Blockade of the Intermediate-Conductance Calcium-Activated Potassium Channel as a New Therapeutic Strategy for Restenosis

Ralf Köhler, PhD; Heike Wulff, PhD; Ines Eichler, MD; Marlene Kneifel; Daniel Neumann; Andrea Knorr; Ivica Grgic; Doris Kämpfe; Han Si, MSc; Judith Wibawa; Robert Real, MD; Klaus Borner, MD; Susanne Brakemeier, MD; Hans-Dieter Orzechowski, MD; Hans-Peter Reusch, MD; Martin Paul, MD; K. George Chandy, MD; Joachim Hoyer, MD

Background—Angioplasty stimulates proliferation and migration of vascular smooth muscle cells (VSMC), leading to neointimal thickening and vascular restenosis. In a rat model of balloon catheter injury (BCI), we investigated whether alterations in expression of Ca^{2+}-activated K^+ channels (K_{Ca}) contribute to intimal hyperplasia and vascular restenosis.

Methods and Results—Function and expression of K_{Ca} in mature medial and neointimal VSMC were characterized in situ by combined single-cell RT-PCR and patch-clamp analysis. Mature medial VSMC exclusively expressed large-conductance K_{Ca} (BK_{Ca}) channels. Two weeks after BCI, expression of BK_{Ca} was significantly reduced in neointimal VSMC, whereas expression of intermediate-conductance K_{Ca} (IKCa1) channels was upregulated. In the aortic VSMC cell line, A7r5 epidermal growth factor (EGF) induced IKCa1 upregulation and EGF-stimulated proliferation was suppressed by the selective IKCa1 blocker TRAM-34. Daily in vivo administration of TRAM-34 to rats significantly reduced intimal hyperplasia by ≈40% at 1, 2, and 6 weeks after BCI. Two weeks of treatment with the related compound clotrimazole was equally effective. Reduction of intimal hyperplasia was accompanied by decreased neointimal cell content, with no change in the rate of apoptosis or collagen content.

Conclusions—The switch toward IKCa1 expression may promote excessive neointimal VSMC proliferation. Blockade of IKCa1 could therefore represent a new therapeutic strategy to prevent restenosis after angioplasty. **(*Circulation*. 2003; 108:1119-1125.)**

Key Words: angioplasty ■ restenosis ■ ion channels

Percutaneous balloon angioplasty, an intervention to relieve arterial stenosis and improve blood flow, is complicated by vascular restenosis within weeks as the result of proliferation of vascular smooth muscle cells (VSMC) and consequent renarrowing of the vessel lumen.[1] Complex interactions between numerous growth-stimulating molecules have been proposed to promote migration and proliferation of VSMC,[2] leading to neointima formation. Proliferating VSMC are characterized by alterations in functional plasticity as they switch from a contractile phenotype to a dedifferentiated phenotype.

Ca^{2+}-activated K^+ channels (K_{Ca}) are important regulators of VSMC function.[3,4] Mature VSMC predominantly express the calcium-activated large-conductance channel (BK_{Ca} or maxi K),[4] a product of the *Slo* gene,[5] which plays a pivotal role in VSMC relaxation by dampening depolarization-dependent activation of Ca^{2+} channels and Ca^{2+} influx through membrane hyperpolarization.[3,4] In contrast to the vasodilatory function of BK_{Ca}, the role of other K_{Ca} channels in VSMC is incompletely understood. The intermediate-conductance K_{Ca} channel encoded by the *IKCa1* gene (also known as *IK1*, *hSK4*, *KCa4*, and *K_{Ca}3.1* as per the new IUPHAR nomenclature: http://www.iuphar.org/compendium2.htm) has been proposed to be an important regulator of cell proliferation. In lymphocytes and fibroblasts, upregulation of *IKCa1* expression is an essential step in mitogenesis.[6–8]

In the present study, we tested the hypothesis that a reorganization of K_{Ca} channel expression pattern after angioplasty promotes neointimal cell proliferation. After balloon catheter injury (BCI) to rat carotid artery (CA), neointimal VSMC switched K_{Ca} gene expression from *Slo* to *IKCa1*, representing a change from a K_{Ca} subtype mediating vasodilation to a K_{Ca} subtype promoting cell proliferation. Blockade of IKCa1 by the antimycotic clotrimazole (CLT) and its selective derivative TRAM-34[6] resulted in inhibition of epidermal growth factor (EGF)-stimulated VSMC proliferation in vitro and in reduced neointima formation in vivo.

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From the Departments of Nephrology (R.K., I.E., M.K., D.N., A.K., I.G., D.K., H.S., J.W., S.B., J.H.), Clinical Pharmacology and Toxicology (R.R., H.D.-O., M.P.), and Clinical Chemistry (K.B.), Benjamin Franklin Medical Center, Berlin, Germany; the Department of Clinical Pharmacology, Ruhr-Universität Bochum, Germany (H.-P.R.); the Department of Physiology and Biophysics (K.G.C.), University of California, Irvine; and the Department of Pharmacology and Toxicology (H.W.), University of California, Davis.

