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# $K^+$ channel blockers and cytochrome *P*450 inhibitors on acetylcholine-induced, endothelium-dependent relaxation in rabbit mesenteric artery

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**Abstract**

Acetylcholine caused an endothelium-dependent relaxation in isolated rabbit mesenteric small artery in the presence of nitro L-arginine and indomethacin. The acetylcholine-induced relaxation was attenuated by high $K^+$ solution, suggesting that the response is mediated by a membrane potential-sensitive mechanism, presumably an endothelium-derived hyperpolarizing factor. The acetylcholine-induced relaxation was also inhibited with tetraethylammonium, 4-aminopyridine and charybdotoxin, but not with $Ba^{2+}$, apamin, iberiotoxin nor glibenclamide. The relaxation was abolished by a combination of apamin and charybdotoxin, but iberiotoxin could not replace charybdotoxin in this combination. The responses to charybdotoxin and 4-aminopyridine were synergistic but neither apamin nor iberiotoxin increased the effect of 4-aminopyridine. Clotrimazole and proadifen inhibited the acetylcholine-induced relaxation, but these drugs also inhibited the cromakalim-induced relaxation, while protoporphyrin IX inhibited the acetylcholine- but not cromakalim-induced relaxation. 17-Octadecynoic acid and 1-aminobenzotriazole did not affect the response to acetylcholine. Four regioisomers of epoxyeicosatrienoic acids did not relax endothelium-denuded artery. A gap junction inhibitor 18α-glycyrrhetinic acid attenuated the relaxation to acetylcholine. It is suggested that in rabbit mesenteric artery, the acetylcholine-induced, nitric oxide- and prostacyclin-independent relaxation is mainly mediated by 4-aminopyridine- and charybdotoxin-sensitive $K^+$ channels and that the relaxation is not mediated through cytochrome *P*450 enzyme metabolites. The contribution of heterocellular gap junctional communication to the relaxation is discussed. © 1999 Elsevier Science B.V. All rights reserved.

*Keywords:* Acetylcholine; Cytochrome *P*450 inhibitor; Endothelium; 18α-glycyrrhetinic acid; $K^+$ channel blocker; Nitro L-arginine

## 1. Introduction

Acetylcholine elicits endothelium-dependent relaxation and hyperpolarization of vascular smooth muscles by release of nitric oxide (NO) or its related compounds, prostacyclin and endothelium-derived hyperpolarizing factor (EDHF) from endothelial cells (Palmer et al., 1987; Chen et al., 1988; Campbell et al., 1996). It is thought that the acetylcholine-induced relaxation in the presence of NO synthase and cyclooxygenase inhibitors is accounted for by the actions of EDHF, which produces a hyperpolarization in smooth muscle cells (Zygmunt et al., 1994; Dong et al., 1997). The contribution of EDHF to the acetylcholine-induced, endothelium-dependent relaxation seems to depend on species, tissue and contractile agonist used (Nagao et al., 1992; Cowan et al., 1993; Fujimoto and Itoh, 1997).

EDHF elicits its effect through the opening of $K^+$ channels in the smooth muscle (Murphy and Brayden, 1995; Campbell et al., 1996; Hashitani and Suzuki, 1997). Studies with $K^+$ channel blockers on identifying subtypes of $K^+$ channels involved have not given a consistent result. The acetylcholine-induced relaxation and hyperpolarization of smooth muscles in the presence of NO synthase and cyclooxygenase inhibitors are inhibited by an ATP-sensitive $K^+$ channel blocker glibenclamide in rabbit cerebral artery and aorta (Brayden, 1990; Cowan et al., 1993) but not in other arteries (Murphy and Brayden, 1995; Chen and Cheung, 1997; Eckman et al., 1998). The responses to acetylcholine are blocked by apamin, a small conductance $Ca^{2+}$-sensitive $K^+$ channel blocker (Garcia-Pascual et al., 1995; Murphy and Brayden, 1995) and

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charybdotoxin, a blocker of several subtypes of $K^+$ channels (Cowan et al., 1993; Dong et al., 1997). Thus, the response seems to involve at least two distinct $K^+$ channels. However, in guinea-pig carotid and rat hepatic arteries, apamin and charybdotoxin does not individually modify the acetylcholine-induced, EDHF-mediated relaxation or hyperpolarization, whereas the combination of two toxins abolishes them (Corriu et al., 1996b; Zygmunt and Högestätt, 1996; Zygmunt et al., 1997). Furthermore, 4-aminopyridine, a delayed rectifier $K^+$ channel blocker, reduces the EDHF-mediated relaxation in guinea-pig coronary but not cerebral arteries (Petersson et al., 1997; Yamanaka et al., 1998). $Ba^{2+}$, an inward rectifier $K^+$ channel blocker, inhibits the EDHF-mediated responses in rat hepatic and rabbit cerebral arteries (Brayden, 1990; Edwards et al., 1998) but not in guinea-pig coronary and rabbit mesenteric arteries (Murphy and Brayden, 1995; Eckman et al., 1998). These conflicting results may explain that multiple EDHFs are generated from the endothelium and activate various $K^+$ channels, being dependent on tissue source and species. In addition, acetylcholine activates $Ca^{2+}$-sensitive $K^+$ channels in endothelial cells, leading to hyperpolarization which promotes the generation of EDHF by facilitating entry of $Ca^{2+}$ (Lückhoff and Busse, 1990; Chen and Cheung, 1992; Edwards et al., 1998; Ohashi et al., 1999). Thus, there is a possibility that $K^+$ channel blockers may act on endothelial cells to inhibit the acetylcholine-induced generation of EDHF.

