

Selective blockade of endothelial Ca^{2+} -activated small- and intermediate-conductance K^{+} -channels suppresses EDHF-mediated vasodilation

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1 Activation of Ca^{2+} -activated K^{+} -channels (K_{Ca}) has been suggested to play a key role in endothelium-derived hyperpolarizing factor (EDHF)-mediated vasodilation. However, due to the low selectivity of commonly used K_{Ca} -channel blockers it is still elusive which endothelial K_{Ca} -subtypes mediate hyperpolarization and thus initiate EDHF-mediated vasodilation.

2 Using the non-cytochrome P450 blocking clotrimazole-derivatives, 1-[(2-chlorophenyl) diphenylmethyl]-1*H*-pyrazole (TRAM-34) and 2-(2-chlorophenyl)-2,2-diphenylacetonitrile (TRAM-39) as highly selective IK1-inhibitors, we investigated the role of the intermediate-conductance K_{Ca} (rIK1) in endothelial hyperpolarization and EDHF-mediated vasodilation.

3 Expression and function of rIK1 and small-conductance K_{Ca} (rSK3) were demonstrated *in situ* in single endothelial cells of rat carotid arteries (CA). rIK1-currents were blocked by TRAM-34 or TRAM-39, while rSK3 was blocked by apamin. In current-clamp experiments, endothelial hyperpolarization in response to acetylcholine was abolished by the combination of apamin and TRAM-34.

4 In phenylephrine-precontracted CA, acetylcholine-induced NO and prostacyclin-independent vasodilation was almost completely blocked by ChTX, CLT, TRAM-34, or TRAM-39 in combination with the SK3-blocker apamin. Apamin, TRAM-34, and CLT alone or sulphaphenazole, a blocker of the cytochrome P450 isoform 2C9, were ineffective in blocking the EDHF-response.

5 In experiments without blocking NO and prostacyclin synthesis, the combined blockade of SK3 and IK1 reduced endothelium-dependent vasodilation.

6 In conclusion, the use of selective IK1-inhibitors together with the SK3-blocker apamin revealed that activation of both K_{Ca} , rIK1 and rSK3 is crucial in mediating endothelial hyperpolarization and generation of the EDHF-signal while the cytochrome P450 pathway seems to play a minor or no role in rat CA.

British Journal of Pharmacology (2003) **138**, 594–601. doi:10.1038/sj.bjp.0705075

Keywords: Endothelium; Ca^{2+} -activated K^{+} -channels; EDHF; TRAM-34; clotrimazole

Abbreviations: APA, apamin; CA, carotid artery; CAEC, carotid artery endothelial cells; ChTX, charybdotoxin; CLT, clotrimazole; CYP, cytochrome P450; eNOS, endothelial nitric oxide synthase; K_{Ca} , Ca^{2+} -activated K^{+} -channel; rIK1, rat intermediate-conductance Ca^{2+} -activated K^{+} -channel; rSK3, rat small-conductance Ca^{2+} -activated K^{+} -channel; SPZ, sulphaphenazole; TRAM-34, 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole, TRAM-39, 2-(2-chlorophenyl)-2,2-diphenylacetonitrile

Introduction

The endothelium controls vascular tone by the release of vasoactive factors such as nitric oxide, prostacyclin, and the endothelium-derived hyperpolarizing factor (EDHF; Pohl *et al.*, 1986; Feletou & Vanhoutte, 2000). In the control of endothelial-mediated vasodilation, the activation of ion channels plays a substantial role by regulating endothelial membrane potential and Ca^{2+} -homeostasis in response to stimulation by humoral factors or haemodynamic forces (Adams *et al.*, 1989; Nilius & Droogmans, 2001). Particularly, Ca^{2+} -activated K^{+} -channels (K_{Ca}) mediate endothelial hyperpolarization in response to humoral stimulation (Köhler

et al., 2000; Nilius & Droogmans, 2001). This hyperpolarization provides the electrochemical driving force for Ca^{2+} entry which is important for the Ca^{2+} -dependent synthesis of vasodilating factors (Lückhoff & Busse, 1990). Moreover, endothelial hyperpolarization has been shown to directly induce vasorelaxation due to an electrotonic spread of hyperpolarization to vascular smooth muscle cells *via* myoendothelial gap junctions (von der Weid & Beny, 1993; Edwards *et al.*, 1999; Chaytor *et al.*, 2001). It was concluded that this electrotonic spread of hyperpolarization contributes to EDHF-signal transduction in addition to other presumed EDHFs such as products of the cytochrome P450 (CYP) pathway (Fisslthaler *et al.*, 1999). However, the contribution of these CYP-generated metabolites of arachidonic acid to EDHF signalling seems to vary considerably in different

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regions of the vascular tree and the species studied (Mombouli & Vanhoutte, 1997).

