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Cellular target of voltage and calcium-dependent K^+ channel blockers involved in EDHF-mediated responses in rat superior mesenteric artery

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1 We have investigated the cellular target of K^+ channel blockers responsible for the inhibition of the EDHF-mediated relaxation in the rat mesenteric artery by studying their effects on tension, smooth muscle cell (SMC) membrane potential and endothelial cell Ca^{2+} signal ($[Ca^{2+}]_{endo}$).

2 In arteries contracted with prostaglandin $F_{2\alpha}$ (2.5–10 μM), relaxation evoked by ACh (0.01–3 μM) was abolished by a combination of charybdotoxin (ChTX, 0.1 μM) plus apamin (Apa, 0.1 μM) and was inhibited by $68\pm6\%$ ($n=6$) by 4-aminopyridine (4-AP, 5 mM).

3 ACh (0.001–3 μM) increased $[Ca^{2+}]_{endo}$ and hyperpolarized SMCs with the same potency, the pD_2 values were equal to 7.2 ± 0.08 ($n=4$) and 7.2 ± 0.07 ($n=9$), respectively. SMCs hyperpolarization to ACh (1 μM) was abolished by high K^+ solution or by ChTX/Apa. It was decreased by $66\pm5\%$ ($n=6$) by 4-AP.

4 The increase in $[Ca^{2+}]_{endo}$ evoked by ACh (1 μM) was insensitive to ChTX/Apa but was depressed by $58\pm16\%$ ($n=6$) and $27\pm4\%$ ($n=7$) by raising external K^+ concentration and by 4-AP, respectively.

5 The effect of 4-AP on $[Ca^{2+}]_{endo}$ was not affected by increasing external K^+ concentration. In Ca-free/EGTA solution, the transient increase in $[Ca^{2+}]_{endo}$ evoked by ACh (1 μM) was abolished by thapsigargin (1 μM) and was decreased by $75\pm7\%$ ($n=5$) by 4-AP.

6 These results show that inhibition of EDHF-evoked responses by 4-AP may be attributed to a decrease in the Ca^{2+} release activated by ACh in endothelial cells. The abolition of SMCs hyperpolarization to ACh by ChTX/Apa is not related to an interaction with the $[Ca^{2+}]_{endo}$.

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Abbreviations: ACh, acetylcholine; ANOVA, analysis of variance; 4-AP, 4-aminopyridine; Apa, apamin; BK_{Ca}, large conductance Ca^{2+}-activated K^+ channel; ChTX, charybdotoxin; 3,4-DAP, 3,4-diaminopyridine; EC, endothelial cell; EDHF, Endothelium-Derived Hyperpolarizing Factor; Em, membrane potential; ER, endoplasmic reticulum; IK_{Ca}, intermediate conductance Ca^{2+}-activated K^+ channel; Indo-1 AM, indo-1 acetoxymethylester; IP_3, inositol triphosphate; L-NOARG, N^{ω}-nitro-L-arginine; NO, nitric oxide, $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; SK_{Ca}, small conductance Ca^{2+}-activated K^+ channel; SMC, smooth muscle cell; TEA, tetraethylammonium; WKY, Wistar Kyoto rat

Introduction

Acetylcholine (ACh) stimulates an endothelium-dependent relaxation of pre-contracted arteries, which has been reported to be mediated by several factors as nitric oxide (NO) (Furchgott & Zawadzki, 1980) and prostacyclin (Moncada & Vane, 1978). In most arteries, the relaxation evoked by ACh is not completely abolished by nitric oxide synthase and cyclo-oxygenase inhibitors and is accompanied by the hyperpolarization of the smooth muscle cell membrane. These responses are attributed to the release of an endothelium-derived hyperpolarizing factor (EDHF) (Taylor & Weston, 1988; Feletou & Vanhoutte, 1988). Its chemical nature and its mechanism of action remain elusive.

It is known that the release of EDHF is activated by an increase of intracellular calcium concentration in the endothelial cells (ECs), which is initiated by the release of Ca^{2+} stores (Chen & Suzuki, 1990, Fukao *et al*., 1995) and maintained by the influx of Ca^{2+} from the extracellular space (Fukao *et al*., 1997). Although voltage-dependent Ca^{2+} channels have been described in ECs (Bossu *et al*., 1992), they do not contribute to the Ca^{2+} regulation (Himmel *et al*., 1993). The Ca^{2+} influx activated by agonists in ECs occurs through non-selective cation channels (Nilius, 1990). It is sensitive to the membrane potential (Lückhöff & Busse, 1990), which affects the driving force for Ca^{2+}. The hyperpolarization of the membrane of ECs favours the Ca^{2+} influx (Lückhöff & Busse, 1990), while depolarization reduces the plateau phase of the Ca^{2+} signal induced by an agonist (for review Nilius *et al*., 1997).