It has been proposed that epoxyeicosatrienoic acids, cytochrome *P*450 metabolites of arachidonic acid, are EDHFs in several blood vessels, since (1) endothelial cells produce epoxyeicosatrienoic acids from arachidonic acid and their release is stimulated by cholinomimetics (Campbell et al., 1996), (2) epoxyeicosatrienoic acids have endothelium-independent vasorelaxing and hyperpolarizing effects, which are inhibited by $K^+$ channel blockers as well as high $K^+$ solution (Hecker et al., 1994; Campbell et al., 1996), and (3) certain inhibitors of cytochrome *P*450 enzymes reduce agonist-induced, NO- and prostacyclin-independent responses which are mediated by endothelium (Bauersachs et al., 1994; Lischke et al., 1995; Dong et al., 1997). However, since some cytochrome *P*450 enzyme inhibitors have an inhibitory effect on several subtypes of $K^+$ channels in vascular and non-vascular tissues (Alvarez et al., 1992; Edwards et al., 1996; Zygmunt et al., 1996; Yamanaka et al., 1998), the effect of these drugs can be explained by blockage of $K^+$ channels in smooth muscles rather than an inhibition of the EDHF production. In addition, epoxyeicosatrienoic acids are not always vasorelaxants in arteries in which acetylcholine elicits endothelium-dependent relaxation (Graier et al., 1996; Zygmunt et al., 1996; Yamanaka et al., 1998).

It has been proposed that arachidonic acid derivative anandamide and $K^+$ may also represent EDHF in some (Randall et al., 1996; Edwards et al., 1998) but not in other blood vessels (White and Hiley, 1997; Quignard et al., 1999). Moreover, Zakhary et al. (1996) have described that an endothelial product carbon monoxide could account for the NO- and prostacyclin-independent relaxation of pig pulmonary arteries.

Functional and electrophysiological studies with inhibitors of gap junctional communication between the endothelium and smooth muscle have suggested that EDHF is transferrd from the endothelium to smooth muscle cells or endothelial hyperpolarization is directly conducted to the smooth muscle via gap junctions (Chaytor et al., 1998; Taylor et al., 1998; Yamamoto et al., 1999).

We have reported that in rabbit mesenteric small artery, endothelium-dependent relaxation in response to acetylcholine is highly resistant to nitro L-arginine (L-NA, a NO synthase inhibitor) and indomethacin (a cyclooxygenase inhibitor) (Fujimoto and Itoh, 1997). Therefore, in these arteries, (1) we have studied effects of $K^+$ channel blockers on the acetylcholine-induced relaxation in the presence of L-NA and indomethacin, (2) to challenge the hypothesis that EDHF is a cytochrome *P*450 metabolite of arachidonic acid, we have studied effects of various *P*450 enzyme inhibitors and arachidonyl trifluoromethyl ketone, an inhibitor of phospholipase $A_2$, on the acetylcholine-induced relaxation and (3) effects of 18α-glycyrrhetinic acid, a gap junction inhibitor, have been investigated on the relaxation evoked by acetylcholine.

## 2. Materials and methods

### *2.1. Vascular smooth muscle preparations and tension measurement*

Male Japan white rabbits (Kitayama Labs, Japan), weighing 1.9–2.3 kg, were anesthetized with pentobarbitone sodium (Nembutal, 40 mg/kg, i.v.) and killed by rapid exsanguination. The mesentery was removed and placed in Krebs-Henseleit bicarbonate (KHB) buffer (composition in mM: NaCl 114, KCl 4.7, $CaCl_2$ 2.5, $MgCl_2$ 1.2, $KH_2PO_4$ 1.2, $NaHCO_3$ 25 and dextrose 10) oxygenated with 5% $CO_2$ in $O_2$. Mesenteric small artery corresponding to a second order branch from the superior mesenteric artery was cut into rings, 2 mm long, and suspended between two metal pins under a resting tension of 300 mg in 5 ml of warmed (37°C) and oxygenated KHB buffer for isometric recordings. The KHB buffer contained 2 μM propranolol to avoid β-adrenoceptor-mediated relaxation. Acetylcholine-induced relaxation was determined in KHB buffer containing 100 μM L-NA and 10 μM indomethacin to avoid the possible productions of NO and prostacyclin, respectively. During an equilibration period of 60–90 min, the preparations were repeatedly stretched until a stable resting tension was obtained. Thereafter, the preparation was contracted 3 times with 1–10 μM norepinephrine for 10 min at 40-min intervals. In some experiments, the endothelium was removed by gentle rubbing of

the intimal surface with a metal wire. Successful removal of the functional endothelium from the preparations was confirmed by the response to acetylcholine (1 μM) during contractions elicited by norepinephrine (1 μM).