Thus far, pharmacological studies investigating the role of endothelial hyperpolarization in EDHF-signalling have commonly used K_{Ca} -channel blockers such as the bee venom apamin (APA) to block small-conductance K_{Ca} (SK) and the scorpion venom charybdotoxin (ChTX) to block large-conductance K_{Ca} (BK) and intermediate-conductance K_{Ca} (IK1) (Quignard *et al.*, 2000; Dong *et al.*, 2000; Coleman *et al.*, 2001). However, conclusions drawn from these studies are limited as ChTX is poorly selective and may inhibit voltage-gated K^+ -channels (K_v) and BK in the endothelium (Fan & Walsh, 1999; Papassotiropou *et al.*, 2000) as well as in the vascular smooth muscle (Waldron & Cole, 1999). Also the use of other K_{Ca} -blockers such as clotrimazole (CLT) which inhibits IK1 in endothelium (Köhler *et al.*, 2001) and in activated T-lymphocytes (Logsdon *et al.*, 1997; Wulff *et al.*, 2000) can not be considered an alternative pharmacological tool. CLT has been reported to block BK-, ATP-sensitive K^+ -channels (K_{ATP}), and Ca^{2+} -signalling in vascular smooth muscle (Rittenhouse *et al.*, 1997; Vanheel & Van de Voorde, 1997; Tofukuji *et al.*, 1998) and effectively inhibits CYP enzymes, thus making it difficult to distinguish whether EDHF-type vasodilation is mediated by eicosanoids or endothelial hyperpolarization (Fisslthaler *et al.*, 1999). Therefore, more selective K_{Ca} -channel inhibitors of IK1 are needed to ascertain the contribution of different endothelial K_{Ca} -subtypes in EDHF-mediated dilation. Recently, CLT-derivatives, TRAM-34 and TRAM-39, were developed (Wulff *et al.*, 2000) which specifically block IK1 at nanomolar concentrations without inhibiting CYP activity. In these compounds the imidazole ring of CLT which is essential for cytochrome P450 inhibition has been replaced by a pyrazole (TRAM-34) or an acetonitrile group (TRAM-39).

To assess the functional importance of especially the endothelial IK1 in EDHF-mediated vasodilations we performed patch-clamp experiments in endothelial cells of rat carotid arteries *in situ* and measurements of EDHF-mediated vasodilation using the new CLT-derivatives, TRAM-34 and TRAM-39. In addition, we demonstrate that TRAM-34 did not interfere with the function of K^+ -channels in vascular smooth muscle cells.

The present study revealed that endothelial hyperpolarizing K_{Ca} -currents and EDHF-mediated vasodilations were almost completely blocked by APA in combination with CLT, TRAM-34, or TRAM-39. Moreover, CLT alone or the CYP inhibitor sulphaphenazole were ineffective in blocking EDHF-mediated vasodilations. Thus the present study indicates that activation of both the endothelial K_{Ca} , rIK1 and rSK3 channels, is essential in generating the EDHF-signal whereas CYP-generated arachidonic acid metabolites apparently do not contribute to EDHF-mediated vasodilations in rat carotid arteries.

Methods

Carotid artery endothelial cells

Freshly isolated carotid arteries (CA) from male Sprague–Dawley rats (350–400 g) were cut open longitudinally and

fixed on a holding capillary to give direct access to the luminal surface. For *in situ* harvesting of endothelial cells, vessel slices were pre-incubated with 0.05% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA) in phosphate buffered saline (PBS) without Ca^{2+}/Mg^{2+} for up to 15 min (Köhler *et al.*, 2001). For wash out, CA were superfused with PBS for 5 min. Under microscopic control, a single EC was selectively fixed with the patch pipette and mechanically detached from the vessel wall.

Carotid artery vascular smooth muscle cells

Vascular smooth muscle cells of rat carotid (CASMC) were isolated as previously described (Gollasch *et al.*, 1992) with some modifications. Briefly, the vessels were cleaned of adventitial tissue and placed in Dulbecco's modified Eagle's medium (Biochrom KG, Berlin, Germany) containing 1.5 mg ml⁻¹ collagenase typ II, 0.5 mg ml⁻¹ elastase typ IIA, and 0.5 mg trypsin inhibitor (all Sigma, Deisenhofen, Germany) and were incubated for 30 min in a humidified atmosphere of 5% CO₂. After cleaning of remaining adventitial tissue, vessels were cut into small segments of 1–2 mm in length and segments were further digested for 1 h under gentle agitation until single cells were dispersed. Cells were washed twice in PBS and immediately used for patch-clamp experiments.

