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Role of endothelial cell hyperpolarization in EDHF-mediated responses in the guinea-pig carotid artery

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1 Experiments were performed to identify the potassium channels involved in the acetylcholine-induced endothelium-dependent hyperpolarization of the guinea-pig internal carotid artery. Smooth muscle and endothelial cell membrane potentials were recorded in isolated arteries with intracellular microelectrodes. Potassium currents were recorded in freshly-dissociated smooth muscle cells using patch clamp techniques.

2 In single myocytes, iberiotoxin (0.1 μM)-, charybdotoxin (0.1 μM)-, apamin (0.5 μM)- and 4-aminopyridine (5 mM)-sensitive potassium currents were identified indicating the presence of large- and small-conductance calcium-sensitive potassium channels (BK_{Ca} and SK_{Ca}) as well as voltage-dependent potassium channels (K_V). Charybdotoxin and iberiotoxin inhibited the same population of BK_{Ca} but a conductance specifically sensitive to the combination of charybdotoxin plus apamin could not be detected. 4-aminopyridine (0.1–25 mM) induced a concentration-dependent inhibition of K_V without affecting the iberiotoxin- or the apamin-sensitive currents.

3 In isolated arteries, both the endothelium-dependent hyperpolarization of smooth muscle and the hyperpolarization of endothelial cells induced by acetylcholine or by substance P were inhibited by 5 mM 4-aminopyridine.

4 These results indicate that in the vascular smooth muscle cells of the guinea-pig carotid artery, a conductance specifically sensitive to the combination of charybdotoxin plus apamin could not be detected, comforting the hypothesis that the combination of these two toxins should act on the endothelial cells. Furthermore, the inhibition by 4-aminopyridine of both smooth muscle and endothelial hyperpolarizations, suggests that in order to observe an endothelium-dependent hyperpolarization of the vascular smooth muscle cells, the activation of endothelial potassium channels is likely to be required.

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Keywords: 4-Aminopyridine; apamin; charybdotoxin; endothelium; EDHF; iberiotoxin; potassium channels; smooth muscle

Abbreviations: 4-AP, 4-aminopyridine; BK_{Ca}, large conductance calcium-activated potassium channels; carboxy-PTIO, 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide; 1-EBIO, 1-ethyl-2-benzimidazolinone; Kv, voltage dependent potassium channels; SK_{Ca}, small conductance calcium-activated potassium channels

Introduction

The vascular endothelium controls tone in the underlying smooth muscle cells by releasing various factors including nitric oxide (NO) (Furchgott & Zawadzki, 1980), prostacyclin (Moncada & Vane, 1979) and an unidentified endothelium-derived hyperpolarizing factor (EDHF) (Félétou & Vanhoutte, 1988; Taylor & Weston, 1988). Many observations combine to suggest that the EDHF-induced hyperpolarization of the vascular smooth muscle involves the opening of potassium channels. Thus, it is associated with a decrease in membrane resistance (Bolton *et al*., 1984; Chen & Suzuki 1989a,b), inversely related to the extracellular $K+$ concentration and cannot be observed at $K+$ concentrations higher than 25 mM (Chen & Suzuki, 1989a; Nagao & Vanhoutte, 1992; Corriu *et al*., 1996a). Furthermore, endothelium-dependent hyperpolarizations are associated with an increase in rubidium efflux (Taylor *et al*., 1988) and prevented by non-selective inhibitors of potassium channels such as tetraethylammonium and tetrabutylammonium (Chen *et al*., 1991; Nagao & Vanhoutte, 1992; Van de Voorde *et al*., 1992).

In various tissues, apamin (a specific inhibitor of small conductance calcium-activated potassium channels) alone or in combination with charybdotoxin (a non-specific inhibitor of calcium-activated potassium channels), inhibits the responses attributed to EDHF (Murphy & Brayden, 1995; Garland & Plane, 1996; Corriu *et al*., 1996a; Zygmunt & Höggestätt, 1996; Chataigneau *et al*., 1998; Yamanaka *et al*., 1998; Quignard *et al*., 1999a). These toxins seem selective as they inhibit EDHF-mediated responses without affecting relaxations or hyperpolarizations produced by endothelial nitric oxide or prostacyclin. Furthermore, the increase in endothelial intracellular calcium produced by acetylcholine was not affected by the two toxins (Yamanaka *et al*., 1998). Therefore, it has been assumed that the target(s) for the two toxins is on the vascular smooth muscle.

However, calcium-activated potassium channels are also expressed in endothelial cells (Marchenko & Sage, 1996) and in the rat hepatic artery and the rabbit aortic valve, the combination of charybdotoxin plus apamin inhibits endothelial cell hyperpolarization produced by acetylcholine (Edwards *et al*., 1998; Ohashi *et al*., 1999). Furthermore, in rat mesenteric artery, charybdotoxin and apamin block EDHF responses if selectively applied to the endothelium (Doughty *et*

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al., 1999). Thus, the endothelial actions of the toxins might be responsible for the inhibition of EDHF. Finally, another potassium channel blocker, 4-aminopyridine, inhibits acetylcholine-induced endothelium-dependent hyperpolarization in the isolated coronary artery of the guinea-pig (Eckman *et al*., 1998). However, in the same blood vessel, 4-aminopyridine inhibits also acetylcholine-induced endothelial cell hyperpolarization (Chen & Cheung 1992a), suggesting again that the endothelial action of the potassium channel blocker could be responsible for the inhibition of EDHF.

The present experiments were therefore designed to study the different types of potassium channels expressed in freshly isolated smooth muscle cells from the guinea-pig carotid artery and to determine the role of endothelial cells in the inhibitory effect of potassium channel blockers.

Methods

Male Hartley guinea-pigs (250–300 g) were anaesthetized by intraperitoneal administration of pentobarbitone (200 mg kg^{-1}) and euthanized by exsanguination.

Patch-clamp studies

The media of guinea-pig carotid artery was dissected from cleaned arteries. The smooth muscle cells were dissociated enzymatically (Quignard *et al*., 1999a). Whole-cell potassium currents were recorded at room temperature using the patch-clamp technique. The cells were superfused with a solution containing (in mM): NaCl 125, KCl 5, CaCl2 2, MgCl2 1.2, HEPES 10 and glucose 11. In order to record K_v current, a calcium-free solution intracellular solution was used with the following composition (in mM): KCl 130, MgCl2 2, ATP 3, GTP 0.5, HEPES 25, EGTA 10, Glucose 11. In order to record K_{Ca} currents, the concentration of EGTA was reduced to 1 mM and CaCl2 (0.5 mM) was added. For the inside-out configuration the intra-pipette solution was (in mM): KCl 130, MgCl2 2, HEPES 10, $CaCl_2$ 2, Glucose 11 and the extracellular medium: KCl 130, MgCl2 2, ATP 3, GTP 0.5, HEPES 25, $CaCl_2$ 0.01, Glucose 11. For the outside-out configuration these last two solutions were exchanged.