Correspondence to Dr R. Köhler, UKBF, Hindenburgdamm 30, 12200 Berlin, Germany. E-mail koe@zedat.fu-berlin.de

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Methods

Balloon Catheter Injury and Treatment Protocols

Under the aegis of a protocol approved by the local Animal Care and Use Committee, Sprague-Dawley rats (weight, 350 to 450 g) were subjected to BCI of the left CA by use of a 2F Fogarty embolectomy catheter (Baxter Scientific).[9] Rats were killed 2 weeks (n=5) after BCI, and left and right CA were excised. Separate groups (each n=4 to 11) were treated with daily subcutaneous injections of TRAM-34 (1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole; 120 mg/kg per day) or the vehicle (peanut oil) for 1, 2, and 6 weeks after BCI. Another group (n=7) was treated with CLT (120 mg/kg per day) for 2 weeks. TRAM-34 and CLT serum levels and TRAM-34 concentrations in liver and subcutaneous fat were quantitatively determined by bioassay.[10]

Neointimal thickening was determined in paraffin-embedded and differential nonserial cross sections stained with hematoxylin and eosin to visualize nuclei and cytoplasm, or with Sirius red to detect collagen. Histomorphological analysis was done with the use of a computerized analysis system (Scion Image) in a blinded manner.

Mature and Neointimal VSMC and Patch-Clamp Experiments

Isolation of mature and neointimal VSMC, whole-cell patch-clamp experiments in situ, and data analysis were performed as described previously.[11–13]

Reverse Transcription and Single-Cell RT-PCR

Reverse transcription of mRNA from single-cell samples and "multiplex" single-cell RT-PCR were performed as described previously.[11,12] Primer pairs for small K_{Ca} (*rSK1–3*), *rIKCa1*, and endothelial nitric oxide synthase (*reNOS*) as endothelial cell markers are stated elsewhere.[12] First and "nested" primer pairs for *rSlo* and myosin heavy chain (*rMyHC*) as VSMC markers spanned intronic sequences, and identity of PCR products was verified by sequencing: Primer, *rSlo*: F5′-GGACTTAGGGGATGGTGGTT-3′; R5′-GGGATGGAGTGGACAGAGGA-3′; nested:F5′-TTTACCGGCT-G AGAGATGCC-3′; R5′-TGTGAGGAGTGGGAGGAATGA-3′; (GenBank *accession*:-AF135265) *rMyHC*: F5′-CATCAATGCCAA-CCGCAG-3′; R5′-TCCCGAGCATCCATTTCTTC-3′; nested: F5′-AGGCCACTGAGAGCAATGAG-3′; R5′-TCAATAACTCTACG-GCCTCCA-3′. (GenBank *accession*:-X16262).

Detection of Apoptosis

Apoptotic nuclei in the neointima were detected by TUNEL method (Apoptaq-Plus; Qbiogene). Slices were counterstained with methyl green.

In Vitro Proliferation Studies

To induce growth arrest, rat aortic VSMC (cell line: -A7r5) were kept in serum-free medium for 48 hours before stimulation with EGF (20 ng/mL) in the presence or absence of TRAM-34, CLT, TRAM-7 (1-tritylpyrrolidine), or IbTX. At 5% to 10% confluence, photomicrographs of cells were taken in fixed fields before and 48 hours after stimulation, and the percent increase in cell count was calculated.

RNA Isolation and Quantitative Real-Time RT-PCR

Cells were harvested at 2 hours or 48 hours after stimulation by scraping. RNA was isolated with TRIZOL and was reverse-transcribed with M-MLV reverse transcriptase (both Life Technologies). Expression was quantified with an ABI-Prism-7700 Sequence Detection System (Perkin-Elmer ABI), using intron-spanning primers and internal oligonucleotides labeled with 6-carboxy-fluorescein on the 5′end and 6-carboxytetramethylrhodamine on the 3′end. Identity of PCR products was verified by sequencing. Linearity of each PCR assay was confirmed by serial dilutions of cDNA; primer and internal oligonucleotides: rIKCa1: F5′-CTGAGAGG-CAGGCTGTCAATG-3′; R5′-ACGTGTTTCTCCGCCTTGTT-3′; P5′-AAGATTGTCTGCTTGTGCACCGGAGTC-3′; rMyHC: F5′-CATCAATGCCAACCGCAG-3′; R5′-TCCCGAGCATCCATTT-CTTC-3′; P5′-TGAGGCCATGGGCCGTGAGG -3′; rat glyceraldehyde-3-phosphate dehydrogenase (rGAPDH): F5′-CGGCACAGT-CAAGGCTGAG-3′; R5′-CAGCATCACCCCATTTGATGT-3′; P5′-CCCATCACCATCTTCCAGGAGCGA-3′ (GenBank accession: -AB017801).

Each 25-μL PCR reaction consisted of 500 nmol forward and reverse primer, 150 nmol probe, 3 μL cDNA, and 1×TaqMan Universal Master Mix (Perkin-Elmer ABI). PCR parameters were 50°C×2 minutes, 95°C×10 minutes, and 50 cycles at 95°C×15 seconds, 60°C×1 minute.

