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Mechanism of Acetylcholine-induced Endothelium-dependent Relaxation in the Rabbit Carotid Artery by M3-receptor Activation

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The present study were designed to characterize the action mechanisms of acetylcholine (ACh)-induced endothelium-dependent relaxation in arteries precontracted with high K^+ (70 mM). For this, we simultaneously measured both muscle tension and cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$), using fura-2, in endothelium-intact, rabbit carotid arterial strips. In the artery with endothelium, high K^+ increased both $[Ca^{2+}]_i$ and muscle tension whereas ACh (10 μM) significantly relaxed the muscle and increased $[Ca^{2+}]_i$. In the presence of N^G-nitro-L-arginine (L-NAME, 0.1 mM), ACh increased $[Ca^{2+}]_i$ without relaxing the muscle. In the artery without endothelium, high K^+ increased both $[Ca^{2+}]_i$ and muscle tension although ACh was ineffective. 4-DAMP (10 nM) or atropine (0.1 μM) abolished ACh-induced increase in $[Ca^{2+}]_i$ and relaxation. The increase of $[Ca^{2+}]_i$ and vasorelaxation by ACh was siginificantly reduced by either 3 μM gadolinium, 10 μM lanthanum, or by 10 μM SKF 96365. These results suggest that in rabbit carotid artery, ACh-evoked relaxation of 70 mM K^+-induced contractions appears to be mediated by the release of NO. ACh-evoked vasorelaxation is mediated via the M_3 subtype, and activation of the M_3 subtype is suggested to stimulate nonselective cation channels, leading to increase of $[Ca^{2+}]_i$ in endothelial cells.

Key Words: Acetylcholine, Vasorelaxation, Nitric oxide, Rabbit carotid artery

INTRODUCTION

Vasodilation produced by acetylcholine (ACh), bradykinin, ATP and histamine has been shown to be dependent on the presence of vascular endothelial cells (Furchgott & Zawadzki, 1980; Moncada et al, 1991). Several endothelium-derived vasodilator factors have been identified. These include endothelium-dependent relaxing factor (EDRF), prostaglandins and endothelium-derived hyperpolarizing factor. Calcium ions are one of the most common signal transduction elements in numerous cell types, including endothelial cells (Miller & Vanhoutte, 1992). Endothelial cells produce nitric oxide (NO) through the activation of endothelial constitutive NO synthase (ecNOS) and induce vasodilation (Moncada et al, 1991). It was reported that NO production by ecNOS was controlled by increases in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) (Busse & Mulsch, 1990; Moncada et al, 1991; Koyama et al, 2002).

Several EDRF-releasing factors such as, ACh increase in $[Ca^{2+}]_i$ in cultured endothelial cells. Endothelial cells respond to various agonists by a biphasic $[Ca^{2+}]_i$ variation: a transient peak followed by a sustained elevation, corresponding respectively to Ca^{2+} release from internal stores and Ca^{2+} entry from the extracellular spaces (Nilius et al, 1997; Tsuchida et al, 2000). These agonist-induced $[Ca^{2+}]_i$ increases have been reported to control NO production. The elucidation of the various Ca^{2+} entry pathways in endothelial cells is still in its infancy.

Previous studies have shown that voltage-dependent Ca^{2+} channels are absent in endothelial cells (Adams, 1994). On the other hand two Ca^{2+} permeable non-specific cation channels have been found in endothelial cells (Nilius et al, 1998). Among those, nonselective cation channels (NSCC) are of considerable interest since they are also believed to play a key role in Ca^{2+} influx in endothelium (Nilius et al, 1997).

The present study was undertaken 1) to determine whether ACh-induced vasorelaxation of isolated carotid artery is accompanied by an increase in endothelial cell $[Ca^{2+}]_i$, 2) to characterize the identity of muscarinic receptors subtypes by use of selective muscarinic antagonists, and 3) to determine whether increases in endothelial cell $[Ca^{2+}]_i$ is mediated by NSCC.