Data were recorded with pClamp6 software (Axon Instruments, U.S.A.) through a RK-400 amplifier (Biologic, France). Passive capacitive currents and leakage currents were subtracted using the P/4 protocol. The cells that showed a leakage current larger than 20 pA, when clamped at a holding potential of −100 mV, were not studied.

Microelectrode studies

The internal carotid artery of the guinea-pig was dissected and cleaned of adherent connective tissues and then was pinned to the bottom of an organ chamber the adventitia upward to record smooth muscle membrane potential. In order to record the membrane potential of endothelial cells, the main carotid artery was dissected with the internal carotid artery and slit open at its junction with the internal branch. Microelectrodes were inserted through this gap and directed toward the endothelial cells of the internal carotid artery as described by Edwards *et al*. (1998). The tissues were superfused (at 37°C) with a modified Krebs-Ringer bicarbonate solution of the following composition (in mM): NaCl 118.3, KCl 4.7, CaCl2 2.5, MgSO4 1.2, KH2PO4 1.2, NaHCO3 25, glucose 11.1 and EDTA 0.026. Transmembrane potential was recorded by using glass capillary microelectrodes (tip resistance of 30–90 MΩ) filled with KCl (3 M) and connected to the headstage of a recording amplifier (intra 767, WPI). Successful impalements were signalled by a sudden negative drop in potential from the baseline (zero potential reference) followed by a stable negative potential for at least 3 min. All the experiments were performed in the presence of N^{ω}-nitro-L-arginine and indomethacin to inhibit nitric oxide synthase and cyclooxygenase, respectively (Corriu *et al*., 1996b).

Binding

In order to determine the pharmacological profile of carbenoxolone, its inhibitory properties toward the activity of various enzymes and its binding toward various receptors and ionic channels were investigated. These studies were performed by CEREP (Celle L'Evescault, France: http//www.cerep.fr).

Drugs

The following drugs were used: acetylcholine, carbenoxolone, substance-P, indomethacin, N^{ω}-L-nitro-arginine, thiorphan, (Sigma); charybdotoxin, apamin (Latoxan,); 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO, Alexis Biochem); 1-ethyl-2-benzimidazolinone (1-EBIO, Aldrich). Perindoprilat and cromakalim were synthesized in Institut de Recherches Servier (Suresnes, France).

Statistics

Data are shown as mean ± s.e.mean; *n* indicates the number of cells in which membrane potential was recorded. Statistical analysis was performed using Student's *t*-test for paired or unpaired observations. Differences were considered to be statistically significant when P was less than 0.05.

Results

Patch-clamp studies

Currents in the presence of intracellular calcium (0.5 mM) The capacity of the carotid arterial myocytes was 27.4 ± 2.9 pF ($n = 60$). With a holding potential of −100 mV, depolarization of the myocytes induced a large whole-cell outward current which was partially inhibited by iberiotoxin (or charybdotoxin), by apamin or by 4-aminopyridine, indicating that it was the resultant of currents carried by different potassium channel types (Figure 1). After inhibition of the current by iberiotoxin (0.1 μM, Figure 1), addition of apamin (0.5 μM) further reduced the current induced by a step-depolarization to +60 mV. Subsequent, additional exposure to charybdotoxin (0.1 μM) did not induce a further inhibition of the whole-cell current but rather an increase which averaged +2 ± 0.3% ($n = 4$, Figure 1). 4-Aminopyridine inhibited the residual current (Figure 1, $n = 4$).

With a holding potential of 0 mV (to inactivate voltage-dependent potassium channels, K_V), and with a high concentration of intracellular calcium (0.5 μM), depolarization of the myocytes induced a noisy outward current (Figure 2). The current became activated at potentials more positive than +10 mV and its density at +60 mV was 14.6 ± 2.2 pA/pF ($n = 31$). This current was partially inhibited by charybdotoxin (0.1 μM, current density inhibited: 10.6 ± 1.9 pA/pF, per cent inhibition: 75.1 ± 6.2, $n = 7$) or by iberiotoxin (0.1 μM; current density inhibited: 10.4 ± 2.1 pA/pF, per cent inhibition: 80.1 ± 3.3, $n = 21$; Figure 2). The addition of iberiotoxin after

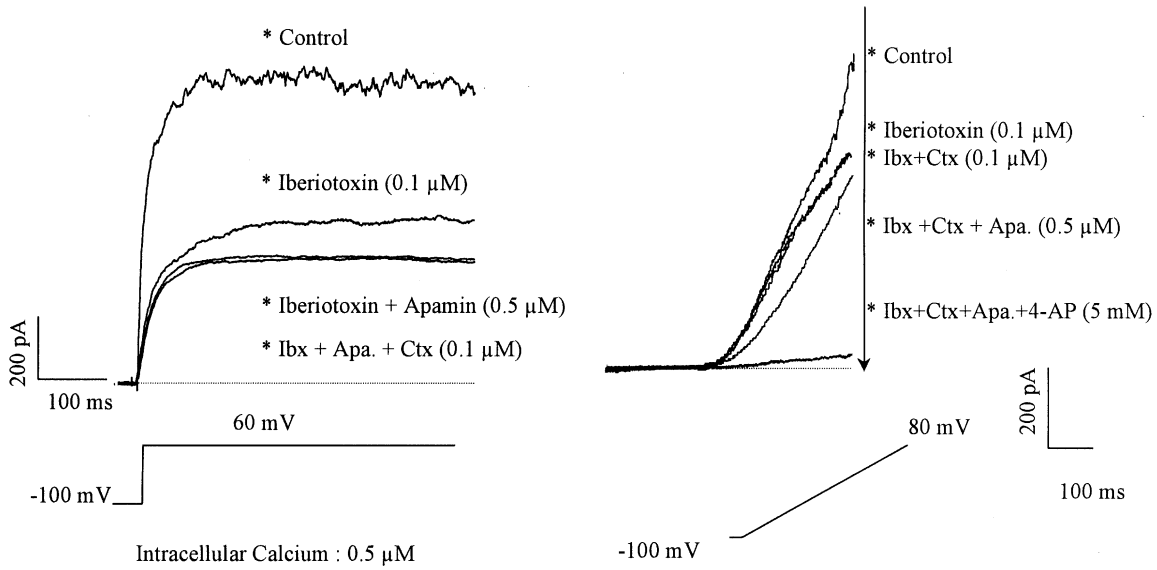

Figure 1 Effect of the combination of different inhibitors of calcium-activated potassium channel in freshly isolated smooth muscle cells of the guinea-pig carotid artery. Left panel: original traces of the large outward current observed in the presence of intracellular calcium (0.5 μM) for a step depolarization from -100 to $+$ 60 mV (whole cell configuration of the patch-clamp technique). This current is partially inhibited by the presence of iberiotoxin (Ibx: 0.1 μM). The addition of apamin (Apa.: 0.5 μM) produces a further inhibition. The subsequent addition of charybdotoxin (Ctx: 0.1 μM) does not produce any further inhibition. However, the addition of 4-aminopyridine produces additional inhibition of the current (data not shown for the sake of clarity). Right panel: original traces of the large global currents observed in presence of intracellular calcium (0.5 μM) for a ramp depolarization from -100 to $+80$ mV (whole cell configuration of the patch-clamp technique). This current is partially inhibited by the presence of iberiotoxin (0.1 μM). The addition of charybdotoxin (0.1 μM) does not produce any further inhibition. However, the subsequent addition of apamin (0.5 μM) produces a further inhibition. The addition of 4-aminopyridine produces additional inhibition of the current.