### 2.2. Relaxation responses to acetylcholine, sodium nitroprusside, cromakalim and epoxyeicosatrienoic acids

The arteries were contracted with norepinephrine at 0.9 μM to 75%–85% ($EC_{75}$) of the maximum norepinephrine response to study response to vasorelaxants, since we had previously found that mean $EC_{75}$ values for the norepinephrine-induced contraction were 0.9 μM (variation in means was less than 15%) either in the endothelium-intact artery in the presence of L-NA and indomethacin or in the endothelium-denuded artery (Fujimoto and Itoh, 1997). After the contraction in response to norepinephrine had reached steady state, cumulative concentration-response curves for acetylcholine were made as follows; two sequential concentration–response curves were made with intervals of 120–180 min between curves. One of paired preparations was treated with inhibitors of $K^+$ channels for 30–60 min, cytochrome *P*450 enzymes for 60–120 min, phospholipase $A_2$ for 90 min and of myoendothelial gap junctions for 60 min before the second curves were determined. Another untreated preparation was used as control. In some experiments, the endothelium-intact mesenteric arteries were contracted with 15.9 and 35.9 mM KCl and further contraction was made with norepinephrine to obtain a contraction similar to that elicited by norepinephrine alone. The final concentrations of norepinephrine were $0.75 \pm 0.05$ μM ($n = 8$) and $0.30 \pm 0.08$ μM ($n = 12$) in the 15.9 mM KCl- and 35.9 mM KCl-stimulated tissues, respectively. Concentration–response curves for sodium nitroprusside-, cromakalim- and epoxyeicosatrienoic acid-induced relaxations were also made in the same way, but in the endothelium-denuded preparation. At the end of the experiment, papaverine (0.1 mM) was added to obtain the maximum relaxation. Since papaverine relaxed the preparations back to almost baseline tension, relaxation responses to the vasorelaxants were expressed as a percentage of the papaverine-induced relaxation (deduction of the tension obtained with papaverine from the norepinephrine-elicited contraction). $EC_{50}$ value is the molar concentration producing 50% of the maximum acetylcholine response in the particular concentration–response curves.

### 2.3. Drugs and solutions

The following drugs were dissolved in distilled water and diluted with the KHB buffer; acetylcholine chloride (Sigma, St. Louis, MO, USA), 1-aminobenzotriazole (Aldrich Chem., Milwaukee, WI, USA), 4-aminopyridine (Sigma), apamin (Sigma), barium chloride (Wako, Osaka, Japan), charybdotoxin (Peptide Institute, Minoh, Japan), iberiotoxin (Sigma), $N^G$-nitro L-arginine (L-NA, Peptide Institute), (−)-norepinephrine bitartrate (Sigma), proadifen HCl (Sigma), DL-propranolol HCl (Sigma), sodium nitroprusside (Wako) and tetraethylammonium chloride (Wako). Cromakalim (Sigma), indomethacin (Sigma) and 17-octadecynoic acid (Sigma) were dissolved in ethanol. Clotrimazole (Sigma), glibenclamide (Sigma), 18α-glycyrrhetinic acid (Sigma), protoporphyrin IX (Sigma) and arachidonyl trifluoromethyl ketone (AACOCF3, Research Biochemicals International, Natic, MA, USA) were dissolved in dimethyl sulfoxide (Sigma). The final concentrations (less than 0.1%) of this solvent in the bathing medium had no noticeable effect on muscle contraction or relaxation. 5,6-, 8,9-, 11,12- and 14,15-Epoxyeicosatrienoic acids obtained as an oil dissolved in ethanol (Sigma) were 10 times concentrated by evaporation in $N_2$ gas at room temperature immediately before use.

### 2.4. Statistical analysis

All values are presented in terms of the means ± S.E. of the number ($n$) of observations. Analysis by Student's *t*-test was performed for paired or unpaired observations as appropriate. $P$ values less than 0.05 were considered significant.

## 3. Results

### 3.1. Effect of KCl on relaxation responses to acetylcholine and cromakalim

In the presence of L-NA and indomethacin, acetylcholine (10 nM to 10 μM) elicited concentration- and endothelium-dependent relaxation in mesenteric small artery stimulated by norepinephrine (Fig. 1A). $EC_{50}$ value and maximum response for acetylcholine-induced relaxation are in Table 1. These results were consistent with our previous data (Fujimoto and Itoh, 1997). When the artery was contracted with a combination of norepinephrine (0.75 ± 0.05 μM) and KCl (15.9 mM), the response to acetylcholine was markedly reduced, and raising the extracellular $K^+$ concentration to 35.9 mM in the presence of norepinephrine ($0.30 \pm 0.08$ μM) eliminated the response. Cromakalim (10 nM–3 μM) relaxed endothelium-denuded arteries in a concentration-dependent fashion (Fig. 1B). The relaxation was abolished by the combination of norepinephrine and KCl (35.9 mM).

### 3.2. Effects of $K^+$ channel blockers on the response to acetylcholine and sodium nitroprusside

Tetraethylammonium (1 and 10 mM) reduced concentration-dependently the ability of the tissues to relax in response to acetylcholine in the presence of L-NA and indomethacin (Fig. 2A). $Ba^{2+}$ (0.1 mM) did not alter the acetylcholine-induced relaxation (Fig. 2B). 4-Aminopyri-

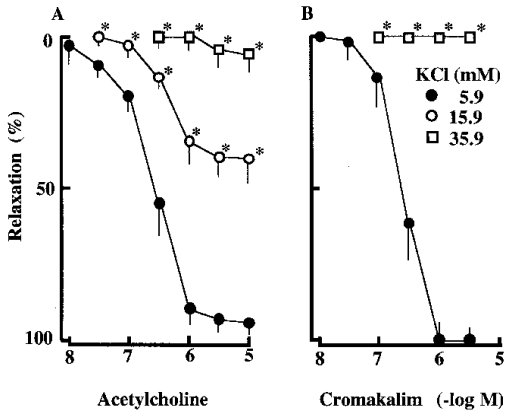


Fig. 1. Cumulative concentration–response curves for acetylcholine-(A) and cromakalim-(B) induced relaxation in mesenteric artery. The acetylcholine-induced relaxation in the presence of L-NA and indomethacin was determined in the endothelium-intact artery and cromakalim-induced response was determined in the endothelium-denuded artery. The artery was contracted with norepinephrine in the presence of KCl (5.9 mM ●, 15.9 mM ○, 35.9 mM □). Ordinate; papaverine (0.1 mM)-induced relaxations are expressed as 100%, and the absolute values for the response to papaverine were 383 ± 19 mg ($n = 20$, ●), 334 ± 21 mg ($n = 8$, ○) and 349 ± 38 mg ($n = 12$, □). Vertical bars represent S.E. of means. $^*P < 0.05$ vs. control (●).

