Yonsei Medical Journal
Vol. 40, No. 4, pp. 331−338, 1999

The Involvement of K^+ Channels and the Possible Pathway of EDHF in the Rabbit Femoral Artery

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Abstract

Experiments were designed to characterize the cellular mechanisms of action of endothelium-derived vasodilator substances in the rabbit femoral artery. Acetylcholine (ACh, $10^{-8}-10^{-5}$ M) induced a concentration-dependent relaxation of isolated endothelium-intact arterial rings precontracted with norepinephrine (NE, 10^{-6} M). The ACh-induced response was abolished by the removal of endothelium. N^G-nitro-L-arginine (L-NAME, 10^{-4} M), an inhibitor of NO synthase, partially inhibited ACh-induced endothelium-dependent relaxation, whereas indomethacin (10^{-5} M) showed no effect on ACh-induced relaxation. 25 mM KCl partially inhibited ACh-induced relaxation by shifting the concentration-response curve and abolished the response when combined with L-NAME and NE. In the presence of L-NAME, ACh-induced relaxation was unaffected by glibenclamide (10^{-5} M) but significantly reduced by apamin (10^{-6} M), and almost completely blocked by tetraethylammonium (TEA, 10^{-3} M), iberiotoxin (10^{-7} M) and 4-aminopyridine (4-AP, 5×10^{-3} M). The cytochrome P450 inhibitors, 7-ethoxyresorufin (7-ER, 10^{-5} M) and miconazole (10^{-5} M) also significantly inhibited ACh-induced relaxation. Ouabain (10^{-6} M), an inhibitor of Na^+, K^+-ATPase, or K^+-free solution, also significantly inhibited ACh-induced relaxation. ACh-induced relaxation was not significantly inhibited by 18-α-glycyrrhetinic acid (18 α-GA, 10^{-4} M). These results of this study indicate that ACh-induced endothelium-dependent relaxation of the rabbit femoral artery occurs via a mechanism that involves activation of Na^+, K^+-ATPase and/or activation of both the voltage-gated K^+ channel (K_v) and the large-conductance, Ca^{2+}-activated K^+ channel (BK_{Ca}). The results further suggest that EDHF released by ACh may be a cytochrome P450 product.

Key Words: Rabbit femoral artery, endothelium-dependent relaxation, acetylcholine, EDHF, K^+ channel

INTRODUCTION

The vascular endothelium is an important regulator of vascular tone. In order to control vascular tone, endothelial cells, following stimulation with autacoids or under shear stress, produce various vasodilators. Among these, an endothelium-derived hyperpolarizing factor (EDHF) that induces smooth muscle relaxation via membrane hyperpolarization has been described.[1,2] Considerable evidence also shows that several receptor-dependent agonists such as acetylcholine (ACh), bradykinin, histamine and substance P release EDHF that causes vascular smooth muscle hyperpolarization which has yet to be identified.[3-5] Several studies have demonstrated that the electrophysiological and pharmacological properties of EDHF differ from endothelium-derived relaxing factor (EDRF) in many respects.[6,7] For instance, EDHF-mediated relaxation and membrane hyperpolarization are resistant to inhibitors of the L-arginine-nitric oxide pathway such as oxyhaemoglobin, methylene blue or N^G-nitro-L-arginine methyl ester.[8,9]

The contribution of EDHF to endothelium-dependent relaxation appears to be dependent on tissue source, species, and agonist employed to induce vasorelaxation.[7,10] In addition, it is generally considered that EDHF may be of greater importance in resistance than in the large conduit arteries and may play a significant role in the determination of peripheral vascular resistance.[6] Although EDHF has not yet been identified, it has been shown that K^+ channel

Received February 8, 1999
Accepted June 24, 1999
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This study was supported by the Kim Myung Sun Memorial Fund for 1996.
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opening is responsible for ACh-induced hyperploarization in vascular smooth muscle, thereby being responsible for vasorelaxation.[6] For example, in the conduit arteries, such as the rabbit abdominal aorta and carotid artery, EDHF-mediated relaxation is inhibited by charybdotoxin,[11,12] whereas in the resistance arteries, such as the rabbit mesenteric and guinea-pig coronary arteries, the effects of EDHF have been shown to be apamin-sensitive.[13,14] However, despite extensive efforts, no consensus view on the type of K^+ channel opened by EDHF has as yet been established.