charybdotoxin or the addition of charybdotoxin after iberiotoxin did not induce any further inhibition that either toxin alone (current density inhibited 9.8 ± 2.1 and 10.6 ± 2.9, pA/pF, per cent inhibition: 78.2 ± 4.5 and 75.6 ± 6.1, $n=3$ and 5 for the combination of charybdotoxin plus iberiotoxin and the combination iberiotoxin plus charybdotoxin, respectively).

A potassium current sensitive to apamin (0.5 μm) could also be observed in 50% of the cells (10 out of 20, current density at 60 mV: 2.6 ± 2.1 pA/pF, $n=10$, Figure 3). In the presence of iberiotoxin to block BKCa, the apamin-sensitive current could still be recorded (1.8 ± 2.1 pA/pF, $n=6$; Figure 1).

Currents in the presence of a low intracellular calcium concentration At a holding potential of -100 mV and with a low intracellular calcium concentration, step-depolarizations induced a slowly-inactivating current (Figure 4). Its activation threshold was -30 mV and the current density was 4.5 ± 1.5 pA/pF ($n=20$) for a step-depolarization to $+20$ mV. 4-Aminopyridine (0.1–25 mM) inhibited this current in a concentration-dependent manner (Figures 4 and 7). The 4-aminopyridine-sensitive potassium current was voltage-dependent. The voltage for half-inactivation calculated from the steady state curve of the current inhibited by 4-aminopyridine (5 mM) averaged -24 ± 1.5 mV ($n=3$, Figure 4). An iberiotoxin-sensitive current could be recorded only for depolarizations to potentials more positive than $+20$ mV (data not shown). 4-Aminopyridine (5 mM) did not affect the potassium currents sensitive to iberiotoxin or apamin (data not shown).

Currents in inside-out and outside-out patches Unitary currents through large-conductance, calcium-sensitive potassium channels were recorded in inside-out and outside-out membrane patches. The amplitude of the current and the channel open probability were dependent on the holding potential. With the inside-out configuration, from the current-voltage relationship only one single channel type with a unitary conductance of 280 ± 20 pS ($n=5$) was observed. With the outside-out configuration, at a stable holding potential of $+30$ mV the open probability of the unitary curent was reduced by the presence of iberiotoxin (0.1 μM, 0.15 ± 0.05 and 0.02 ± 0.01, $n=3$ in control and presence of iberiotoxin, respectively; Figure 5). Under the same conditions, application of 4-aminopyridine (5 mM) did not modify the current kinetics (open probability 0.10 ± 0.04 and 0.09 ± 0.04, $n=2$, in control and presence of 4-aminopyridine, respectively; Figure 5).

Intracellular microelectrode studies

Smooth muscle cells In the presence of N^{ω}-nitro-L-arginine (100 μM) and indomethacin (5 μM), the resting membrane potential of smooth muscle cells in isolated guinea-pig carotid arteries was -52.7 ± 0.9, $n=74$ and acetylcholine (1 μM) induced an endothelium-dependent hyperpolarization (17.1 ± 1.0 mV, $n=25$; Figure 6). In the presence of a higher bath concentration of potassium (35 mM), the cell membrane was depolarized to -32.8 ± 1.9 mV ($n=9$) and acetylcholine did not evoke significant changes in membrane potential ($n=3$). In potassium-free solution the membrane potential was -49.8 ± 4.3 mV ($n=6$) and acetylcholine induced a significantly larger hyperpolarization (38.0 ± 3.7 mV; $n=4$) than in the control solution.

4-Aminopyridine alone (up to 5 mM) did not significantly affect the resting membrane potential of vascular smooth muscle cells (-50.9 ± 1.1 mV, $n=21$). At 1 and 5 mM, it produced a concentration-dependent inhibition of the acetylcholine-induced hyperpolarization (Figures 6 and 7). However, even in the presence of 5 mM 4-aminopyridine there was a residual hyperpolarization ($5.1+0.9$ mV, $n=15$) which was not affected by the presence of the NO scavenger, carboxy-PTIO (10 μM, hyperpolarization 5.1 ± 0.6 mV, $n=4$), char-

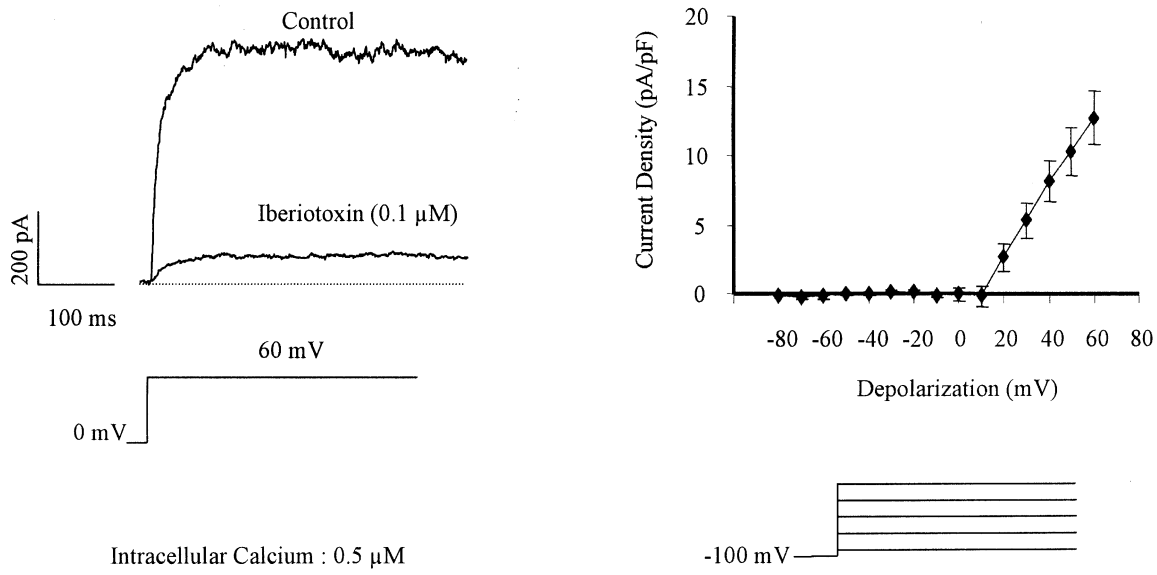

Figure 2 Iberiotoxin-sensitive current in freshly isolated smooth muscle cells of the guinea-pig carotid artery. Left panel: original traces of the large outward current observed in the presence of intracellular calcium (0.5 μM) for a step depolarization from 0 to +60 mV and its inhibition by the presence of iberiotoxin (0.1 μM, whole cell configuration of the patch-clamp technique). Right panel: Current-voltage relationship of the iberiotoxin-sensitive current ($n=5$).