METHODS

Tissue preparations:

Male rabbits (1.5~2 kg) were killed by a sharp blow to the neck and exsanguination. The carotid arteries were isolated and cut into helical strips of 1.5- to 2-mm width and 8 to 10-mm length and placed in physiological salt

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ABBREVIATIONS: ACh, acetylcholine; L-NAME, NG-nitro-L-arginine; NO, nitric oxide; EDRF, endothelium-derived relaxing factor.

solution (PSS) of the following composition (in mM): NaCl 136.9, KCl 5.4, $CaCl_2$ 1.5, $MgCl_2$ 1.2, $NaHCO_3$ 23.8, EDTA 0.01, glucose 5.5. Ethylenediaminetetraacetic acid (EDTA) at 0.01 mM was also added to chelate the contaminated heavy metal ions. High K^+ solution was made replacing NaCl with equimolar KCl. These solutions were saturated with 95% O_2 and 5% CO_2 at 37°C to maintain pH at 7.4.

Fura-2 loading and simultaneous measurements of muscle force and $[Ca^{2+}]_i$

$[Ca^{2+}]_i$ was measured according to the method described by Tsuchida et al (2000) using the fluorescent Ca^{2+} indicator fura-2. Muscle strips were exposed to the acethoxymethyl ester of fura-2 (fura-2/AM, 10 μM) in the presence of 0.02% cremophor EL for 5~6 hr at room temperature (22~24°C). The muscle strips was then transfered to the tissue bath at 37°C. The muscle strips was illuminated alternately (48 Hz) with 340 nm and 380 nm light, and the ratio of 500 nm fluorescence induced by 340 nm excitation (F340) and that induce by 380 nm excitation (F380) was detected with a spectrophotometer (CAF-110, Japan Spectroscopic, Tokyo, Japan). Increase in ratio induced by 70 mM K^+ was considered as a reference response (100%).

Chemicals

The chemicals used were acetylcholine chloride, atropine sulphate, pirenzepine, 4-diphenyl acetpxy N-methyl piperidine (4-DAMP) and 11-[[2-(diethylamino)methyl]-1-piperidinyl]acetyl]-5,11-dyhydro-6H-pyrido[2,3-b][1,4]benzodiaze pine-6-one (AF-DX 116), gadolinium chloride, lanthanum chloride, SKF 96365, tropicamide (Sigma Chemical).

Statistics

The results of the experiments are expressed as mean± S.E.M. Unpaired Student's t-test was used for statistical analysis of the results and the number of preparations taken from separate animals was indicated by n. Significant tests were performed by Student's paired or unpaired t test. P values of less than 0.05 were considered significant.

RESULTS

As shown in Fig. 1, 70 mM K^+ induced sustained increases in $[Ca^{2+}]_i$ and muscle tension in carotid arterial strips with endothelium. After 70 mM K^+-stimulated responses were reached to a steady-state level, addition of ACh (10 μM) induced sustained increase in $[Ca^{2+}]_i$ and relaxation (Fig 1A). Pretreatment with 0.1 mM L-NAME (10 min), completely inhibited ACh-induced relaxation, while they had no significant effect on the $[Ca^{2+}]_i$ elevations.

As shown in Fig. 2, 0.1 μM, 1 μM, and 10 μM ACh was added cumulatively during the 70 mM K^+-induced contraction and the relationship between ACh-induced additional increase in $[Ca^{2+}]_i$ and decrease in muscle tension was obtained (Fig. 2, left panel). It was found that there is a positive correlation between endothelial $[Ca^{2+}]_i$ and relaxation (Fig. 2, right panel) (correlation coefficient = 0.89, $p<0.01$).