Threshold cycles (Ct) were calculated by means of TaqMan software (ABI, User Bulletin No. 2). Real-time RT-PCR signals for rIKCa1 and rMyHC were standardized to rGAPDH by use of the equation $CtX - Ct_{rGAPDH} = \Delta Ct$. The equation, $\Delta Ct_{w/o} - \Delta CtX = \Delta\Delta Ct$, was used to determine changes in expression, where the ΔCt_X−value (EGF-stimulated) was subtracted from the control $\Delta Ct_{w/o}$−value (w/o=without stimulus) of the same experiment. Fold increases in expression were calculated by the equation $2^{\Delta\Delta Ct}$=fold change.

Statistical Analysis

Data are given as mean±SEM. If appropriate, the Wilcoxon rank sum test or χ^2 analyses were used to assess differences between groups. Values of $P<0.05$ were considered significant.

Results

Alterations in K_{Ca} Functional Expression in Neointimal VSMC After BCI

To measure functional K_{Ca} channel expression, we performed whole-cell patch-clamp experiments on freshly isolated mature VSMC and neointimal VSMC in situ after BCI.[12,13] Mature VSMC (n=14) from normal CA exhibited an outward Ca^{2+} activated and voltage-dependent K^+ current with characteristics of the cloned BK_{Ca} channel.[4,5,13] The outward K^+ current was small at negative membrane potentials, increased steeply at positive membrane potentials, and was blocked by the selective BK_{Ca} inhibitor iberiotoxin (IbTX) (Figure 1A, left panel), with a potency (K_D 11±3 nmol/L, Figure 1A, left inset) similar to the cloned BK_{Ca} channel. The selective SK_{Ca} blocker apamin (APA, 1 μmol/L), and the IK_{Ca} blockers TRAM-34 (1 μmol/L) and CLT (1 μmol/L)[6,7,14] had no effect on this current (data not shown). A small residual voltage-gated Ca^{2+}-independent (K_V) K^+ current (1.1±0.2 pA/pF at 0 mV) was sensitive to 2 mmol/L 4-aminopyridine (Figure 1A, right panel). The voltage dependence of the composite BK_{Ca} plus K_v current in mature VSMC, normalized for cell capacitance (I_K [pA/pF]), is shown in Figure 1D.

Two weeks after BCI, neointimal VSMC (n=30) had a substantially altered K^+ current pattern. The majority of neointimal VSMC (19/30) expressed two K_{Ca} currents (Figure 1B, left panel) with properties resembling BK_{Ca} and IK_{Ca} channels. The IK_{Ca} component seen at negative potentials was eliminated by the selective IK_{Ca} inhibitor TRAM-34, leaving a residual BK_{Ca} current that could be suppressed by IbTX (Figure 1B, left panel). BK_{Ca} currents were absent in 11 of 30 neointimal VSMC that contained only IK_{Ca} currents (Figure 1B, right panel, and Figure 1C) with properties similar to the cloned and native IK_{Ca} channels.[6–8,11,12,14–16,18] These IK_{Ca} currents were blocked by TRAM-34 in a dose-dependent fashion, 500 nmol/L TRAM-34 completely abolishing the current (Figure 1B, right panel). The dose-response curves in Figure 1C demonstrate that charybdotoxin (ChTX; K_D 5±1 nmol/L), TRAM-34 (K_D 10±2 nmol/L), and CLT (K_D 31±4

