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Mechanism of L-NAME-Resistant Endothelium-Dependent Relaxation Induced by Acetylcholine in Rabbit Renal Artery

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In the rabbit renal artery, acetylcholine (ACh, 1 nM~10 μM) induced endothelium-dependent relaxation of arterial rings precontracted with norepinephrine (NE, 1 μM) in a dose-dependent manner. N^G-nitro-L-arginine (L-NAME, 0.1 mM), an inhibitor of NO synthase, or ODQ (1 μM), a soluble guanylate cyclase inhibitor, partially inhibited the ACh-induced endothelium-dependent relaxation. The ACh-induced relaxation was abolished in the presence of 25 mM KCl and L-NAME. The cytochrome P450 inhibitors, 7-ethoxyresorufin (7-ER, 10 μM), miconazole (10 μM), or 17-octadecynoic acid (17-ODYA, 10 μM), failed to inhibit the ACh-induced relaxation in the presence of L-NAME. 11,12-epoxyeicosatrienoic acid (11,12-EET, 10 μM) had no relaxant effect. The ACh-induced relaxation observed in the presence of L-NAME was significantly reduced by a combination of iberiotoxin (0.3 μM) and apamin (1 μM), and almost completely blocked by 4-aminopyridine (5 mM). The ACh-induced relaxation was antagonized by P_{2Y} receptor antagonist, cibacron blue (10 and 100 μM), in a dose-dependent manner. Furthermore, 2-methylthio-ATP (2MeSATP), a potent P_{2Y} agonist, induced the endothelium-dependent relaxation, and this relaxation was markedly reduced by either the combination of iberiotoxin and apamin or by cibacron blue. In conclusion, in renal arteries isolated from rabbit, ACh produced non-NO relaxation that is mediated by an EDHF. The results also suggest that ACh may activate the release of ATP from endothelial cells, which in turn activates P_{2Y} receptor on the endothelial cells. Activation of endothelial P_{2Y} receptors induces a release of EDHF resulting in a vasorelaxation via a mechanism that involves activation of both the voltage-gated K^+ channels and the Ca^{2+}-activated K^+ channels. The results further suggest that EDHF does not appear to be a cytochrome P450 metabolite.

Key Words: Acetylcholine, Renal artery, Endothelium-dependent relaxation, EDHF, K^+ channel, P_{2Y} receptor

INTRODUCTION

The vascular endothelial cells play an important role in maintaining the vascular homeostasis by liberating several vasodilator substances, including nitric oxide (NO), prostacyclin (PGI_2), and endothelium-derived hyperpolarizing factor (EDHF) (Adeagbo & Triggle, 1993). There is also considerable evidence 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. Several studies have demonstrated that the electrophysiological and pharmacological properties of EDHF differ from endothelium-derived relaxing factor (EDRF) in many respects. For instance, EDHF-mediated relaxation and membrane hyperpolarization are resistant to inhibitors of the L-arginine-NO pathway such as oxyhaemoglobin, methylene blue or N^G-nitro-L-arginine methyl ester (Holzmann et al, 1994).

Although EDHF is believed to activate K^+ channels in vascular smooth muscles, no consensus has been reached on the identity of the K^+ channles activated by EDHF. Studies with K^+ channel blockers have produced conflicting results. For example,

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in the conduit arteries, such as rabbit abdominal aorta and carotid artery, EDHF-mediated relaxation is inhibited by charybdotoxin (Cowan et al, 1993; Lischke et al, 1995), 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 (Hecker et al, 1994; Murphy & Baryden, 1995). Furthermore, in the rat hepatic and guinea pig carotid arteries, the action of EDHF was abolished by a combination of charybdotoxin plus apamin, but unaffected by either apamin or charybdotoxin alone (Corriu et al, 1996; Zygmunt & Hogestatt, 1996).

Several recent studies suggested that cytochrome P450 epoxygenase in endothelial cells contributed to the production of EDHF (Harder et al, 1995; Campbell et al, 1996). These enzymes are known to generate vasorelaxant products like epoxyeicosatrienoic acids (EETs) from arachidonic acid. Modulation of cytochrome P450 enzyme activities has resulted in corresponding changes in endothelium-dependent relaxation (Harder er al, 1995). Furthermore, cytochrome P450 inhibitors such as proadifen and clotrimazole have been shown to inhibit EDHF-mediated hyperpolarization and relaxation (Hecker et al, 1994; Campbell et al, 1996; Chen & Cheung, 1996). However, more recent evidence casts doubt on this contention, because 17-ODYA, a suicide-substrate inhibitor of cytochrome P450 epoxygenase responsible for the production of EETs, has no effect on EDHF-mediated responses in the rat hepatic artery (Zygmunt et al, 1996). Therefore, the nature of EDHF has yet to be identified.

