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Role of SK_{Ca} and IK_{Ca} in endothelium-dependent hyperpolarizations of the guinea-pig isolated carotid artery

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1 This study was designed to determine whether the endothelium-dependent hyperpolarizations evoked by acetylcholine in guinea-pig carotid artery involve a cytochrome *P*450 metabolite and whether they are linked to the activation of two distinct populations of endothelial K_{Ca} channels, SK_{Ca} and $IK_{Ca.}$

2 The membrane potential was recorded in the vascular smooth muscle cells of the guinea-pig isolated carotid artery. All the experiments were performed in the presence of N^{ω}-L-nitro arginine (100 μM) and indomethacin (5 μM).

3 Under control conditions (Ca^{2+}: 2.5 mM), acetylcholine (10 nM to 10 μM) induced a concentration- and endothelium-dependent hyperpolarization of the vascular smooth muscle cells. Two structurally different specific blockers of SK_{Ca}, apamin (0.5 μM) or UCL 1684 (10 μM), produced a partial but significant inhibition of the hyperpolarization evoked by acetylcholine whereas charybdotoxin (0.1 μM) and TRAM-34 (10 μM), a nonpeptidic and specific blocker of $IK_{Ca,}$ were ineffective. In contrast, the combinations of apamin plus charybdotoxin, apamin plus TRAM-34 (10 μM) or UCL 1684 (10 μM) plus TRAM-34 (10 μM) virtually abolished the acetylcholine-induced hyperpolarization.

4 In the presence of a combination of apamin and a subeffective dose of TRAM-34 (5 μM), the residual hyperpolarization produced by acetylcholine was not inhibited further by the addition of either an epoxyeicosatrienoic acid antagonist, 14,15-EEZE (10 μM) or the specific blocker of BK_{Ca}, iberiotoxin (0.1 μM).

5 In presence of 0.5 mM Ca^{2+}, the hyperpolarization in response to acetylcholine (1 μM) was significantly lower than in 2.5 mM Ca^{2+}. The EDHF-mediated responses became predominantly sensitive to charybdotoxin or TRAM-34 but resistant to apamin.

6 This investigation shows that the production of a cytochrome *P*450 metabolite, and the subsequent activation of BK_{Ca}, is unlikely to contribute to the EDHF-mediated responses in the guinea-pig carotid artery. Furthermore, the EDHF-mediated response involves the activation of both endothelial IK_{Ca} and SK_{Ca} channels, the activation of either one being able to produce a true hyperpolarization.
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Abbreviations: BK_{Ca}, large-conductance calcium-activated potassium channels; EDHF, endothelium-derived hyperpolarizing factor; IK_{Ca}, intermediate-conductance calcium-activated potassium channels; SK_{Ca}, small-conductance calcium-activated potassium channels

Introduction

Endothelium-dependent hyperpolarizations are blocked by the combination of two toxins, apamin, a selective inhibitor of the small-conductance Ca^{2+}-activated K^+ channel: (SK_{Ca}), and charybdotoxin, a nonselective inhibitor of intermediate-conductance Ca^{2+}-activated K^+ channel (IK_{Ca}), large-conductance Ca^{2+}-activated K^+ channel (BK_{Ca}) as well as some voltage-dependent K^+ channels (K_V) (Garcia *et al*., 1991; Garland & Plane, 1996; Corriu *et al*., 1996a; Chataigneau *et al*., 1998; Edwards *et al*., 1998; 2000). The effect of apamin can be mimicked by scyllatoxin, a structurally distinct SK_{Ca} inhibitor, indicating that SK_{Ca} are involved in endothelium-dependent hyperpolarizations (Corriu *et al*., 1996a). However, iberiotoxin cannot substitute for charybdotoxin (Zygmunt & Högestätt, 1996; Chataigneau *et al*., 1998) excluding a pivotal role for BK_{Ca} in many EDHF-mediated responses. Pharmacological proofs of the involvement of IK_{Ca} were obtained with maurotoxin, a blocker of both IK_{Ca} and $K_{V,}$ and more convincingly with recently developed specific and nonpeptidic blockers of IK_{Ca}, TRAM-34 and TRAM-39 (Wulff *et al*., 2000; 2001) as they fully mimic the effects of charybdotoxin, at least in rat arteries (Crane *et al*., 2003; Eichler *et al*., 2003; Hinton & Langton, 2003; Sandow *et al*., 2004). In most blood vessels, these two channels, SK_{Ca} and IK_{Ca}, are thought to be located on the endothelial cells and are activated by an initial increase in the endothelial intracellular calcium concentration (Busse *et al*., 2002).

However, many questions, concerning the effects of the inhibitors of endothelium-dependent hyperpolarizations,

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remain unanswered. For instance, as charybdotoxin blocks both IK_{Ca} and BK_{Ca}, it is difficult to determine whether or not this toxin prevents the hyperpolarization of the endothelial cell and/or that of the smooth muscle cells, which could have been evoked by the diffusion of an endothelium-derived diffusible factor such as an epoxyeicosatrienoic acid, an arachidonic acid metabolite generated *via* the cytochrome *P*450 monooxygenase pathway (Campbell *et al*., 1996; Fisslthaler *et al*., 1999; Edwards *et al*., 2001). In order to assess properly the involvement of a putative epoxyeicosatrienoic acid in endothelium-derived hyperpolarizing factor (EDHF)-mediated responses, the specific blockade of both IK_{Ca} and SK_{Ca} is required.