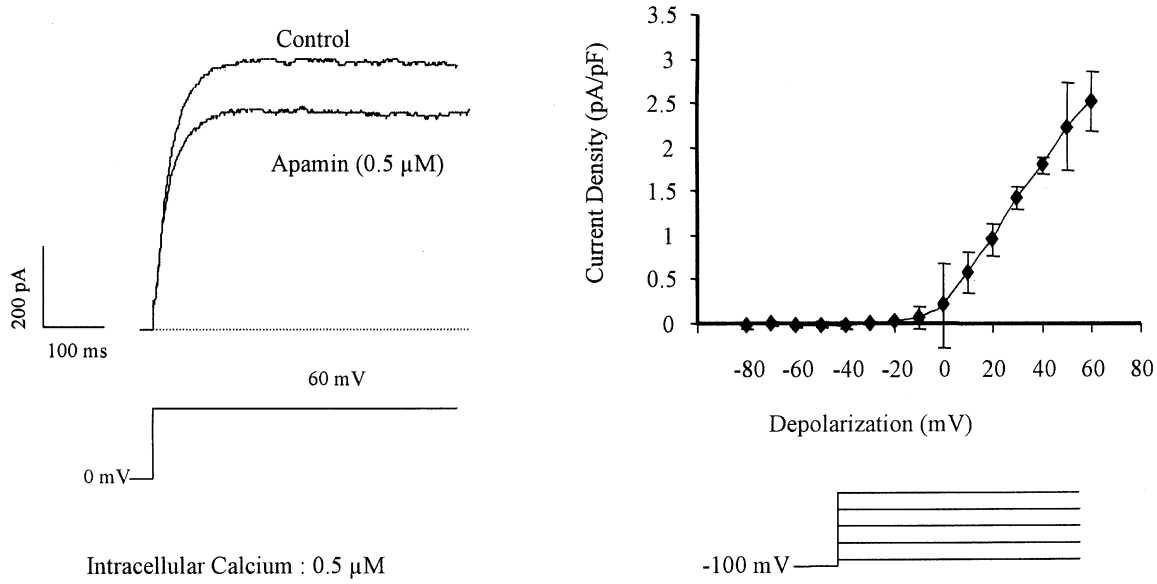

Figure 3 Apamin-sensitive current in freshly isolated smooth muscle cells of the guinea-pig carotid artery. Left panel: original traces of the large outward current observed in the presence of intracellular calcium (0.5 μM) for a step depolarization from 0 to +60 mV and its partial inhibition by the presence of apamin (0.5 μM, whole cell configuration of the patch-clamp technique). Right panel: Current-voltage relationship of the apamin-sensitive current ($n=4$).

ybdotoxin (0.1 μM, hyperpolarization 4.2 ± 1.1 mV, $n=3$) or apamin (0.1 μM, hyperpolarization 4.7 ± 0.6 mV, $n=8$).

Under control conditions, substance P did not evoke reproducible endothelium-dependent hyperpolarization when smooth muscle cells were impaled from the adventitial side of the guinea-pig carotid artery (data not shown; $n=4$). However, in the presence of thiorphan (1 μM) and perindoprilat (1 μM), which did not modify the resting membrane potential (-51.4 ± 0.6 mV, $n=11$), substance *P* (0.1 μM) produced consistent and reproducible hyperpolarizations (15.6 ± 0.4 mV, $n=11$, Figure 6). These were transient (144 ± 8 s, $n=5$) in contrast to the sustained hyperpolarizations produced by acetylcholine. There was no cross-desensitisation between acetylcholine and substance *P* (data not shown). 4-Aminopyridine (5 mM) produced a significant inhibition of the hyperpolarization evoked by substance *P* ($3.9\pm$ 0.8 mV, $n=6$, resting membrane potential -51.2 ± 1.7 mV, $n=6$, Figure 6).

4-Aminopyridine (5 mM) did not significantly affect the hyperpolarization to cromakalim (10 μM; 19.5 ± 2.4 mV, $n=3$ and 19.9 ± 1.3 mV, $n=7$ in control and presence of 4-aminopyridine, respectively).

Endothelial cells In presence of of N^{ω}-nitro-L-arginine (300 μM) and indomethacin (10 μM), the resting membrane potential of carotid artery endothelial cells was -58.7 ± 0.4 mV ($n=4$). Acetylcholine (10 μM), substance *P* (100 nM) and levcromakalim (10 μM) produced a hyperpolarization of the

