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Mitogenic modulation of Ca^{2+}-activated K^+ channels in proliferating A7r5 vascular smooth muscle cells

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1 Modulation of Ca^{2+}-activated K^+ channels (K_{Ca}) has been implicated in the control of proliferation in vascular smooth muscle cells (VSMC) and other cell types. In the present study, we investigated the underlying signal transduction mechanisms leading to mitogen-induced alterations in the expression pattern of intermediate-conductance K_{Ca} in VSMC.

2 Regulation of expression of $IK_{Ca}/rK_{Ca}3.1$ and $BK_{Ca}/rK_{Ca}1.1$ in A7r5 cells, a cell line derived from rat aortic VSMC, was investigated by patch-clamp technique, quantitative RT–PCR, immunoblotting procedures, and siRNA strategy.

3 PDGF stimulation for 2 and 48 h induced an 11- and 3.5-fold increase in $rK_{Ca}3.1$ transcript levels resulting in a four- and seven-fold increase in IK_{Ca} currents after 4 and 48 h, respectively. Upregulation of $rK_{Ca}3.1$ transcript levels and channel function required phosphorylation of extracellular signal-regulated kinases (ERK1/2) and Ca^{2+} mobilization, but not activation of p38-MAP kinase, c-Jun NH(2)-terminal kinase, protein kinase C, calcium-calmodulin kinase II and Src kinases.

4 In contrast to $rK_{Ca}3.1$, mRNA expression and functions of $BK_{Ca}/rK_{Ca}1.1$ were decreased by half following mitogenic stimulation. Downregulation of $rK_{Ca}1.1$ did not require ERK1/2 phosphorylation or Ca^{2+} mobilization.

5 In an *in vitro*-proliferation assay, knockdown of $rK_{Ca}3.1$ expression by siRNA completely abolished functional IK_{Ca} channels and mitogenesis.

6 Mitogen-induced upregulation of $rK_{Ca}3.1$ expression is mediated *via* activation of the Raf/MEK- and ERK-signaling cascade in a Ca^{2+}-dependent manner. Upregulation of $rK_{Ca}3.1$ promotes VSMC proliferation and may thus represent a pharmacological target in cardiovascular disease states characterized by abnormal cell proliferation.
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Abbreviations: BK_{Ca}, large-conductance K_{Ca}; IbTx, Iberiotoxin; IK_{Ca}, intermediate-conductance; K_{Ca}, Ca^{2+}-activated K^+ channel; TRAM-34, 1-[(2-chlorophenyl)diphenylmethyl]-1*H*-pyrazole

Introduction

In the course of disease, excessive proliferation of vascular smooth muscle cells (VSMC) plays an important pathophysiological role during arteriosclerosis, neointimal proliferation following angioplastic intervention (Racusen *et al*., 1999), transplant vasculopathies (McBride *et al*., 1988), and vascular remodeling as a consequence of arterial hypertension (Endemann & Schiffrin, 2004). In several studies, numerous growth-stimulating molecules such as ET1, PDGF and EGF to name some have been proposed to promote the observed proliferation, migration, and subsequent dedifferentiation of VSMC in such disease states (Newby & Zaltsman, 2000). In addition, alterations in ion channel function have also been proposed to control proliferation of several cell types (Nilius & Droogmans, 2001) including VSMC by enhancing intracellular Ca^{2+} signaling and affecting cell cycle progression (Afroze & Husain, 2001). For instance, upregulation of receptor/second messenger-operated channels of the TRP gene family has been related to increased Ca^{2+} signaling in proliferating VSMC *in vitro* (Golovina *et al*., 2001; Yu *et al*., 2004). Cl^- channels and K^+ channels also seem to play a role during these modulations as these channels set the membrane potential which in turn controls the driving force for Ca^{2+} entry (Nilius & Droogmans, 2001). Consequently, inhibition of such Cl^- and K^+ channels have been shown to prevent mitogenesis *in vitro* (Eggermont *et al*., 2001; Jager *et al*., 2004; Grgic *et al*., 2005).