In addition, in many vessels, including in the guinea-pig carotid artery, each toxin alone produces no or minor inhibition of EDHF-mediated responses while the toxin combination abolishes the responses (Corriu *et al*., 1996a; Chataigneau *et al*., 1998). Two interpretations of these findings are possible. Endothelial SK_{Ca} and IK_{Ca} channels could be activated similarly with each system capable of generating a "full" response. Alternatively, and as previously suggested, the endothelial channel could be a novel channel, possibly a heteromultimer composed of IK1 and SK1-3 α-subunits, which requires the presence of both toxins in order to be inhibited (Zygmunt *et al*., 1997; Ding & Triggle, 2000; Ding *et al*., 2003). Indeed, in a heterologous expression system, the SK3 α-subunit, known to be expressed in endothelial cells and thought to be involved in EDHF-mediated responses (Burnham *et al*., 2002; Ding *et al*., 2003; Eichler *et al*., 2003; Taylor *et al*., 2003), can interact with SK1 and SK2 α-subunits to form heteromeric SK_{Ca} channels (Ishii *et al*., 1997; Monaghan *et al*., 2004). Similarly, IK1 α-subunit could form heteromultimers with corresponding SK1, SK2 or SK3 products (Castle, 1999), although it is not known whether or not these resulting heteromers are formed in native cells.

The purpose of the present work was to further clarify the respective roles of IK_{Ca} and SK_{Ca} in the endothelium-dependent hyperpolarizations evoked by acetylcholine in the guinea-pig carotid artery. This was made possible using the recently synthesized nonpeptide inhibitors of IK_{Ca} and SK_{Ca}, TRAM-34 (a clotrimazole derivative, Wulff *et al*., 2000) and UCL 1684 (a dequalinium-related compound, Campos-Rosa *et al*., 2000). Also, the possible involvement of an arachidonic acid metabolite generated *via* the cytochrome *P*450 monooxygenase pathway was investigated using the recently described epoxyeicosatrienoic acid antagonist, 14,15-EEZE (Gauthier *et al*., 2002). A preliminary account of these findings has been presented to the BPS (Gluais *et al*., 2003; Edwards *et al*., 2004).

Methods

Male Hartley guinea-pigs (250–300 g) were killed with an overdose of pentobarbitone (200 mg kg^{-1}, i.p.) and the internal carotid arteries with their branches were dissected free. Segments of artery (1 cm in length) were cleaned of adherent connective tissues and pinned down to the bottom of an organ chamber (0.5 ml in volume) superfused at a constant flow (2 ml min^{-1} and 37°C) with modified Krebs–Ringer bicarbonate solution of the following composition (in mM): NaCl 118.3, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, glucose 11.1 and ethylenediamine tetra-acetic acid (EDTA) 0.026 (buffered with 95% O_2 and 5% CO_2, pH 7.4). In some experiments, referred to as low-calcium experiments, the concentration of $CaCl_2$ was 0.5 mM. Transmembrane potentials were recorded from the adventitial side of the internal carotid arteries with glass capillary microelectrodes (tip resistance of 30 to 90 MΩ) filled with KCl (3 M) and connected to the headstage of a recording amplifier (World Precision Instrument (intra 767), New Haven, CT, U.S.A.); an Ag/AgCl pellet, in contact with the bathing solution and directly connected to the amplifier, served as the reference electrode. The signal was continuously monitored on an oscilloscope (Gould DSO 405, Valley view, OH, U.S.A.) and recorded using a pClamp software (Axon instrument, Foster City, CA, U.S.A.). 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. The acetylcholine-induced hyperpolarizations that, in this artery, are fully endothelium-dependent (Corriu *et al*., 1996a, b) were analysed using pClampfit (Axon instrument, Foster City, CA, U.S.A.) and hyperpolarization values are expressed as the maximal amplitude between resting membrane potential and the membrane potential in the presence of the hyperpolarizing drugs (peak amplitude). Drugs were added by continuous superfusion *via* the Krebs solution reservoir. The preincubation time was at least 20 min with the various inhibitors studied.

Drugs

The following drugs were used: acetylcholine, indomethacin, N^{ω}-nitro-L-arginine, 6,10-diaza-3(1,3),8(1,4)-dibenzena-1,5(1,4)-diquinolinacyclodecaphane (UCL 1684; Sigma, La Verpillère, France); synthetic charybdotoxin, apamin and iberiotoxin (Latoxan, Rosans, France); 1-[(2-chlorophenyl) diphenylmethyl]-1*H*-pyrazole (TRAM-34) and 1,3-dihydro-1-[2-hydroxy-5-(trifluoromethyl)phenyl]-5-(trifluoromethyl)-2*H*-benzimidazol-2-one (NS1619) were generous gifts from Dr H. Wulff (University of California Irvine, CA, U.S.A.) and Dr S.P. Olesen (Neurosearch Laboratory, Glostrup, Denmark), respectively; 14, 15-epoxyeicosa-5(Z)-enoic acid (14, 15 EEZE) was synthesized in the department of Biochemistry of the University of Texas Southwestern Medical School (Dallas, U.S.A.) and cromakalim by the Institut de Recherches Servier (Suresnes, France). The drugs were prepared as concentrated stock solutions and subsequently diluted with the Krebs solution. Indomethacin was dissolved in deionized water and an equimolar concentration of Na_2CO_3, 14, 15 EEZE and cromakalim were dissolved in ethanol (final concentration: 1%). UCL 1684, TRAM-34 and NS1619 were dissolved in DMSO (final concentrations: 1.1, 1 and 0.1%, respectively). All other drugs were dissolved in deionized water.

