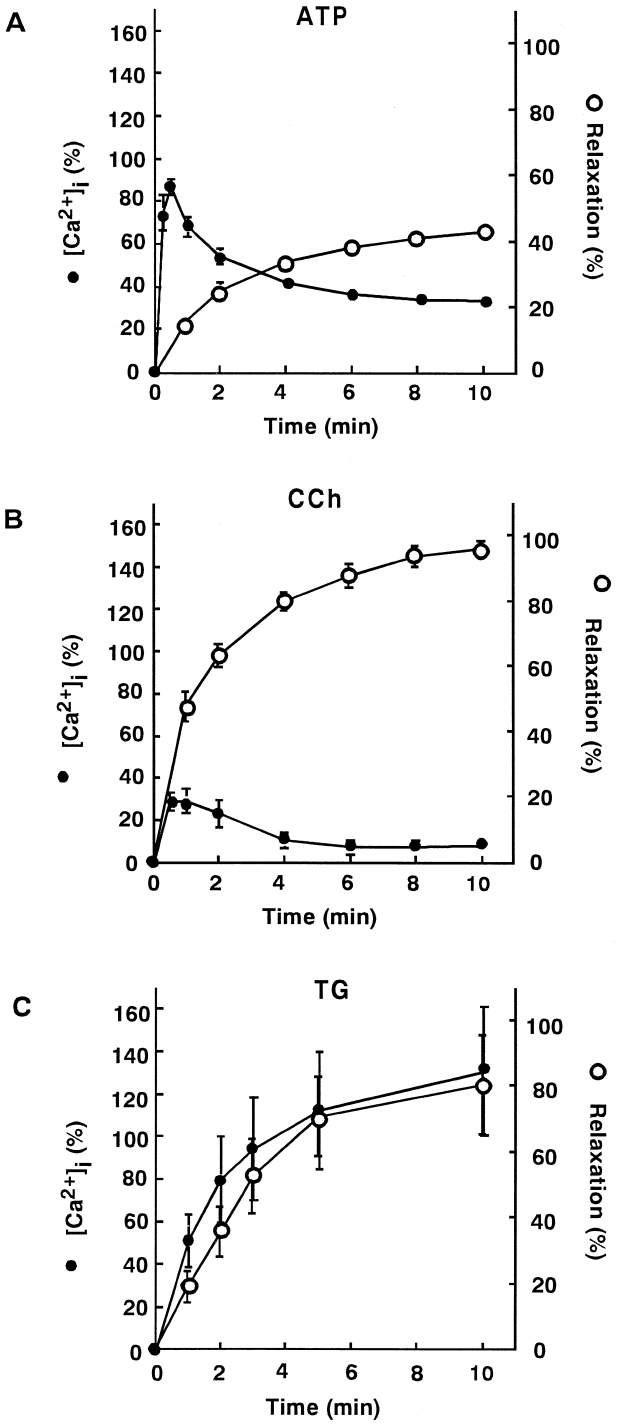

Fig. 3. Time courses of the increase in $[Ca^{2+}]_i$ and the muscle relaxation induced by 10 μM ATP (A), 1 μM carbachol (B) and 1 μM thapsigargin (C). Changes in $[Ca^{2+}]_i$ were monitored by measuring the fura-PE3 fluorescence ratio (R340/380). The maximum response to 10 μM ATP, obtained before starting the experiment, was taken as 100% and the resting $[Ca^{2+}]_i$ level as 0%. Relaxation is expressed as percentage of the maximum relaxation induced by 1 μM carbachol, obtained before starting the experiment. Each point represents the mean ± S.E. ($n = 4$).

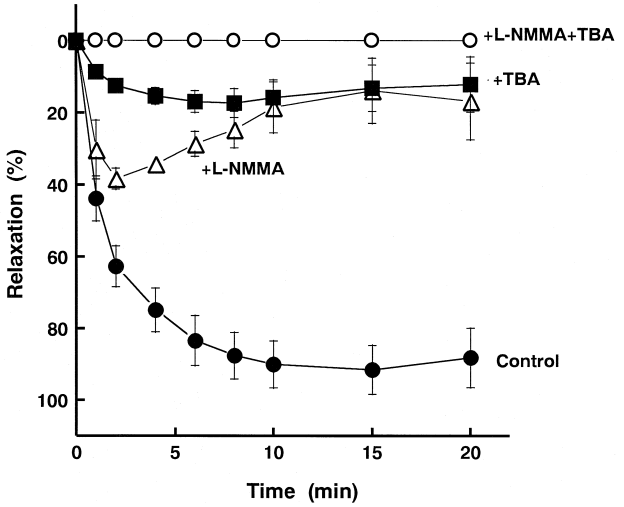

Fig. 4. The effects of L-NMMA and tetra-*n*-butylammonium (TBA) on the muscle relaxation induced by 1 μM carbachol in endothelium-denuded aorta attached to the valve. Rabbit aorta had been precontracted with 1 μM phenylephrine. Each value is expressed as percentage of the maximum relaxation induced by 1 μM carbachol, obtained before starting the experiment. Each point represents the mean ± S.E. ($n = 4$).