dine (0.1 and 0.5 mM) diminished the response (Fig. 2C). Charybdotoxin (10 nM and 100 nM) shifted the concentration-response curve for acetylcholine to the right and upwards (Fig. 2D). The remaining relaxation due to acetylcholine in the presence of 4-aminopyridine (0.5 mM) was inhibited by charybdotoxin (100 nM, Fig. 2C) but not by apamin (100 nM, Fig. 2C) nor by iberiotoxin (100 nM, data not shown). The remaining relaxation due to acetylcholine in the presence of charybdotoxin (100 nM, Fig. 2D) was abolished by apamin (100 nM), which did not alter the response to acetylcholine in the absence of

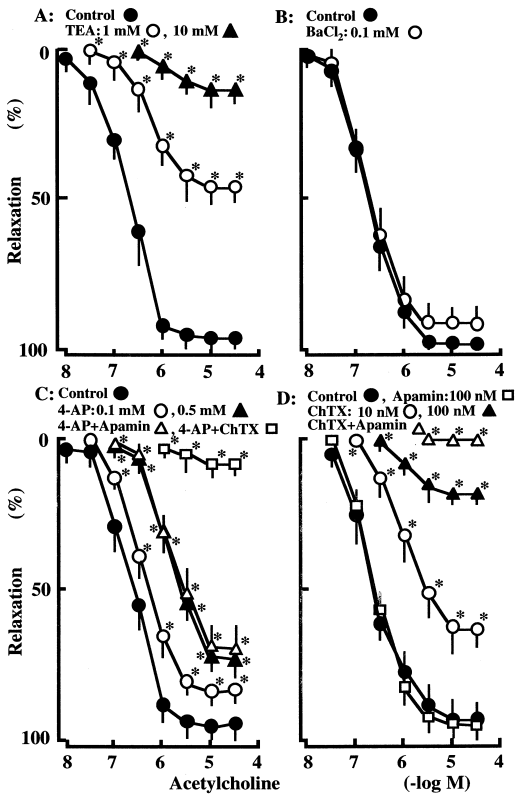


Fig. 2. Effects of $K^+$ channel blockers on acetylcholine-induced, endothelium-dependent relaxation of mesenteric artery in the presence of L-NA and indomethacin. The artery was treated for 30–60 min with the following $K^+$ channel blockers before and during the determination of the concentration–response curve for acetylcholine. (A); tetraethylammonium (TEA: 1 mM ○, 10 mM ▲), (B); $Ba^{2+}$ (0.1 mM ○), (C); 4-aminopyridine (4-AP: 0.1 mM ○, 0.5 mM ▲), apamin (100 nM △) or charybdotoxin (ChTX: 100 nM □) in the presence of 4-AP (0.5 mM), (D); apamin (100 nM □), charybdotoxin (ChTX: 10 nM ○, 100 nM ▲), a combination of ChTX (100 nM) and apamin (100 nM, △). (●), Control. Ordinate; the relaxation due to papaverine (0.1 mM) is expressed as 100% (absolute values; 406 ± 34 mg, $n = 15$). Vertical bars represent S.E. of means ($n = 4$–22). $^*P < 0.05$ vs. control.

Table 1
Effects of iberiotoxin, glibenclamide, arachidonyl trifluoromethyl ketone (AACOCF3), 17-octadecynoic acid and 1-aminobenzotriazole on acetylcholine-induced, NO- and prostacyclin-independent relaxation in mesenteric artery

Results are expressed as means ± S.E. of means of $n$ observations with rings from different arteries.

The endothelium-intact preparation was constracted with norepinephrine, and concentration–response curves for acetylcholine-induced relaxation were determined in the presence of L-NA (100 μM) and indomethacin (10 μM).

| | $n$ | $EC_{50}$ value (μM) | Maximum relaxation (%)[a] |
|---|---|---|---|
| Control | 30 | 0.223 ± 0.059 | 95 ± 2 |
| Iberiotoxin, 100 nM | 6 | 0.218 ± 0.057 | 89 ± 2 |
| Glibenclamide, 10 μM | 5 | 0.198 ± 0.037 | 93 ± 2 |
| AACOCF3, 10 μM | 6 | 0.208 ± 0.039 | 88 ± 2 |
| 17-Octadecynoic acid, 50 μM | 8 | 0.268 ± 0.048 | 91 ± 1 |
| 1-Aminobenzotriazole, 2 mM | 5 | 0.211 ± 0.044 | 90 ± 3 |

[a]Maximum acetylcholine responses are expressed as a percentage of the relaxation induced by papaverine (0.1 mM).

charybdotoxin (Fig. 2D). Iberiotoxin (100 nM) alone or in the combination with apamin (100 nM) did not alter the response to acetylcholine (data not shown). Glibenclamide (10 μM) did not alter the response. $EC_{50}$ values and maximum responses for acetylcholine-induced relaxation in the presence of iberiotoxin and glibenclamide are in Table 1. The basal vascular tone and contractile response to norepinephrine were not significantly affected by these $K^+$ channel blockers at concentrations used.