Statistics

Data are shown as mean ± s.e.m.; *n* indicates the number of cells in which membrane potential was recorded. Comparisons *versus* control were performed statistically using an analysis of variance (ANOVA1 or ANOVA2 followed by the Dunnett's *t*-test or Bonferroni post-tests) or by use of Student's *t*-test for paired or unpaired observations, as appropriate. Differences were considered to be statistically significant when the *P*-value was less than 0.05.

Results

All the experiments were performed in the presence of N^{ω}-nitro-L-arginine (100 μM) and indomethacin (5 μM) in order to inhibit nitric oxide synthases and cyclooxygenases, respectively.

Charybdotoxin and apamin

The resting membrane potential of the vascular smooth muscle cells was -51.0 ± 0.9 mV ($n = 41$). Noncumulative addition of acetylcholine (1 nM–10 μM) induced a concentration-dependent hyperpolarization, which was maximal at the highest concentration tested and reached 23.2 ± 1.4 mV (Figure 1).

The presence of apamin (0.5 μM) did not significantly affect the resting membrane potential whereas charybdotoxin (0.1 μM) alone or in combination with apamin produced a small but statistically significant depolarization (Table 1). The endothelium-dependent hyperpolarization was not modified by the presence of charybdotoxin at any of the acetylcholine concentrations tested. In contrast, apamin partially but significantly inhibited the hyperpolarization induced by 1 and 10 μM of acetylcholine. The combination of charybdotoxin plus apamin was significantly more effective than apamin alone and abolished the endothelium-dependent hyperpolarization to acetylcholine at virtually all the concentrations tested (Figure 1).

TRAM-34

TRAM-34 (5 or 10 μM), a nonpeptidic and selective blocker of IK_{Ca}, did not significantly affect the resting membrane potential of the vascular smooth muscle cells whereas the highest concentration of TRAM-34 (10 μM) in combination with apamin produced a small but significant reduction of the resting membrane potential (Table 1).

TRAM-34 alone, at either 5 or 10 μM, did not affect the hyperpolarizations in response to acetylcholine (Figure 2). The combination of apamin plus TRAM-34 (5 μM) partially inhibited the hyperpolarizations but this inhibition was not significantly different from that evoked by acetylcholine in the presence of apamin alone (Figure 2). However, the combination of apamin plus TRAM-34 (10 μM) virtually abolished the hyperpolarization to acetycholine. The inhibition produced by this combination was significantly different from that induced by apamin alone and was undistinguishable from that produced by the combination of apamin plus charybdotoxin (Figure 2).

UCL 1684

UCL 1684 (1 or 10 μM), a nonpeptidic and selective blocker of SK_{Ca}, did not affect the resting membrane potential of the vascular smooth muscle cells whereas in combination with TRAM-34 (10 μM), UCL 1684 (10 μM) produced a small but significant reduction of the resting membrane potential (Table 1).

UCL 1684 at 1 μM did not induce any significant changes in the concentration–response to acetylcholine (Figure 3). At 10 μM, UCL 1684 produced a partial but significant inhibition of the hyperpolarization, an effect which was similar to that evoked by apamin alone.

The combination of UCL 1684 (1 μM) plus TRAM-34 (10 μM) significantly inhibited the hyperpolarization induced

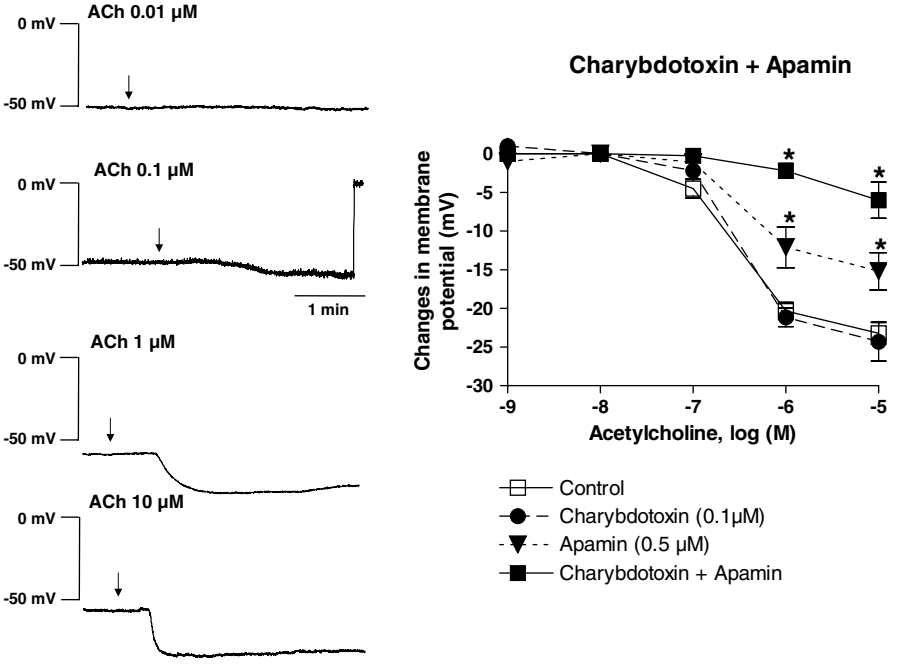

Figure 1 Acetylcholine (ACh) concentration-dependently induced an endothelium dependent hyperpolarization in the guinea-pig isolated carotid artery. Effects of charybdotoxin and apamin. Left: Original traces; Right: Concentration–response curves to acetylcholine (1 nM to 10 μM) obtained in control conditions and in presence of charybdotoxin (0.1 μM), apamin (0.5 μM) and their combination. Data are shown as mean $\pm$ s.e.m. ($n = 2$–3 for acetylcholine 1 and 10 nM and 4 to 7 for acetylcholine 0.1 to 10 μM, and indicates the number of different animals from which the arteries were taken). The asterisks indicate a statistically significant difference *versus* the control values ($P < 0.05$). Besides, for the two same concentrations of acetylcholine (1 and 10 μM), the charybdotoxin plus apamin group is significantly different from the two other groups (charybdotoxin alone and apamin alone) and the apamin group is significantly different from the charybdotoxin group.