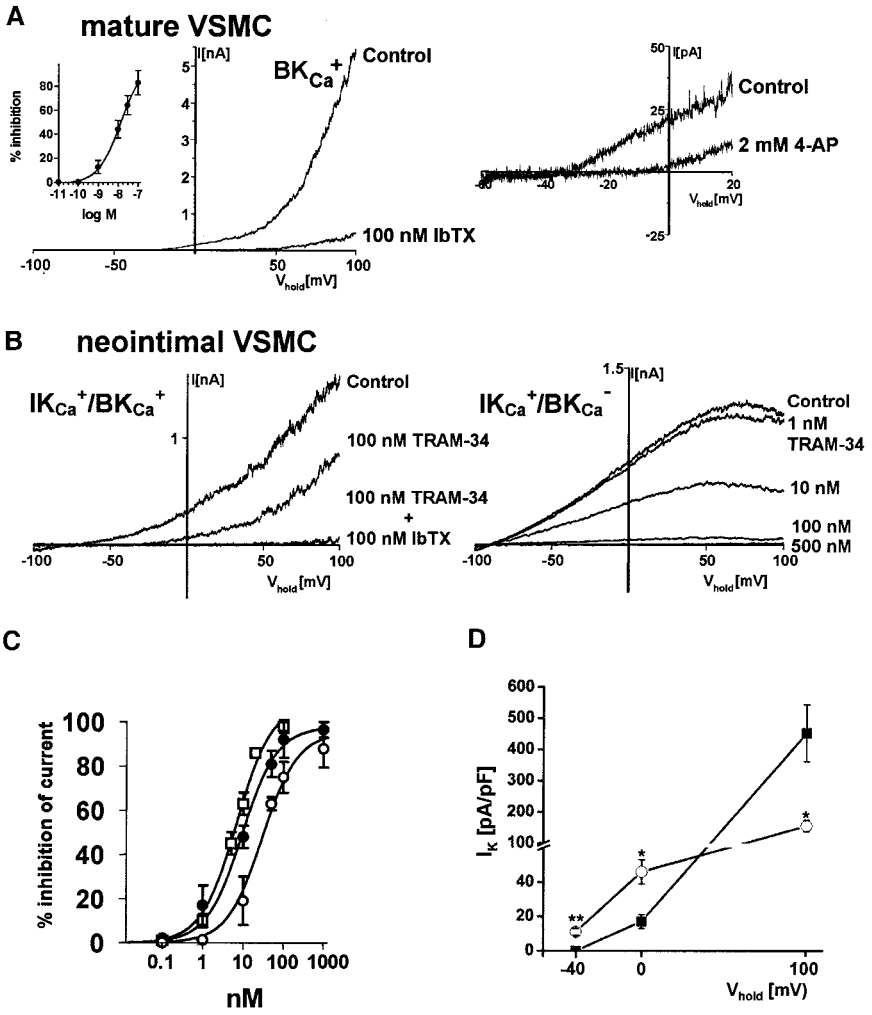

Figure 1. Mature VSMC express BK_{Ca} currents; neointimal VSMC express IK_{Ca} currents at week 2 after BCI. A, Left panel, BK_{Ca} currents in mature VSMC and channel blockade by 100 nmol/L IbTX. Inset, Concentration-dependent blockade of BK_{Ca} currents by IbTX (n=4 to 5). Right panel, Voltage-gated K^+ currents in mature VSMC recorded with a Ca^{2+}-free pipette solution and blockade by 4-AP. B, Left panel, Mixed BK_{Ca} and IK_{Ca} currents in neointimal VSMC at week 2 after BCI and blockade of IK_{Ca} currents by TRAM-34 and BK_{Ca} currents by IbTX. Right panel, Concentration-dependent blockade of IK_{Ca} currents by TRAM-34 in a cell expression pure IKCa1 current. C, Pharmacology of IK_{Ca} currents; TRAM-34 (n=6 to 7, ●), CLT (n=3 to 5; ○), and ChTX (n=3 to 4; □). D, Quantitative analysis of IK_{Ca} and BK_{Ca} currents in mature VSMC (■) and neointimal VSMC (○). *$P<0.05$,**$P<0.01$, Wilcoxon rank sum test.

nmol/L) blocked these currents with potencies similar to the cloned IKCa1 channel.[6,14,18] The currents were not affected by TRAM-7 (1 μmol/L), an inactive analog of TRAM-34, or by the SK blocker APA (1 μmol/L) or the K_v blocker 4-aminopyridine (2 mmol/L) (not shown). When normalized for membrane capacitance (Figure 1D), the mean K^+ current in neointimal VSMC was significantly increased at −40 and 0 mV and reduced at +100 mV, compared with mature VSMC, reflecting the shift from BK_{Ca} expression in mature VSMC to a mixture of IK_{Ca} and BK_{Ca} in neointimal cells.

Alterations in BKCa and IKCa1 mRNA Expression in Neointimal VSMC After BCI Correlate With Changes in Functional Expression

We used "multiplex" single-cell RT-PCR to determine whether the changes in functional BK_{Ca} and IK_{Ca} expression after BCI correlated with alterations in mRNA levels for the *Slo* and *IKCa1* genes, respectively. The VSMC marker MyHC was detected in all mature VSMC (34/34) and in all neointimal VSMC (18/18) 2 weeks after BCI. Endothelial cell–specific eNOS expression was not detected in any of the cell samples, demonstrating that our VSMC samples are not contaminated with endothelial cells. None of the negative controls (n=24) yielded any PCR products.

Mature VSMC that functionally express BK_{Ca} and not IK_{Ca} channels (Figure 1A) contained substantial quantities of *Slo* mRNA (87%; 54/62) and no *IKCa1* mRNA (0/27; Figure 2). Two weeks after BCI, the K_{Ca} gene expression pattern in neointimal VSMC was altered (Figure 2), in keeping with the changes observed in the amplitude of BK_{Ca} and IK_{Ca} currents in these cells (Figure 1B and 1D). *Slo* transcripts were detected less frequently (24/67; −36%) and *IKCa1* transcripts more frequently (42/67; −63%) than in mature VSMC (χ^2 analysis; $P<0.01$ and $P<0.001$, respectively). Transcripts of *SK1-SK3* genes were not detected in mature or neointimal VSMC (data not shown). These results indicate that changes in *Slo* and *IKCa1* mRNA levels after BCI contribute to the changes in BK_{Ca} and IK_{Ca} functional expression in VSMC.

EGF-Induced Upregulation of IKCa1 Expression and Proliferation of VSMC

The switch from BK_{Ca} expression in mature VSMC to a mixture of IK_{Ca} and BK_{Ca} in neointimal cells after BCI may reflect a change from a contractile to a proliferating phenotype. To test this hypothesis, we examined IK_{Ca} function and *IKCa1* mRNA expression in the aortic VSMC cell line A7r5 after mitogenic stimulation with EGF. Forty-eight hours after stimulation, the amplitude of the K^+ current increased 3-fold