3.4. Effects of L-NMMA and tetra-n-butylammonium on aortic relaxation induced by carbachol

Since carbachol, unlike ATP and thapsigargin, did not cause relaxation in the endothelium-denuded rabbit aorta without aortic valve, we further examined the mechanisms of the endothelium-dependent relaxation caused by carbachol.

As shown in Fig. 4, pretreatment with L-NMMA (100 μM) largely (60–80%) inhibited the relaxation induced by carbachol (1 μM). The combined treatment with L-NMMA (100 μM) and tetra-*n*-butylammonium (1 mM), a non-selective K^+ channel inhibitor, completely inhibited that relaxation. On the other hand, in the aorta without endothelium ($n = 4$), L-NMMA (100 μM) alone or in combination with tetra-*n*-butylammonium (1 mM) had no effect on the muscle tension induced by phenylephrine (1 μM).

4. Discussion

Carbachol (1 μM), ATP (10 μM) and thapsigargin (1 μM) increased the endothelial $[Ca^{2+}]_i$ of rabbit aortic valve and caused relaxation in the phenylephrine-contracted rabbit aorta attached to an aortic valve. The increases in $[Ca^{2+}]_i$ were abolished when the surface of the valve was rubbed with cotton swabs to remove endothelial cells, suggesting that the fluorescent signal obtained from aortic valve is contributed exclusively to endothelial cells. Endothelial cells can be identified by their specific uptake of fluorescent acetylated low-density lipoprotein (DiI-Ac-LDL) (Netland et al., 1985). Morphological study has shown that fura-2 and DiI-Ac-LDL fluorescence overlap each other in porcine aortic valve (Aoki et al., 1994), confirming the suggestion that the cellular component which contributed to $[Ca^{2+}]_i$ signal should be exclusively endothelial cells.

After relaxation was induced with carbachol, ATP or thapsigargin, the addition of 100 μM L-NMMA increased muscle tension to a level higher than that before the addition of these relaxants. These results suggest that NO is the major factor released from the endothelium of aortic valve. Not only these relaxants, but phenylephrine also, induced a small transient increase in $[Ca^{2+}]_i$. Furthermore, L-NMMA (100 μM) augmented the phenylephrine-induced contraction in the aorta attached to an aortic valve. Thus, it seems likely that the endothelium of aortic valve has α_1-adrenoceptors, stimulation of which increases the release of NO. L-NMMA restored muscle tension to a level higher than that before the addition of carbachol, ATP or thapsigargin, possibly because L-NMMA also antagonized the relaxant effect of phenylephrine. Another possibility is that there is a continuous, spontaneous release of NO which is inhibited by L-NMMA.

In some preparations, ATP (10 μM) or thapsigargin (1 μM) induced a slight relaxation in the aorta in the absence of aortic valve. This relaxation was not inhibited by L-NMMA (100 μM). It has been reported that ATP acts directly on smooth muscle to induce relaxation in rabbit aorta denuded of endothelium via stimulation of the P_3 purinoceptor (Chinellato et al., 1992, 1994). This mechanism may be responsible for the relaxation induced by ATP in rabbit aorta denuded of endothelium. Simultaneous addition of cyclopiazonic acid, an inhibitor of Ca^{2+}-ATPase on endoplasmic reticulum, and 1,4-dihydro-2,6-dimethyl-5-nitro-4-[2-(trifluoromethyl) phenyl]-3-pyridinecarboxilic acid methyl ester (Bay K 8644), an activator of L-type Ca^{2+} channels, has been shown to induce relaxation in rabbit aorta without endothelium and precontracted with phenylephrine and this effect has been attributed to the activation of Ca^{2+}-dependent K^+ channels as a result of Ca^{2+} overload (Omote and Mizusawa, 1996). Activation of Ca^{2+}-dependent K^+ channel may be responsible for the relaxation induced by thapsigargin in rabbit aorta denuded of endothelium.

As shown in Fig. 4, although the relaxation induced by carbachol (1 μM) in endothelium-denuded rabbit aorta in the presence of aortic valve was completely inhibited by pretreatment with 100 μM L-NMMA in the sustained phase, the initial transient relaxation was resistant to L-NMMA. However, the transient relaxation was completely inhibited by the combination of L-NMMA and tetra-*n*-butylammonium or by the latter alone. These results suggest that the release of EDHF may also be partly responsible for the carbachol-induced relaxation in the initial phase in endothelium-denuded rabbit aorta attached to an aortic valve.