Table 1 Resting membrane potential of guinea-pig carotid artery smooth muscle cells

Drugs	*Membrane potential in mV (cell number)*	
	Ca^{2+}: 2.5 mM	*Ca^{2+}: 0.5 mM*
Control	−51.0±0.9 (41)	−44.9±1.1$^{\#}$ (16)
Apamin (0.5 μM)	−50.7±0.9 (37)	−45.6±1.0$^{\#}$ (15)
Charybdotoxin (0.1 μM)	−46.8±1.2* (35)	−40.9±1.1$^{\#,*}$ (16)
Apamin + charybdotoxin	−45.7±0.9* (38)	−38.0±2.5$^{\#,*}$ (4)
TRAM-34 (10 μM)	−50.1±1.5 (23)	−43.2±0.6$^{\#}$ (16)
TRAM-34 (10 μM) + apamin	−45.1±1.1* (14)	
UCL 1684 (1 μM)	−54.2±1.3 (9)	
UCL 1684 (1 μM) + TRAM-34 (10 μM)	−51.9±1.6 (8)	
UCL 1684 (10 μM)	−48.6±1.6 (4)	
UCL 1684 (10 μM) + TRAM-34 (10 μM)	−46.2±1.2* (19)	
TRAM-34 (5 μM)	−50.2±1.2 (21)	
TRAM-34 (5 μM) + apamin	−50.4±1.0 (35)	
TRAM-34 (5 μM) + apamin + EEZE (10 μM)	−54.9±1.5§ (16)	
TRAM-34 (5 μM) + apamin + IbTX (0.1 μM)	−44.2±1.2§ (21)	

Data are shown as mean±s.e.m. The asterisk indicates a statistically significant difference with controls. The # sign indicates a difference between 2.5 and 0.5 mM Ca^{2+} while the § sign indicates a statistically significant difference *versus* TRAM-34 (5 μM) + apamin ($P<0.05$). EEZE: 14,15-epoxyeicosa(Z)-enoic acid; IbTX: iberiotoxin.

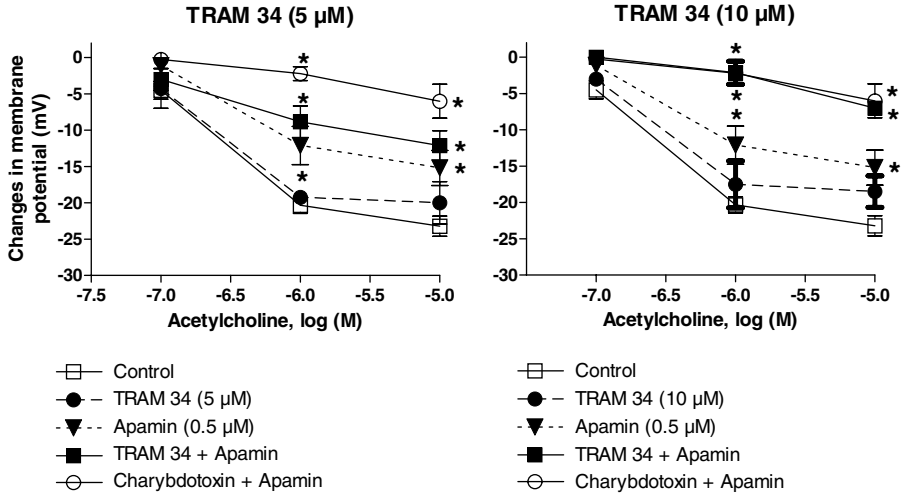

Figure 2 Acetylcholine (0.1–10 μM)-induced endothelium-dependent hyperpolarization in the guinea-pig isolated carotid artery. Effects of TRAM-34 (5 and 10 μM) and apamin (0.5 μM). Left: TRAM-34 (5 μM). Data are shown as mean±s.e.m. ($n=3$ to 9 and indicates the number of different animals from which the blood vessels were taken). The asterisks indicate a statistically significant difference *versus* the control values ($P<0.05$). Besides, for the two same concentrations of acetylcholine (1 and 10 μM), the charybdotoxin plus apamin group is significantly different from the three other groups (TRAM-34 , apamin and TRAM-34 + apamin) and the apamin group as well as the apamin + TRAM-34 groups are significantly different from the TRAM 34 group. Right: TRAM-34 (10 μM; $n=3$ to 7). The asterisks indicate a statistically significant difference *versus* the control values ($P<0.05$). Besides, for the two same concentrations of acetylcholine (1 and 10 μM), the charybdotoxin + apamin and the apamin + TRAM-34 groups are significantly different from the two other groups (TRAM-34 and apamin, alone) and the apamin group is significantly different from the TRAM 34 group.

by acetylcholine (Figure 3) but this effect was significantly less than the inhibition evoked by the combination of apamin plus charybdotoxin. The combination of 10 μM UCL 1684 plus 10 μM of TRAM-34 further inhibited the endothelium-dependent hyperpolarization to acetylcholine. The inhibition achieved was then indistinguishable from that produced by either the combination of apamin plus charybdotoxin or that of TRAM-34 (10 μM) plus apamin (Figure 3).