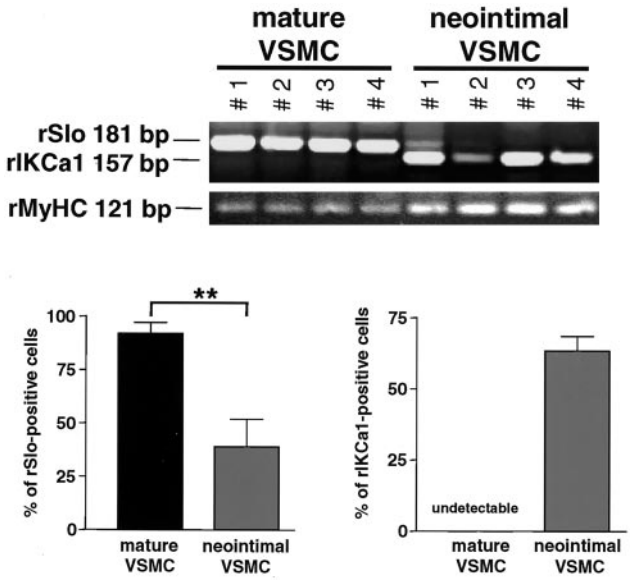

Figure 2. Multiplex single-cell RT-PCR analysis of single mature and VSMC. Ethidium bromide–stained gels of RT-PCR products from representative mature VSMC and neointimal VSMC at week 2 after BCI. rSlo and rIKCa1 (upper panel) and myosin heavy chain rMyHC (lower panel). Columns show quantitative analysis of rSlo and rIKCa1 expression in mature VSMC (rats, n=9) and neointimal VSMC (rats, n=5). $^{**}P<0.01$, Wilcoxon rank sum test.

compared with untreated cells (Figure 3A and Table 1, $P<0.01$). Parallel RT-PCR studies revealed a 6-fold increase in *IKCa1* mRNA levels as early as 2 hours after EGF stimulation and a 3-fold increase after 48 hours (Table 1). Pharmacological studies confirmed that the K^+ currents in EGF-treated cells were indeed IKCa1 (Figure 3B). The currents were not affected by the BK_{Ca} inhibitor IbTX (100 nmol/L) but were blocked by TRAM-34 (K_D 8±1 nmol/L), ChTX (K_D 6±1 nmol/L), and CLT (K_D 30±1 nmol/L) with potencies similar to IKCa1. Thus, the channel expression pattern in EGF-stimulated A7r5 cells resembles that seen in proliferating neointima in vivo.

To test whether the enhanced IK_{Ca} expression in VSMC might have functional consequences, we examined whether the IKCa1 inhibitors TRAM-34 and CLT could suppress EGF-stimulated mitogenesis of A7r5 cells. The cell count increased 145±8% 48 hours after EGF stimulation but only 109±3% in unstimulated A7r5 cells ($P<0.001$). TRAM-34 (IC_{50} 8±4 nmol/L) and CLT (IC_{50} 14±5 nmol/L) suppressed EGF-stimulated proliferation in a dose-dependent fashion, reducing mitogenesis to baseline levels at 100 nmol/L (Figure 3C). The inactive triarylmethane TRAM-7 (1 μmol/L) did not suppress proliferation, indicating that the suppressive effect of TRAM-34 and CLT are not the result of nonspecific toxicity. The BK_{Ca} blocker IbTX (100 nmol/L) also had not effect on proliferation (Figure 3C). These results suggest that upregulation of IK_{Ca} channel expression is required for EGF-induced VSMC proliferation, as has been reported in lymphocyte activation and fibroblast mitogenesis.[6–8,18]

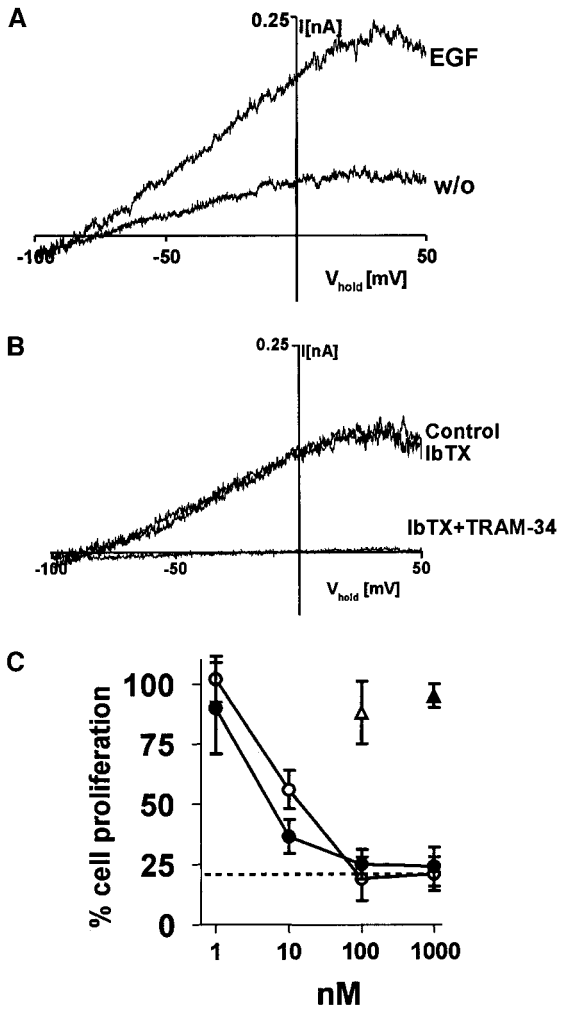

Figure 3. EGF upregulates IKCa1 expression and induces proliferation of the VSMC line, A7r5. A, Representative Ca^{2+}-activated K^+ currents in unstimulated (w/o) and EGF-stimulated cells (48 hours). B, Blockade of IK_{Ca} currents in A7r5 cells by 100 nmol/L TRAM-34 but not by 100 nmol/L IbTX. C, Dose-dependent inhibition of EGF-induced proliferation (percent cell proliferation after 48 hours) by TRAM-34 (n=4 to 10, ●) and CLT (n=6 to 8, ○) but not by inactive TRAM-7 (1 μmol/L; n=3, ▲) or by IbTX (100 nmol/L; n=7; Δ). Dashed line indicates baseline proliferation in A7r5 cells in the absence of EGF.