In various arteries, apamin (an inhibitor of small conductance Ca^{2+}-activated K^+ channel, SK_{Ca}) alone or in combination with charybdotoxin (ChTX) (an inhibitor of large conductance Ca^{2+}-activated K^+ channel, BK_{Ca}, and intermediate conductance Ca^{2+}-activated K^+ channel, IK_{Ca}), inhibits the responses attributed to EDHF (Waldron & Garland, 1994a; Zygmunt & Höggestätt, 1996; Chataigneau *et al*., 1998; Quignard *et al*., 1999) whereas apamin plus

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iberiotoxin, a specific inhibitor of BK_{Ca} channels, do not affect EDHF-mediated relaxation (Waldron & Garland, 1994a; Zygmunt & Höggestätt, 1996). This suggests that activation of IK_{Ca} and SK_{Ca} channels could be responsible for the hyperpolarization of smooth muscle cells (SMCs). However, K_{Ca} channels are extensively expressed in ECs (Demirel *et al*., 1994; Groschner *et al*., 1994; Marchenko & Sage, 1996). It has been proposed that the inhibition of EDHF responses by the combination of ChTX plus apamin could result from their action at the level of IK_{Ca} and SK_{Ca} channels on ECs (Edwards *et al*., 1998; Doughty *et al*., 1999). Voltage-dependent K^+ channels (K_v) have also been proposed to be involved in EDHF-mediated responses. Indeed, 4-aminopyridine (4-AP), a specific delayed rectifier channel blocker, inhibits ACh-induced endothelium-dependent hyperpolarization in coronary artery of the guinea-pig (Eckman *et al*., 1998), but the contribution of this channel subtype is not observed in all arteries (Zygmunt *et al*., 1997). The inhibition by 4-AP of the endothelium-dependent hyperpolarization evoked by ACh in the isolated carotid artery of guinea-pig and in rat hepatic artery has been shown to be associated with an inhibition of the hyperpolarization of ECs (Quignard *et al*., 2000).

The aim of this study was to investigate the site of action of K^+ channel blockers involved in the inhibition of EDHF responses activated by ACh in the rat superior mesenteric artery. Simultaneous measurement of contractile responses and membrane potential was used to record the hyperpolarization and relaxation of SMCs. Since raising cytosolic Ca^{2+} concentration in ECs appears to be the first step in the EDHF pathway, the effect of K^+ channel blockers was investigated on the Ca^{2+} signal in ECs by using front surface fluorimetry in indo-1-loaded artery.

Our results showed that K_v and K_{Ca} are involved in the relaxation induced by ACh in the presence of NO synthase and cyclo-oxygenase inhibitors. Inhibition by ChTX and apamin of the SMCs hyperpolarization evoked by ACh is not related to the interaction of the blockers with the increase in calcium signal in the endothelium. The inhibition of EDHF-evoked responses by 4-AP can be, at least partly, attributed to the inhibition of the Ca^{2+} release in ECs.

Methods

Normotensive Wistar-Kyoto (WKY) male rats (Iffa Credo, L'Arbresle, France) were used. All rats were killed by decapitation at 14 weeks. The superior mesenteric artery was rapidly removed and immersed in physiological solution (composition in mM): NaCl 122, KCl 5.9, $NaHCO_3$ 15, glucose 10, $MgCl_2$ 1.25 and $CaCl_2$ 1.25, gassed with a mixture of 95% O_2-5% CO_2. The superior mesenteric artery was carefully cleaned of all fat and connective tissue. All experiments were performed in the presence of N^{ω}-nitro-L-arginine (L-NOARG) and indomethacin to block the nitric oxide synthase and the cyclo-oxygenase, respectively.

Simultaneous measurement of contractile tension and membrane potential

A segment of the superior mesenteric artery, about 2 mm in length, was inverted and mounted in a wire myograph (Model 500A, Danish Myo Technology A/S, Aarhus, Denmark) as described (Ghisdal *et al*., 1999). Briefly, two 40 μm wires were threaded through the lumen of the vessel segment. One wire was attached to a stationary support driven by a micrometer, while the other was attached to an isometric force transducer. Vessels were maintained under zero force for 60 min. A passive diameter-tension curve was constructed as described (Mulvany & Halpern, 1977). From this curve the effective transmural pressure was calculated. The vessel was set at a tension equivalent to that generated at 0.9 times the diameter of the vessel at 100 mmHg. The bath of the myograph was continuously perfused with physiological solution gassed with a mixture of 95% O_2-5% CO_2 and warmed at 37°C.

Measurement of the smooth muscle membrane potential was made with a glass microelectrode (Clark, Electromedical instruments, type GC 120F-15) filled with 1.5 M KCl and advanced through the luminal surface of the arterial segment with a micromanipulator (Leitz). The input resistance of the microelectrodes varied between 50 and 80 MΩ. Potential differences were measured with reference (reference electrode: Clark, Electromedical instruments, type E208) to the grounded bath by means of a Dagan amplifier (8100, Minneapolis, MN, U.S.A.). Electrical responses were monitored on an oscilloscope (Hitachi, oscilloscope V-252, 20 MHz). Membrane potential and tension were simultaneously recorded with a pen recorder (Kontron, 500 SP). Criteria for a successful impalement were (1) an abrupt drop in voltage on entry of microelectrode into the cell, (2) stable membrane potential for at least 2 min, and (3) a sharp return to zero on withdrawal of the electrode.

After being mounted in the organ chamber, the rings were maintained in gassed physiological solution (see above). Endothelium integrity was assessed at the beginning of each experiment by the application of 1 μM ACh on the plateau of the contraction evoked by noradrenaline (1 μM). When the rings relaxed with success, the preparation was maintained in physiological solution containing indomethacin (10 μM) and L-NOARG (100 μM) at 37°C. Drugs were applied in the perfusion solution. High KCl solutions were obtained by equimolar substitution of Na^+ ions for K^+ ions. When high-KCl solution or K^+ channel blockers were used, the experiment was performed in the presence of phentolamine (1 μM) to rule out the contribution of α-adrenergic transmitter released by nervous ending. ACh concentration-response curves for the change in resting membrane potential were established by the successive application of different concentrations of the agonist with 30 min time intervals between two concentrations in order to avoid the development of tachyphylaxis. The inhibitors used were pre-incubated 10–15 min before application of ACh.