As shown in Fig. 3, the time course of the increase in endothelial $[Ca^{2+}]_i$ induced by thapsigargin (1 μM) corre-

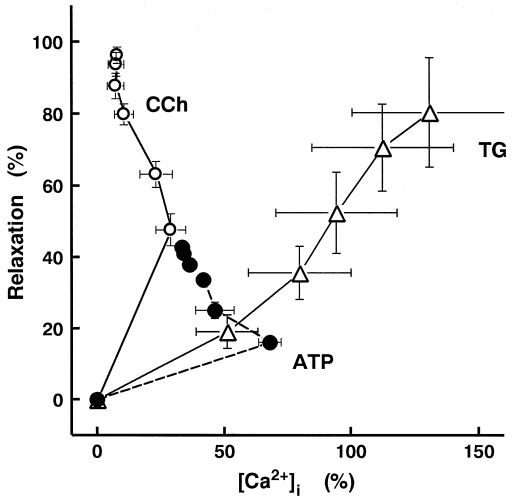

Fig. 5. Relationships between the increase in $[Ca^{2+}]_i$ and the muscle relaxation induced by 10 μM ATP, 1 μM carbachol (CCh) and 1 μM thapsigargin (TG). Data were obtained from Fig. 3. Each point represents the mean ± S.E. ($n = 4$).

lated well with that of the decrease in muscle force. With ATP (10 μM), the increase in $[Ca^{2+}]_i$ was greater than the relaxation at initial phase, but the relaxation was greater than the increase in $[Ca^{2+}]_i$ in the sustained phase. With carbachol (1 μM), a greater relaxation was observed with only a small increase in $[Ca^{2+}]_i$. As shown in Fig. 5, when endothelial $[Ca^{2+}]_i$ was plotted against relaxation, different $[Ca^{2+}]_i$–relaxation relationships were obtained with these agents.

In endothelial cells, the transient component of the increase in $[Ca^{2+}]_i$ induced by receptor agonists reflects the release of Ca^{2+} from internal stores due to an increase in inositol 1,4,5-trisphosphate (IP_3), whereas the sustained component reflects the influx of Ca^{2+} from the external space (Adams et al., 1989). Thapsigargin induces IP_3-independent mobilization of the intracellular Ca^{2+} pool and the influx of Ca^{2+} from the external space (Dolor et al., 1992). It has been reported that receptor agonists and inhibitors of Ca^{2+}-ATPase in the intracellular Ca^{2+} pool activate the same Ca^{2+} influx pathway (Schilling et al., 1992; Thuringeer and Sauvé, 1992; Vaca and Kunze, 1994, 1995; Amano et al., 1997), that is the capacitative Ca^{2+} entry pathway (Putney, 1986). Since $[Ca^{2+}]_i$–relaxation relationships obtained with ATP and thapsigargin are not different in the initial phase, the different mechanisms of mobilization of the intracellular Ca^{2+} pool induced by receptor agonists and thapsigargin may not affect the $[Ca^{2+}]_i$–relaxation relationships. It is possible that differences in the signal transduction mechanisms following receptor activation may be responsible for the difference between the $[Ca^{2+}]_i$–relaxation relationships obtained with receptor agonists and with thapsigargin.

It has been reported that shear stress can increase NO production without any increase in $[Ca^{2+}]_i$ in bovine aortic endothelial cells (Corson et al., 1996). Besides Ca^{2+}-dependent activation of endothelial NO synthase, there may be a Ca^{2+}-independent activity of endothelial NO synthase and/or a change in Ca^{2+} sensitivity of the enzyme in the endothelium of rabbit aortic valve. Venema et al. (1996) recently reported that there is an endothelial NO synthase-associated protein in endothelial cells, and that bradykinin-induced tyrosine phosphorylation of this protein accelerates the translocation of endothelial NO synthase, which results in the activation of endothelial NO synthase. It has also been reported that endothelial NO synthase activation by bradykinin, histamine or ATP produces a transient increase in the amounts of detergent-insoluble caveolin-1, an endogenous endothelial NO synthase-inhibiting protein, and that the agonist-induced activation of endothelial NO synthase appears to involve tyrosine phosphorylation-dependent changes in the interaction of endothelial NO synthase with caveorin-1 (Venema et al., 1997). Thus, it is possible that tyrosine phosphorylation of endothelial NO synthase and/or an other protein such as caveorin-1 may participate in the Ca^{2+}-independent activity of endothelial NO synthase and/or in the change in Ca^{2+} sensitivity of the enzyme, and that these mechanism(s) may be activated by receptor activation.

In conclusion, we found that the endothelium-derived relaxing factor(s) released from aortic valve is mainly NO, and that ATP, carbachol and thapsigargin increased endothelial $[Ca^{2+}]_i$ and released NO although the relationships between $[Ca^{2+}]_i$ and NO release were different, depending on the type of agonists, possibly because of the changes in Ca^{2+} sensitivity of endothelial NO synthase and/or the Ca^{2+}-independent endothelial NO synthase activity.

Acknowledgements

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