TRAM-34 and CLT Suppress BCI-Induced Intimal Hyperplasia In Vivo

IK_{Ca} upregulation in proliferating neointimal VSMC and the effectiveness of IK_{Ca} blockers in suppressing EGF-induced proliferation of A7r5 VSMC suggest that in vivo IK_{Ca} blockade might reduce intimal hyperplasia in CA of rats after BCI. We tested this idea by administration of CLT and TRAM-34 after BCI. Data from these experiments are summarized in Table 2, and representative cross sections of CA are shown in Figure 4.

An initial 2-week trial with CLT (120 mg/kg per day) yielded encouraging results, but the CLT-treated rats gained less weight than the vehicle-treated group, and hepatomegaly developed as the result of reported CLT liver toxicity mediated by inhibition of P450-dependent enzymes. We therefore switched to the selective IK_{Ca} inhibitor TRAM-34 (120 mg/kg

TABLE 1. Mitogenic Regulation of rIKCa1 Expression and Function in A7r5 Cells

			x-Fold Increase					
Cell Treatment	n	rGAPDH (Ct)	rIKCa1 (ΔCt)	rIKCa1 (ΔΔCt)	rMyHC (ΔCt)	Cell Treatment	n (Cells)	I_{IKCa} (pA/pF)
w/o	16	22.6±0.5	12.4±0.4		9.2±0.3	w/o	14	0.8±0.2
EGF (2-h)	17	22.7±0.6	9.6±0.4‡	2.8 (≈6-fold)		EGF	16	3.4±0.6†
EGF (48-h)	11	21.7±0.8	10.8±0.4†	1.6 (≈3-fold)	9.0±0.4			

Real-time RT-PCR analysis of rat intermediate-conductance Ca^{2+}-activated K^+ channel (rIKCa1) and rat myosin heavy chain (rMyHC) expression (left) and IK_{Ca} currents at 0 mV (right) in rat VSMC cell line A7r5 after EGF stimulation for 48 hours. $\Delta Ct = Ct_X - Ct_{rGAPDH}$; $\Delta\Delta Ct = \Delta Ct_{w/o} - \Delta Ct_X$; $2^{\Delta\Delta Ct}$ indicates fold increase in expression; w/o, no stimulation.

†$P<0.01$, ‡$P<0.001$ vs w/o; Wilcoxon rank sum test.

per day), which has no effect on P450-dependent enzymes and does not cause overt acute toxicity after intravenous administration.[6] Although neointima formation progressively increased from week 1 to week 6 after BCI in the vehicle-treated group, the area of the neointimal cell layer in the TRAM-34–treated group was significantly smaller at week 1 (−64%; $P<0.01$), week 2 (−35%; $P<0.01$), and week 6 (−43%; $P<0.01$) after BCI (Figure 4 and Table 2). Two weeks' treatment with CLT also resulted in a pronounced reduction of neointimal formation (−50%; $P<0.001$, Figure 4 and Table 2). The area of the medial smooth muscle cell layer was not different between rats treated with TRAM-34, CLT, or vehicle. The ratio of neointimal/medial areas (N/M) and the wall area bounded by the external elastic lamina (EEL) in TRAM-34–treated and CLT-treated rats were therefore significantly smaller than that of the respective vehicle-treated groups at all times measured after BCI. Reduced neointima formation in TRAM-34–treated animals resulted in larger residual lumina at week 2 (+34%; $P<0.05$) and at week 6 (+44%; $P<0.01$) after BCI compared with vehicle-treated rats. CLT-treated animals also displayed larger residual lumina at 2 weeks (+49%; $P<0.001$) after BCI. When the lumen area of the injured CA (rL) was normalized to that of the uninjured contralateral CA (rL/cL), TRAM-34–treated rats displayed reduced lumen narrowing (higher rL/CL values) at week 2 (−9%; $P<0.01$) and week 6 (−19%; $P<0.01$) than vehicle-treated control animals (−36% at week 2 and −50% week 6). Less lumen narrowing was also observed in CLT-treated animals 2 weeks after BCI (−18%; $P<0.05$).

TRAM-34 treatment caused no visible side effects or macroscopic organ damage during the course of the study. After transient weight loss in the first week as the result of surgery, TRAM-34–treated rats gained weight (30±5 g at week 2; 99±6 g at week 6), similar to the vehicle-treated group (25±4 g at week 2, 90±15 g at week 6). In contrast, the CLT-treated group gained significantly less weight (7±6 g; $P<0.05$) within 2 weeks after BCI.