Measurement of endothelial cell calcium signal

A segment of the superior mesenteric artery, about 2.5 mm in length, was inverted and mounted between two hooks under a tension of 10 mN in a 3 ml cuvette continuously perfused with physiological solution (composition as above) gassed with a 95–5% mixture of O_2 and CO_2 and warmed at 37°C. An isometric force transducer measured the muscle tone.

Endothelium integrity was assessed at the beginning of each experiment by the application of 1 μM ACh on the

plateau of the contraction evoked by 1 μM noradrenaline. Only the segments where the contraction was inhibited by 75% were used. Mesenteric artery rings were then incubated for 3 h at room temperature (22°C) in physiological solution containing 5 μM indo-1 acetoxymethyl ester (indo-1-AM) and 0.05% Cremophor EL. After the loading period, the rings were washed in physiological solution containing L-NOARG (100 μM), indomethacin (10 μM), phentolamine (1 μM) and nimodipine (1 μM) at 37°C for 30 min. Nimodipine, a voltage-dependent calcium channel blocker, was present in the physiological solution to rule out the contribution of smooth muscle Ca^{2+} signal when high KCl solution or K^+ channel blockers were used. ACh concentration-response curves for the changes in cytosolic Ca^{2+} signal of ECs ($[Ca^{2+}]_{endo}$) were established by the cumulative application of increasing concentrations of the agonist. All physiological calcium-free solutions were supplied with 0.2 mM ethylene glycol-bis (b-amino ethyl ether) tetraacetic acid (EGTA).

The cuvette was part of a fluorimeter (CAF, JASCO, Tokyo, Japan) which allowed estimation of the calcium signal. After excitation at 340 nm, the fluorescence signals emitted at 405 nm (F_{405}) and 500 nm (F_{500}) were measured simultaneously with the contractile tension and recorded on a computer by using the data acquisition hardware MacLab and data recording software Chart (AD Instruments Pty Ltd., Castle Hill, Australia). At the end of each experiment, the autofluorescence of the tissue was measured at 405 and 500 nm by quenching the indo-1 fluorescence with $MnCl_2$ (5 mM) and subtracted from F_{405} and F_{500}. The $[Ca^{2+}]_{endo}$ was estimated by the ratio of the fluorescence emitted at 405 and 500 nm.

Drugs

Indo 1-AM was from Calbiochem (EuroBiochem, Bierges, Belgium). Acetylcholine chloride (ACh), 4-aminopyridine (4-AP), apamin (Apa), cremophor EL, L-indomethacin, N^{ω}-nitro-L-arginine (L-NOARG), phentolamine, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and thapsigargin were obtained from Sigma. Stock solution of indomethacin was prepared in 2% Na_2CO_3. Charybdotoxin (ChTX) was from Latoxan (Rosans, France). Nimodipine was from Bayer (Leverkussen, Germany) and stock solution (10 mM) was prepared in ethanol. In the experiments performed with 4-AP, the physiological solution was buffered to pH 7.4 with tris(hydroxy-methyl)-aminomethane (Tris, 5 mM) and N-[2-hydroxy-ethyl]piperazine-N′-[2-ethanesulphonic acid] (HEPES).

Statistics

Results are given as mean ± standard error (s.e.mean). Comparisons were made using Student's *t*-test or by analysis of variance followed by a Bonferroni test (one-way ANOVA), when more than two groups were involved in the comparison. *P* values lower than 0.05 indicated significant differences. EC_{50} values (concentration of an agonist that produces 50% of the maximal effect) were calculated by non-linear curve fitting of the experimental data of the concentration-response curves to the equation:

$$E = \frac{E_{\max} * [A]^{n_H}}{[A]^{n_H} + EC_{50}{}^{n_H}}, \quad (1)$$

where E_{max} is the maximum amplitude of the effect produced by the agonist, $[A]$ is the concentration of the agonist and n_H is the Hill slope (Multifit, Day Computing, Cambridge, UK; KaleidaGraph, Synergy Software, Reading, PA, U.S.A.). The negative logarithm to base 10 of EC_{50} values (pD_2) was used for the statistical analysis.

Results

Effect of K^+ channel blockers on the EDHF-dependent relaxation induced by acetylcholine

In order to investigate the effect of K^+ channel blockers on the relaxation evoked by ACh in rat mesenteric artery, we have performed a series of experiments where ACh (0.01–3 μM) was added cumulatively on the vessels pre-contracted by prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$). The $PGF_{2\alpha}$ concentration (2.5–10 μM) was adapted to produce a contraction equivalent to that evoked by a 100 mM KCl solution (13.2 ± 0.8 mN, $n = 26$). In the presence of L-NOARG (100 μM), indomethacin (10 μM) and phentolamine (1 μM), maximum relaxation to ACh reached 75 ± 2.4% of the contraction (ACh 3 μM); the pD_2 value of ACh was equal to 6.6 ± 0.08 ($n = 13$) (Figure 1). The association of charybdotoxin (ChTX, 0.1 μM) plus apamin (Apa, 0.1 μM) to inhibit K_{Ca} channels produced a contraction of 2 ± 0.3 mN ($n = 4$). In the presence of ChTX/Apa, the concentration of $PGF_{2\alpha}$ was reduced about two times to get a similar level of contraction as in the absence of the blockers. K_{Ca} channel blockers abolished the relaxation induced by ACh (Figure 1), which even produced a slight additional contraction to that evoked by $PGF_{2\alpha}$. 4-Aminopyridine (4-AP, 5 mM) was used