Selectivity of TRAM-34 and UCL 1684

Cromakalim (10 μM), the opener of K-ATP channel, produced a large and sustained hyperpolarization of resting membrane potential of the smooth muscle cells (25.7±2.9 mV, $n=3$). In contrast, NS1619 (10 μM), an activator of BK_{Ca} (Olesen *et al.*, 1994), produced a small but measurable hyperpolarization of the vascular smooth muscle cells (2.2±0.6 mV, $n=3$). TRAM-34 (10 μM), UCL 1684 (10 μM) and their combination did not affect the hyperpolarization produced by either cromakalim (26.0±1.0, 20.7±6.7 and 28.0±4.0 mV, $n=3$, respectively), or NS1619 (1.8±0.2, 2.0±0.6, 1.3±0.7 mV, $n=3$, respectively).

In the presence of the combination of TRAM-34 (5 μM) and apamin, the addition of 14,15-EEZE (10 μM), an antagonist of epoxyeicosatrienoic acid (Gauthier *et al.*, 2002), produced a small but significant hyperpolarization of the smooth muscle cells while the addition of iberiotoxin (0.1 μM) produced a significant depolarization (Table 1). The residual endothelium-dependent hyperpolarization to acetylcholine (1 and 10 μM)

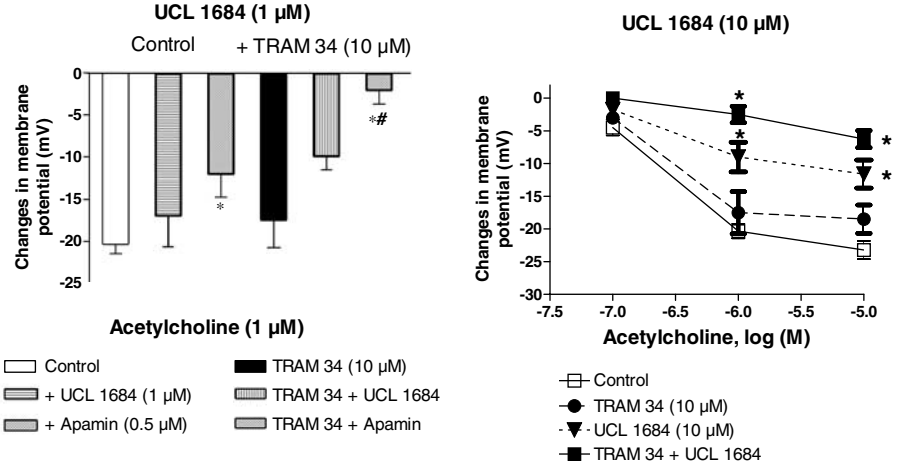

Figure 3 Acetylcholine-induced endothelium-dependent hyperpolarization in the guinea-pig isolated carotid artery. Effects of UCL 1634 and TRAM-34 (10 μM). Left: UCL 1684 (1 μM) and acetylcholine (1μM); Data are shown as mean$\pm$s.e.m. ($n=3$ to 5 and indicates the number of different animals from which the blood vessels were taken). The asterisks indicate a statistically significant difference *versus* the control values ($P<0.05$). The sign # indicates a statistically significant difference between UCL 1684 and apamin. Right: UCL 1684 (10 μM) and acetylcholine (0.1–10 μM, $n=3$ to 7). The asterisks indicate a statistically significant difference *versus* the control values ($P<0.05$). Besides, for the two same concentrations of acetylcholine (1 and 10 μM), the TRAM-34 + UCL 1684 group is significantly different from TRAM-34 group and, for acetylcholine 1 μM, this combination is significantly different from the UCL 1684 group. Additionally, the UCL 1684 group is significantly different from the TRAM 34 group.

observed in the presence of the combination of TRAM-34 (5 μM) and apamin was not significantly affected by the addition 14,15-EEZE (Figure 4). Similarly, the remaining hyperpolarization observed in presence of TRAM-34 (5 μM) plus apamin was not inhibited and was even significantly increased in the presence of iberiotoxin (acetylcholine 1 μM: 8.8$\pm$2.2 and 21.2$\pm$4.0 mV, $n=6$ and 4, in presence of TRAM-34 plus apamin and TRAM-34 plus apamin + iberiotoxin, respectively, $P<0.05$).

Low calcium

In physiological salt solution containing 0.5 mM Ca^{2+}, the smooth muscle was significantly depolarized when compared to that observed in control solution (2.5 mM Ca^{2+}; Table 1). Furthermore, the hyperpolarization produced by acetylcholine (1 μM) was significantly smaller than that observed in presence of 2.5 mM Ca^{2+}.

In the presence of 0.5 mM Ca^{2+}, charybdotoxin (0.1 μM) or TRAM-34 (10 μM) produced a significant inhibition of the acetylcholine (1 μM)-induced hyperpolarization (Figure 5) while in 2.5 mM Ca^{2+} neither charybdotoxin nor TRAM-34 affected the cholinergic responses (Figure 1). In the presence of charybdotoxin or TRAM-34, the amplitude of the hyperpolarizations evoked by acetylcholine was significantly smaller in 0.5 mM than in 2.5 mM Ca^{2+} (Figure 5).