As it is generally accepted that P_{2Y} purinoceptors are located in vascular endothelial cells, the P_{2Y}-mediated relaxation is an endothelium dependent phenomenon (Olsson & Pearson, 1990). Recent studies suggest that activation of P_{2Y} receptors induces endothelium-dependent vasodilation mediated by EDHF (Ralevic & Burnstock, 1991). In the rat mesenteric artery, P_{2Y} receptor-induced vasodilatation is antagonized by a combination of the K^+ channels inhibitors charybdotoxin and apamin, which prevents hyperpolarization and relaxation mediated by EDHF in different blood vessels (Malmsjo et al, 1998; Zygmunt et al, 1996). However, no study has provided direct evidence that EDHF is released by activation of endothelial P_{2Y} receptors.

The aims of this study were to pharmacologically determine (a) whether EDHF contributes to endothelium-dependent vasorelaxation in rabbit renal artery, and (b) the effects of cytochrome P450 inhibitors, K^+ channel blockers, and P_{2Y} antagonist on the ACh-n induced relaxation in order to characterize the nature and mechanism of action of EDHF. The effect of a potent P_{2Y} agonist, 2MeSATP on NE-contracted tissues was also investigated and the results were compared with that of ACh-induced relaxation.

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 renal 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 (1 μM). 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 (1 μM). 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 (1 μM). To evaluate NO-independent vasorelaxation attributed to EDHF, ACh-induced relaxation in the presence of L-NAME (100 μM) was determined after NE preconstriction. Vessels were pre-incubated for 30 min in the presence of the guanylate cyclase inhibitor ODQ (1 μM), the cytochrome P450 substrate and inhibitor 7-ER (10 μM), cytochrome P450 blocker miconazole (10 μM) or 17-ODYA (10 μM), the ATP-sensitive K^+ channel inhibitor glibenclamide (10 μM),

the large-conductance Ca^{2+}-activated K^+ channel blocker iberiotoxin (0.3 μM), the small-conductance Ca^{2+}-activated K^+ channel blocker apamin (1 μM), and the P_{2Y} purinoceptor antagonist cibacron blue (10 and 100 μM). Vessels were then preconstricted with 1 μM NE and concentration-response curves to ACh (1 nM～10 μM) or 2MeSATP (0.1 μM～1 mM) were constructed.

Chemicals

ACh, 4-AP, apamin, cibacron blue, iberiotoxin, 11,12-EET, ETYA, 17-ODYA, 2MeSATP, miconazole and L-NAME were purchased from Sigma (St. Louis, Mo, USA), and ODQ (1H-[1,2,4]oxadiazolo [4,3-a]quinoxaline-1-one) was purchased from Tocris Cookson (Bristol, UK).

Statistics

The results of the experiments are expressed as mean±S.E.M. Student's t test was used for statistical analysis of the results and $p<0.05$ was considered to be significantly different. The number of preparations taken from separate animals was indicated by n.

RESULTS

Characterization of ACh-mediated vasorelaxation

In the endothelium-intact ring segments of a rabbit renal artery precontracted with NE (1 μM), ACh (1 nM～10 μM) elicited concentration-dependent relaxation (Fig. 1). This ACh-induced vasorelaxation was completely abolished by mechanical removal of the endothelium (n=8). The relaxant response was markedly reduced by the NO synthase blocker L-NAME (0.1 mM) or by the soluble guanylate cyclase inhibitor ODQ (1 μM). In arteries contracted with 25 mM KCl plus 1 μM NE in the presence of 0.1 mM L-NAME, the ACh-elicited relaxation was completely abolished, suggesting that the relaxation is mediated in part by an endothelium-dependent hyperpolarization.