To determine whether or not the inhibitory effect of these $K^+$ channel blockers was specific to the acetylcholine-induced, NO- and prostacyclin-independent relaxation, we studied effects of the $K^+$ channel blockers on relaxation in response to an NO donor sodium nitroprusside. Sodium nitroprusside (1 nM–1 μM) relaxed norepinephrine (0.9 μM)-stimulated, endothelium-denuded artery with $EC_{50}$ value and maximum response of 3.08 ± 0.27 nM and 94 ± 2% ($n = 16$), respectively. Neither 4-

aminopyridine (0.5 mM), charybdotoxin (100 nM) nor a combination of charybdotoxin (100 nM) and apamin (100 nM) changed the response to sodium nitroprusside; $EC_{50}$ value and maximum response for sodium nitroprusside were $3.58 \pm 0.19$ nM and $94 \pm 1\%$ ($n = 6$, 4-aminopyridine), $2.96 \pm 0.28$ nM and $92 \pm 4\%$ ($n = 4$, charybdotoxin) and $3.32 \pm 0.35$ nM and $96 \pm 3\%$ ($n = 6$, charybdotoxin plus apamin), respectively. In addition, the response to sodium nitroprusside was not altered by a combination of 4-aminopyridine (0.5 mM) and charybdotoxin (100 nM) ($EC_{50}$ value; $2.90 \pm 0.34$ nM, maximum response; $94 \pm 3\%$, $n = 6$).

### 3.3. Effects of arachidonyl trifluoromethyl ketone (AACOCF3), cytochrome P450 enzyme inhibitors and 18α-glycyrrhetinic acid on the response to acetylcholine, sodium nitroprusside and cromakalim

When the artery was treated with AACOCF3 (10 μM) for 90 min, vascular contractile response to norepinephrine (0.9 μM) was reduced from $335 \pm 26$ mg to $258 \pm 22$ mg ($n = 6$, $P < 0.05$). Therefore, the artery was contracted with 2 μM norepinephrine to $308 \pm 43$ mg ($n = 6$) that was similar to the response to norepinephrine in the absence of AACOCF3. The concentration–response curves for acetylcholine was not altered by AACOCF3 in the artery. The $EC_{50}$ values and maximum responses for the acetylcholine-induced relaxation in the presence of AACOCF3 are in Table 1.

Treatment with clotrimazole and proadifen (400 nM and 2 μM) for 60 min reduced relaxation response to acetylcholine in the presence of L-NA and indomethacin (Fig. 3Aa,b). Protoporphyrin IX (100 nM) abolished the response (Fig. 3Ac). These drugs did not affect the relaxation response to sodium nitroprusside (Fig. 3B). On the other hand, relaxation in response to cromakalim was reduced by clotrimazole (400 nM and 2 μM), abolished by proadifen (400 nM) and unaffected by protoporphyrin IX (100 nM)(Fig. 3C). Treatment with 17-octadecynoic acid (50 μM) for 120 min did not significantly change the acetylcholine-induced relaxation; $EC_{50}$ values and maximum responses for acetylcholine in the presence of 17-octadecynoic acid are in Table 1.

Clotrimazole, proadifen, protoporphyrin IX and 17-octadecynoic acid at concentrations used did not affect the basal vascular tone and the norepinephrine-induced contraction. On the other hand, treatment with 1-aminobenzotriazole (2 mM) for 120 min did not change the basal resting tension but reduced the norepinephrine-induced contraction to $159 \pm 33$ mg ($n = 5$), therefore, the preparation was contracted with 5 μM norepinephrine to $266 \pm 35$ mg ($n = 5$) which was still significantly less than the response to norepinephrine (0.9 μM) alone (norepinephrine at concentrations higher than 5 μM did not cause further contractions). 1-Aminobenzotriazole did not affect

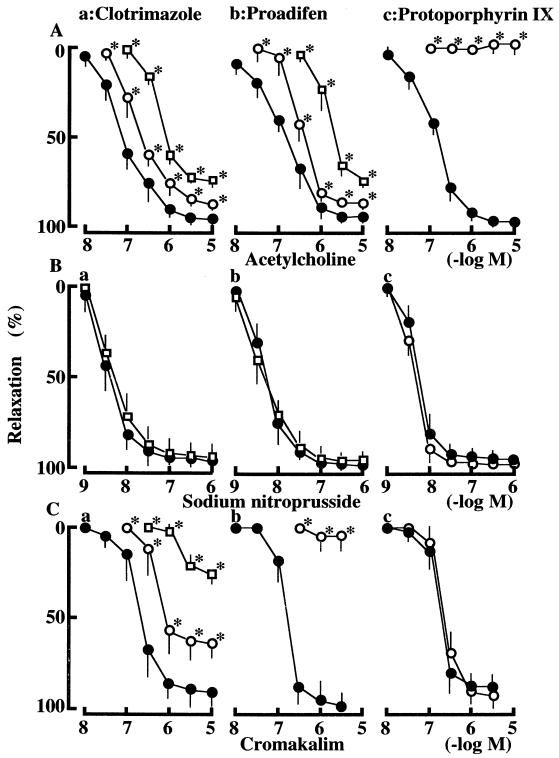


Fig. 3. Effects of clotrimazole (a), proadifen (b) and protoporphyrin IX (c) on acetylcholine-(A), sodium nitroprusside-(B) and cromakalim-(C) induced relaxation in endothelium-intact (A) or -denuded (B, C) arteries. The tissues were exposed for 60 min with clotrimazole (400 nM ○, 2 μM □), proadifen (400 nM ○, 2 μM □)) and protoporphyrin IX (100 nM ○). Control (●). Ordinate; the relaxation due to papaverine (0.1 mM) is expressed as 100%. Vertical bars represent S.E. of means ($n = 4$–8). $^*P < 0.05$ vs. control.

the acetylcholine-induced relaxation in the artery precontracted by 5 μM norepinephrine; $EC_{50}$ values and maximum responses for acetylcholine in the presence of 1-aminobenzotriazole are in Table 1.