Recently, a number of studies reported the involvement of cytochrome P450 enzymes in EDHF-mediated relaxation.[15,16] Cytochrome P450 enzymes are located mainly in the endothelium of blood vessels. These enzymes are known to generate vasorelaxant products like epoxyeicosatrienoic acids (EETs) from arachidonic acid.[15] Modulation of cytochrome P450 enzyme activities have resulted in corresponding changes in endothelium-dependent relaxation.[16] Most of the results representing EDHF as a cytochrome P450 epoxygenase-derived compounds are due to the inhibitory properties on cytochrome P450 inhibitors like SKF 525A. And those inhibitors have been shown to possess a number of other effects, like the inhibition of K^+ channels and Ca^{2+}-ATPase inhibition.[17,18] Therefore, the nature of EDHF has not been identified yet.

The aims of this study were designed to pharmacologically determine (a) whether EDHF contributes to endothelium-dependent vasorelaxation in rabbit femoral artery, and (b) the effects of K^+ channel blockers and cytochrome P450 inhibitors, and the contribution of a Na^+, K^+-ATPase on the ACh-induced relaxation in order to characterize the nature and mechanism of action of EDHF.

MATERIALS AND METHODS

Preparation of artery rings

New Zealand white rabbits (2−3 kg) of either sex were killed by exsanguination after anaesthesia with pentobarbital sodium (30 mg/kg iv). The femoral artery was quickly excised and placed in a cold physiological salt 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. The pH of the solution after saturation with 95% O_2 +5% CO_2 gas mixture was 7.4. The vessels were cut into 1 mm-wide ring segments and were placed in 20 ml tissue baths on 2 L-shaped hooks, one of which was attached to a force transducer for isometric measurement of tension. The vessel tension was recorded on a pen recorder. The baths were thermostatically kept at 37°C. A resting tension of 0.5 g was maintained throughout the experiments. Tissues were allowed to equilibrate for 90 min before each experiment.

Relaxations were studied in preparations contracted by NE (10^{-6} M). When stable contractions were obtained, ACh was added cumulatively to determine the concentration-response relationship. The function of the endothelium was checked at the beginning of each experiment with ACh (10^{-6} M). In some experiments, the endothelium was mechanically removed by gentle rubbing with moistened cotton, and its absence was confirmed by the lack of a relaxant response to ACh (10^{-6} M). Unless otherwise stated, L-NAME (10^{-4} M) was present in the PSS.

Data analysis

Results were expressed as mean±SE. 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

Characterization of vasorelaxation to ACh

In the endothelium-intact ring segments of a rabbit femoral artery precontracted with NE (10^{-6} M), ACh ($10^{-8}-10^{-5}$ M) induced a concentration-dependent relaxation (Fig. 1). This ACh-induced vasorelaxation was completely abolished by mechanical removal of the endothelium (n=8). Treatment with the NO synthase blocker, L-NAME (10^{-4} M) slightly but significantly inhibited the concentration-response curve for ACh. The cyclo-oxygenase blocker, indomethacin (10^{-5} M) did not have any significant effect on ACh-elicited relaxation in the presence of L-NAME (Fig. 1).