Role of cytochrome P450 pathway in ACh-mediated vasorelaxation

The contribution of the cytochrome P450 pathway to ACh-induced relaxation was assessed with 7-ER, cytochrome P450 substrate and inhibitor (10 μM);

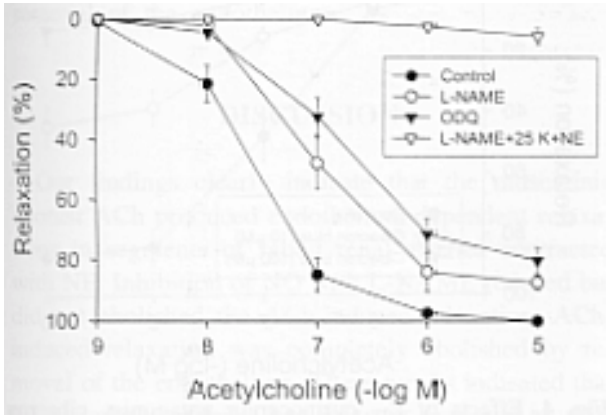

Fig. 1. Average concentration-response curves for the relaxant effects of ACh in the absence (●) and presence of 0.1 mM L-NAME (○) or in the presence of ODQ (▼) or in the presence of 1 μM NE, L-NAME plus 25 mM KCl (▽). Responses are expressed as the percentage of contraction elicited by NE (1 μM) before the addition of ACh. Each point represents mean of 6～10 rings and S.E.M. is shown by vertical bar. L-NAME, N^G-nitro-L-arginine methyl ester hydrochloride.

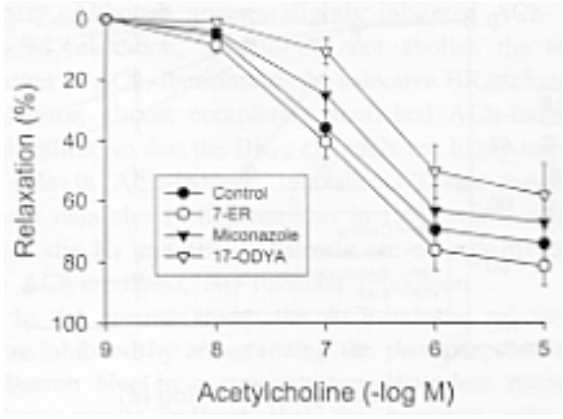

Fig. 2. Effects of cytochrome P450 inhibitors on ACh-induced relaxation in endothelium-intact L-NAME (0.1 mM)-pretreated segments of the rabbit renal artery precontracted with NE (1 μM). ●, Control; ○, in the presence of 7-ER (10 μM); ▼, in the presence of miconazole (10 μM); ▽, in the presence of 17-ODYA (10 μM). Responses are expressed as the percentage of contraction elicited by NE before the addition of ACh. Each point represents mean of 5～8 rings and S.E.M. is shown by vertical bar. L-NAME, N^G-nitro-L-arginine methyl ester hydrochloride.

miconazole, cytochrome P450 blocker (10 μM); or 17-ODYA (10 μM), a suicide-substrate cytochrome P450 mono-oxygenase inhibitor, in the rabbit renal artery pretreated with L-NAME (0.1 mM). ACh-elicited vasorelaxation in the presence of L-NAME was not inhibited by 7-ER, by miconazole or by 17-ODYA (Fig. 2). Application of 11,12-EET (10 μM) to arteries precontracted with NE induced a small relaxation (9.8±3.1%, n=6), which did not differ significantly from the response to vehicle (0.2% ethanol; 5.4±2.3%, n=5), indicating that 11,12-EET had no relaxant effect of its own.

Role of K^+ channels in ACh-mediated vasorelaxation

The contribution of K^+ channels to ACh-induced relaxation was assessed in a renal artery in the presence of L-NAME (0.1 mM). The ACh-induced relaxation was almost completely abolished following inhibition of delayed rectifier K^+ channels with 4-AP (5 mM) (n=8, Fig. 3). The ACh-induced relaxation was also strongly inhibited by an inhibition of large conductance Ca^{2+}-activated K^+ channels with iberiotoxin (0.3 μM; n=5), and further reduced by the combination of iberiotoxin (0.1 μM) and apamin (1 μM) (n=6, Fig. 4). On the other hand, an inhibitor of the ATP-sensitive K^+ channels glibenclamide (10 μM) did not affect the ACh-induced relaxation (n=5, data not shown).

Effect of cibacron blue on ACh-mediated vasorelaxation

The possible involvement of P_{2Y} purinoceptor to ACh-induced relaxation was assessed with cibacron blue, a P_{2Y} receptor antagonist, in the rabbit renal artery pretreated with L-NAME (0.1 mM). The ACh-induced relaxation in the rings precontracted with NE was significantly reduced by a cibacron blue (10 and 100 μM) in a concentration-dependent manner (n=5) (Fig. 4). On the other hand, the selective P_{2X} purinoceptor antagonist PPADS (100 μM) did not affect the ACh-induced relaxation (n=5, data not shown).