18α-Glycyrrhetinic acid (70 μM) for 60 min did not significantly alter the basal vascular tone but reduced the contraction evoked by norepinephrine (0.9 μM). The artery was, therefore, contracted with 5 μM norepinephrine to $258 \pm 31$ mg ($n = 6$) which was similar to the contraction induced by 0.9 μM norepinephrine alone ($317 \pm 46$ mg, $n = 6$). 18α-Glycyrrhetinic acid partially reduced the acetylcholine-induced, endothelium-dependent relaxation in the presence of L-NA and indomethacin (Fig. 4A), but did not change the response to sodium nitroprusside in endothelium-denuded arteries (Fig. 4B). It was not possible to use 18α-glycyrrhetinic acid at concentrations higher than 70 μM in the organ bath due to solubility difficulties.

### 3.4. Effect of epoxyeicosatrienoic acids

In endothelium-denuded artery which had been contracted with norepinephrine (0.9 μM), 5,6-, 7,8-, 11,12- and 14,15-epoxyeicosatrienoic acids (10 μM) were not

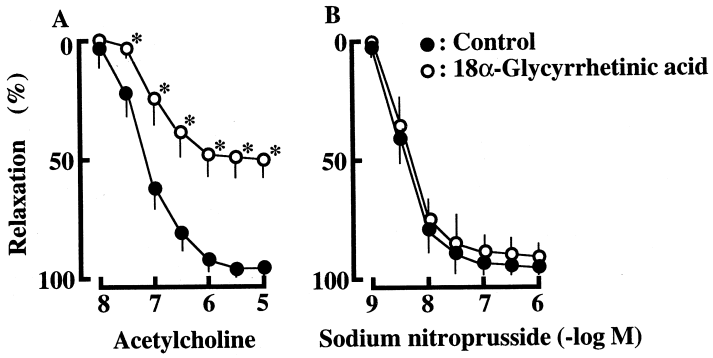


Fig. 4. Effects of 18α-glycyrrhetinic acid on acetylcholine-(A) and sodium nitroprusside-(B) induced relaxation in endothelium-intact (A) or -denuded (B) arteries. The tissues were exposed for 60 min with 18α-glycyrrhetinic acid (70 μM ○) and vehicle (●), and contracted with 5 μM and 0.9 μM norepinephrine, respectively. Acetylcholine-induced relaxation was determined in the presence of L-NA and indomethacin. Ordinate; papaverine (0.1 mM)-induced relaxations are expressed as 100%. Vertical bars represent S.E. of means ($n = 4–5$). $^*P < 0.05$ vs. control.

vasorelaxants ($n = 2$ for each epoxyeicosatrienoic acid, data not shown).

## 4. Discussion

In the present as well as previous studies with rabbit mesenteric small artery (Fujimoto and Itoh, 1997), we attempted to characterize endothelium-dependent relaxation response to acetylcholine in the presence of L-NA (100 μM) and indomethacin (10 μM). Many investigators have also treated blood vessels with L-NA (100 μM) and indomethacin (10 μM) to minimize the contribution of NO and prostacyclin to acetylcholine-induced responses (relaxation and hyperpolarization) (Dong et al., 1997; Eckman et al., 1998; Yamanaka et al., 1998). We found that the acetylcholine-induced, NO- and prostacyclin-independent as well as cromakalim-induced, endothelium-independent relaxation disappeared in the mesenteric artery depolarized by 35.9 mM KCl. These results suggest that the acetylcholine-induced relaxation is mediated by membrane potential-sensitive mechanisms, presumably by EDHF. Similarly, acetylcholine-induced, EDHF-mediated responses have been demonstrated in a number of blood vessels (Chen et al., 1988; Chen and Suzuki, 1989; Lischke et al., 1995).

### *4.1. $K^+$ channel blockers on acetylcholine-induced relaxation*

Since subtypes of $K^+$ channels involved in the EDHF-mediated relaxation and hyperpolarization are not fully understood for the present, we investigated effects of various $K^+$ channel blockers on the relaxation due to acetylcholine in the presence of L-NA and indomethacin. It has been demonstrated that the acetylcholine-induced, EDHF-mediated relaxation or hyperpolarization is inhibited by $Ba^{2+}$ (a blocker of inward rectifier $K^+$ channels) and glibenclamide (an ATP-sensitive $K^+$ channel blocker) in some but not in other arteries of various animals (Brayden, 1990; Murphy and Brayden, 1995; Hashitani and Suzuki, 1997; Eckman et al., 1998). The present experiments show that in rabbit mesenteric small artery, neither $Ba^{2+}$ nor glibenclamide alters the acetylcholine-induced, NO- and prostacyclin-independent relaxation, suggesting that activation of these $K^+$ channels does not contribute to the relaxation. The EDHF-mediated relaxation was reduced by tetraethylammonium (1–5 mM, a non-selective $Ca^{2+}$-sensitive $K^+$ channel blocker) in rat mesenteric artery and aorta (Cowan et al., 1993; Chen and Cheung, 1997), charybdotoxin (100 nM, a blocker of several subtypes of $K^+$ channels) in rabbit carotid and guinea-pig coronary arteries (Dong et al., 1997; Yamanaka et al., 1998) and apamin (0.1–1 μM, a small conductance $Ca^{2+}$-sensitive $K^+$ channel blocker) in bovine coronary and oviductal arteries (Hecker et al., 1994; Garcia-Pascual et al., 1995). However, several lines of evidence have shown that neither charybdotoxin nor apamin affects the relaxation and hyperpolarization mediated by EDHF but a combination of both toxins abolishes the responses in guinea-pig carotid and rat hepatic arteries (Corriu et al., 1996b; Zygmunt and Högestätt, 1996; Zygmunt et al., 1997). On the other hand, as has been reported for the guinea-pig basilar and coronary arteries (Petersson et al., 1997; Yamanaka et al., 1998), we found that in rabbit mesenteric small artery, charybdotoxin partially reduced but apamin did not affect the acetylcholine-induced relaxation, but again a combination of both toxins abolished the response. Similarly, the hyperpolarization produced by acetylcholine was not affected by apamin, partially inhibited by charybdotoxin and abolished by a combination of both toxins (Hashitani and Suzuki, 1997). We and others also found that iberiotoxin (100 nM, a highly selective large conductance $Ca^{2+}$-sensitive $K^+$ channel blocker as compared to charybdotoxin), failed to affect the acetylcholine-induced relaxation in the presence and absence of apamin (Petersson et al., 1997; Yamanaka et al., 1998), suggesting that activation of this type of $Ca^{2+}$-sensitive $K^+$ channel does not contribute to the response. The present results support the previous data that 4-aminopyridine (1–5 mM; a moderately selective