Additional experiments were preformed to test the effects of the putative muscarinic receptor subtypes antagonists against ACh-induced responses (see Table 1). After observing the high K^+-induced changes, addition of 10 μM ACh induced sustained increase in $[Ca^{2+}]_i$ and relaxation (Fig 3A). The M_3 selective receptor antagonist 4-DAMP (10 nM), had no effect on basal $[Ca^{2+}]_i$ and muscle tension but completely inhibited ACh-induced changes (Fig. 3B). In separate experiments, atropine (0.1 μM) also abolished the ACh-induced fluorescence and relaxation. On the other

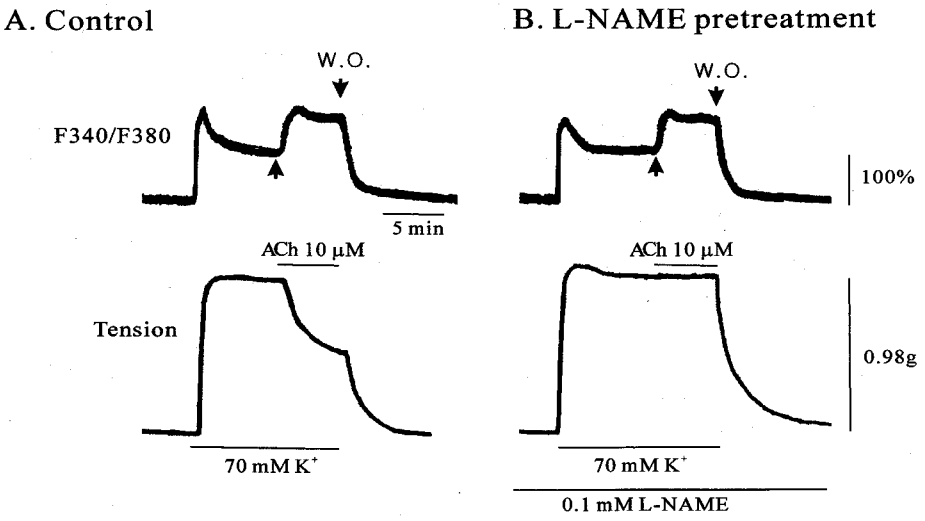

Fig. 1. Typical recordings of the increase in $[Ca^{2+}]_i$ (indicated by F340/F380; upper trace) and muscle tension (lower trace) in response to ACh in carotid arterial strips with endothelium in the absence (A) and presence of 0.1 mM L-NAME (B). Muscle strips were contracted with high K^+ (70 mM) and relaxed by ACh (10 μM). Changes in $[Ca^{2+}]_i$ were monitored by measuring the fura-2 fluorescence ration (F340/F380). The sustained response to high K^+, obtained before application of ACh, was taken as 100% and the resting $[Ca^{2+}]_i$ level as 0%.

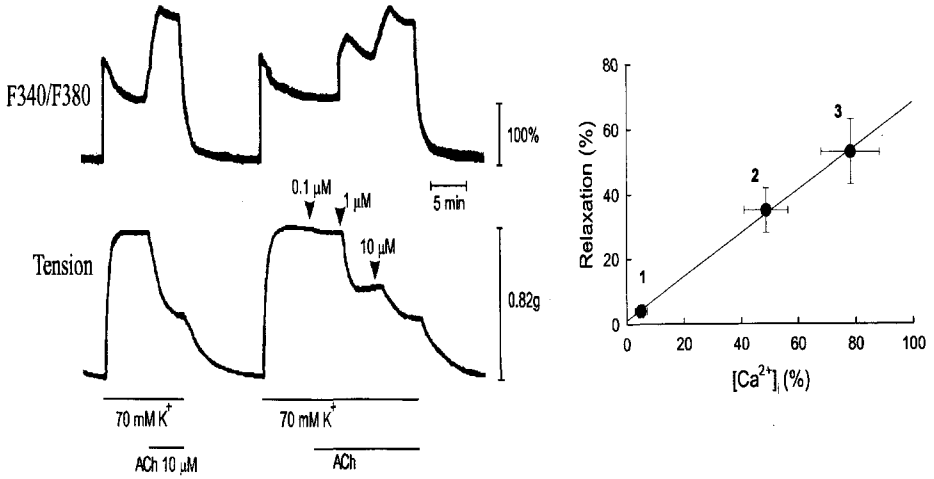

Fig. 2. Relationship between the additional increases in $[Ca^{2+}]_i$ (abscissa) and relaxation (ordinate) induced by ACh in high K^+-stimulated arterial strips with endothelium. ACh was added cumulatively during the contraction induced by 70 mM K^+; 100% represents represent the level before the addition of ACh. The concentration of ACh is indicated by numbers (1, 0.1 μM; 2, 1 μM; 3, 10 μM). Each point represent the mean of 5 to 8 experiments. S.E.M. is shown by vertical and horizontal bar. The regression line was drawn by least-square method (correlation coeffieient = 0.89, $p<0.01$).