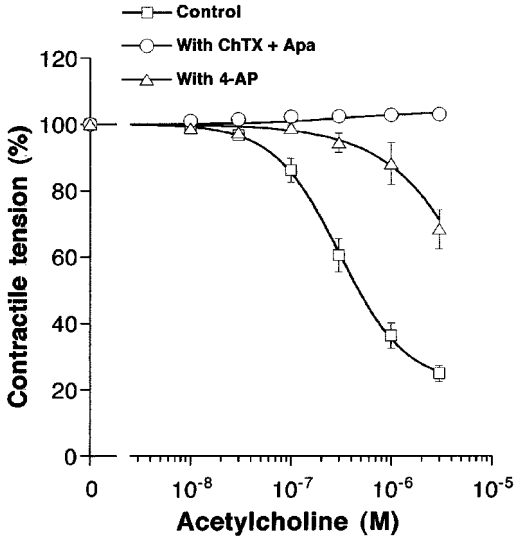

Figure 1 Effect of K^+ channel blockers on the EDHF-dependent relaxation induced by acetylcholine in rat superior mesenteric artery. Concentration-response curves for the effects of acetylcholine on the contractile tension of mesenteric arteries stimulated by prostaglandin $F_{2\alpha}$ in the absence (control, $n = 13$) and in the presence of charybdotoxin and apamin (ChTX + Apa, 0.1 μM; $n = 3$), or 4-aminopyridine (4-AP, 5 mM, $n = 6$). All experiments were performed in the presence of N^{ω}-nitro-L-arginine (100 μM), indomethacin (10 μM) and phentolamine (1 μM).

to block K_v channels. It caused a small increase in tone of 0.3 ± 0.08 mN ($n=6$) and did not affect the contraction to $PGF_{2\alpha}$ significantly. The pre-exposure of the vessels to 4-AP depressed the relaxation to ACh 3 μM by $68 \pm 5.9\%$ ($n=6$) (Figure 1).

Effect of K^+ channel blockers on the hyperpolarization evoked by acetylcholine in smooth muscle cells

In the presence of L-NOARG (100 μM), indomethacin (10 μM) and phentolamine (1 μM), the resting membrane potential (E_m) of main mesenteric artery SMCs was averaged at -46.6 ± 0.3 mV ($n=34$). Exposure of the vessels to ACh (0.001 – 1 μM) induced a concentration-dependent hyperpolarization of SMCs (Figure 2A). The pD_2 value of ACh was equal to 7.2 ± 0.07 ($n=9$). Increasing external KCl concentration depressed the hyperpolarization to ACh in a concentration-dependent manner (Figure 2B). Hyperpolarization was completely abolished in the presence of 40 mM KCl. The concentration of KCl producing 50% inhibition was equal to 12.4 ± 2.2 mM ($n=4$) (Figure 2B). The incubation of artery rings with ChTX plus Apa depolarized SMCs by 7.5 ± 1.5 mV ($n=4$) and abolished the hyperpolarization induced by 1 μM ACh ($P<0.05$; $n=4$) (Figure 3B). In artery rings pre-exposed to 5 mM 4-AP, SMCs were depolarized by 6.3 ± 1.1 mV ($n=6$) and the hyperpolarization evoked by 1 μM ACh was inhibited by $66 \pm 5.2\%$ ($P<0.05$; $n=6$) (Figure 3B). These results confirmed the involvement of K_v and K_{Ca} channels in the EDHF pathway (Corriu *et al*., 1996; Eckman *et al*., 1998).

Effect of acetylcholine on cytosolic Ca^{2+} signal in endothelial cells

The following experiments were designed in order to determine whether the inhibition by K^+ channel blockers of EDHF-mediated relaxation and hyperpolarization could result from an action of the blockers on Ca^{2+} signal in ECs. Endothelial cells Ca^{2+} signal ($[Ca^{2+}]_{endo}$) was recorded in indo-1-loaded arteries incubated in the presence of nimodipine (1 μM) and phentolamine (1 μM). Under these conditions, high KCl solution (100 mM) did not increase calcium signal, as it would be expected if signal arised from SMCs. Figure 4A shows a typical record illustrating the effect of ACh. The muscarinic agonist induced a fast increase in the F_{405}/F_{500} ratio, which was stable for about 2 min. The ratio then slightly decreased: after 6 min, the Ca^{2+} signal levelled at $43 \pm 2.4\%$ ($n=9$) of its peak value (Figure 4A). All effects were corrected for the decrease in the Ca^{2+} signal with time. When the endothelium was mechanically removed, ACh did not affect the Ca^{2+} signal (Figure 4B), attesting of the EC specificity of the changes evoked by ACh.

In intact artery rings, the cumulative application of ACh (0.001 – 3 μM) induced a concentration-dependent increase in $[Ca^{2+}]_{endo}$ (Figure 2A). The maximum effect of ACh was obtained at the concentration of 1 – 3 μM. The pD_2 value of ACh was equal to 7.2 ± 0.08 (Figure 2A; $n=4$). It is worth noting that the concentration-response relations for the hyperpolarization and the elevation of $[Ca^{2+}]_{endo}$ evoked by ACh were perfectly superimposed, with a correlation coefficient close to one (Figure 2A). The participation of EC membrane potential to the increase in $[Ca^{2+}]_{endo}$ induced by ACh (1 μM) was examined by investigating the effect of enhanced extracellular K^+ concentration. Elevation of extracellular K^+ concentration from 5.9 to 100 mM inhibited the Ca^{2+} signal evoked by ACh by $58 \pm 16\%$ ($n=6$; Figure 2B). Inhibition was concentration-dependent and was linear up to 100 mM KCl. The effect of K^+ channel blockers on ACh-evoked $[Ca^{2+}]_{endo}$ is summarized in Figure 3A. Neither the association ChTX plus Apa (0.1 μM) nor 4-AP (5 mM) did modify resting Ca^{2+} signal. The association of ChTX plus