blocker of delayed rectifier $K^+$ channels) inhibited the EDHF-mediated relaxation (Zygmunt et al., 1996; Eckman et al., 1998), and the residual response in the presence of 4-aminopyridine was affected by neither apamin nor iberiotoxin but abolished by charybdotoxin. These results suggest that 4-aminopyridine does not suppress, if any, the charybdotoxin-sensitive $K^+$ channel. Therefore, it seems likely that the relaxation is mainly mediated by at least two distinct 4-aminopyridine-sensitive and charybdotoxin-sensitive $K^+$ channels. In addition, we are of opinion that 4-aminopyridine and apamin have different mechanisms by which the inhibitory effect of charybdotoxin on the acetylcholine-induced relaxation is affected. However, the possibility should not be excluded that 4-aminopyridine and apamin affected a single subtype of $K^+$ channels.

Agonists like acetylcholine also induce membrane hyperpolarization in endothelial cells, which is initially due to the release of $Ca^{2+}$ from its store sites and to an activation of $Ca^{2+}$-sensitive $K^+$ channels (Lückhoff and Busse, 1990; Wang et al., 1996). The hyperpolarization results in an increase in the driving force for transmembrane $Ca^{2+}$ influx, thereby leading to an increase in amounts of intracellular $Ca^{2+}$ which is thought to play a crucial role in the EDHF production and/or release from the endothelium (Chen and Suzuki, 1990; Fukao et al., 1997b). The acetylcholine-induced hyperpolarization in endothelial cells was inhibited by tetraethylammonium and charybdotoxin in guinea-pig coronary artery and rabbit aorta (Chen and Cheung, 1992; Wang et al., 1996). The response was abolished by a combination of apamin and charybdotoxin in endothelial cells of rat hepatic artery and rabbit aortic valve (Edwards et al., 1998; Ohashi et al., 1999). In addition, Rusko et al. (1992) have demonstrated that charybdotoxin-sensitive $K^+$ channels are present in the rabbit aortic endothelium. Therefore, we can not rule out the possibility that the $K^+$ channel blockers used can partly reduce the EDHF production via blockage of $K^+$ channels and of $Ca^{2+}$ influx on the endothelium and therefore inhibit the acetylcholine-induced relaxation of smooth muscles. However, the inhibition by apamin and charybdotoxin of the relaxation was not accompanied by an inhibition of the increase in intracellular $Ca^{2+}$ concentrations in the endothelium (Yamanaka et al., 1998). Thus, it seems likely that the effects of these blockers are mainly due to their action on smooth muscle cells.

### *4.2. Arachidonic acid metabolism on acetylcholine-induced relaxation*

It has been suggested that in some blood vessels, EDHF is one of the epoxyeicosatrienoic acids, arachidonic acid metabolites produced by cytochrome *P*450 enzymes in endothelial cells (Hecker et al., 1994; Campbell et al., 1996; Graier et al., 1996), whereas in other vessels this seems unlikely (Corriu et al., 1996a; Zygmunt et al., 1996; Eckman et al., 1998). Quinacrine, an inhibitor of phospholipase $A_2$ that generates arachidonic acid, inhibited agonist-induced, NO- and prostacyclin-independent relaxation in porcine and rat coronary arteries (Bauersachs et al., 1994; Hecker et al., 1994). In contrast, in guinea-pig carotid artery, the hyperpolarization produced by acetylcholine was not affected by quinacrine (Corriu et al., 1996a). Fukao et al. (1997a) who have found that quinacrine inhibits hyperpolarization produced by pinacidil, an ATP-sensitive $K^+$ channel opener, suggest that the drug may act as a blocker of the effects of EDHF in smooth muscles rather than as the phospholipase $A_2$ inhibitor in the endothelium. On the other hand, Adeagbo and Henzel (1998) reported that AACOCF3 (3 μM), a more specific inhibitor of $Ca^{2+}$-activated phospholipase $A_2$ decreased acetylcholine-induced, NO- and prostacyclin-independent dilatation in perfused rat mesenteric vascular bed. On the contrary, we found that AACOCF3 (10 μM) did not alter the acetylcholine-induced relaxation, as Yamanaka et al. (1998) had shown before for guinea-pig coronary artery. It is suggested that AACOCF3-sensitive phospholipase $A_2$-mediated release of arachidonic acid, if any, is just marginal for the response to acetylcholine in these arteries.