Nonproliferating VSMCs predominantly express the calcium-activated large-conductance K^+ channel (BK_{Ca} or maxi K), a product of the $K_{Ca}1.1$ gene (Atkinson *et al*., 1991) (as per the new IUPHAR nomenclature: http://www.iuphar.org/compendium2.htm; a.k.a. *Slo*). BK_{Ca} plays a pivotal role in VSMC relaxation by dampening depolarization-dependent activation of Ca^{2+}-channels and Ca^{2+}-influx *via* membrane hyperpolarization (Waldron & Cole, 1999). In a recent study, we demonstrated that neointimal, proliferating VSMCs downregulate BK_{Ca} and instead express the intermediate-conductance K_{Ca}

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channel (IK_{Ca}) (Kohler *et al*., 2003) encoded by the *K_{Ca}3.1* gene (as per the new IUPHAR nomenclature; a.k.a. *IKCa1*, *KCNN4*, *IK1*, *hSK4*). This IK_{Ca} channel has been proposed to be an important regulator of proliferation in several cell types (Khanna *et al*., 1999; Pena *et al*., 2000; Wulff *et al*., 2000). We could additionally show that selective inhibition of this IK_{Ca} channel substantially reduced neointima formation in rat carotid arteries *in vivo* and cultured rat VSMC *in vitro* (Kohler *et al*., 2003).

In the present study, we aimed to investigate the underlying signal transduction pathways of growth factor-induced reorganization of K_{Ca} gene expression in A7r5 cells. In particular, we tested whether IK_{Ca} deficiency induced by silencing of *rK_{Ca}3.1* expression with K_{Ca}3.1-specific siRNA inhibits A7r5 cell proliferation. Our studies demonstrated that PDGF-BB induced a rapid and long-lasting upregulation of *rK_{Ca}3.1* expression and IK_{Ca} channel function which required intracellular calcium and an intact Raf/MEK- and extracellular signal-regulated kinases (ERK)-signaling cascade. In contrast, BK_{Ca}/Slo expression was significantly downregulated. Strikingly, during mitogen-induced proliferation, siRNA-induced silencing of *rK_{Ca}3.1* abolished A7r5 cell proliferation *in-vitro*.

Methods

Materials

Cell culture media were obtained from Biochrom (Berlin, Germany). Unless otherwise stated, antibodies were from New England Biolabs (Ipswich, MA, U.S.A.). The MEK inhibitors PD98059 (20 μM) or U0126 (10 μM), the selective inhibitor of *c-Jun* N-terminal kinase (JNK) SP600125 (20 μM), the p38 MAP kinase inhibitor SB203580 (10 μM) were purchased from Tocris (Cologne, Germany), 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetate-acetoxymethyl ester (BAPTA-AM, 10 μM) and TMB-8 (100 μM, both intracellular calcium antagonists), chelerythrine (1 μM, protein kinase C (PKC) inhibitor), KN-93 (40 μM, calcium-calmodulin-dependent kinase II (CaMKII) inhibitor), wortmannin (0.1 μM, PI3 kinase inhibitor) or the Src tyrosine kinase inhibitor PP2 (2 μM) were from Sigma-Aldrich (Deisenhofen, Germany).

Cell culture

Transformed VSMC, cell line-A7r5, were cultured as previously described (Kohler *et al*., 2003). Prior to stimulation A7r5 cells were kept in serum-free medium for 48 h to induce growth arrest.

Patch-clamp experiments

Whole-cell patch-clamp experiments and data analysis were performed as described previously (Papassotiriou *et al*., 2000; Kohler *et al*., 2001). For activation of K_{Ca} currents, A7r5 cells were dialyzed with a KCl-pipette solution containing 3 μM $[Ca^{2+}]_{free}$ (mM): 140 KCl, 1 $MgCl_2$, 2 ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1.90 $CaCl_2$, and 5 *N*-[2-hydroxyethyl]piperazine-*N*′-[2-ethanesulfonic acid] (HEPES), pH 7.2. In another set of experiments, pipette solution contained 0.72 or 1.36 mM $CaCl_2$ and 2 mM EGTA yielding 0.1 and 0.4 μM $[Ca^{2+}]_{free}$ or 100 μM $[Ca^{2+}]$ without EGTA. The NaCl bath solution contained (mM): 137 NaCl, 4.5 Na_2HPO_4, 3 KCl, 1.5 KH_2PO_4, 0.4 $MgCl_2$ and 0.7 $CaCl_2$, pH 7.4.