Effect of 2MeSATP on ACh-mediated vasorelaxation

In endothelium-intact NE-contracted rabbit renal artery rings under L-NAME (0.1 mM), 2MeSATP also produced concentration-dependent relaxations. The relaxant response was significantly reduced either

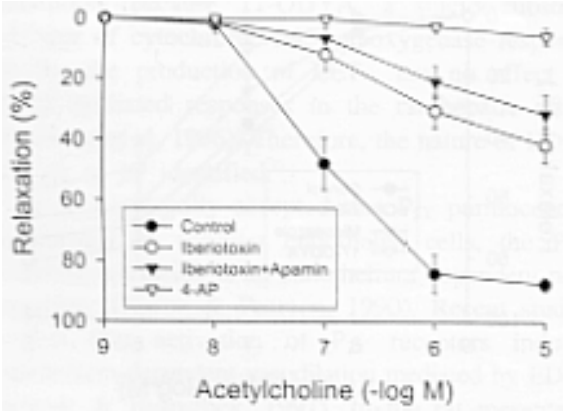

Fig. 3. Effects of K^+ channel blockers on ACh-induced relaxation in endothelium-intact L-NAME (0.1 mM)-pretreated segments of the rabbit renal artery precontracted with NE (1 μM). ●, Control; ○, in the presence of iberiotoxin (0.3 μM); ▼, in the presence of iberiotoxin (0.3 μM) plus apamin (1 μM); ▽, in the presence of 4-AP (5 mM). Responses are expressed as the percentage of contraction elicited by NE before the addition of ACh. Each point represents mean of 5~8 rings and S.E.M. is shown by vertical bar. L-NAME, N^G-nitro-L-arginine methyl ester hydrochloride.

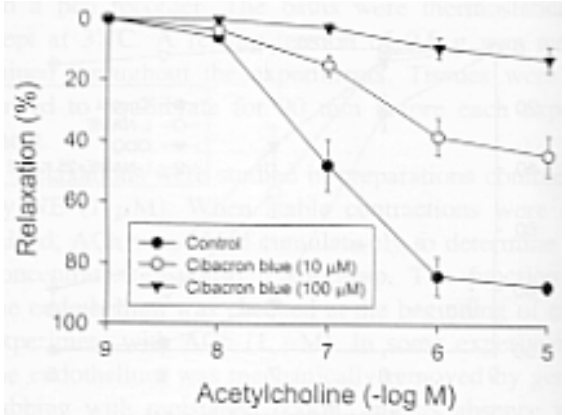

Fig. 4. Effects of P_{2Y} purinoceptor antagonist, cibacron blue on ACh-induced relaxation in endothelium-intact L-NAME (0.1 mM)-pretreated segments of the rabbit renal artery precontracted with NE (1 μM). ●, Control; ○, in the presence of 10 μM cibacron blue; ▼, in the presence of 100 μM cibacron blue. Responses are expressed as the percentage of contraction elicited by NE before the addition of ACh. Each point represents mean of 6 rings and S.E.M. is shown by vertical bar. L-NAME, N^G-nitro-L-arginine methyl ester hydrochloride.

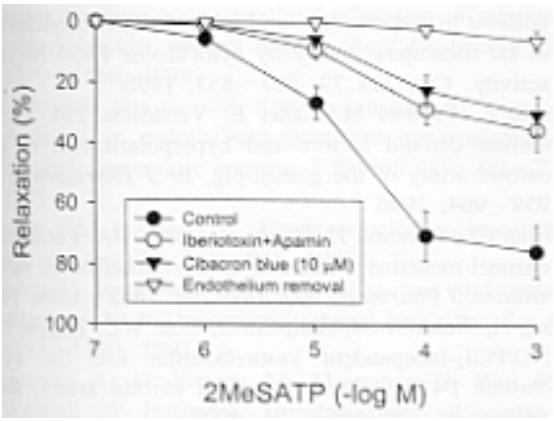

Fig. 5. Average concentration-response curves for the relaxant effects of 2MeSATP, a potent P_{2Y} agonist, in endothelium-intact ring segments of the rabbit renal artery precontracted with NE (1 μM). ●, Control; ○, in the presence of iberiotoxin (0.3 μM) plus apamin (1 μM); ▼, in the presence of 10 μM cibacron blue. (▽) Endothelium was removed. Responses are expressed as the percentage of contraction elicited by NE before the addition of ACh. Each point represents mean of 5~6 rings and S.E.M. is shown by vertical bar. L-NAME, N^G-nitro-L-arginine methyl ester hydrochloride.

by iberiotoxin (0.3 μM, n=6) or by cibacron blue (10 μM, n=5) (Fig. 5). This 2MeSATP-induced vasorelaxation was completely abolished by mechanical removal of the endothelium.