17-Octadecynoic acid (5–100 μM) and 1-aminobenzotriazole (2 mM), suicide substrate inhibitors of cytochrome *P*450 enzymes, had no effect on relaxation or hyperpolarization response to acetylcholine in some blood vessels (present study, Corriu et al., 1996a; Zygmunt et al., 1996; Fukao et al., 1997a; Van de Voorde and Vanheel, 1997; Vanheel and Van de Voorde, 1997), but in other arteries, 17-octadecynoic acid (2–3 μM) inhibited the agonist-induced, EDHF-mediated responses (Fulton et al., 1995; Popp et al., 1996; Dong et al., 1997; Chataigneau et al., 1998). A part of the inhibitory effect of these agents seems to be due to a non-specific inhibition of $K^+$ channels in smooth muscles (Edwards et al., 1996). On the other hand, we found that the response was attenuated by other cytochrome *P*450 enzyme inhibitors including clotrimazole, proadifen and protoporphyrin IX. These drugs did not affect the sodium nitroprusside-elicited relaxation, a result showing that the capacity for relaxation of smooth muscles is not impaired by these drugs. It has been found that clotrimazole and proadifen interfere with various subtypes of $K^+$ channels and inhibit responses to $K^+$ channel openers (Alvarez et al., 1992; Zygmunt et al., 1996; Vanheel and Van de Voorde, 1997). Therefore, the inhibitory properties of these drugs on the response to acetylcholine can be explained by the inhibition of the action of EDHF on smooth muscles rather than by the inhibition of endothelial cytochrome *P*450 enzymes. On the other hand, protoporphyrin IX abolished relaxation response to acetylcholine, without having effect on ATP-sensitive $K^+$ channel opener-induced relaxation (this study, Graier et al., 1996), indicating that the drug did not influence ATP-sensitive $K^+$ channels. The drug was also found to inhibit $Ca^{2+}$ ionophore A23187-induced, NO- and prostacyclin-independent relaxation in bovine but not in porcine coro-

nary arteries (Graier et al., 1996), suggesting that the specific cytochrome *P*450 enzyme involved may vary in different tissues. However, at present it remains unclear whether or not the inhibition by protoporphyrin IX of the acetylcholine-induced relaxation is accounted for by its inhibitory effect on cytochrome *P*450 enzymes. On the other hand, protoporphyrin IX was also found to inhibit endothelial heme oxygenase, which synthesized carbon monoxide (Zakhary et al., 1996). Carbon monoxide has been found to induce vasodilatation in perfused rat heart (McFaul and McGrath, 1987) and cause a hyperpolarization of the membrane potential of human intestinal smooth muscles (Farrugia et al., 1993). Based on these and present results, it is suggested that carbon monoxide may mediate the acetylcholine-induced relaxation in the presence of L-NA and indomethacin, although further studies are needed to address this issue.

5,6-, 8,9-, 11,12- and 14,15-Epoxyeicosatrienoic acids (less than 10 μM) acted on smooth muscles to produce hyperpolarization and relaxation, which were inhibited by high $K^+$ solution and by $K^+$ channel blockers (Hecker et al., 1994; Campbell et al., 1996; Popp et al., 1996). These data support the idea that the epoxyeicosatrienoic acids represent EDHF. However, in rabbit mesenteric, rat hepatic, guinea-pig carotid and coronary arteries these epoxyeicosatrienoic acids at similar concentration ranges did not cause hyperpolarization and relaxation (this study, Zygmunt et al., 1996; Chataigneau et al., 1998; Yamanaka et al., 1998). Furthermore, unlike responses to acetylcholine, those to 11,12-epoxyeicosatrienoic acid were inhibited by glibenclamide or iberiotoxin in rat mesenteric and guinea-pig coronary arteries (Fukao et al., 1997a; Eckman et al., 1998). These data suggest that the epoxyeicosatrienoic acids do not represent a NO- and prostacyclin-independent factor released by acetylcholine in these blood vessels.

### *4.3. Gap junctional component of acetylcholine-induced relaxation*

It has been found that in guinea-pig mesenteric artery, 18β-glycyrrhetinic acid, an inhibitor of gap juctional communication between smooth muscle and endothelial cells, inhibits acetylcholine-induced hyperpolarization of smooth muscle cells without affecting the hyperpolarizing response of the endothelium to acetylcholine (Yamamoto et al., 1999). In addition, functional studies have demonstrated that gap juctional inhibitors including 18α-glycyrrhetinic acid inhibit acetylcholine- but not sodium nitroprusside-induced relaxation of rabbit conduit arteries (Chaytor et al., 1998; Taylor et al., 1998). It was found that 18α-glycyrrhetinic acid at 70 μM, a concentration which did not affect the response of mesenteric artery smooth muscle to sodium nitroprusside attenuated the acetycholine-induced, endothelium-dependent relaxation. Thus, the present results supported the hypothesis that myoendothelial gap junctions may at least in part contribute to the response to acetylcholine.

In summary, in isolated rabbit mesenteric small artery, acetylcholine elicited endothelium-dependent relaxation in the presence of L-NA and indomethacin. It is suggested that 4-aminopyrine- and charybdotoxin-sensitive $K^+$ channels are predominantly involved in the acetylcholine-induced relaxation. The relaxation is unlikely mediated by the cytochrome *P*450 metabolite of arachidonic acid, but may be accounted for by heterocellular gap junctional communication.

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