RNA isolation and quantitative RT–PCR

Cells were harvested 2 or 48 h after stimulation, RNA was isolated using the high pure RNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany) and RNA was subsequently reverse transcribed using M-MLV reverse transcriptase (Life Technologies, Eggenstein, Germany). Expression levels were quantified with an ABI-Prism-7700 Sequence Detection System (ABI, Darmstadt, Germany) 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:

rK_{Ca}3.1 (product size: 217 bp): F5′-CTGAGAGGCAGGCT GTCAATG-3′ (813–833);
R5′-ACgTgTTTCTCCgCCTTgTT-3′ (1010–1029);
P5′-AAGATTGTCTGCTTGTGCACCGGAGTC-3′ (926–952)
rK_{Ca}1.1 (product size: 181 bp): F5′-TTTACCGGCTGAGA GATGCC-3′ (3926–3945);
R5′-TGTGAGGAGTGGGAGGAATGA-3′ (4086–4106);
P5′-ACCTCAGCACCCCCAGCCAGTG-3′ (3947–3968)
rat glyceraldehyde-3-phosphate dehydrogenase (*rGAPDH*) (product size: 101 bp):
F5′-CGGCACAGTCAAGGCTGAG-3′ (1014–1032);
R5′-CAGCATCACCCCATTTGATGT-3′ (1094–1114);
P5′-CCCATCACCATCTTCCAGGAGCGA-3′ (1060–1083)

GenBank™ accession: *rK_{Ca}3.1*: AF156554; *rK_{Ca}1.1*: AF135265; *rGAPDH*: AB017801.

Each 25 μl PCR reaction contained 500 nM forward and reverse primer, 150 nM probe, 3 μl cDNA, and 1 × TaqMan Universal Master Mix (ABI, Darmstadt, Germany). PCR parameters were 50°C × 2 min, 95°C × 10 min, and 50 cycles at 95°C × 15 s, 60°C × 1 min.

Threshold cycles (C_t) were calculated using TaqMan®-software (ABI-User-Bulletin-#2). Real-time RT–PCR signals for *rK_{Ca}3.1* and *rK_{Ca}1.1* were standardized to *rGAPDH* by using the equation: $C_{t_x} - C_{t_{rGAPDH}} = \Delta C_t$. The equation, $\Delta C_{t_{w/o}} - \Delta C_{t_x} = \Delta\Delta C_t$, was used to determine changes in expression, where the $\Delta C_{t_{w/o}}$-value (growth factor stimulated) was subtracted from the control $\Delta C_{t_{w/o}}$-value (w/o = without stimulus) of the same experiment. Fold increases in expression were calculated by the equation, $2^{\Delta\Delta C_t}$ = fold change.

siRNA experiments and in vitro *proliferation studies*

Synthetic siRNA directed against *rK_{Ca}3.1* (sense: 5′-GGAG GUCCAGCUGUUCAUGtt-3′, antisense: 5′-CAUGAACAG CUGGACCUCCtt-3′) and scrambled negative control siRNA (sense: 5′-CAUUCACUCAGGUCAUCAGtt-3′, antisense: 5′-CUGAUGACCUGAGUGAAUGtt-3′), which does not interfere with any known mRNA, were synthesized by Ambion (Cambridgeshire, U.K.). A7r5 cells were transfected for 4 h

with 25 nM of the siRNAs using siPORT™ Amine (Ambion) in accordance with the manufacturer's protocol. At this concentration, functional expression of *$rK_{Ca}3.1$* was effectively eliminated for up to 72 h. Lower (10 nM) or higher (50 nM) concentrations of siRNAs resulted in inefficient transfection or cell death, respectively. Prior to transfection with siRNA-Ctrl or siRNA-*$rK_{Ca}3.1$*, VSMC were kept in serum-free DMEM for 48 h. Stimulation of these cells with FCS (10%) or PDGF-BB (10 ng ml^{-1}) was performed directly following the transfection. At 10–20% confluence, photo-micrographs of cells were taken in fixed fields before and 48 h after stimulation and the percent increase in cell count was calculated.