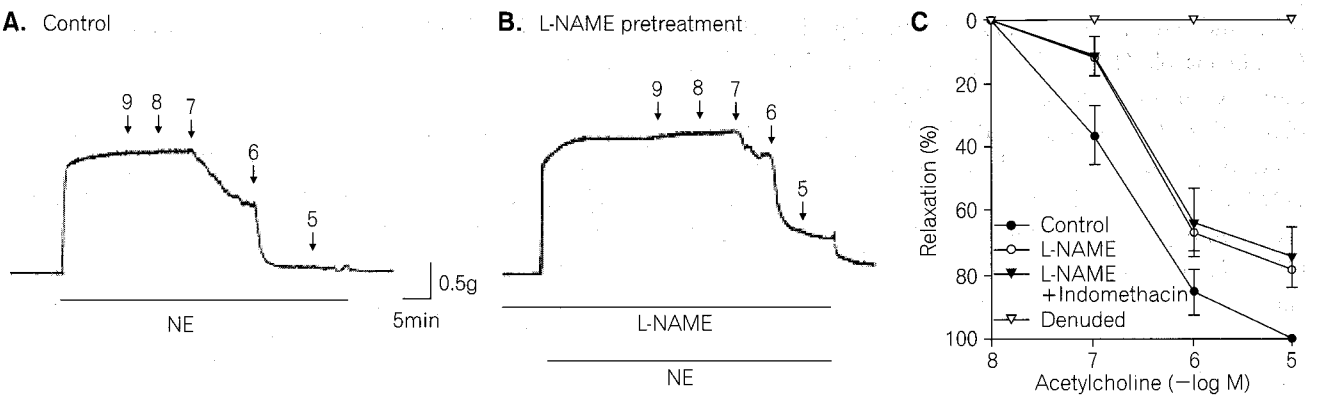

Fig. 1. Traces showing endothelium-dependent relaxations elicited by -log concentrations of acetylcholine (ACh) in rabbit femoral arteries contracted by norepinephrine (NE, 10^{-6} M) in the absence (A) and presence (B) of L-NAME (10^{-4} M). C, average concentration- response curves for the relaxant effects of ACh in the absence (●) and presence of L-NAME (○) or L-NAME plus indomethacin (10^{-5} M, ▼). (▽) Endothelium was removed. Responses are expressed as the percentage of contraction elicited by NE before the addition of ACh. Points represent mean and vertical lines show S.E. mean of 6−10 experiments. L-NAME, N^G-nitro-L-arginine methyl ester hydrochloride.

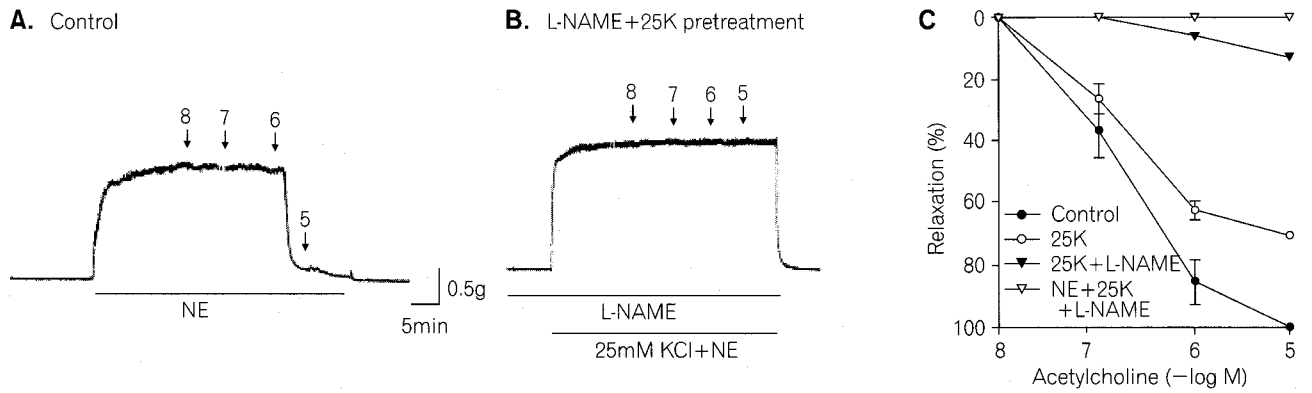

Fig. 2. Traces showing endothelium-dependent relaxations elicited by -log concentrations of acetylcholine (ACh) in rabbit femoral arteries contracted by norepinephrine (NE, 10^{-6} M) in the absence (A) and presence (B) of L-NAME (10^{-4} M) plus 25 mM KCl. C, average concentration-response curves for acetylcholine (ACh) in rabbit femoral artery precontracted with NE (○) or 25 mM KCl (●) or in the presence of NE plus L-NAME (10^{-4} M, ▼), or NE, L-NAME plus 25 mM KCl (▽). Responses are expressed as the percentage of contraction elicited by either NE or 25 mM KCl before the addition of ACh. Points represent mean and vertical lines show S.E. mean of 6−8 experiments. L-NAME, N^G-nitro-L-arginine methyl ester hydrochloride.