Patch-clamp experiments

Whole-cell currents in electrically isolated CAEC and isolated CASMC were recorded with an EPC-9 (HEKA) patch-clamp amplifier using voltage ramps (duration: 1000 ms) from –100 mV to +50 mV as described previously (Köhler *et al.*, 2000). Membrane potential changes in response to acetylcholine (200 nM) were recorded in the electrically coupled CAEC *in situ* by using the current-clamp mode of the EPC-9 amplifier. Patch pipettes had a tip resistance of 2–4 M Ω in symmetrical KCl solution. For activation of K_{Ca} -currents, CAEC and CASMC were dialysed with a KCl-pipette solution containing 3 μ M [Ca^{2+}]_{free} (mM): 135 KCl, 4 MgCl₂, 2 ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 1.91 CaCl₂, and 5 N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid] (HEPES), pH 7.2. For measurements of currents through voltage-gated K^+ -channels (K_v) in CASMC and current-clamp experiments in CAEC, the pipette solution was prepared with 0.86 mM CaCl₂ ([Ca^{2+}]_{free} = 100 nM). The NaCl bath solution contained (mM): NaCl 137, Na₂HPO₄ 4.5, KCl 3, KH₂PO₄ 1.5, MgCl₂ 0.4 and CaCl₂ 0.7, pH 7.4. Whole-cell currents through ATP-sensitive K^+ -channels (K_{ATP}) in CASMC were measured, as previously described (Petkov *et al.*, 2001). The bathing solution contained (in mM) NaCl 90, KCl 50, CaCl₂ 1, MgCl₂ 1 and HEPES 10, pH 7.4. The pipette solution contained (in mM) KCl 140, CaCl₂ 1 (free Ca^{2+} 20 nM), MgCl₂ 1, EGTA 10, HEPES 10, GTP 0.2, ADP 0.1 and ATP 0.1 (free ATP = 0.0053), pH 7.2. Under these conditions and at holding potentials more negative than –26 mV, K_{ATP} -currents were inwardly directed.

Experiments were performed at room temperature. Data analysis was performed as previously described (Köhler *et al.*, 2001).

Reverse transcription and single-cell-RT-PCR

Subsequent to patch-clamp experiments EC were transferred to a tube containing 1 μ l 'first-strand' buffer, 0.5 μ l dNTPs (10 mM each), 1 μ l 'random'-hexamer primer (100 μ M), 1 μ l DTT (0.1 M), and 0.5 μ l RNase-inhibitor (40 U μ l⁻¹). After one freeze-thaw cycle to induce breakdown of the cell membrane, 0.5 μ l SuperScript[®] RT (200 U μ l⁻¹; Life Technologies) was added and the final volume (~10 μ l) was incubated for 1 h at 37°C. Single-cell RT-PCR was performed as described previously (Köhler *et al.*, 2001). First and 'nested' primer pairs (TIB MOL BIOL, Berlin) for rat endothelial nitric oxide synthase (eNOS) as endothelial cell marker, small K_{Ca} (rSK3), and intermediate K_{Ca} (rIK1) were selected to span intronic sequences and identities of PCR products were verified by sequencing using an ABI 377 automatic sequencer (ABI Prism). Forward and reverse primer: rIK1: first: 5'-GAGAGGCAGGCTGTCAATG-3'; 5'-GGGAGTCCTTCCTTCGAGTG-3'; nested: 5'-CATCACGTTCTGACCATTG-3'; 5'-GTGTTTCTCCGCCTTGTGA-3'; rSK3: first: 5'-AACCCCTCCAGCTCTTCAGT-3'; 5'-TGTGGTAGGCGATGATCAAAA-3'; nested: 5'-GATAACCATGCCACCAGAC-3'; 5'-ATTTTCAGGGCCAAACGAAAAC-3'; eNOS: first: 5'-GCACTATGGGGTCTGTTC-CA-3'; 5'-CTTCATCCAGCTCCATGCT-3'; nested: 5'-CCAGCTCTGCTCCTCAGAAGG-3'; 5'-ATGGATGAGC-CAACTCAAGG-3'. In a single-cell sample, cDNA of rIK1 and rSK3 were co-amplified along with eNOS cDNA. A first 'multiplex' PCR was performed in a final volume of 50 μ l containing 5 μ l PCR-buffer (10 \times), 2 μ l dNTPs (10 mM each), 3 μ l MgCl₂ (50 mM), 1 μ l of each sense- and antisense-primer (10 pmol), ~10 μ l RT-product, and 0.5 μ l Taq DNA polymerase (5 U μ l⁻¹; all Life Technologies) using a Biozym Maxicycler PTC 9600. After initial denaturation for 5 min at 94°C, PCR amplification was carried out for 30 s at 94°C, 1 min at 55°C, 1 min at 72°C, and 10 min at 72°C for 50 cycles. In a second 'multiplex' PCR round with 'nested' primers, 5 μ l of the first PCR product were used for reamplification (45 cycles, annealing temperature: 60°C). PCR products were analysed on a 2% agarose gel containing ethidium bromide. A 50 bp DNA ladder served as molecular weight markers.