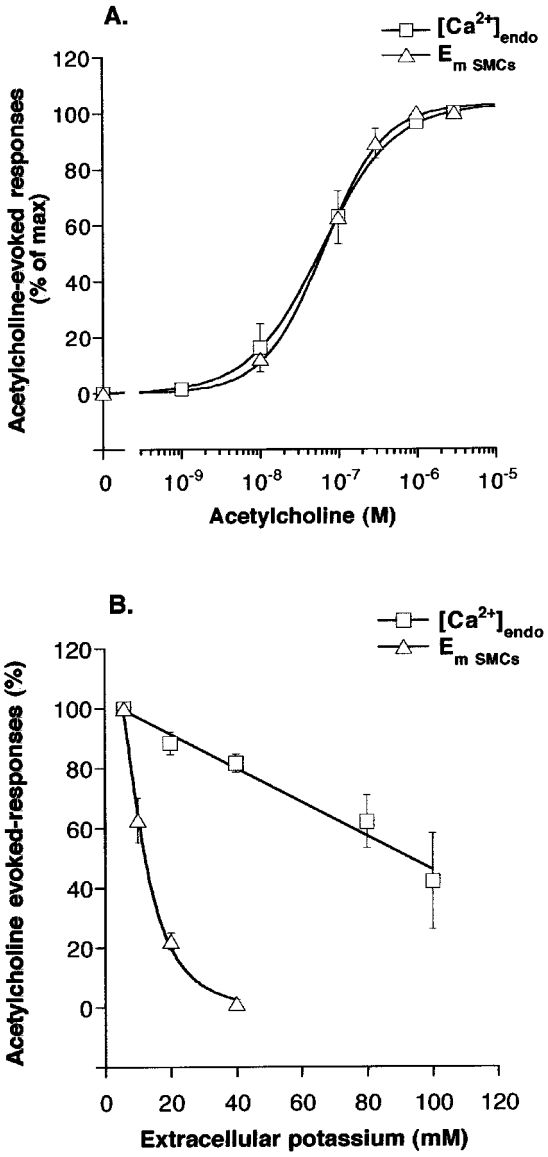

Figure 2 Effect of increase in extracellular K^+ concentration on changes of endothelial cells Ca^{2+} and SMCs membrane potential evoked by ACh. (A) Concentration-response curves to acetylcholine (0.001 – 3 μM) were established in unstimulated mesenteric arteries. Change in membrane potential of smooth muscle cells (Em_{SMCs}, $n=9$) and increase in Ca^{2+} signal in endothelial cells ($[Ca^{2+}]_{endo}$, $n=4$) are expressed as a percentage of the maximal responses to ACh (% of max). Data are presented as means ± s.e.mean. (B) Effect of varying extracellular K^+ concentration on the increase in Ca^{2+} signal in endothelial cells ($[Ca^{2+}]_{endo}$, $n=6$) and the hyperpolarization of SMCs (Em_{SMCs}, $n=4$) induced by acetylcholine (ACh, 1 μM). Data are expressed as percent of the responses in the presence of 5.9 mM KCl and are presented as means ± s.e.mean.

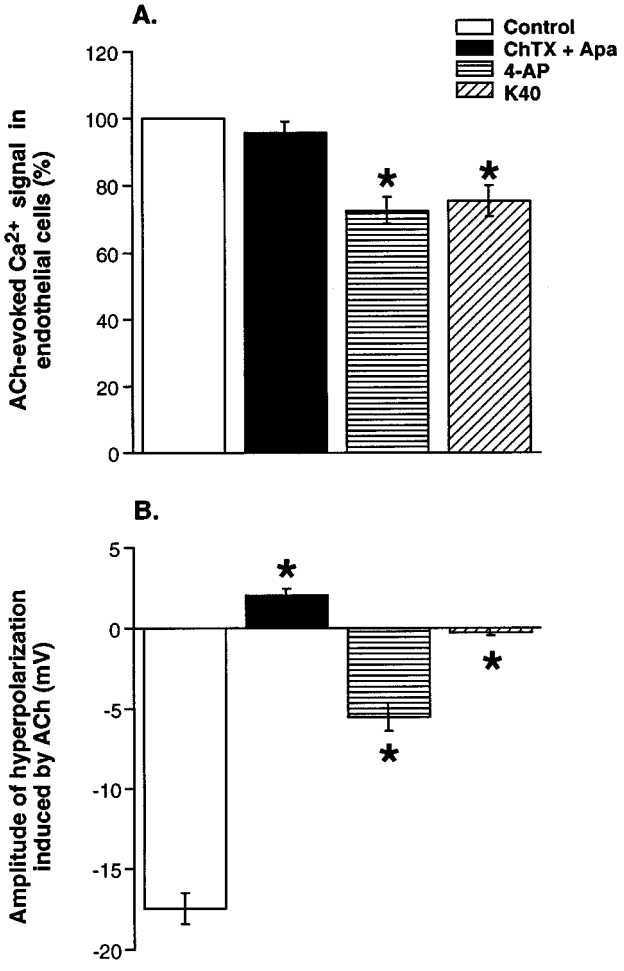

Figure 3 Comparison of the effects of K^+ channel blockers on acetylcholine-evoked changes in endothelial cells Ca^{2+} signal (A) and SMCs membrane potential (B). Responses to ACh (1 μM) were measured in the absence (control) and in the presence of charybdotoxin and apamin (ChTX + Apa, 0.1 μM), 4-aminopyridine (4-AP, 5 mM) or in physiological solution containing 40 mM of KCl (K40). Endothelial cells Ca^{2+} signal was expressed as a percentage of the maximum amplitude of acetylcholine-evoked responses in the absence of test drugs. Data are presented as means ± s.e.mean. Asterisks denote a statistically significant difference from control values ($P<0.05$).