In the presence of either 2.5 or 0.5 mM of Ca^{2+}, apamin (0.5 μM) produced a significant inhibition of the hyperpolarization evoked by acetylcholine (1 μM). However, in the presence of apamin, the amplitude of the acetylcholine-induced hyperpolarizations, as observed in 0.5 and 2.5 mM Ca^{2+}, were not significantly different (Figures 1 and 5).

In the presence of the combination of charybdotoxin plus apamin, the acetylcholine-induced hyperpolarization was abolished under both Ca^{2+} conditions (acetylcholine 1 μM: 2.2$\pm$1.0 and -0.3 ± 0.9 mV, $n=5$ and 4, in 2.5 and 0.5 mM Ca^{2+}, respectively).

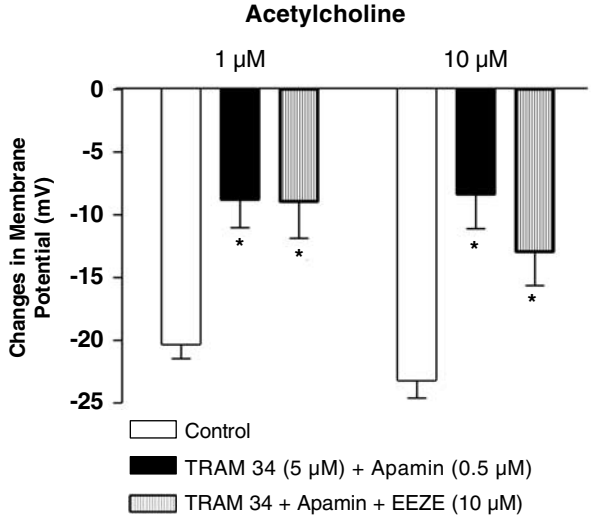

Figure 4 Acetylcholine (1 and 10 μM)-induced endothelium-dependent hyperpolarization in the presence of the combination of TRAM-34 (5 μM) plus apamin (0.5 μM). Effect of 14,15-EEZE (10 μM). Data are shown as mean$\pm$s.e.m. ($n=3$ and indicates the number of different animals from which the blood vessels were taken). The asterisks indicate a statistically significant difference *versus* the control values ($P<0.05$).

Discussion

This study confirms that, in the guinea-pig carotid artery, SK$_{Ca}$ and IK$_{Ca}$ independently but simultaneously play a key role in the concentration-dependent and endothelium-dependent hyperpolarization in response to acetylcholine.

UCL 1684 and TRAM-34, two nonpeptide blockers of SK$_{Ca}$ and IK$_{Ca}$, respectively (Campos-Rosa *et al.*, 2000; Wulff *et al.*, 2000), fully mimicked the effects of apamin and charybdotoxin. However, the micromolar concentrations of TRAM-34 and UCL 1684, required in the present study to block the

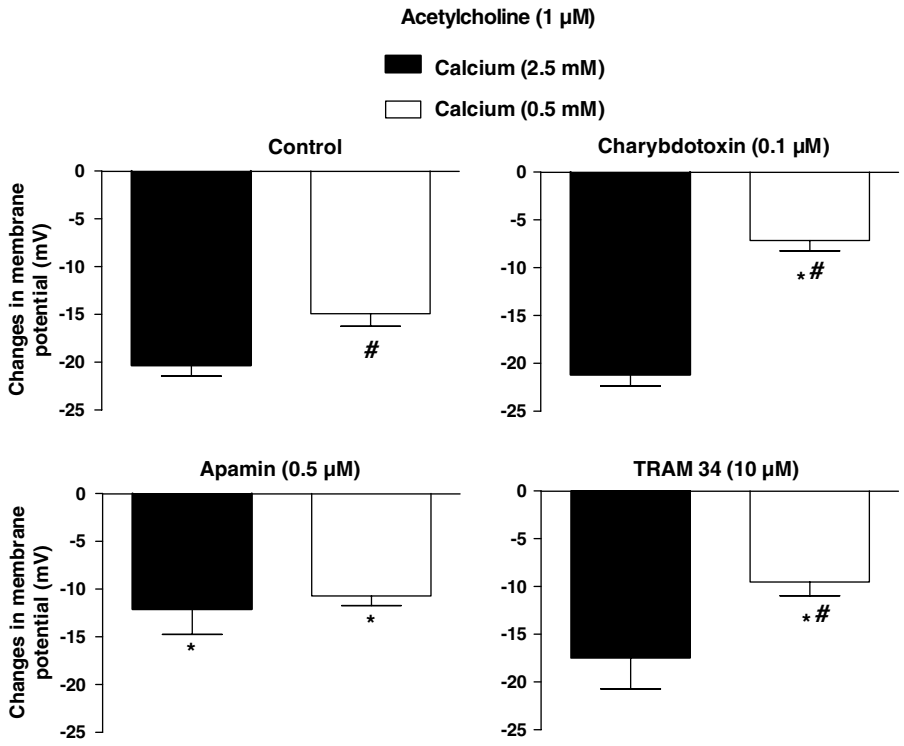

Figure 5 Acetylcholine (1 μM)-induced endothelium-dependent hyperpolarization in the presence of either 2.5 or 0.5 mM Ca^{2+}. Effects of apamin (0.5 μM), charybdotoxin (0.1 μM) or TRAM-34 (5 μM). Data are shown as mean $\pm$ s.e.m. ($n = 5$–6 and indicates the number of different animals from which the vessels were taken). The asterisk indicates a statistically significant difference between control and the presence of a potassium channel blocker while the # sign indicates a difference between 2.5 and 0.5 mM Ca^{2+} ($P < 0.05$).