In rings constricted with 25 mM KCl, the relaxation responses to ACh were significantly attenuated; the concentration-relaxation curve was shifted to the right and the maximal response was significantly reduced compared to those parameters in rings constricted with NE (Fig. 2). ACh-induced relaxation obtained in rings contracted with 25 mM KCl was significantly reduced by L-NAME (10^{-4} M). In the presence of 10^{-4} M L-NAME, in arteries contracted with 25 mM KCl plus 10^{-6} M NE, ACh-elicited relaxation was completely abolished, suggesting that it was mediated by an endothelium-dependent hyperpolarization.

Role of cytochrome P 450 pathway in ACh-mediated vasorelaxation

The contribution of the cytochrome P 450 pathway to ACh-induced relaxation was assessed with 7-ER (10^{-5} M) or mconazole (10^{-5} M) in the rabbit femoral artery pretreated with L-NAME (10^{-4} M). 7-ER, cytochrome P 450 substrate and inhibitor, significantly reduced the amplitude of relaxation induced with ACh (10^{-6} M; Control: 79.3±4.0%,

n=6 and 7-ER: 32.2±2.4%, n=6) (Fig. 3A, B and C). Miconazole (10^{-5} M), a cytochrome P 450 blocker, also significantly inhibited ACh-induced relaxation (Control and miconazole: 45.7±5.4%, n=5). However, ACh-induced vasorelaxation in the presence of L-NAME was not inhibited following putative inhibition lipoxygenase pathways with either caffeic acid (10^{-5} M) or (10^{-5} M) or ETYA (10^{-5} M) (data not shown).

Role of K^+ channels in ACh-mediated vasorelaxation

The contribution of K^+ channels to ACh-induced relaxation was assessed in a femoral artery pretreated with L-NAME (10^{-4} M). ACh-induced relaxation was almost completely abolished following putative inhibition of delayed rectifier K^+ channels with 4-AP (5×10^{-3} M; Control: 79.9±4.9%, and 4-AP: 0%, n=6) (Fig. 4A, B and C). ACh-induced relaxation was also strongly inhibited following inhibition of large conductance Ca^{2+}-activated K^+ channels with TEA (10^{-3}