Apa had no effect on the response elicited by 1 μM ACh. In the presence of 4-AP, the Ca^{2+} signal evoked by ACh (1 μM) was decreased by 27 ± 4% ($n=7$, $P<0.05$ *vs* control). In order to determine whether the effect of 4-AP could result from a depolarization of ECs, the external K^+ concentration was elevated to 40 mM. Under this condition, the Ca^{2+} signal evoked by ACh (1 μM) was reduced by 24 ± 4.6% ($n=7$) but it was still inhibited by 4-AP by 22 ± 2% ($n=6$).

In the second series of experiments, we have tested the hypothesis that 4-AP could affect the intracellular calcium release process stimulated by acetylcholine. In this aim, the artery rings were incubated for 10 min in Ca^{2+}-free/EGTA physiological solution containing 40 mM of KCl. This produced a decrease in Ca^{2+} signal of 10 ± 1% ($n=8$). The addition of ACh (1 μM) then evoked a rapid but transient increase in $[Ca^{2+}]_{endo}$, which returned to the baseline values within 2 min (Figure 5A). The magnitude of the calcium peak represented 51 ± 6% ($n=8$) of the maximum increase in Ca^{2+} signal evoked by ACh in the presence of Ca^{2+} and K^+ 40 mM. It was completely inhibited by thapsigargin (1 μM) (Figure 5B; $n=4$), an inhibitor of the Ca^{2+}-ATPase pump of the endoplasmic reticulum (Lytton *et al.*, 1991). In the presence of 4-AP, the transient calcium peak elicited by ACh was inhibited by 75 ± 7% ($n=5$, $P<0.05$ *vs* control). The readmission of Ca^{2+} in the medium produced a rapid and large increase in $[Ca^{2+}]_{endo}$ (Figure 5A). The amplitude of the increase in Ca^{2+} signal evoked by the readmission of Ca^{2+} was enhanced in the presence of thapsigargin (Figure 5B) but was decreased by 22 ± 4% in the presence of 4-AP ($n=5$, $P<0.05$ *vs* control) (Figure 5C).

Discussion

The present results showed that inhibition of EDHF-mediated relaxation to ACh by the K^+ channel blocker 4-AP results from the interaction of the blocker with the ECs Ca^{2+} signal. On the opposite, ChTX and Apa, which completely blocked SMCs hyperpolarization to ACh, did not affect the increase in $[Ca^{2+}]_{endo}$ evoked by the muscarinic agonist.

EDHF mediated relaxation

In WKY superior mesenteric artery, as in several arteries, ACh induces an endothelium-dependent relaxation that is resistant to nitric oxide and cyclo-oxygenase inhibitors, and is associated with the hyperpolarization of SMCs (Waldron & Garland, 1994b; Ghisdal *et al.*, 1999). Inhibition of hyperpolarization and relaxation to ACh by KCl (Chen & Suzuki, 1989; Adeagbo & Triggle, 1993) or a combination of apamin and charybdotoxin, but not apamin and iberiotoxin (Waldron & Garland, 1994a; for review Feletou & Vanhoutte, 1999) is considered as a finger print of EDHF-mediated responses.

Fluorescence studies in indo-1-loaded arteries confirmed the previous observations by Chen & Suzuki (1990) and by Fukao *et al.* (1997) that the hyperpolarization evoked by ACh is initiated by thapsigargin-sensitive Ca^{2+} release from intracellular Ca^{2+} pool and maintained by Ca^{2+} influx pathway distinct from L-type Ca^{2+} channels coupled to the depletion of intracellular stores. The Ca^{2+} influx in ECs is controlled by the membrane potential as indicated by the observation that increasing extracellular K^+ concentration during agonist stimulation diminished the rise in $[Ca^{2+}]_{endo}$ (Kamouchi *et al.*, 1999; Wang & van Breemen, 1999; Knot *et al.*, 1999). The close correlation that was found between the effect of ACh on $[Ca^{2+}]_{endo}$ and E_m of SMCs indicates that blunting the Ca^{2+} signal in ECs could cause a proportional reduction in the EDHF-evoked SMCs hyperpolarization. Thus, the different sensitivity to external K^+ of ACh-evoked $[Ca^{2+}]_{endo}$ and SMCs hyperpolarization suggests that inhibition of $[Ca^{2+}]_{endo}$ signal could contribute to, but could not be the only determinant of the K^+-sensitivity of the relaxation evoked by EDHF.