We used a functional bioassay[10] to determine the levels of TRAM-34 and CLT in serum samples obtained at varying times (1, −2, −4, −6, −8, −12, −24 hours) after injection. CLT plasma levels peaked 4 hours after administration (2 μmol/L) and then progressively decreased over the next 24 hours (6 hours: 1.2 μmol/L; 12 hours: 600 nmol/L; 24 hours: 350 nmol/L). TRAM-34 levels in plasma peaked 1 hour after administration (1 μmol/L) and then dropped more rapidly than CLT (2 hours: 500 nmol/L; 4 and 6 hours: 200 nmol/L; 8 hours: 150 nmol/L; 24 hours: 120 nmol/L, 48 hours: 100 nmol/L). The continued presence of low levels (70 nmol/L) of TRAM-34 in the plasma 48 hours after injection suggested that the compound was partitioning into a "deep compartment" from which it was slowly being released back into the blood. The presence of 5 μmol/L TRAM-34 in the liver and 200 nmol/L in the subcutaneous fat at the 48-hour time point indicates that the highly lipophilic TRAM-34 (log$P$=4.0

TABLE 2. Effect of TRAM-34 and CLT on Intimal Hyperplasia After BCI

Treatment Group	n	Neointimal Area, mm²	Medial Area, mm²	Wall Area Bounded by EEL, mm²	N/M	Residual Lumen Area, mm²	rL/cL	Nuclei Count, Cell No.	Rate of Apoptosis, %	Collagen Content, %
Vehicle										
1 wk	4	0.011±0.010	0.084±0.005	0.095±0.007	0.13±0.01	0.22±0.01	0.93±0.04	159±17		
2 wk	11	0.097±0.006	0.099±0.003	0.195±0.009	1.05±0.06	0.16±0.01	0.64±0.05	984±82	0.9±0.6	11±2
6 wk after BCI	5	0.169±0.008	0.091±0.008	0.260±0.011	1.92±0.22	0.13±0.01	0.50±0.02	1525±79	<<1	19±3
TRAM-1										
1 wk	4	0.004±0.001*	0.084±0.004	0.088±0.004	0.05±0.01†	0.21±0.02	0.96±0.05	47±16*		
2 wk	6	0.063±0.005†	0.095±0.002	0158±0.006†	0.66±0.05†	0.21±0.02*	0.91±0.06†	601±36†	1.0±0.6	15±2
6 wk after BCI	5	0.096±0.018†	0.082±0.006	0.179±0.022†	1.15±0.18†	0.18±0.01†	0.81±0.06†	612±97‡	<<1	11±4
CLT										
2 wk after BCI	7	0.049±0.010‡	0.102±0.004	0.151±0.009†	0.49±0.10‡	0.24±0.02†	0.82±0.05*	407±82‡	0.6±0.5	13±1

EEL indicates external elastic lamina; N/M, ratio of neointimal/medial areas; and rL/cL, ratio of residual/contralateral lumens.

*$P<0.05$, †$P<0.01$, ‡$P<0.001$ vs vehicle, Wilcoxon rank sum test.

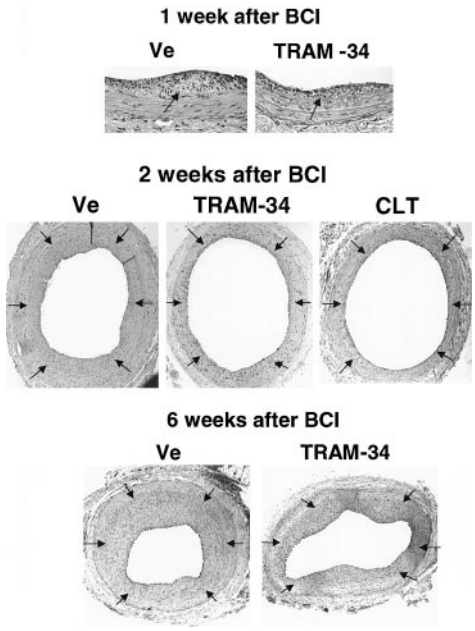

Figure 4. TRAM-34 and CLT reduce neointima formation after BCI. Upper panel, Representative cross sections of carotid arteries stained with hematoxylin and eosin after treatment with TRAM-34 or vehicle (Ve) at week 1 after BCI; original magnification ×200; arrows indicate neointima/media borders. Middle panel, Representative cross sections after treatment with TRAM-34, CLT, or Ve at week 2 after BCI; original magnification ×50. Lower panel, Representative cross sections after treatment with TRAM-34, CLT, or Ve at week 6 after BCI.

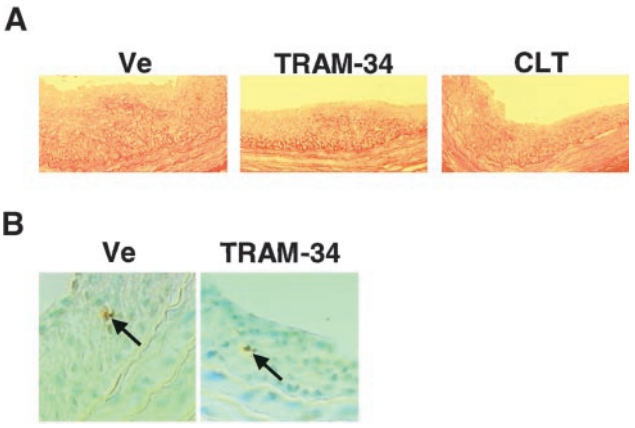

Figure 5. TRAM-34 and clotrimazole had no effect on collagen content or rate of apoptosis in the neointima after BCI. A, Representative cross sections stained with Sirius red (collagen stain) after treatment with TRAM-34, CLT, or vehicle (Ve) at week 2 after BCI; original magnification ×100. B, Representative cross sections stained by use of the TUNEL method for detection of apoptotic nuclei in the neointima after treatment with TRAM-34, CLT, or Ve at week 2 after BCI; sections were counterstained with methyl green to visualize all nuclei; original magnification ×400; arrows indicate apoptotic nuclei.