Pressure myograph experiments

CA segments of 3–4 mm in length were cannulated with micropipettes in an experimental chamber mounted on the stage of a Zeiss Axiovert 100. Vessel diameter was continuously monitored with a video camera. The bath and perfusion solution contained (in mM): NaCl 145.0, NaH₂PO₄ 1.2, KCl 4.7, MgSO₄ 1.2, CaCl₂ 2.0, glucose 5.0, pyruvate 2.0 and MOPS buffer 3.0 along with 1 g 100 ml⁻¹ bovine serum albumin (pH 7.4 at 37°C). To block NO and prostacyclin synthesis the NO-synthase inhibitor N^G-nitro-L-arginine (L-NNA, 100 μ M) and the cyclo-oxygenase inhibitor indomethacin (10 μ M) were added to both the bath and perfusion solution. CA were pressurized to 80 mmHg with a pressure myograph system (J.P. Trading P100) and continuously perfused at a flow rate of 0.6 ml min⁻¹ and at constant intraluminal pressure. After an initial equilibration period for 40 min, CA were precontracted with 1 μ M phenylephrine in the bath solution. After development of stable tone, NO- and

prostacyclin-independent vasodilatory responses were determined by perfusion with increasing concentrations of acetylcholine (ACh, 1 nM to 1 μ M) alone or with 200 nM APA in combination with 100 nM charybdotoxin (ChTX), TRAM-34, TRAM-39, or CLT (all 1 μ M). In different subsets of experiments, vessels were preincubated with 10 μ M CLT or 10 μ M sulphaphenazole (SPZ), a selective CYP 2C9 inhibitor (Fleming *et al.*, 2001) for 30 min. In an additional subset of experiments, the bath and perfusion medium was prepared without NO-synthase- and cyclo-oxygenase inhibitor. Diameter changes were expressed as a percentage of the maximal dilation measured in response to 1 μ M sodium nitroprusside (SNP). TRAM-34 and TRAM-39 were a kind gift from Heike Wulff (University of California Irvine, CA, U.S.A.). Levromakalim, the active enantiomer of cromakalim (BRL 38227), was obtained from TOCRIS (Ballwin, MO, U.S.A.). All other standard chemicals and toxins were obtained from Sigma.

Statistical analysis

Data are given as mean \pm s.e.mean. The Mann-Whitney U-test was used to assess differences between groups. *P*-values < 0.05 were considered significant.

Results

Inhibition of endothelial K_{Ca} by new clotrimazole-derivatives

In *in situ* whole-cell patch-clamp experiments, K_{Ca} -currents in EC of rat carotid arteries (CAEC) were activated by cell dialysis with 3 μ M [Ca²⁺]_{free} via the patch pipette. They exhibited characteristics of both rIK1 and rSK3 regarding K⁺-selectivity, slight inward-rectification, and Ca²⁺-dependence (Köhler *et al.*, 2001). In the presence of TRAM-34 (100 nM), the selective IK1-blocker (Wulff *et al.*, 2000), the K_{Ca} -current was reduced to 60–70% of the maximal current amplitude under control conditions (Figure 1A). The remaining TRAM-34-insensitive K_{Ca} -current was almost completely inhibited by the rSK3-blocker APA (200 nM) thus showing a substantial contribution of rSK3 to K_{Ca} -currents in the cells. In the presence of apamin (200 nM) to eliminate SK3-currents, TRAM-34 blocked the rIK1-current with a potency (K_D 11 \pm 2 nM) similar to the cloned channel (Wulff *et al.*, 2000) (Figure 1C). The other IK1-blocker TRAM-39 had similar blocking effects on rIK1-currents although with a slightly higher K_D of 20 \pm 3 nM (Figure 1B,C).

In a separate set of experiments, we conducted current-clamp experiments in the electrically coupled CAEC *in situ* (Köhler *et al.*, 2001) and measured ACh (200 nM) induced hyperpolarizations (Figure 1D). Resting membrane potentials were -38 ± 2 mV ($n=10$). After addition of ACh (200 nM) the membrane potential shifted by -29 ± 3 mV ($n=7$) to a more negative value of -61 ± 2 mV ($n=7$). When APA (200 nM) was added thereafter, we observed a small depolarization to -51 ± 4 mV ($n=6$). An additional strong depolarization to -17 ± 6 mV ($n=6$) was achieved by adding TRAM-34 (1 μ M, Figure 1E). When APA and TRAM-34 were applied first followed by ACh, endothelial hyperpolarization was almost completely suppressed ($n=3$; Figure 1F).