Table 1. Summary of effects of muscarinic antagonists for inhibition of $[Ca^{2+}]_i$ increase and contraction evoked by ACh in rabbit carotid artery strips precontracted with 70 mM K^+

Condition	% increase in $[Ca^{2+}]_i$	Relaxation (%)	n
Control	53.3±7.8	48.1±6.7	8
Atropine (0.1 μM)	0±0**	0±0**	4
Pirenzepine (0.1 μM)	51.5±5.8	47.7±6.4	5
AF-DX 116 (1 μM)	49.5±6.1	45.4±6.1	5
4-DAMP (10 nM)	0±0**	0±0**	5
Tropicamide (1 μM)	50.5±6.3	48.3±7.3	4

$[Ca^{2+}]_i$ and muscle tension are expressed by relative values taking the value in the resting carotid artery as 0% and the high K^+-stimulated carotid artery as 100%. Each value represents mean ±S.E.M. n, number of experiments. **: Significantly different from the value before the addition of muscarinic antagonists with $p<0.01$.

hand, pretreatment with either the M_1 selective receptor antagonist pirenzepine (0.1 μM), the M_2 selective receptor antagonist AF-DX 116 (1 μM), or the M_4 antagonist tropicamide (1 μM), showed no inhibitory effect on ACh-induced responses.

Next we examined the effects of NSCC antagonists on ACh-induced changes (see Table 2). Typical recordings of the response to ACh in renal arterial strips in the absence and presence of gadolinium are shown in Fig. 4. Addition of ACh (10 μM) induced significant increases in $[Ca^{2+}]_i$ and relaxation in renal arterial strips with endothelium. Gadolinium (stretch-activated cation channel blocker, 3 μM), had no effect on basal $[Ca^{2+}]_i$ and muscle tension but significantly reduced ACh-induced changes. After removal of gadolinium, ACh-induced responses was returned to normal (n=4, data not shown). Addition of either lanthanum (10 μM) or SK& F 96365 (10 μM), also gave similar results as gadolinium.

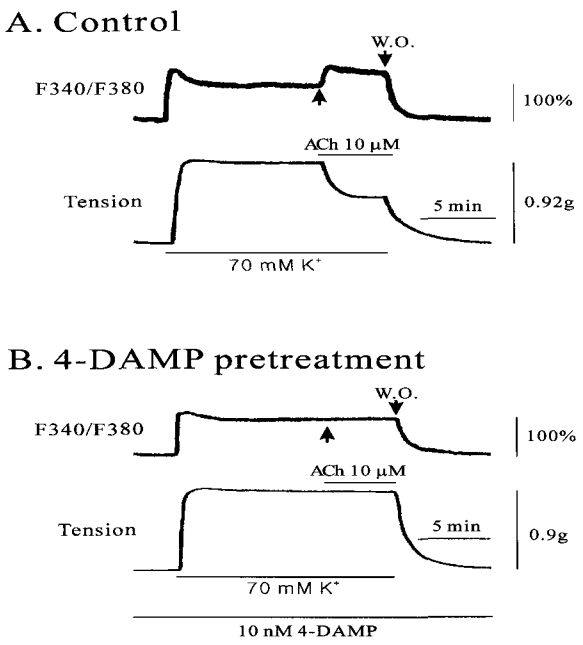

Fig. 3. Typical recordings of the increase in $[Ca^{2+}]_i$ (indicated by F340/F380; upper trace) and muscle tension (lower trace) in response to ACh in carotid arterial strips with endothelium in the absence (A) and presence of 0.1 μM 4-DAMP (B). Muscle strips were contracted with high K^+ (70 mM) and relaxed by ACh (10 μM). Changes in $[Ca^{2+}]_i$ were monitored by measuring the fura-2 fluorescence ration (F340/F380). The sustained response to high K^+, obtained before application of ACh, was taken as 100% and the resting $[Ca^{2+}]_i$ level as 0%.