DISCUSSION

Our findings clearly indicate that the muscarinic agonist ACh produced endothelium-dependent relaxations in segments of rabbit renal arteries contracted with NE. Inhibition of NO with L-NAME reduced but did not abolished the ACh-induced relaxation. ACh-induced relaxation was completely abolished by removal of the endothelium. These results indicated that the endothelium-dependent relaxant response to ACh in the rabbit renal artery is partly mediated by NO-independent factor, probably EDHF. Moreover, the ACh-induced relaxation was completely prevented by L-NAME in the presence of high extracellular K^+, suggesting that the EDHF may be released from endothelial cells contributing to endothelium-dependent relaxation. Similar observations have been made with the rabbit carotid artery and the rat renal artery (Dong et al, 1997; Jiang et al, 2000).

Although the nature of EDHF has not yet been elucidated, recent studies indicate that EDHF may be a cytochrome P450 product. In the present study, we provided evidence that the cytochrome P450 inhibitors: 7-ER, miconazole, or 17-ODYA, did not modulate NO-independent relaxation to ACh in the rabbit renal artery. Our results are in general agreement with those of Zygmunt et al (1996) and Fukao et al (1997) in which cytochrome P450 inhibitors were without effect on EDHF-mediated hyperpolarization and relaxation. It is therefore unlikely that cytochrome P450 mono-oxygenase enzyme is involved in the formation of EDHF in the rabbit renal artery.

In the present 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 renal artery. EDHF-mediated relaxation in the rat hepatic artery is inhibited by 4-AP and charybdotoxin (Zygmunt & Hogestatt, 1996; Zygmunt et al, 1997). Since charybdotoxin inhibits not only BK_{Ca}, but also K_v channels, this leads to the conclusion that the K_v channels are a target for EDHF. In this study, 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 renal artery. Although apamin slightly 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, 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 renal artery, both the K_v and BK_{Ca} channels are mostly involved in ACh-mediated, NO-resistant relaxation.

In the present study, the ACh-induced relaxation was inhibited by antagonizing the purinoceptors with cibacron blue in a concentration-dependent manner. These results indicate that the response may be evoked by the activation of P_{2Y} purinoceptors. The significant inhibition of the ACh-induced relaxation is likely to be due to a specific action, because cibacron blue has been demonstrated to be a selective P_{2Y} receptor antagonist in vascular smooth muscles (Hopwood & Burnstock, 1987). A number of studies reported that P_{2Y} purinoceptors are typically located on the endothelial cells, and the activation of endothelial P_{2Y} induced the release of EDHF (Olsson & Pearson, 1990; Malmsjo et al, 1999). Vasoactive

substances such as noradrenaline, bradykinin, ACh and serotonin are known to release ATP from endothelial cells (Shinozuka et al, 1994; Yang et al, 1994). These results indicated that the released ATP from endothelial cells might activate P_{2Y} purinoceptors on the endothelial cells to induce the EDHF mediated responses.

In the present study, the selective P_{2Y} receptor agonist 2MeSATP, like ACh, induced relaxation of NE-precontracted rabbit renal artery. This relaxation appears to occur by activation of P_{2Y} purinoceptors located on endothelial cells with subsequent involvement of EDHF because this agent induced vasorelaxation, which was blocked by iberiotoxin plus apamin, and was antagonized by cibacron blue and abolished by endothelial denudation. Furthermore, in rat mesenteric arteries, Malmsjo et al (1998) have reported that stimulation of P_{2Y} receptors triggers endothelium-dependent relaxations, which are inhibited by this toxin combination in the presence of L-NAME and indomethacin. Recent studies also suggest that activation of P_{2Y} receptors induces endothelium-dependent vasodilation mediated by EDHF (You et al, 1997; McMurray et al, 1998; Malmsjo et al, 1999). Therefore it is suggested that 2MeSATP activates P_{2Y} receptors to induce the release of EDHF in endothelial cells of rabbit renal arteries. However, further investigations are needed to draw any conclusions on the nature of purinergic subtype receptors present on endothelial cells.

In conclusion, we have demonstrated that in the rabbit renal artery, the released ATP from endothelial cells activates endothelial P_{2Y} receptors in an autocrine manner to induce the release of EDHF. This in turn induces vasorelaxation via a mechanism that involves activation of both the K_v and BK_{Ca} channels.

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