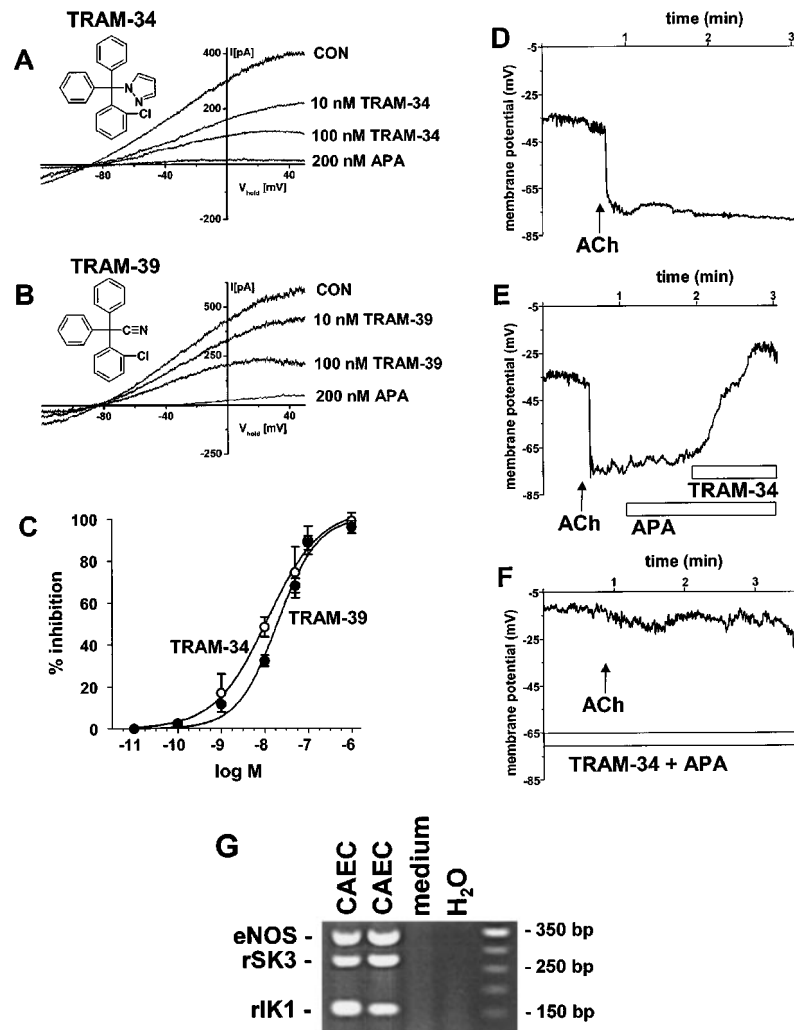


Figure 1 Representative whole-cell recordings of CAEC from rat carotid artery (CA) and inhibition by selective IK1 and SK3 blockers. For K_{Ca} -activation CAEC were dialyzed with a 140 mM K^+ pipette solution containing $3 \mu\text{M}$ $[\text{Ca}^{2+}]_{\text{free}}$. Bathing solution contained 4.5 mM K^+ . Inhibition of Ca^{2+} -activated K^+ -currents by TRAM-34 (A) and TRAM-39 (B) at indicated concentrations. TRAM-34- and TRAM-39-insensitive Ca^{2+} -activated K^+ -currents were inhibited by APA. (C) Concentration-dependent blockade of apamin-resistant K_{Ca} -currents by TRAM-34 ($n=4-6$) and TRAM-39 ($n=4-6$) determined at membrane potential of 0 mV and in the presence of 200 nM apamin. Data points were fitted to the Boltzmann equation. Values are given as mean \pm s.e.mean. (D-F) Representative current-clamp recordings of endothelial membrane potentials and ACh-induced hyperpolarization and its inhibition by APA (200 nM) and TRAM-34 (1 μM). (G) Representative 'multiplex' RT-PCR analysis of single EC from rat CA *in situ*. Ethidium bromide-stained gels of RT-PCR products of eNOS and the K_{Ca} -genes, rSK3 and rIK1 from 2 EC, one medium sample as negative control and one non-template control (H_2O); right lane, molecular weight marker.

Subsequent to patch-clamp experiments, expression of the K_{Ca} -genes rIK1 and rSK3 in the same CAEC was verified by 'multiplex'-single-cell RT-PCR analysis (Figure 1G). Other small K_{Ca} -subtypes such as rSK1 and rSK2 or the rBK were not considerably expressed in CAEC (Köhler *et al.*, 2001).

To test whether TRAM-34 affects the function of K^+ -channels in vascular smooth muscle of rat carotid artery, we measured K^+ -currents through BK-, K_V - and K_{ATP} -channels in the presence or absence of TRAM-34. TRAM-34 (10 μM) had apparently no effect on the function of BK-channels in CASMC while BK-currents were almost completely blocked by the selective BK-blocker iberiotoxin (100 nM, Figure 2A). Similarly, TRAM-34 (10 μM) exerted no apparent blocking effects on K_V or levromakalim-induced K_{ATP} -currents in

CASMC whereas the K_V -blocker 4-aminopyridine (2 mM) or the K_{ATP} -blocker glibenclamide (10 μM) were effective, respectively (Figure 2B,C).