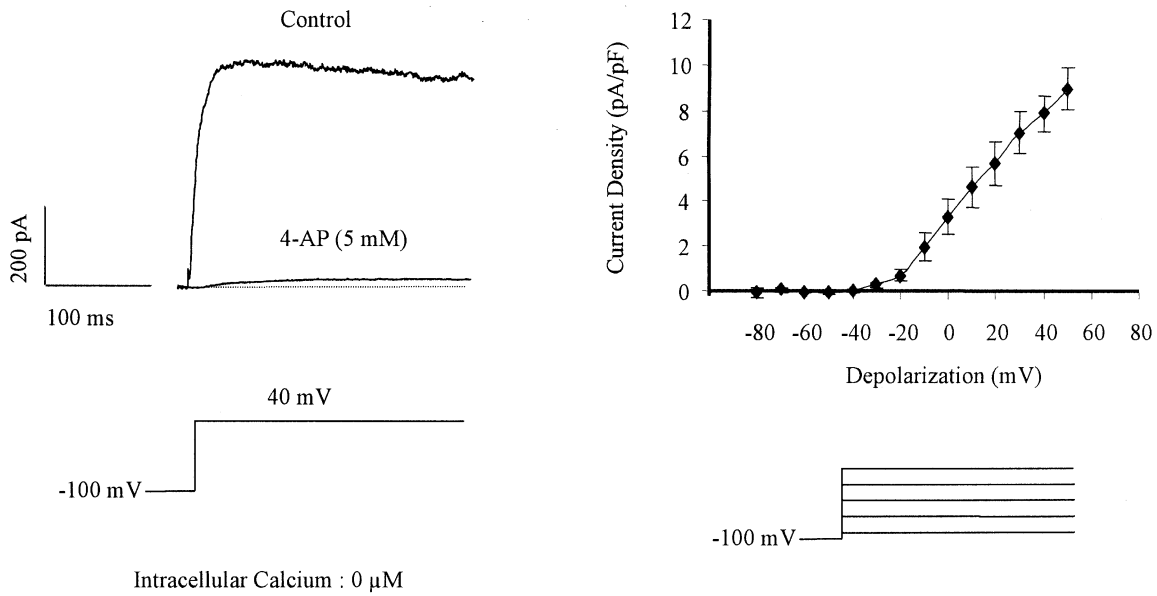

Figure 4 4-Aminopyridine-sensitive current in freshly isolated smooth muscle cells of the guinea-pig carotid artery. Left panel: original traces of the large outward current observed in the presence of very low concentration of intracellular calcium for a step depolarization from −100 to 40 mV and its inhibition by the presence of 4-aminopyridine (4-AP: 5 mM, whole cell configuration of the patch-clamp technique). Right panel: Current-voltage relationship of the 4-aminopyridine-sensitive current ($n=7$).

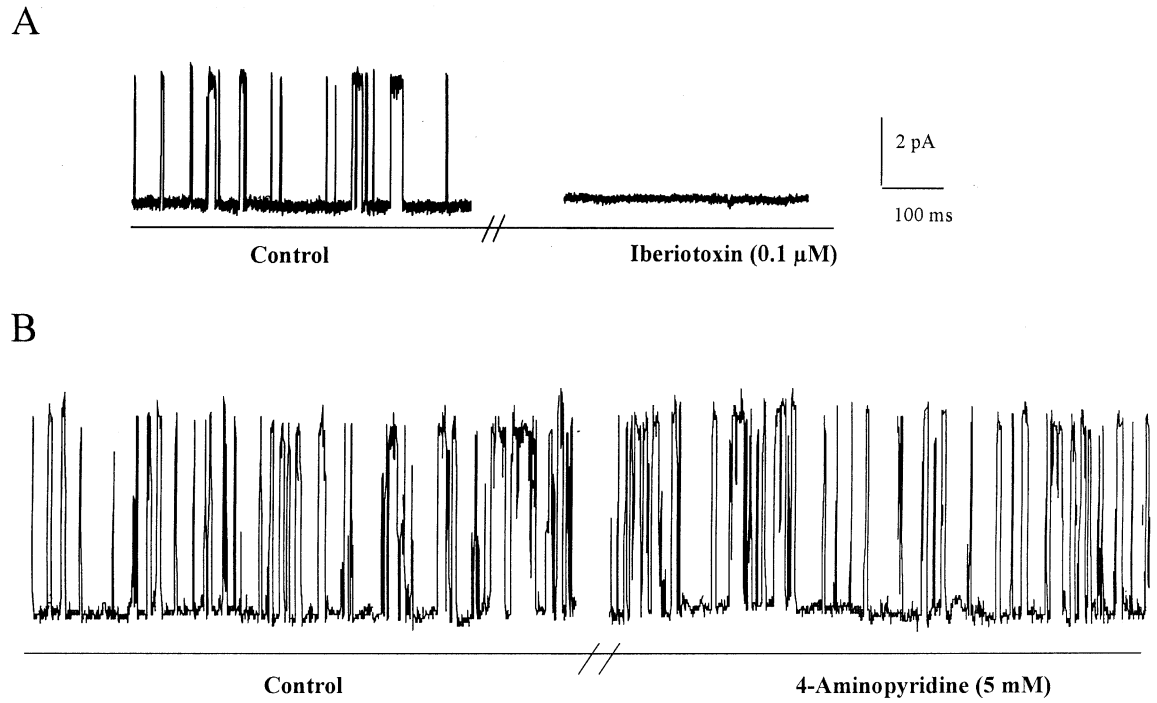

Figure 5 Currents recorded with the outside-out configuration of the patch-clamp technique. At a stable holding potential of +30 mV, unitary currents through large-conductance, calcium-sensitive potassium channels are recorded. (A) The unitary current is abolished by the presence of iberiotoxin (0.1 μM); (B) Under the same conditions, application of 4-aminopyridine (5 mM) did not modify the current kinetics.

endothelial cells (19.4±0.3, 18.4±0.3 and 24.5±0.9 mV, $n=4$, respectively). Under these conditions, brief exposure to 100 nM substance *P* did not show marked tachyphylaxis over the time-course of the experiment (Figure 8). Similarly, the responses to 10 μM acetylcholine and levcromakalim were reproducible. However, after 5 min exposure to 5 mM 4-AP, the hyperpolarizations to acetylcholine and to substance *P* were significantly inhibited while the response to levcromakalim remained unaffected (Figure 8).

The gap junction inhibitor, carbenoxolone (100 μM), did not modify the resting membrane potential of the internal carotid artery endothelial cells (−57.3±0.5 mV, $n=5$). In the presence of carbenoxolone, acetylcholine (10 μM) hyperpolarized the endothelial cells by 20.5±0.6 mV ($n=5$). 4-Aminopyridine (5 mM) did not produce any significant changes in the resting membrane potential (−56.6±0.5 mV; $n=5$) but significantly inhibited the hyperpolarization produced by acetylcholine (6.0±0.9 mV;