endothelium-dependent hyperpolarizations fully, were significantly higher than the concentrations reported to be effective in the early publications demonstrating the activity of these agents (TRAM-34 and the cloned IK1 channel: K_d of 20 to 25 nM; UCL 1684 and SK_{Ca} channel: IC_{50} of 4 to 10 nM, in rat chromaffin cells or in rabbit blood cells; Dunn, 1999; Campos-Rosa *et al*., 2000; Malik-Hall *et al*., 2000; Wulff *et al*., 2000; 2001). In functional work dedicated to the study of endothelium-dependent hyperpolarizations, the range of concentration used, if higher than that reported in the cellular assays, was still lower than that required in the present study (Andersson *et al*., 2000; Eichler *et al*., 2003; Hinton & Langton, 2003; Crane *et al*., 2003).

In the guinea-pig carotid artery, a high concentration of TRAM-34 may have been required to inhibit the endothelium-dependent hyperpolarization because, besides the activation of SK_{Ca} and IK_{Ca}, an additional mechanism is involved. It is well established that epoxyeicosatrienoic acids, products of cytochrome *P*450 monooxygenases, are EDHFs in coronary arteries (see Busse *et al*., 2002). TRAM-34 is a derivative of clotrimazole, a potent inhibitor of both IK_{Ca} and cytochrome *P*450, which has been designed to show minimum inhibitory activity toward cytochrome *P*450 monooxygenase (inactive up to 10 μM; Alvarez *et al*., 1992; Castle, 1999; Wulff *et al*., 2000; 2001). Nevertheless, the high concentration used in the present study may have jeopardized the selectivity of TRAM-34. In order to test this hypothesis, a subthreshold concentration of TRAM-34 (5 μM, in combination with apamin) was studied in the presence or absence of either the epoxyeicosatrienoic acid inhibitor, 14,15-EEZE, a selective blocker of 11,12 epoxyeicosatrienoic acid (Gauthier *et al*., 2002), or iberiotoxin, the selective blocker of BK_{Ca}, the potassium channel target of epoxyeicosatrienoic acids on smooth muscle cells (Campbell *et al*., 1996; Fisslthaler *et al*., 1999; Quilley & McGiff, 2000; Edwards *et al*., 2001). However, neither the presence of 14,15-EEZE or iberiotoxin produced any additional inhibitory effects. Paradoxically, in the presence of iberiotoxin, the hyperpolarization was enhanced. This enhanced hyperpolarization observed in presence of iberiotoxin could be linked, at least in part, to the 6 mV depolarization produced by the toxin. In the guinea-pig carotid artery, the absolute value of the endothelium-dependent hyperpolarization is correlated with the value of the resting membrane potential (Chataigneau *et al*., 1998).

It could still be argued firstly that, since epoxyeicosatrienoic acids activate smooth muscle BK_{Ca}, they could induce relaxation and repolarization of contracted arteries but not the true hyperpolarization of the smooth muscle cells, and secondly that, in guinea-pig arteries, TRAM-34, at the concentration of 5 μM, could have already fully blocked the cytochrome *P*450 monooxygenase, but not IK_{Ca}, hence explaining the absence of any further inhibitory effects of either 14,15-EEZE or iberiotoxin. As this work involves the measurement of membrane potential and includes neither functional studies of vessel tone nor the measurement of cytochrome *P*450 activity, these two points cannot be refuted with the data shown in the present paper. However, earlier studies have shown that, in guinea-pig carotid, coronary and basilar arteries various inhibitors of cytochrome *P*450 monooxygenase did not affect endothelium-dependent relaxation

and/or hyperpolarizations and that epoxyeicosatrienoic acids, up to 3 μM, failed to produce relaxation and/or hyperpolarizations (Corriu *et al*., 1996b, Chataigneau *et al*., 1998; Eckman *et al*., 1998; Petersson *et al*., 1998; Yamanaka *et al*., 1998). Taken into conjunction, all these studies strongly suggest that, in guinea-pig arteries, the endothelial production of epoxyeicosatrienoic acids does not play a major role in the endothelium-dependent hyperpolarization elicited by acetylcholine.

Furthermore, as TRAM-34 and UCL 1684 (either alone or combination) did not affect the robust hyperpolarization in response to the K_{ATP} channel opener cromakalim, nor the small hyperpolarization to the BK_{Ca} channel opener, NS 1619 (Olesen *et al*., 1994), the two compounds can be considered, under the present experimental conditions, to act solely as specific blockers of IK_{Ca} and SK_{Ca}, respectively. The differences in potency could be attributed to species differences since previous functional studies were performed in rat while the present work was performed in guinea-pig.

The two structurally different specific inhibitors of SK_{Ca}, the bee venom peptide apamin and the nonpeptide dequalinium derivative, UCL 1684 (Campos-Rosa *et al*., 2000), *per se* produced a partial inhibition of the hyperpolarization. In contrast, the inhibitors of IK_{Ca}, the scorpion venom peptide charybdotoxin and the nonpeptide clotrimazole derivative TRAM-34 (Wulff *et al*., 2000) were each completely ineffective alone. The inhibitory effects of the two structurally different SK_{Ca} blockers confirm a predominant role for SK_{Ca} in the resting guinea-pig carotid artery (Chataigneau *et al*., 1998) and are in agreement with previous studies performed in other arteries such as the rabbit and rat mesenteric arteries (Murphy & Brayden, 1995; Chen & Cheung, 1997, Crane *et al*., 2003) as well as the bovine coronary artery (Drummond *et al*., 2000).