Inhibition of EDHF-mediated vasodilations by blockade of endothelial K_{Ca}

After preincubation with 100 μM L-NNA and 10 μM indomethacin, intraluminally applied ACh ($n=31$) induced a dose-dependent and EDHF-type vasodilation, with a maximal reduction of 13% of PE-induced tone at the highest concentration of ACh (1 μM) tested (Figure 3A). ACh-induced EDHF-mediated vasodilations at all ACh concentrations up to 1 μM ACh were almost eliminated by the

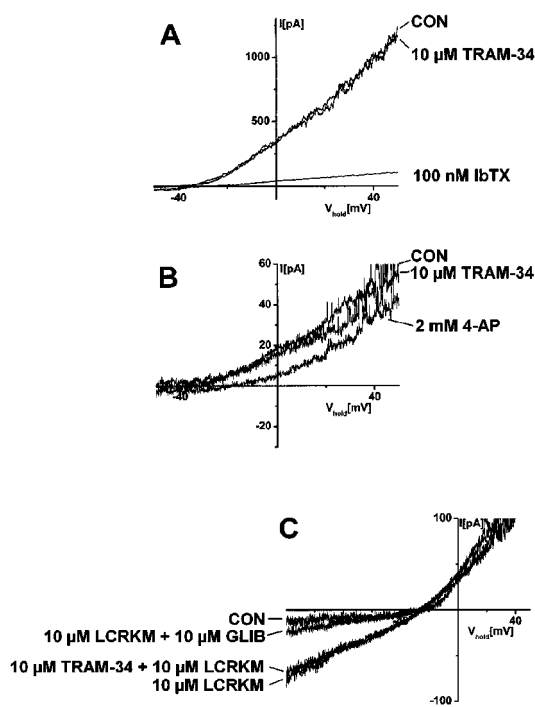


Figure 2 TRAM-34 did not affect the function of BK-, K_V -, and K_{ATP} -channels in CASMC. (A) Whole-cell recordings of BK-currents in the presence or absence of TRAM-34 (10 μ M) and blockade of BK-currents by selective BK-blocker iberiotoxin (IbTX, 100 nM). For K_{Ca} -activation CASMC were dialysed with a 140 mM K^+ pipette solution containing 3 μ M $[Ca^{2+}]_{free}$. Bathing solution contained 4.5 mM K^+ . The experiment shown is representative of seven experiments with similar results. (B) Whole-cell recordings of K_V -currents in the presence or absence of TRAM-34 (10 μ M) and blockade of K_V -currents by the K_V -blocker 4-aminopyridine (4-AP, 2 mM). CASMC were dialysed with a 140 mM K^+ -pipette solution containing 0.1 μ M $[Ca^{2+}]_{free}$. The experiment shown is representative of ten experiments with similar results. Bathing solution used for measurements of BK- and K_V -currents contained 4.5 mM K^+ . (C) Levromakalim (LCRKM, 10 μ M)-induced whole-cell currents through K_{ATP} -channels in CASMC in absence and presence of TRAM-34 (10 μ M) and blockade of K_{ATP} -currents by the K_{ATP} -blocker glibenclamide (GLIB, 10 μ M). CASMC were dialysed with a 140 mM K^+ -pipette solution containing 0.1 μ M $[Ca^{2+}]_{free}$ and 5.3 μ M $[ATP]_{free}$. Bathing solution contained 50 mM K^+ . Note that under these conditions and at holding potentials more negative than -26 mV, K_{ATP} currents were inwardly directed. The experiment shown is representative of eight experiments with similar results.

combination of APA with CLT ($n=5$), TRAM-34 ($n=5$), or TRAM-39 ($n=9$, Figure 3A,B). Similar to the blocking effects of APA in combination with TRAM-34, TRAM-39, or CLT, EDHF-type vasodilations were almost abolished by the combination of ChTX (100 nM) and APA (200 nM) ($n=5$; Figure 3C). When ACh was applied in combination with only one K_{Ca} -blocker, APA (2 μ M; $n=7$) or TRAM-34 (1 μ M; $n=7$), EDHF-type vasodilations were not significantly different from those induced in the absence of K_{Ca} -blockers (Figure 3C).

To test whether EDHF-type vasodilations could be reduced by inhibiting CYP-generated EDHF, vessels were preincubated with CLT (10 μ M, $n=5$), which blocks CYP in addition to its blocking effects on the IK1, or with the CYP 2C9 specific inhibitor sulphaphenazole (SPZ, 10 μ M, $n=3$)