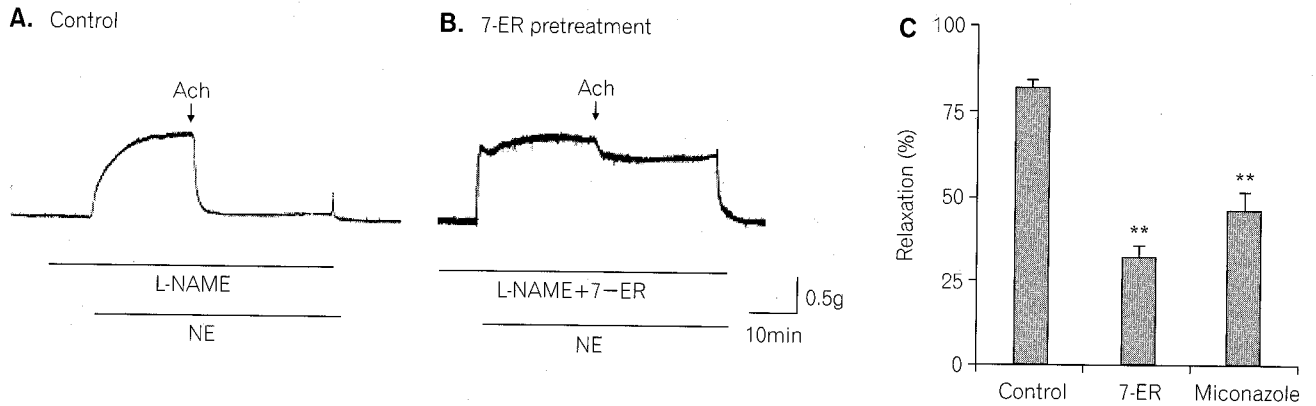

*Fig. 3. Representative traces showing the inhibitory effects of 7-ethoxyresorufin (7-ER) on acetylcholine (ACh)-induced relaxation in a rabbit femoral artery precontracted with norepinephrine (NE, 10^{-6} M) (A and B). Pretreatment with 7-ER (10^{-5} M) for 10 min. C, summarized data showing inhibitory effect of 7-ER and miconazole (10^{-5} M) on ACh-induced relaxation. The experiments were performed in the presence of L-NAME (10^{-4} M). Columns which are the mean ±SE mean from 7 separate experiments, represent the effects under control conditions (Control) and in the presence of 7-ER. **p <0.01 vs control.*

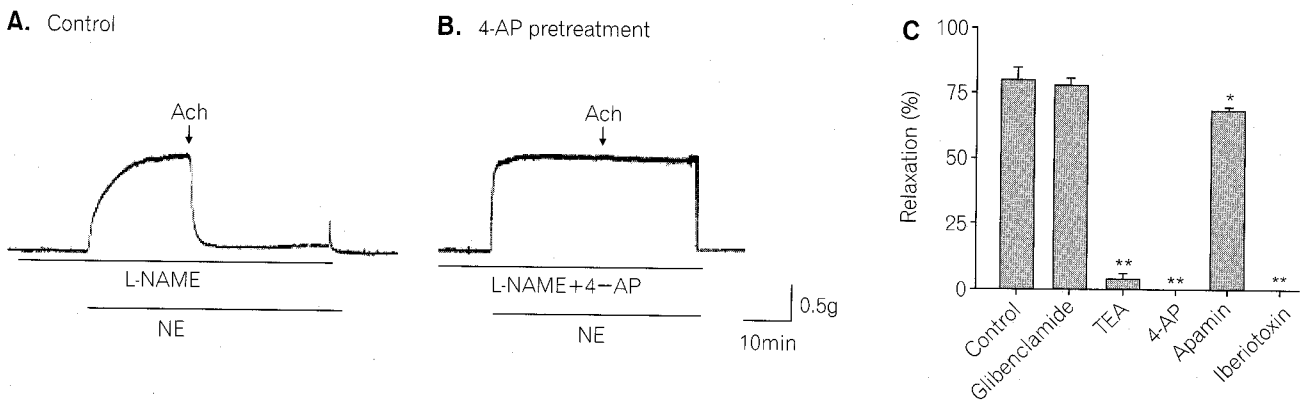

*Fig. 4. Effects of 4-AP on the relaxation elicited by acetylcholine (ACh, 10^{-6} M) in a rabbit femoral artery precontracted with norepinephrine (NE, 10^{-6} M) in the presence of L-NAME (10^{-4} M). Original tracings show the ACh-induced relaxation in the absence (A) and presence of 4-AP (5×10^{-3} M, B). C, summarized data showing the inhibitory effects of K^+ channel blockers on ACh-induced relaxation. Columns which are the mean ±SE mean from 6−10 separate experiments, represent the effects under control conditions (Control) and in the presence of glibenclamide (10^{-5} M), TEA (10^{-3} M), 4-AP (5×10^{-3} M), apamin (10^{-6} M), or iberiotoxin (10^{-7} M). *p <0.05, **p <0.01 vs control.*

M; control and 4.0±1.7%, n=8) or iberiotoxin (10^{-7} M; control and 0%, n=6) (Fig. 4C). Blockade of the small conductance Ca^{2+}-activated K^+ channel with apamin (10^{-6} M) also significantly reduced ACh-induced relaxation (Control and 68.3±1.0%, n=6). In contrast to the effects of Ca^{2+}-activated K^+ channel blockers, the inhibitor of the ATP-sensitive K^+ channels, glibenclamide (10^{-5} M) did not affect the relaxation of ACh (n=8).