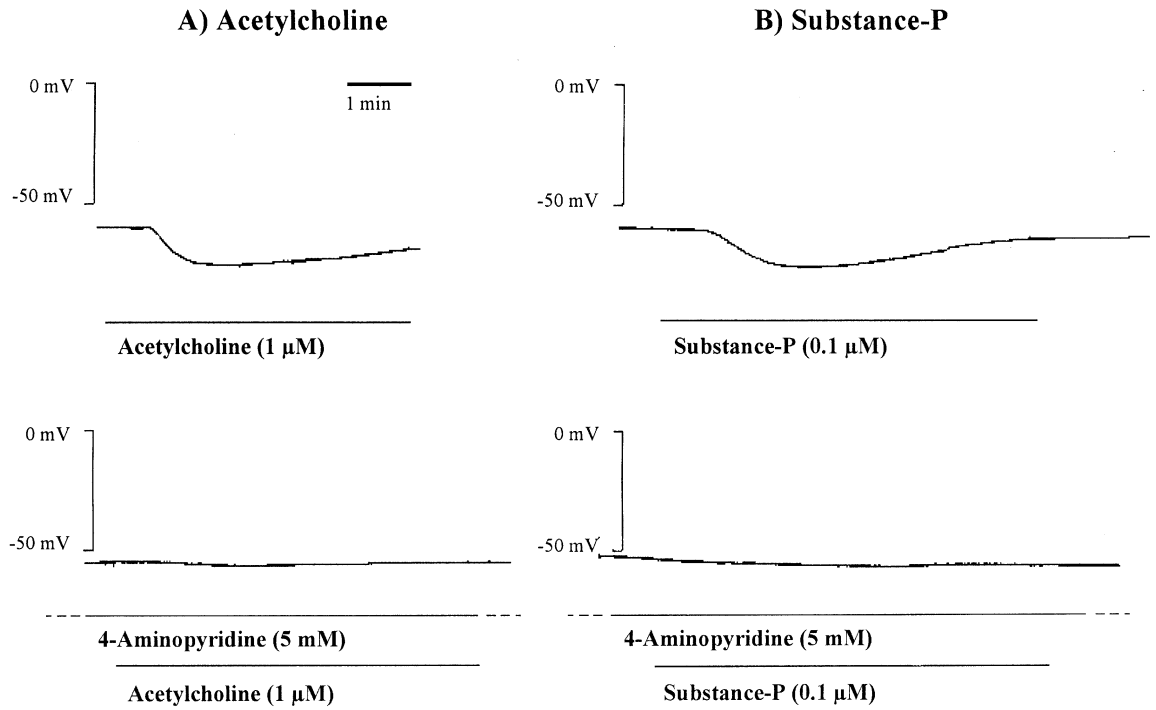

Figure 6 Endothelium-dependent hyperpolarization produced by acetylcholine (1 μM, left panel) and substance *P* (0.1 μM, right panel) in an isolated guinea-pig internal carotid artery, in presence of L-nitroarginine (100 μM) and indomethacin (5 μM). 4-Aminopyridine (5 mM) produces a significant inhibition of the hyperpolarizations produced by both mediators [experiments involving substance *P* were performed in the presence of thiorphan (1 μM) and perindoprilat (1 μM)].

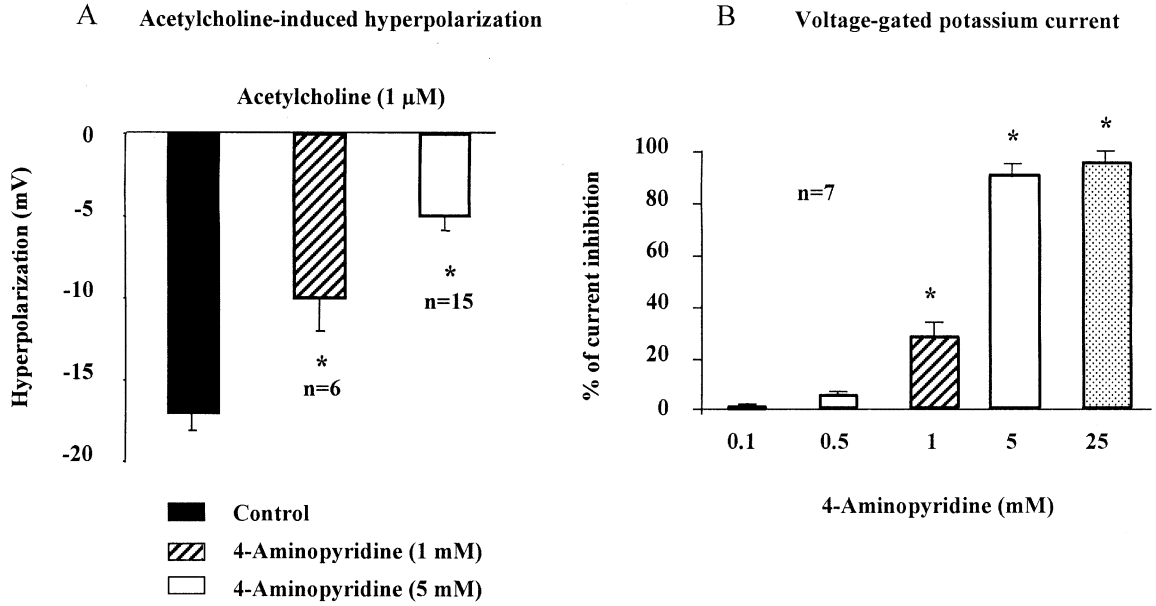

Figure 7 4-Aminopyridine and the smooth muscle cells of the guinea-pig carotid artery. Left panel: endothelium-dependent hyperpolarization of the smooth muscle cells of isolated fragments of internal carotid artery, measured with intracellular microelectrode. Right panel: voltage-gated potassium currents in freshly isolated smooth muscle cells of the guinea-pig carotid artery: presence of very low concentration of intracellular calcium for a step depolarization from -100 to 40 mV (whole cell configuration of the patch-clamp technique). 4-Aminopyridine induces a concentration-dependent inhibition of the acetylcholine-induced hyperpolarization and of the voltage-gated potassium currents.

$n=5$, $P<0.05$). In contrast, the hyperpolarization produced by 1-EBIO (600 μM) observed in presence of carbenoxolone was not affected by 4-aminopyridine (Figure 9).

Binding In binding experiments, carbenoxolone (0.1–100 μM), at the highest concentration, tested induced a displacement superior to 50% of mibolerone (59%; ligand of the testosterone receptor) and of picrotoxinine (82%; chloride channel) but exhibited little or no affinity for the other 35 other receptors (including muscarinic) and ion channels (including potassium) tested (data not shown).