Western blotting

Immunoblotting were performed as described previously (Reusch *et al*., 2001b) applying an anti-IK_{Ca} primary antibody (1 : 200, Sigma, Germany) and phospho-specific antibodies against ERK1/2, phospho-specific Akt and respective secondary antibodies (Cell Signaling Technology, Frankfurt, Germany).

Statistical analysis

Data are given as mean ± s.e. If appropriate, the Student's *t*-test or ANOVA and Bonferroni *post hoc* test were used to assess differences between groups. *P*-values of $P<0.05$ were considered significant.

Results

Growth factor-induced upregulation of IK_{Ca} function and $rK_{Ca}3.1$ expression in A7r5 cells

Numerous growth factors such as PDGF and EGF induce VSMC proliferation *in vitro* and have also been demonstrated to play a role in neointima formation *in vivo* (Newby & Zaltsman, 2000). However, these complex remodeling processes are only incompletely understood. Growth factor-induced K^+ channel activity like IK_{Ca} upregulation might, for example play a pivotal intermediate step during these processes. To test this hypothesis, we compared IK_{Ca} function and *$rK_{Ca}3.1$* expression in A7r5 cells before, 2, 4 and 48 h after stimulation with PDGF-BB (10 ng ml^{-1}). Prior to stimulation, the amplitude of 'total' K_{Ca} currents was rather small, whereas following PDGF stimulation, the amplitude of the K_{Ca} current increased five-fold at 4 h and remained upregulated for at least 48 h. Cell capacitance was not altered by either PDGF or FCS treatment (data not shown). Original traces of K_{Ca} currents, mean K_{Ca} currents normalized to cell capacitance, and time course of upregulation of K_{Ca} currents are shown in Figure 1a, left, middle and right panels.

The PDGF-induced current was indeed a K^+-selective and Ca^{2+}-activated current since in the presence of low (4.5 mM) or high (140 mM) extracellular K^+ concentrations, the current reversed near the K^+ equilibrium potential of –89 or at 0 mV (Figure 1b, left panel). Cell dialysis with varying amounts of free Ca^{2+} (0.1–100 μM) resulted in a concentration-dependent increase in K^+ currents with an EC_{50} of $\approx$400 nM (Figure 1b, middle and right panel). In addition, the current was almost completely blocked by applying the selective IK_{Ca} inhibitor TRAM-34 (100 nM) (Wulff *et al*., 2000; Eichler *et al*., 2003) at negative membrane potential, leaving a small TRAM-34-insensitive K^+ current component at positive membrane potentials (Figure 1c). The remaining K^+ current was completely sensitive to the BK_{Ca} selective blocker IbTx (100 nM). Thus, the data with selective pharmacological inhibitors indicate that K_{Ca} currents in PDGF-stimulated A7r5 cells are largely mediated by IK_{Ca} currents with a small contribution of BK_{Ca} currents. Note that since the combination of TRAM-34 and IbTx treatment completely blocked total K^+ currents, A7r5 cells appear to lack substantial other voltage-gated K^+ currents irrespective of PDGF stimulation. This K_{Ca}-expression pattern in mitogen-stimulated A7r5 cells resembles that seen in proliferating neointimal VSMC *in vivo* rather than that observed in contractile nonproliferating VSMC (Kohler *et al*., 2003).

To test whether the high amplitude of IK_{Ca} functions after PDGF treatment is a consequence of new protein synthesis, we performed Western blot analysis using a specific IK_{Ca} antibody. This Western blot analysis revealed that PDGF stimulation for 48 h induced significant amounts of the IK_{Ca} protein while the IK_{Ca} protein was not detectable in the nonstimulated cells (Figure 1d).