In the rat brain cortex, the binding of [^{125}I]charybdotoxin is increased in a dose-dependent manner by apamin (Zygmunt *et al*., 1997) suggesting that a novel channel with the two binding sites could be expressed. The endothelial potassium channel, responsible for EDHF-mediated responses, could be a heteromultimer composed of both SK and IK1 α-subunits since these subunits can theoretically coassemble (Castle, 1999). Both IK_{Ca} and SK_{Ca} are activated by a rise in intracellular Ca^{2+}, but IK1, when first cloned and characterized, was described as a unique K_{Ca} channel with a very high affinity for Ca^{2+} (Joiner *et al*., 1997). Even if this report has been tempered by more recent results, IK1 still appears slightly more sensitive to Ca^{2+} ($K_d = 0.1$–$0.3\,\mu$M; Ishii *et al*., 1997; Joiner *et al*., 1997; Logsdon *et al*., 1997; Ghanshani *et al*., 2000) than SK1 ($K_d = 0.7\,\mu$M; Xia *et al*., 1998), SK2 ($K_d = 0.6\,\mu$M; Hirschberg *et al*., 1998) or SK3 ($K_d = 0.6\,\mu$M; Barfod *et al*., 2001) channels. The principal mechanism that sustains the opening of endothelial K_{Ca} channels, following agonist stimulation, is the capacitive Ca^{2+} entry elicited by the depletion of Ca^{2+} stores (Marchenko & Sage, 1993; Sedova *et al*., 2000; Nilius & Droogmans, 2001). Earlier studies as well as the present one have shown that endothelium-dependent hyperpolarizations are dependent on the extracellular calcium concentration (Chen & Suzuki, 1990). It was further hypothesized that if, in the guinea-pig carotid artery, an heteromultimer (constituted of SK and IK1 α-subunits) is involved in the acetylcholine-induced hyperpolarization, the endothelium-dependent hyperpolarization observed in 0.5 mM Ca^{2+} should still be similarly sensitive to the combination of apamin and charybdotoxin whereas if homotetramers of IK1 and SK channels are expressed, IK_{Ca} should become preferentially activated.

In presence of 0.5 mM Ca^{2+}, charybdotoxin and TRAM-34, which were ineffective in control solution (2.5 mM Ca^{2+}), both produced a marked inhibition of the acetylcholine-induced endothelium-dependent hyperpolarization (% inhibition of the hyperpolarization: -52 and -36% for charybdotoxin and TRAM-34, respectively) while, in contrast, apamin was less effective in presence of 0.5 mM Ca^{2+} than in control condition (% inhibition of the hyperpolarization: -41 and -28% in 2.5 and 0.5 mM Ca^{2+}, respectively). Charybdotoxin and TRAM-34 are structurally different IK_{Ca} blockers, which do not share the same binding site (Wulff *et al*., 2000; 2001). In presence of 0.5 mM Ca^{2+}, the inhibition produced by each of these two blockers was revealed and was larger than that produced by apamin, which strongly suggests that, under these experimental conditions, IK_{Ca} became preferentially activated. These data are consistent with the hypothesis that, in the guinea-pig carotid artery, the targets of charybdotoxin (or TRAM-34) and apamin are homomeric IK_{Ca} and SK_{Ca} and do not support the existence of SK and IK heterotetramers. These results are in agreement with a recent report demonstrating that IK1 does not form heteromeric entity with SK1, although the latter coassemble with either SK2 or SK3 (Monaghan *et al*., 2004).

The present study demonstrates that, in the guinea-pig carotid artery, the activation of either IK_{Ca} or SK_{Ca} produces a true hyperpolarization (i.e. driving the membrane potential below the resting membrane potential toward the equilibrium value for potassium ions). In contrast, a recent work involving the mesenteric artery of the rat has shown that the activation of SK_{Ca} produced hyperpolarization whereas IK_{Ca} could only evoke repolarization (Crane *et al*., 2003). Since the activation of either SK_{Ca} or IK_{Ca} is virtually voltage-independent (Burnham *et al*., 2002; Bychkov *et al*., 2002), Crane *et al*. (2003) have suggested that the $[Ca^{2+}]_i$ in the microdomains surrounding SK_{Ca} and IK_{Ca} must be differently regulated. In the guinea-pig carotid artery, such a differential regulation of $[Ca^{2+}]_i$ may also explain the predominant role of SK_{Ca} observed in 2.5 mM Ca^{2+} although a higher level of expression of this channel when compared to IK_{Ca} can also be a plausible explanation. Additionally, the many discrepancies reported in the literature, concerning the relative contribution of SK_{Ca} and IK_{Ca} in endothelium-dependent hyperpolarizations, could be related to the differential cell- or species-dependent expression of endogenously expressed dominant-negative isoform of SK3, such as the recently described SK3-1C, a dominant-negative suppressor of SK_{Ca} and IK_{Ca} (Kolski-Andreaco *et al*., 2004).

In conclusion, these results suggest that the EDHF response is triggered *via* the combined opening of endothelial IK_{Ca} and SK_{Ca} channels. Furthermore, in this artery the production of a cytochrome *P*450 metabolite, and the subsequent activation of BK_{Ca}, is unlikely to contribute in the EDHF-mediated responses. The two selective, nonpeptide blockers of IK_{Ca} and SK_{Ca}, TRAM-34 and UCL 1684, respectively, should allow the design of *in vivo* experimental protocols that could help to clarify the potential physiological and pathophysiological role of EDHF-mediated responses.

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