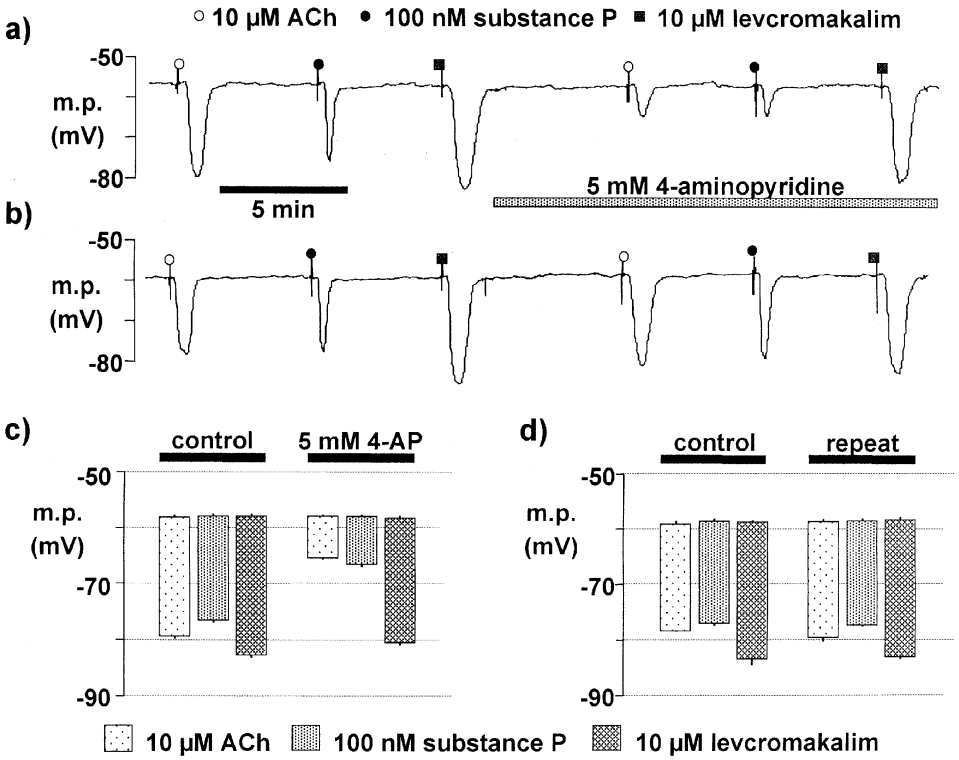

Figure 8 4-Aminopyridine (4-AP) and the endothelial cells of the guinea-pig carotid artery. (a) Original trace showing the hyperpolarizations of an endothelial cell from an isolated fragment of internal carotid artery in response to acetylcholine (ACh: 10 μM), substance *P* (100 nM) and levcromakalim (10 μM) before and after the administration of 4-aminopyridine (5 mM). (b) Time control showing the reproducibility of the hyperpolarizations of an endothelial cell in response to acetylcholine, substance *P* and levcromakalim. (c) Summary showing the average membrane potential (m.p.) of the endothelial cells and the average hyperpolarization produced by acetylcholine, substance *P* and levcromakalim, in control conditions and in the presence of 4-aminopyridine. (d) Summary showing the average membrane potential (m.p.) of the endothelial cells and the average hyperpolarization for time controls. Data are shown as means ± s.e.mean ($n=4$). 4-Aminopyridine (5 mM) induces a significant inhibition of the acetylcholine- and substance *P*-induced endothelial hyperpolarizations.

Discussion

Calcium-sensitive K^+ channels in the smooth muscle

In the present study, two different types of calcium-dependent potassium channel were observed in the smooth muscle cells. Using the whole-cell configuration, a voltage-dependent noisy outward current sensitive to iberiotoxin was recorded. Under the patch configurations inside-out and outside-out, a large unitary conductance potassium channel was observed, the open probability of which was significantly diminished by the same toxin. These observations thus demonstrate the presence of the channel known as 'BK_{Ca}' (Kuriyama *et al.* 1995). Under the experimental conditions of the present study, charybdotoxin and iberiotoxin each inhibited the same population of potassium channels.

In the presence of intracellular calcium, an apamin-sensitive current was also detected in some muscle cells, even in the presence of iberiotoxin. Such a finding could indicate the presence of the small conductance, calcium-sensitive potassium channel known as SK_{Ca}. However, the current carried by classical SK_{Ca} channels should be voltage-independent (Latorre *et al.* 1989), whereas the observed current was voltage-sensitive with an activation threshold of approximately -30 mV. It thus resembles that reported in the smooth muscle cells of the renal arterioles of the rat (Gebremedhin *et al.* 1996).

A conductance specifically sensitive to the combination of charybdotoxin plus apamin, and which was distinct from BK_{Ca}, could not be recorded in the isolated smooth muscle cells of the guinea-pig carotid artery. This confirms previous observations in the rat hepatic artery (Zygmunt *et al.* 1997) and comforts the hypothesis that the inhibitory effect of the combination of these two toxins on EDHF-mediated responses

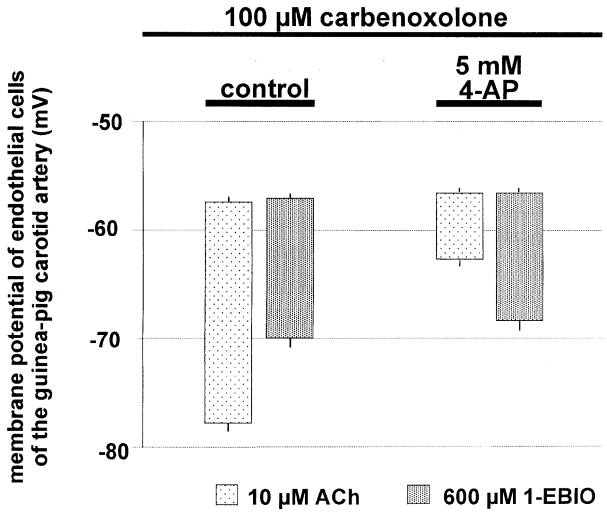

Figure 9 Membrane potential of endothelial cells of the guinea-pig carotid artery in presence of carbenoxolone. Summary showing the average membrane potential (m.p.) of the endothelial cells and the average hyperpolarization produced by acetylcholine (10 μM) and 1-EBIO (600 μM) in control conditions and in the presence of 4-aminopyridine (5 mM). Data are shown as means ± s.e. mean ($n \geqslant 4$). 4-Aminopyridine (5 mM) induces a significant inhibition of the acetylcholine-induced endothelial hyperpolarizations but does not significantly affect 1-EBIO.

could be attributed to an effect on the endothelial cells (Edwards *et al*., 1998; Ohashi *et al*., 1999; Doughty *et al*., 1999). However, it cannot be excluded that the presence of a stimulant (EDHF) might be required to observe this conductance in the smooth muscle cells.

K^+-channel inhibition by 4-aminopyridine

In the present study, a current typical of that carried by delayed rectifier channels (K_V) was present in the smooth muscle cells of the guinea-pig carotid artery. It exhibited a threshold voltage for activation of approximately -30 mV and inactivated with slow kinetics. However, the rapidly inactivating (type A) channel was not observed under our experimental conditions (for review see Kuriyama *et al*., 1995). The recorded current ($I_{K(V)}$) could be inactivated using a holding potential of 0 mV and was inhibited by 4-aminopyridine (for review, see Faraci *et al*., 1998).

Inhibition of EDHF by 4-AP

In the guinea-pig carotid artery, acetylcholine and substance *P* induced an endothelium-dependent hyperpolarization which has been attributed to EDHF (Corriu *et al*., 1996a; Chataigneau *et al*., 1998) and which is inversely related to the extracellular potassium concentration. In the guinea-pig carotid artery, previous reports suggested that endothelium-dependent hyperpolarization in response to substance *P* were observed exclusively when impalements were performed from the intimal side of the vessel (Zhang *et al*., 1994; Corriu *et al*., 1996b). The present study shows that in the presence of inhibitors of neutral endopeptidase and angiotensin converting enzyme, the hyperpolarization to substance *P* can be recorded even when the impalements are performed from the adventitial side. The presence of thiorphan and perindoprilat prevents enzymatic degradation of substance *P* by the vascular wall, thus permitting it to reach the endothelial cells even when given from the adventitial side. The transient nature of the hyperpolarization to substance *P* can be attributed to a rapid desensibilization of the endothelial tachykinin receptors since acetylcholine still can induce hyperpolarization in the presence of substance *P*.