Parallel studies applying quantitative RT–PCR technology revealed an about 11-fold increase in *$rK_{Ca}3.1$* transcript levels following 2 h PDGF stimulation (Figure 2a), thus clearly preceding the observed upregulation in IK_{Ca} function. At 48 h following PDGF stimulation, *$rK_{Ca}3.1$* transcript level was still 3.5-fold increased over unstimulated A7r5 cells (Figure 2a). Stimulation with 10% FCS for 48 h similarly upregulated IK_{Ca} function (Figure 1a, left and middle panels) and *$rK_{Ca}3.1$* mRNA expression (Figure 2a). In contrast, stimulating A7r5 cells with thrombin (1 U ml^{-1}), another potent mitogen for A7r5 cells (McNamara *et al*., 1996), was ineffective in either augmenting IK_{Ca} current amplitude (0.4 ± 0.1 pA pF^{-1} at 0 mV, $n=11$) or upregulating expression of *$rK_{Ca}3.1$* (-0.6 ± 0.3 $\Delta\Delta C_t$, $n=7$).

PDGF-induced upregulation of IK_{Ca} function and $rK_{Ca}3.1$ expression requires activation of the Raf/MEK and ERK-signaling cascade

The participation of the MAP kinases ERK1/2 has been shown to play an important role in VSMC proliferation *in vitro* and *in vivo* (Kingsley *et al*., 2002). Interestingly, the Ras/Raf/MEK/ERK-signaling cascade has also been involved in the mitogen-induced modulations of IK_{Ca} functions in rat fibroblasts (Pena *et al*., 2000) and of IK_{Ca} functions and *$K_{Ca}3.1$* expression in human endothelial cells (Grgic *et al*., 2005). In T lymphocytes, promoter analysis of the *$K_{Ca}3.1$* gene revealed that activation of *$K_{Ca}3.1$* gene expression requires DNA binding of the transcription factor AP1 (Ghanshani *et al*., 2000) which has been shown to be a downstream target of ERK1/2 kinase activity (Park & Levitt, 1993). With respect to *$rK_{Ca}3.1$* expression, treatment of A7r5 cells with the MEK-inhibitor PD98059 (20 μM) for 30 min prior to stimulation with PDGF abolished the growth factor-mediated upregulation of *$rK_{Ca}3.1$* transcript levels at 2 h (Figure 2a) and of IK_{Ca} current amplitude at 48 h (Figure 2b and c). Similar data were obtained with the MEK inhibitor U0126 (10 μM, data not shown). Pretreatment of A7r5 cells with either the JNK inhibitor SP600125 or the p38-MAP kinase inhibitor