Involvement of endothelial cell K_v channels in the EDHF-mediated responses

The present results showed that 4-AP partially inhibited the endothelium-dependent relaxation and hyperpolarization of

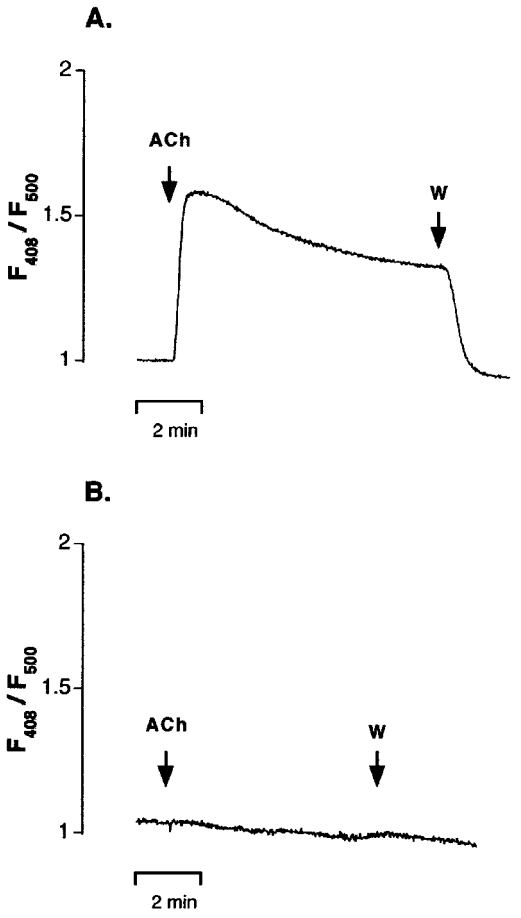

Figure 4 Endothelial cells Ca^{2+} signal in indo-1-loaded mesenteric artery. Representative experimental traces showing the increase in indo-1 fluorescence ratio (F_{405}/F_{500}) induced by acetylcholine (ACh, 1 μM) in the presence (A) and in the absence (B) of the endothelium. Fluorescence ratio obtained after subtracting the autofluorescence was normalised to the value measured before addition of ACh. In the presence of an intact endothelium, the muscarinic agonist induced a fast increase in the F_{405}/F_{500} ratio, which returned to the basal level after wash (W). When the endothelium was mechanically removed, ACh did not affect Ca^{2+} signal. ACh was applied as indicated.

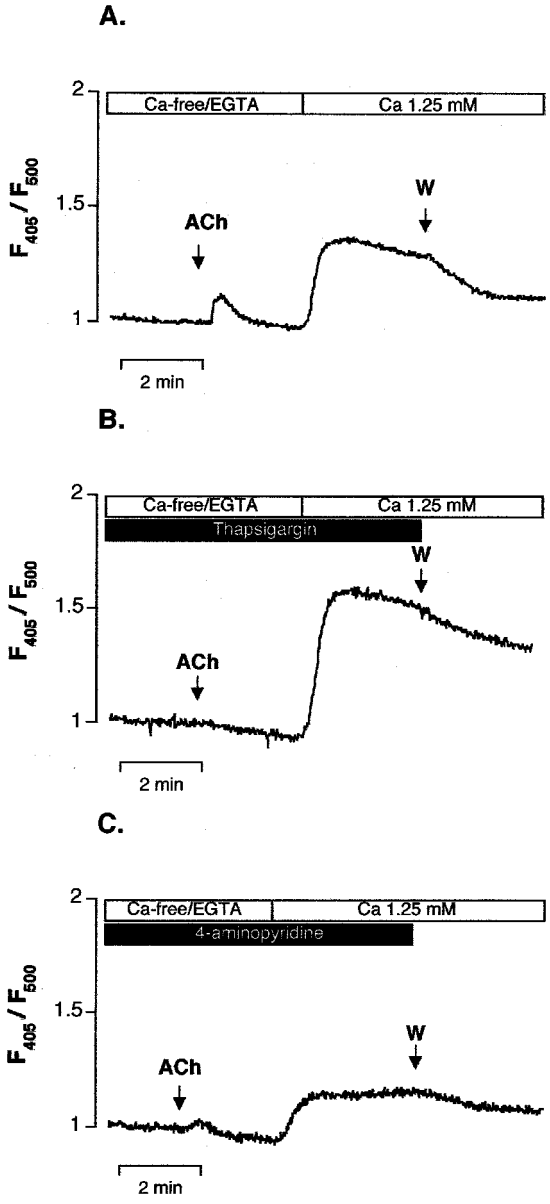

Figure 5 Effect of 4-aminopyridine on intracellular Ca^{2+} release stimulated by acetylcholine in indo-1-loaded endothelial cells. Representative experimental traces showing the increase in indo-1 fluorescence ratio (F_{405}/F_{500}) evoked by acetylcholine (ACh, 1 μM) in arteries pre-incubated for 10 min in Ca^{2+}-free/EGTA solution in the presence of 40 mM of K^+. Fluorescence ratio corrected for the autofluorescence was normalized to the value measured before addition of ACh. Readmission of Ca^{2+} (Ca 1.25 mM) to the solution evoked an increase in fluorescence ratio. (A) Control condition, (B) in the presence of the thapsigargin (1 μM) and (C) in the presence of 4-aminopyridine (5 mM). (W) Indicates the washout of the artery.