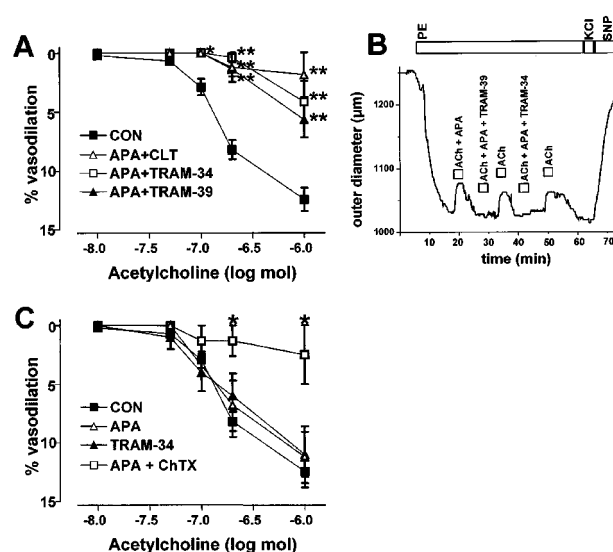


Figure 3 Inhibition of EDHF-type vasodilations in rat carotid arteries by K_{Ca} -blockers. (A) Inhibition of EDHF-type vasodilator responses by combinations of K_{Ca} -blockers as indicated. K_{Ca} -blocker concentrations: 200 nM APA, 1 μ M CLT, 1 μ M TRAM-34, 1 μ M TRAM-39. Values are given as mean \pm s.e.mean. * $P < 0.05$, ** $P < 0.01$, Mann-Whitney U -test. (B) Original trace illustrating EDHF-type vasodilator responses to intraluminal application of 0.2 μ M ACh and in combination with APA (200 nM) or with APA and TRAM-34 (1 μ M) or with APA and TRAM-39 (1 μ M). Vessels were pre-constricted with 1 μ M phenylephrine (PE). Endothelium-independent vasodilation and maximal vasoconstriction were induced by extraluminal application of 1 μ M sodium nitroprusside (SNP) and 60 mM KCl, respectively. Boxes indicate exposure intervals followed by wash-out (right upper lane). (C) Dose-dependent EDHF-type vasodilator response in response to ACh and inhibition by the combination of APA (200 nM) and ChTX (100 nM) but not by APA (2 μ M) or TRAM-34 (1 μ M) alone.

for 30 min. We found that under these conditions ACh-induced vasodilations were not substantially altered by CLT or SPZ when compared to controls (Figure 4A). However, when ACh was then applied in combination with APA (200 nM, $n=4$) in the presence of CLT, EDHF-type vasodilations were almost abolished (Figure 4B).

In the absence of inhibitors of NO and prostacyclin synthesis we found that the combination of APA and TRAM-34 reduced maximal ACh-induced dilation at all ACh concentrations up to 1 μ M ACh (Figure 5). TRAM-34 alone was ineffective in blocking ACh-induced dilation (Figure 5).

Discussion

In the present study, the new IK1 inhibitors TRAM-34 and TRAM-39 have been employed to investigate the functional role of the endothelial rIK1 in NO- and prostacyclin-independent EDHF-type vasodilation of rat carotid arteries. The findings provide new evidence that activation of the endothelial rIK1 in synergy with the rSK3 is essential in generating the EDHF-response.

In *in situ* whole-cell patch-clamp experiments in native EC from rat CA, rIK1-currents were selectively and dose-

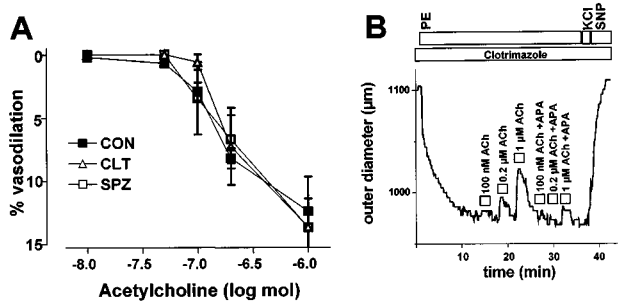


Figure 4 (A) Dose-dependent EDHF-type vasodilations in rat carotid arteries in the presence of CLT (10 μ M) or SPZ (10 μ M). (B) Original tracings illustrating dose-dependent EDHF-type vasodilator responses in the presence of CLT (10 μ M) alone and in combination with APA (200 nM). Values are given as mean \pm s.e.mean.

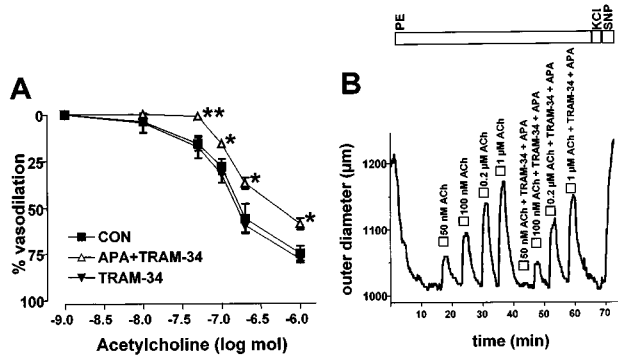


Figure 5 Inhibition of ACh-induced vasodilations by the K_{Ca} -blockers APA (200 nM) and TRAM-34 (1 μ M) in the absence of inhibitors of NO-synthase and cyclo-oxygenase. (A) Dose-response curves of ACh-induced vasodilations in the presence ($n=8$) or absence ($n=6$) of APA (200 nM) and TRAM-34 (1 μ M) or in the presence of TRAM-34 (1 μ M) alone. Values are given as mean \pm s.e.mean. * $P < 0.05$, ** $P < 0.01$, Mann-Whitney U -test. (B) Original trace illustrating dose-dependent vasodilations to intraluminal application of ACh alone and of ACh in combination with APA (200 nM) and TRAM-34 (1 μ M).