Effect of ouabain on ACh-mediated vasorelaxation

In the presence of ouabain (10^{-7} to 10^{-6} M), an inhibitor of Na^+, K^+-ATPase, the ACh-induced relaxation in rings precontracted with NE was significantly attenuated (Fig. 5A, B and C). Ouabain did not induce a contractile response in femoral artery rings. When femoral artery rings were incubated in K^+-free solution, which is another way to inhibit the Na^+, K^+-ATPase, ACh did not cause relaxation (Fig. 5C).

Effect of 18 α-GA on ACh-mediated vasorelaxation

In the presence of L-NAME (10^{-4} M), 18-α-glycyrrhetinic acid (18 α-GA, 10^{-4} M), the gap

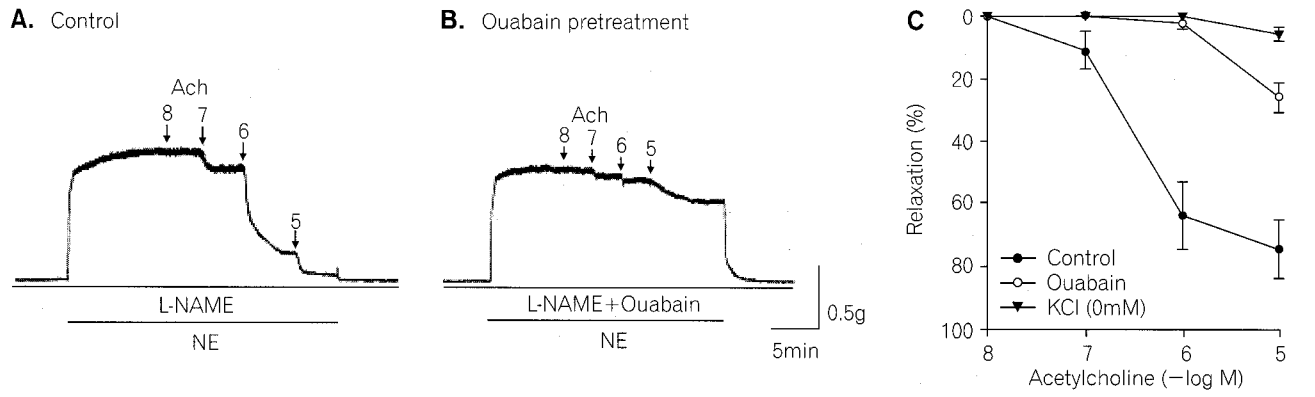

Fig. 5. Effect of the Na^+, K^+-ATPase inhibitor ouabain or K^+-free solution on relaxation induced by acetylcholine (ACh) in endothelium-intact rabbit femoral artery precontracted with norepinephrine (NE, 10^{-6} M) plus L-NAME (10^{-4} M). Original tracings show the ACh-induced relaxation in the absence (A) and presence of ouabain (10^{-6} M, B). C, summarized data showing inhibitory effects of ouabain and K^+-free solution on ACh-induced relaxation. (●) Control; (○) in the presence of ouabain (10^{-6} M, 30 min); (▼) in the presence of K^+-free solution. Points are the mean and vertical lines show S.E. mean from 6−8 separate experiments. L-NAME, N^G-nitro-Larginine methyl ester hydrochloride.