In the present study, the endothelium-dependent hyperpolarization of smooth muscle cells by acetylcholine or substance *P* was inhibited by 4-aminopyridine. Although EDHF-mediated changes resistant to this agent have been described in some vessels (Petersson *et al*., 1997; Murphy & Brayden 1995; Ohlmann 1997), 4-aminopyridine inhibits responses attributed to EDHF in the guinea-pig coronary, porcine coronary and rat mesenteric arteries (Eckman *et al*. 1998; Shimizu & Paul, 1998; Cheung *et al*., 1999). A possible explanation for these apparent discrepancies could be the use of different concentrations of 4-aminopyridine. Thus, the present study clearly shows that substantial inhibition of EDHF requires a concentration of 5 m 4-aminopyridine whereas 1 mM produces only minor inhibition (Nishiyama *et al*., 1998).

In millimolar concentrations, 4-aminopyridine can, depending on the tissue studied, block or activate muscarinic receptors (Urquhart & Broadley, 1991; Navarro-Polanco & Sanchez-Chapula, 1997) but an action on these is unlikely to explain its inhibition of EDHF. Thus, at 5 mM, 4-aminopyridine did not significantly reduce the affinity of acetylcholine in the guinea-pig trachea (pD_2: -5.6 ± 0.1 and -6.1 ± 0.1, $n=4$ in absence and presence of 5 mM of 4-aminopyridine, respectively; unpublished observations). Furthermore, the inhibition of endothelium-dependent hyperpolarizations to substance *P* (which interacts with NK1 receptors) cannot be attributed to an inhibitory effect at muscarinic receptors. Under the present experimental conditions, 4-aminopyridine is unlikely to interact with the other populations of potassium channel studied. Indeed, this compound did not affect the smooth muscle hyperpolarizations produced by cromakalim, indicating that it does not inhibit K_{ATP}. Furthermore, in the patch-clamp experiments performed on isolated smooth muscle cells of the guinea-pig carotid artery, 4-aminopyridine did not inhibit $I_{BK(Ca)}$, $I_{SK(Ca)}$ or the inwardly rectifying potassium channel (Quignard *et al*., 1999b).

Therefore, as the concentration of this agent required to inhibit $I_{K(V)}$ was the same as that necessary to antagonize EDHF, it could indicate that the target for EDHF is the smooth muscle K_V. However, its activation threshold of -30 mV means that this channel is unlikely to be able to sustain a hyperpolarization which shifts the membrane potential into the region of -80 mV.

Endothelial cell actions of 4-aminopyridine

Impalement of endothelial cells with microelectrodes showed that acetylcholine or substance *P* produced endothelial cell hyperpolarizations which were also markedly inhibited by 4-aminopyridine. These findings confirm those of an earlier study in which 4-aminopyridine was found to inhibit the endothelial hyperpolarization produced by acetylcholine in the guinea-pig coronary arteries (Chen & Cheung, 1992a). Carbenoxolone, a succinate salt of glycyrrhetinic acid, is an inhibitor of gap junction (Yamamoto *et al*., 1998; 1999; Taylor *et al*., 1998). This compound has also been described as an inhibitor of both cyclic GMP- and cyclic AMP-dependent phosphodiesterases (Vapaatalo *et al*., 1978), of Na^+/K^+-ATPase (Terasawa *et al*., 1992) and 11β-hydroxysteroid-dehydrogenase (Walker *et al*., 1991). However, in binding experiments, carbenoxolone exhibited little or no affinity for 35 different receptors (including muscarinic) and ion channels (including potassium). Furthermore, in the guinea-pig carotid artery and in the rat hepatic and mesenteric arteries, the effect of carbenoxolone and GAP 27 (a peptide inhibitor of gap junction; Chaytor *et al*., 1998) on EDHF-mediated responses were similar (Edwards *et al*., 1999a). Collectively these data suggest that under our experimental conditions the effect of carbenoxolone should most likely being attributed to gap junction inhibition than to other undesirable side effects.

Since this inhibition of substance *P*-induced endothelial cell hyperpolarization by 4-aminopyridine, is still observed in the presence of carbenoxolone, the recorded changes in membrane potential must have originated in the endothelial cells and not have been conducted to the endothelium from the smooth muscle cells (Dora *et al*., 1997). Furthermore, 1-EBIO a specific opener of calcium activated potassium channel of intermediate conductance (Cai *et al*., 1998; Jensen *et al*., 1998) produced an endothelial hyperpolarization in presence of carbenoxolone. This benzimidazolone does not activate BK_{ca} and does not hyperpolarize smooth muscle cells (Edwards *et al*., 1999b) further suggesting that the membrane potential is recorded from endothelial cells.

Recent studies (Edwards *et al*., 1998; Ohashi *et al*., 1999) have shown that the site of action of the combination of charybdotoxin plus apamin is likely to be small and intermediate conductance Ca^{2+}-sensitive potassium channels (SK_{Ca} and IK_{Ca}, respectively) present on endothelial cells. 4-AP did not inhibit the effects of 1-

EBIO indicating that IK_{Ca} are not affected by the potassium channel blocker. Furthermore, in the present study, the inhibitory effect of 4-aminopyridine was not increased by apamin or charybdotoxin, suggesting that 4-aminopyridine acts on endothelial cell but independently to the targets of these two toxins possibly at a step proximal to the activation of SK_{Ca} and IK_{Ca}. A detailed analysis of the exact site of action of 4-aminopyridine was beyond the scope of this study. However, agonist-induced hyperpolarization of endothelial cells is dependent upon the influx of extracellular calcium and the release of calcium from intracellular pools (Mehrke & Daut, 1990; Chen & Cheung, 1992b). It is therefore tempting to speculate that 4-aminopyridine inhibits this endothelial cell Ca^{2+} release process, evidence for which was recently reported (Wood & Gillespie, 1998).

Conclusions

The present study suggests that the agonist-induced smooth muscle hyperpolarization attributed to EDHF in the guinea-pig internal carotid artery is dependent on the hyperpolarization of vascular endothelial cells. The site of the inhibitory action of 4-aminopyridine is unlikely to be the smooth muscle delayed rectifier channel. Instead, the effects of this agent are probably exerted on the endothelial cell, also the site at which charybdotoxin and apamin inhibit EDHF.

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