versus 3.5 for CLT) accumulates in these tissues. Thus, after subcutaneous administration in peanut oil, TRAM-34 was slowly released into the blood stream, resulting in serum levels sufficient to suppress proliferation (in vitro: IC_{50} 8 nmol/L) over a 24- to 48-hour period.

To understand the mechanism by which TRAM-34 and CLT reduced neointima formation, we investigated cell proliferation, apoptosis, and extracellular matrix (collagen) content. The neointimal nuclei count, a measure of cell proliferation, was reduced by −70% ($P<0.05$) after 1 week, by −39% ($P<0.01$) after 2 weeks, and by −61% ($P<0.001$) after 6 weeks of TRAM-34 treatment.

A similar reduction (−59%, $P<0.001$) in the neointimal nuclei count was observed in the CLT-treated group at 2 weeks after BCI. However, the collagen content and the rate of apoptosis (percentage of apoptotic nuclei) in the neointima was not different in TRAM-34–treated and CLT-treated rats compared with vehicle-treated control animals (Table 2 and Figure 5A and 5B). Taken together, our results demonstrate that in vivo IK_{Ca} blockade reduces neointima formation and vessel narrowing through inhibition of VSMC proliferation.

Discussion

Using the rat balloon catheter injury model, we demonstrate that neointimal formation after angioplasty is associated with a switch in K_{Ca} channel expression from exclusive BK_{Ca} expression in mature contractile VSMC to downregulated BK_{Ca} and upregulated IK_{Ca} expression in proliferating neointimal VSMC. A similar upregulated IK_{Ca} channel expression pattern was observed in proliferating aortic VSMC A7r5 cells after stimulation with EGF, and selective IK_{Ca} blockade suppressed A7r5 mitogenesis in vitro, suggesting that IK_{Ca} channels play an important role in VSMC proliferation. Consistent with this idea, in vivo blockade of IK_{Ca} channels reduced BCI-triggered neointimal formation and vessel renarrowing, which suggests a novel therapeutic strategy for the prevention of restenosis after angioplasty.

Neointimal proliferation and IK_{Ca} upregulation after BCI is mediated by numerous mitogenic factors. Using the aortic VSMC line A7r5 as a model system, we demonstrated that mitogenic doses of EGF augment *IKCa1* RNA and IK_{Ca} current amplitude. Upregulated IK_{Ca} expression has been similarly reported to contribute to the proliferation of mitogen-stimulated fibroblasts[8] and human T lymphocytes.[6,7,14,18] Enhanced IKCa1 expression might therefore be a functional characteristic of proliferating and dedifferentiated cells.[8,15]

IK_{Ca} channels may promote VSMC mitogenesis by enhancing the electrochemical driving force for Ca^{2+} influx through membrane hyperpolarization and thus sustain a high intracellular Ca^{2+} concentration required for gene transcription, as has been reported in lymphocytes and fibroblasts.[6,7,18] *IKCa1* may play a more important role than BK_{Ca} in shaping Ca^{2+} signals of proliferating VSMC because its higher Ca^{2+} affinity[3,4,6,7,11,13–16] would result in channel opening and membrane hyperpolarization in response to subtle increases in the intracellular Ca^{2+} concentration. Induction of *IKCa1* expression might thus be a required step for neointimal VSMC proliferation after BCI. Consistent with such a role, IKCa1 blockade by CLT and the specific inhibitor TRAM-34 suppressed the proliferation of cultured VSMC. IKCa1 blockers may therefore have therapeutic value for preventing neointimal proliferation and restenosis after BCI.

In a rat model of BCI, in vivo administration of CLT significantly reduced neointimal thickening, but the trial was discontinued after 2 weeks because of reduced weight gain and the development of severe hepatomegaly, presumably because of liver toxicity[17] caused by blockade of cytochrome P450–dependent enzymes.[6] A subsequent trial with TRAM-34, an *IKCa1* selective inhibitor that does not block cytochrome-P450 enzymes or exhibit acute toxicity,[6] significantly reduced neointimal hyperplasia and vessel narrowing without causing visible signs of organ damage. The therapeutic effect of TRAM-34 was due to inhibition of neointimal cell proliferation and not due to increased apoptosis or decreased matrix formation. In conclusion, targeting IKCa1 channels in proliferating VSMC with TRAM-34 might have therapeutic utility in the prevention of restenosis after angioplasty and for the treatment of other cardiovascular disorders characterized by abnormal VSMC proliferation.

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Blockade of the Intermediate-Conductance Calcium-Activated Potassium Channel as a New Therapeutic Strategy for Restenosis

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