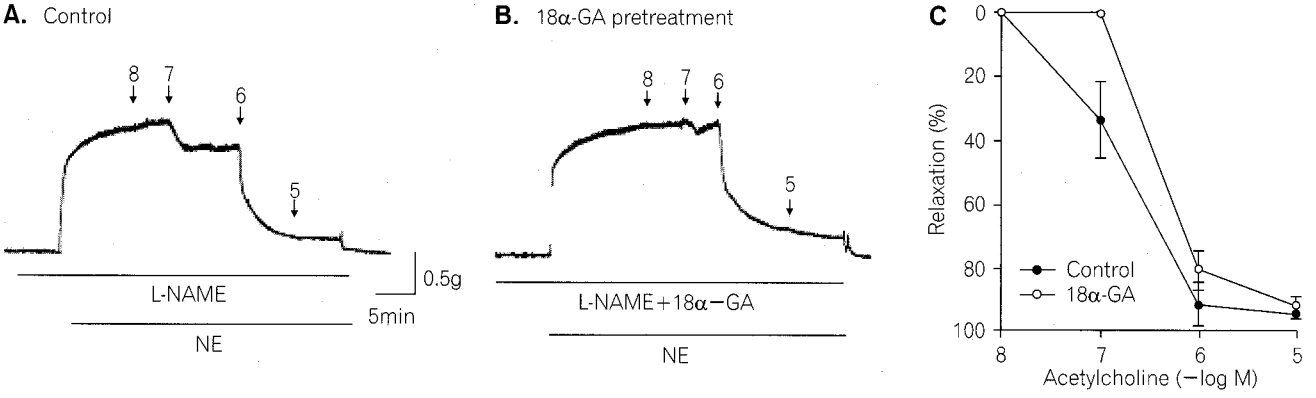

Fig. 6. Traces showing endothelium-dependent relaxation elicited by -log concentrations of acetylcholine (ACh) in rabbit femoral arteries contracted by norepinephrine (NE, 10^{-6} M) in the absence (A) and presence (B) of 18 α-glycyrrhetinic acid (18 α-GA, 10^{-4} M). C, summarized data showing the inhibitory effects of 18 α-GA on ACh-induced relaxation. (●) Control; (○) in the presence of 18 α-GA (10^{-4} M, 30 min). The experiments were performed in the presence of L-NAME (10^{-4} M). Results are mean and vertical lines show S.E. mean of 8−12 arteries and are expressed relative to the NE-elicited contractions. L-NAME, N^G-nitro-Larginine methyl ester hydrochloride.

junction inhibitor did not significantly attenuate endothelium-dependent relaxation to ACh in rings of a precontracted rabbit femoral artery (Fig. 6).

DISCUSSION

Our findings clearly indicate that endothelium-dependent relaxation induced by ACh in rabbit femoral artery involves the release of non-prostanoid/NO endothelial factor (EDHF) which relaxes the underlying smooth muscle by activating both the K_v and K_{Ca} channels, and/or by activation of Na^+, K^+-ATPase pump.

In this study, ACh induced a concentration-dependent relaxation, which was completely abolished by removal of the endothelium and was only partially attenuated by L-NAME. Indomethacin did not change the relaxant response to ACh and combined inhibition of prostanoids and NO did not have any additional inhibitory effect compared to that of L-NAME alone, thus excluding the participation of one factor in the absence of the other. These results suggested that ACh-induced relaxation of the rabbit femoral artery is endothelium-dependent and rules out an involvement of prostacyclin in the relaxation response to ACh. Moreover, ACh-elicited relaxation was largely reduced when extracellular K^+ was increased, and completely prevented by L-NAME in the presence of high extracellular K^+ and NE. Therefore, these results indicated that the endothelium-dependent, relaxant response to ACh in the rabbit femoral artery is mediated by at least 2 different factors, namely NO and EDHF.

Although the nature of EDHF has not yet been elucidated, recent studies indicate that EDHF may be a cytochrome P 450 product.[16,17] In this study, we provide evidence that the cytochrome P 450 inhibitors, 7-ER and miconazole modulate NO/prostacyclin-independent relaxation to ACh in the rabbit femoral artery. Our results are in general agreement with those of Hecker et al.[13] and Harder et al.[16] in which cytochrome P 450 inhibitors significantly inhibited the NO/prostacyclin-independent relaxation to bradykinin in the porcine coronary artery and in the rabbit carotid artery.

In the presence of L-NAME, ACh induced an NO/prostacyclin-resistant relaxation in the rabbit femoral artery, suggesting that EDHF released from endothelial cells contributes to endothelium-dependent relaxation. The cellular mechanism underlying ACh-mediated relaxation of vascular smooth muscle is not fully understood, especially with respect to the subtype of K^+ channels involved. EDHF mediates the activation of both large conductance Ca^{2+}-activated K^+ (BK_{Ca}) channels and delayed rectifier K^+ (K_v) channels in the rat hepatic artery,[19] but it activates BK_{Ca} channels in the rabbit carotid artery,[20] the small conductance Ca^{2+}-activated K^+ (SK_{Ca}) channels in the rabbit mesenteric artery,[13,14] or the ATP-sensitive K^+ (K_{ATP}) channels in the rabbit basilar artery.[21] These data suggest that the subtypes of K^+ channels involved in EDHF-mediated relaxation of arteries appear to be both tissue- and species dependent. However, subtypes of K^+ channels involved in EDHF-mediated relaxation are not fully understood.