DISCUSSION

We have demonstrated that (1) ACh induced both endothelium-dependent relaxation and increase in $[Ca^{2+}]_i$ and (2) both M_3 muscarinic receptor antagonist and NSCC antagonists inhibited the ACh-induced responses. These findings suggest that the NO production by endothelial cells requires Ca^{2+} entry from the extracellular spaces via the activation of NSCC and does dependent on the global increase in cytosolic Ca^{2+} concentration.

The present study used fura-2 fluorescence to examine changes in $[Ca^{2+}]_i$ induced by ACh in rabbit carotid arteries. ACh increased $[Ca^{2+}]_i$ and reduced the muscle tension in high K^+-stimulated tissues. In the present study, it was found that ACh induced additional increases in $[Ca^{2+}]_i$, which preceded the endothelium-dependent relaxation in the strips precontracted with high K^+. Moreover we demonstrated that there was a positive correlation between the increase in $[Ca^{2+}]_i$ and vascular relaxation. It is possible that ACh-induced increases in endothelial $[Ca^{2+}]_i$ obtained from muscle strips with endothelium (which also contain smooth muscle cells) may be underestimated, because ACh decreases $[Ca^{2+}]_i$ in smooth muscle cells by NO released from endothelium (Sato et al, 1990; Tsuchida et al, 2000). The application of the NOS antagonist L-NAME blocked ACh-induced relaxation but no effect on $[Ca^{2+}]_i$. Using strips without endothelium, we confirm that ACh affected neither resting tension or basal $[Ca^{2+}]_i$ level. These finding suggest that ACh has no effect on intracellular $[Ca^{2+}]_i$ in smooth muscle cell and susceptibility of smooth muscle in response to nitric oxide as EDRF. It may be conclude that the ACh-induced additional increases in $[Ca^{2+}]_i$ is due to the increases in endothelial $[Ca^{2+}]_i$ and that synthesis of NO is regulated by the amount of $[Ca^{2+}]_i$ in the endothelial cells.

It is unclear which subtypes of muscarinic receptors in rabbit carotid artery mediates the relaxation caused by ACh. The stimulation of muscarinic receptors on the endothelium in most vascular beds produces muscle relaxation on the release of NO, but there is controversy regarding which muscarinic receptor is involved in the release of NO in different vascular beds (Komori & Suzuki, 1987; Chiba & Tuskada, 1996; Wu et al, 1997). In the present study, four selective muscarinic subtype receptor antagonists were evaluated to differenciate which muscarinic receptor is responsible for the increase in $[Ca^{2+}]_i$ and relaxation from endothelium of rabbit carotid arteries. We demonstrated that the ACh-induced relaxation was inhibited by 4-DAMP (M_3 receptor subtype antagonists), and left unaffected by

Table 2. Summary of the effects of NSCC antagonists for inhibition of $[Ca^{2+}]_i$ increase and contraction evoked by ACh in rabbit carotid artery strips precontracted with 70 mM K^+

Condition	% increase in $[Ca^{2+}]_i$	Relaxation (%)	n
Control	55.4±7.1	48.4±7.2	5
Gadolinium (3 μM)	20.3±6.5**	8.8±2.8**	5
Lanthanum (10 μM)	22.3±6.2**	11.2±3.9**	5
SK&F 96365 (10 μM)	30.2±5.8	19.7±4.7*	5

$[Ca^{2+}]_i$ and muscle tension are expressed by relative values taking the value in the resting carotid artery as 0% and the high K^+-stimulated carotid artery as 70%. Each value represents mean± S.E.M. n, number of experiments. *, **: Significantly different from the value before the addition of nonselective cation channel antagonists with $p<0.05$ and $p<0.01$, respectively.