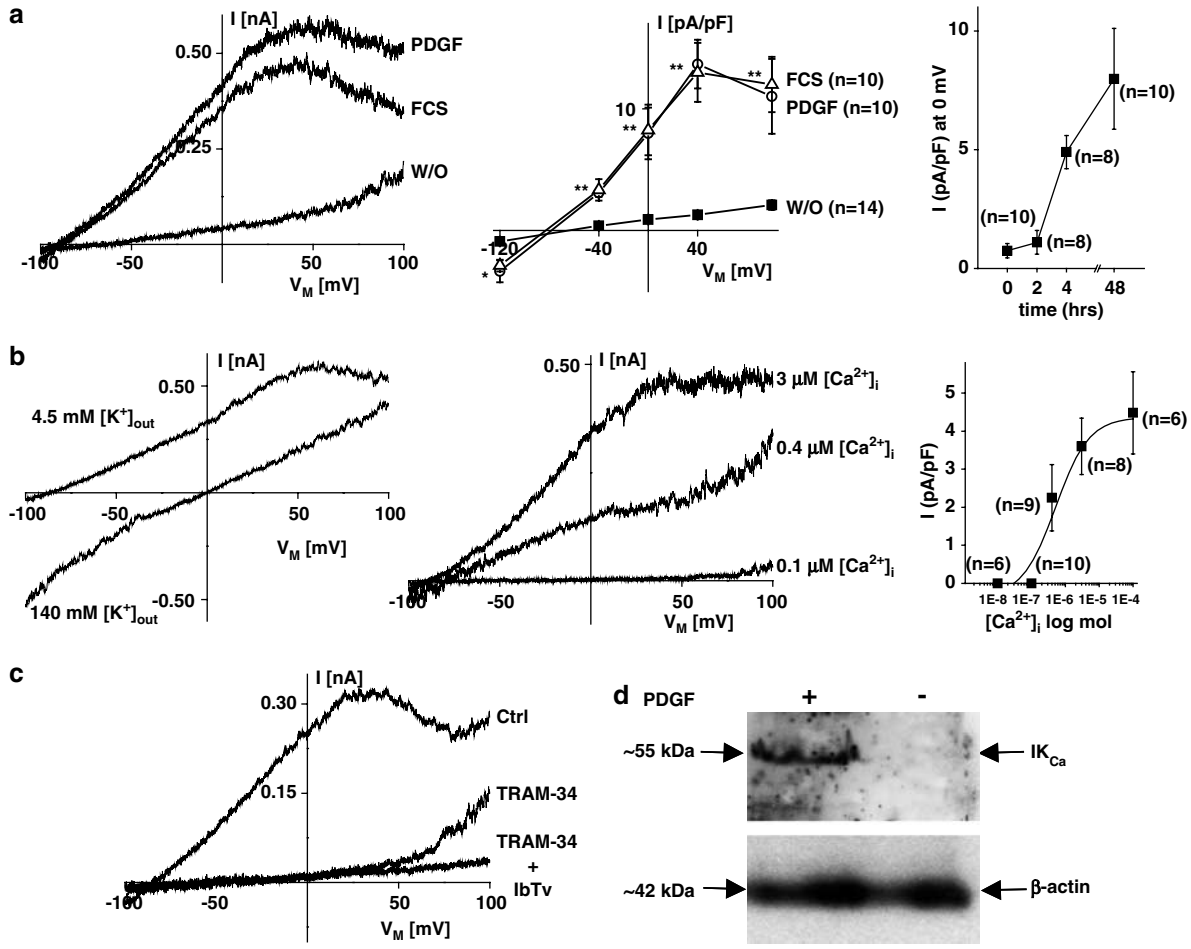

Figure 1 Mitogenic stimulation induces mRNA expression of *$rK_{Ca}3.1$* and increases IK_{Ca} currents in A7r5 cells. (a) Left panel, representative whole cell recordings of K_{Ca} currents in unstimulated (w/o) A7r5 cells and after stimulation with PDGF (10 ng ml^{-1}) or FCS (10%). Middle panel, mean K_{Ca} currents (activated by cell dialysis with a 3 μM-free Ca^{2+} concentration and normalized to cell capacitance) in PDGF-stimulated or -unstimulated (w/o) A7r5 cells. Data points represent mean $\pm$ s.e., $^*P<0.05$; $^{**}P<0.01$ (ANOVA); right panel, amplitude of K_{Ca} currents, at membrane potential of 0 mV before and 2, 4 and 48 h after PDGF stimulation, respectively. (b) Left panel, PDGF-induced K_{Ca} currents in the presence of low (4.5 mM) and high (140 mM) extracellular K^+, respectively. Reversal potentials of ≈ -89 and 0 mV indicate K^+ selectivity of the Ca^{2+}-activated current. Middle panel, original whole-cell recordings showing Ca^{2+} dependence of PDGF-induced K_{Ca} currents. Right panel, concentration-dependent increase of mean IK_{Ca} currents by Ca^{2+} (EC_{50} 400 nM). (c) Mixed BK_{Ca} and IK_{Ca} currents in A7r5 cells after PDGF stimulation and blockade of IK_{Ca} currents by TRAM-34 (100 nM) and BK_{Ca} currents by IbTX (100 nM). (d) Western blotting analysis of the IK_{Ca} protein expression in A7r5 cells with (+) PDGF (10 ng ml^{-1}) stimulation for 48 h or without (−) PDGF stimulation. The blot shown in the upper panel was probed with an antibody against rIK_{Ca}. Equal loading is demonstrated with the antibody detecting β-actin in the lower panel.

SB203580 was without any effect on PDGF-induced upregulation of *$rK_{Ca}3.1$* transcript levels (Figure 2a) or IK_{Ca} current amplitudes (data not shown).