In this study, ACh-induced relaxation was abolished by 4-AP, a specific inhibitor of K_v channels, suggesting that K_v channels are involved in ACh-mediated relaxation of the rabbit femoral artery. EDHF-mediated relaxation in the rat hepatic artery is inhibited by 4-AP and charybdotoxin.[22] Since charybdotoxin inhibits not only BKCa, but also K_v channels, this leads to the conclusion that the K_v channels are a target for EDHF. In this study, 3 K^+ channel blockers with different selectivity for subtypes of Ca^{2+}-activated K^+ (K_{Ca}) channels were used to determine which subtypes of K_{Ca} channels play a functional role in the relaxation response to ACh in the rabbit femoral artery. Although apamin significantly inhibited ACh-induced relaxation, apamin did not abolish the relaxation to ACh. Iberiotoxin, the selective BK_{Ca} channel inhibitor, almost completely abolished ACh-induced relaxation, and TEA also significantly inhibited ACh-induced relaxation, so that the BK_{Ca} channels are likely to play a role in ACh-induced relaxation. Taken together, these data clearly indicate that in rabbit femoral artery, both the BK_{Ca} and K_v channels are involved in ACh-mediated, NO/prostacyclin-resistant relaxation.

In the presence of L-NAME, ouabain elicited a significant inhibition of the ACh-induced endothelium-dependent relaxation. This concentration of ouabain exerted no direct contractile response in the rabbit femoral artery. This suggests that a basal activity of the Na^+, K^+-ATPase pump is probably not involved in the maintenance of femoral artery tone, as shown previously for vascular smooth mus-

cle.[23] Because the above data suggested that relaxation might be mediated by activation of the Na^+, K^+-ATPase pump, experiments were performed in the absence of K^+, which is known to inhibit the pump. ACh did not cause relaxation when arteries were incubated in K^+-free solution. It has already been suggetsed that ouabain inhibits ACh-induced endothelium-dependent vasodilation and EDHF-mediated endothelium-dependent hyperpolarization.[23,24] These findings suggested that ACh-induced relaxation is mediated by activation of Na^+, K^+-APTase pump. Although we cannot rule out an effect of ouabain on the synthesis/release of EDHF, this seems unlikely since ouabain also inhibited relaxation to exogenous NO in horse penile arteries.[24] Whether the endothelial factor activating both the K_{Ca} and K_v channels is the same as that stimulating the Na^+, K^+-ATPase pump in the femoral artery remains to be determined.

The importance of the gap junction between endothelial and smooth muscle cells has often been assessed by substances such as heptanol and halothane which block gap junctions in cardiac muscle.[25] Their effectiveness in smooth muscle, however, has been questioned.[26] Recently, glycyrrhetinic acid has been shown to block gap junctional communication in smooth muscles.[27,28] This study has shown that the L-NAME insensitive component of ACh-induced relaxation was not attenuated by 18 α-GA, suggesting that the electrical coupling between smooth muscle and endothelial cells is not involved. These findings are in accord with previous studies showing an absence of functional coupling between endothelial and smooth muscle cells.[29] The present findings therefore suggest that the electrical coupling between endothelial and smooth muscle cells is not involved in ACh-induced, endothelium-dependent relaxation in the rabbit femoral artery.

In conclusion, we have demonstrated that in the rabbit femoral artery, ACh-evoked relaxation of NE-induced contractions appears to be mainly mediated by EDHF. The diffusable EDHF released by ACh may be a cytochrome P 450-epoxygenase metabolite and EDHF-induced relaxation may involve the activation of Na^+, K^+-ATPase pump and/or by opening both the K_v and K_{Ca} channels.

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