SMCs to ACh in rat mesenteric artery. Similar effect has been reported in guinea-pig coronary artery and in porcine coronary artery (Shimizu & Paul, 1997; Eckman *et al.*, 1998). Fluorescence studies in indo-1-loaded artery revealed that 4-AP also inhibited ACh-evoked increase in $[Ca^{2+}]_{endo}$, suggesting that the inhibition of SMCs hyperpolarization by 4-AP could be caused by an effect of the blocker on the endothelium. ECs have K_v channels (Takeda *et al.*, 1987; Hogg *et al.*, 1999; Dittrich & Daut, 1999), which are involved in the EC hyperpolarization induced by ACh in the guinea-pig coronary artery (Chen & Cheung, 1992; Quignard *et al.*, 2000). Inhibition by 4-AP of ACh-evoked increase in $[Ca^{2+}]_{endo}$ could result from the inhibition by 4-AP of endothelium K_v channels and the consecutive depolarization of ECs. This hypothesis had to be rejected following the observation that clamping the membrane potential of ECs with high-KCl solution did not affect the inhibition of Ca^{2+} signal by 4-AP. In addition, ChTX/Apamin did not affect the endothelial cell Ca^{2+} response stimulated by ACh. K_v 1.2 and K_v 1.3 channels, present in mesenteric artery (Xu *et al.*, 1999), are sensitive to ChTX (Grissmer *et al.*, 1994). It is then highly likely that endothelial cell K_v 1.2 and K_v 1.3 channels do not contribute to the ACh response. Interestingly, experiments performed in Ca^{2+} free solution revealed that the pre-exposure of the arteries to 4-AP strongly inhibited the transient thapsigargin-sensitive increase in $[Ca^{2+}]_{endo}$ stimulated by ACh, suggesting that 4-AP could interact with the intracellular calcium release process activated by ACh. It has been shown in bovine aortic ECs that Ca^{2+} release from IP_3-

sensitive stores is modulated by a K^+ counter-ion system present in the membrane of endoplasmic reticulum (ER). An inward movement of K^+ through the ER membrane could facilitate the sustained release of Ca^{2+} during IP_3 -induced mobilisation from internal stores (Wood & Gillespie, 1998). These intracellular K^+ channels can be blocked by K^+ channel blockers like TEA, 4-AP or 3,4-DAP, which reduce the IP_3 response to a level not significantly different to that of complete K^+ replacement (Wood & Gillespie, 1998). After incubation in Ca^{2+} free condition and challenge with ACh, re-admission of Ca^{2+} in the solution evoked a rapid increase in $[Ca^{2+}]_{endo}$. This response also was depressed in the presence of 4-AP. At the opposite, emptying Ca^{2+} stores with thapsigargin led to an increased capacitative Ca^{2+} entry. The inhibition of the Ca^{2+} re-admission process by 4-AP is then not in the line of a thapsigargin-like action of 4-AP at the level of the Ca^{2+} pump of the ER, as has been suggested by Ishida & Honda (1993). Indeed, the latter effect would lead to the emptying of intracellular Ca^{2+} stores and the increase in the capacitative Ca^{2+} entry, as observed with thapsigargin.

Since EC hyperpolarization to ACh results from the activation by Ca^{2+} of K_{Ca} channels (Wang *et al*., 1996; Ohashi *et al*., 1999), the inhibition of endothelial cells Ca^{2+} signal by 4-AP explains the observation by Quignard *et al*. (2000) that 4-AP inhibits ACh-evoked hyperpolarization of ECs. The close relation between ACh-evoked SMCs hyperpolarization and increase in $[Ca^{2+}]_{endo}$ suggests that inhibition of Ca^{2+} signal in ECs by 4-AP can be responsible for about 27 % inhibition of the SMCs hyperpolarization. Additional effect of 4-AP is thus required to justify the total 58% inhibition on EDHF-mediated SMCs hyperpolarization.

Involvement of endothelial cell K_{Ca} channels in the EDHF-mediated response

Involvement of K_{Ca} channels in the EDHF-mediated relaxation has been suggested by the effect of the K_{Ca} blockers ChTX/Apa, which abolish EDHF-mediated hyperpolarization in several arteries (Waldron & Garland, 1994a; Zygmunt & Höggestätt, 1996, Corriu *et al*., 1996; Prieto *et al*., 1998; Quignard *et al*., 1999; Doughty *et al*., 1999). The present results showed that, in rat superior mesenteric artery, ChTX/Apa inhibited the relaxation and the hyperpolarization to ACh but did not affect $[Ca^{2+}]_{endo}$.

Doughty *et al*. (1999) showed that, in third-order superior mesenteric artery of the rat, ChTX and apamin block EDHF-mediated relaxation only when they are applied intraluminally. ChTX/Apa-sensitive K^+ channels are indeed present in vascular ECs (Marchenko & Sage, 1996) and are responsible for the hyperpolarization evoked by ACh in ECs (Wang *et al*., 1996; Ohashi *et al*., 1999). The present study ruled out the possibility that the combination of these toxins abolishes SMCs hyperpolarization by inhibiting Ca^{2+} signal in ECs, but cannot exclude that endothelial cells ChTX/Apa-sensitive-K_{Ca} channels are involved in the EDHF pathway, downstream the increase of Ca^{2+} concentration in ECs. Edwards *et al*. (1998) reported that in rat hepatic and mesenteric artery ACh opens ChTX and apamin-sensitive K^+ channels in ECs, leading to the release of K^+ in the myo-endothelial space. Accumulation of K^+ in myo-endothelial space has been reported to hyperpolarize the endothelium by increasing outward current through inward rectifying K^+ channels. Hyperpolarization could then be transmitted to SMCs through myo-endothelial gap junctions (Doughty *et al*., 2001). Activation by K^+ of SMCs Na^+/K^+-ATPase could also be responsible for the hyperpolarization of SMCs (Doughty *et al*., 2000; Dora & Garland, 2001).

It is concluded that voltage- and Ca^{2+}-dependent K^+ channels are involved in EDHF-mediated relaxation evoked by acetylcholine in the rat superior mesenteric artery. Our results showed that inhibition of EDHF responses by 4-AP can be, at least partly, attributed to an inhibition of the intracellular Ca^{2+} release process activated by ACh in ECs. The present study ruled out the possibility that ChTX and apamin abolish the EDHF-dependent hyperpolarization and relaxation by acting on endothelial cell Ca^{2+} signal process.

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