independently blocked by TRAM-34 and TRAM-39 as well as by CLT. TRAM-34 was found to be the most potent inhibitor followed by TRAM-39 and CLT. The K_D values for rIK1-blockade in rat CAEC and the rank order of potency were similar to those reported for cloned human IK1 and the IK1 in activated human T-lymphocytes (Wulff *et al.*, 2000). In contrast to inhibition of the endothelial rIK1, these CLT-derivatives had no blocking effects on endothelial rSK3 in rat CAEC. Moreover, TRAM-34 had also been shown to have no blocking effects on the cloned BK, several types of cloned voltage-gated K^+ - and, inward rectifying K^+ -channels, and Ca^{2+} -release activated Ca^{2+} -currents (Wulff *et al.*, 2000) and did not interfere with the function of BK-, K_V -, and K_{ATP} -channels in CASMC as shown in the present study. Thus in our hands, the apparent high selectivity of these compounds to block exclusively IK1 makes them an important pharmacological tool to define the functional role of the endothelial IK1 in endothelium-dependent control of vascular tone.

In a variety of small and large arteries such as guinea-pig cerebral and carotid arteries, rat mesenteric arteries, and pig coronary arteries the functional role of endothelial K_{Ca} in EDHF-mediated vasodilations was tested by the combination of the toxins APA and ChTX (Petersson *et al.*, 1997; Doughty *et al.*, 1999; Quignard *et al.*, 1999; 2000). The synergistic inhibitory action of these two toxins on EDHF-type vasodilations led to the conclusion that opening of both endothelial K_{Ca} and the subsequent hyperpolarization is required to generate EDHF-type vasodilations in response to endothelial stimulation. In the present study we confirmed that the combination of APA and ChTX, when selectively applied to the endothelium of rat CA, abolished the EDHF-type dilatory response to ACh. Moreover, we showed here that a similar inhibition of EDHF-type dilation was achieved by combining APA with the other commonly used IK1 blocker CLT. These observations indicate that both endothelial K_{Ca} are involved in endothelial hyperpolarization and the subsequent EDHF-type vasodilatory response. However, experimental evidence at this point is still limited. In particular ChTX could have exerted its blocking action also by inhibiting BK in vascular smooth muscle and voltage-gated K^+ -channels in endothelial and vascular smooth muscle cells. This might result in changes in resting potentials of both cell types and thus in a decreased ability to hyperpolarize. Regarding CLT, the inhibition of EDHF-type vasodilations could have been exerted by interfering with BK- or ATP-sensitive K^+ -channels (K_{ATP}) in vascular smooth muscle (Rittenhouse *et al.*, 1997; Vanheel & Van de Voorde, 1997) or with the CYP pathway and thus the synthesis of metabolites of arachidonic acid which also have been shown to serve as EDHFs in various vessel types (Fisslthaler *et al.*, 1999). Thus, in our hands, the poor selectivity of ChTX or CLT limits their usefulness to define the role of a specific K^+ -channel or the CYP-pathway in EDHF-type vasodilations.

The major finding of the present study is that inhibition of the endothelial rIK1 by the highly selective TRAM-34 or TRAM-39 combined with rSK3 blockade by APA effectively abolished EDHF-type vasodilations in rat CA. This clearly supports the conclusion that in rat CA activation of both rIK1 and rSK3 together is essential in generating the EDHF-signal in response to endothelial stimulation. Moreover, rIK1- and SK3-mediated endothelial hyperpolarization seems to be a relevant and additive component of ACh-induced vasodilation in addition to the vasodilators NO and prostacyclin, since K_{Ca} -channel blockade considerably diminished the endothelium-dependent dilations even when NO and prostacyclin generation was not blocked.

CYP-generated metabolites of arachidonic acid such as epoxyeicosatrienoic acids have been proposed to serve as EDHFs in the porcine coronary artery and human umbilical vein endothelium (Fisslthaler *et al.*, 1999; Welsh & Segal, 2000; Bolz *et al.*, 2000). Moreover, a CYP isozyme homologous to CYP 2C8/9 has been identified as an EDHF synthase. However, in the present study inhibition of CYP by CLT or SPZ alone was without effect on EDHF-type vasodilations. Therefore, in rat CA CYP-generated arachidonic acid metabolites presumably do not contribute to EDHF-mediated vasodilations and thus apparently do not serve as EDHFs. This is in agreement with observations of other investigators who also found strongly varying or even no contribution of CYP-generated arachidonic metabolites

depending on the vascular bed studied (Drummond *et al.*, 2000; Andersson *et al.*, 2000; Ungvari & Koller, 2001; Coleman *et al.*, 2001).

In conclusion, inhibition of endothelial hyperpolarization through blockade of both IK1 and SK3 channels prevents EDHF-mediated vasodilation. This indicates that activation of endothelial K_{Ca} is essential for NO- and prostacyclin-independent vasodilation whereas arachidonic acid metabo-

lites of the CYP pathway seem to play a minor or no role in rat carotid arteries.

This work was supported by the Deutsche Forschungsgemeinschaft (FOR 341/1 and 341/7). The authors wish to thank Heike Wulff for the gift of TRAM-34 and TRAM-39 and for helpful discussions on the manuscript.

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(Received September 24, 2002

Revised October 15, 2002

Accepted October 29, 2002)