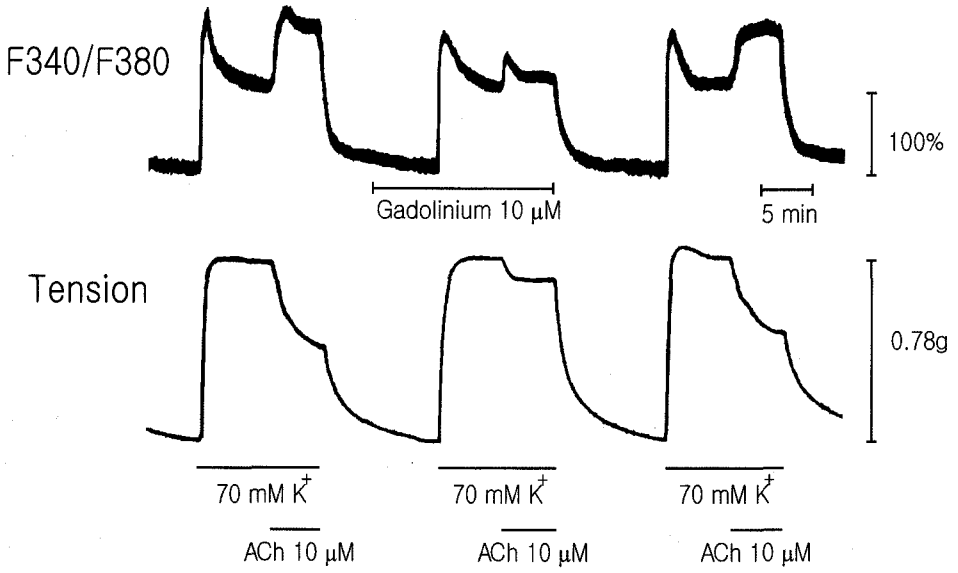

Fig. 4. Typical recordings of the increase in $[Ca^{2+}]_i$ (indicated by F340/F380; upper trace) and muscle tension (lower trace) in response to ACh in carotid arterial strips with endothelium in the absence and presence of 3 μM gadolinium. Muscle strips were contracted with high K^+ (70 mM) and relaxed by ACh (10 μM). Changes in $[Ca^{2+}]_i$ were monitored by measuring the fura-2 fluorescence ration (F340/F380). The sustained response to high K^+, obtained before application of ACh, was taken as 100% and the resting $[Ca^{2+}]_i$ level as 0%.

either pirenzepine (M_1 antagonist), AF-DX 116 (M_2 antagonist) or tropicamide (M_4 antagonist). This is consistent wither other findings (Chiba & Tsukada, 1996). Thus it is likely that both increase in $[Ca^{2+}]_i$ and relaxation is mediated by stimulating the M_3-subtype receptor in the rabbit carotid artery.

Endothelial cells lack voltage-sensitive Ca^{2+} channels and Ca^{2+} influx may occur through NSCC (Adams, 1994; Nilius et al, 1998). It has been also reported that NSCC is agonist-activated Ca^{2+}-permeable channel and that inhibition of these channels decreases the agonist-induced Ca^{2+} plateau in endothelial cells (Viana et al, 1998; Kamouchi et al, 1999). These properties strongly suggest that activation of NSCC increases $[Ca^{2+}]_i$ and may therefore play a role as Ca^{2+} influx pathway which is responsible for sustained elevation of $[Ca^{2+}]_i$ during agonist stimulation. In the present study, ACh-induced both increases in $[Ca^{2+}]_i$ and relxation was significantly reduced by either gadolinium, lanthanum or SK&F 96365. The concentration of NSCC inhibitors used here was in the range reported to block NSCC and utilized by others to test participation of NSCC in response to agonist stimulation or mechanical stress (Caldwell et al, 1998; Nilius et al, 1998; Koyama et al, 2002). Thus, the ability of ACh to increase Ca^{2+} influx into endothelial cells through receptor-activated NSCC can be expected to contribute to activation of ecNOS and production of EDRF (NO) from these cells.

In summary, in rabbit carotid artery, ACh-evoked relaxation of 70 mM K^+-induced contractions appears to be mediated by the release of NO. ACh-evoked vasorelaxation is mediated via the M_3 subtype, and activation of the M_3 subtype is suggested to stimulate nonselective cation channels, leading to increase of $[Ca^{2+}]_i$ in endothelial cells.

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