To determine whether the observed upregulation of *$rK_{Ca}3.1$* expression in A7r5 cells is mediated *via* activation of the Raf/MEK/ERK- or other MAP kinase-signaling cascades, we measured PDGF-induced phosphorylation of MAP kinases ERK1/2, c-Jun NH(2)-terminal kinase (JNK), or p38 MAP kinase. PDGF treatment resulted in pronounced MEK and ERK1/2 phosphorylation, which was maximal at 5–10 min and declined within 100 min to basal levels (Figure 3a). Other mitogens, that is EGF (10 ng ml^{-1}) and thrombin (1 U ml^{-1})-induced ERK phosphorylation similar to PDGF. While the time course and degree of ERK phosphorylation after stimulation with EGF and PDGF were similar, thrombin-induced ERK phosphorylation appeared weaker and more transient (please refer to online Supplementary information).

Phosphorylation of terminal JNK or p38 MAP kinase was not detectable after PDGF stimulation whereas challenging A7r5 cells with FCS induced phosphorylation of all three MAP kinases in these cells (data not shown). Incubation with MEK inhibitors PD98059 or U0126 for 30 min prior to PDGF stimulation dose dependently inhibited ERK-1/2 phosphorylation (Figure 3a and b), whereas the p38-MAP kinase inhibitor SB203580 (Figure 3a) and the JNK inhibitor SP600125 (data not shown) were without any effects. Previous studies confirmed subsequent translocation of phosphorylated ERK-1/2 into the nucleus where numerous transcription factors could become phosphorylated or activated (data not shown, Schauwienold *et al*., 2003). Interestingly, PDGF stimulation also activated the phosphatidylinositol-3-kinase (PI-3 kinase)-

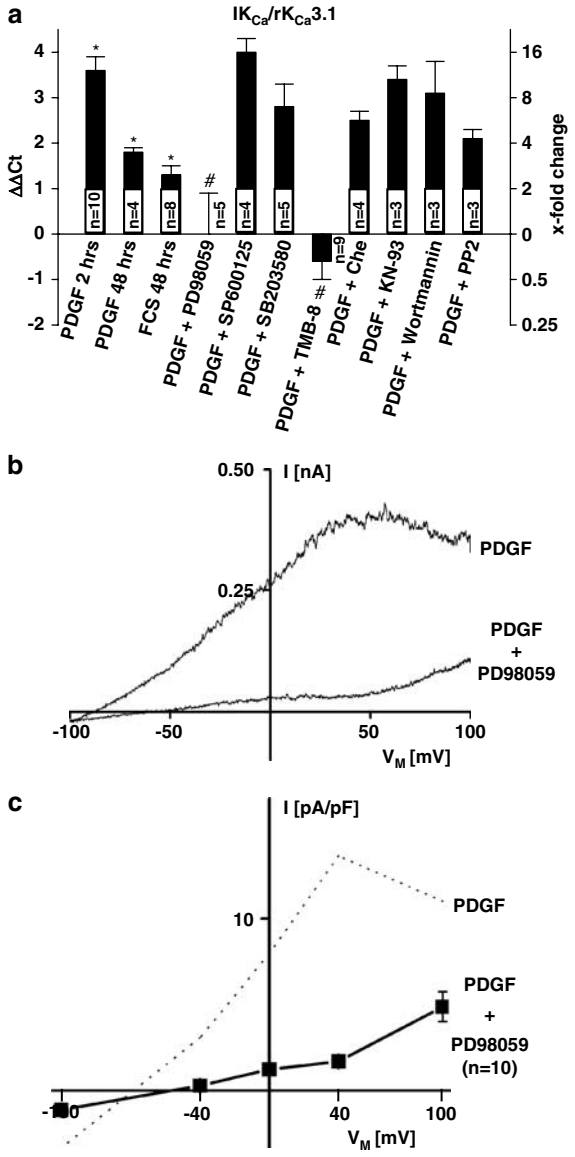

Figure 2 Effects of MAP kinase blockers and other signal transduction modulators on mRNA expression levels of IK_{Ca}/$rK_{Ca}3.1$ in mitogen-stimulated A7r5 cells. (a) Semiquantitative RT–PCR analysis of $rK_{Ca}3.1$ mRNA expression levels following stimulation with PDGF for 2 and 48 h and with FCS (10%) for 48 h as well as following PDGF stimulation for 2 h after pretreatment with signal transduction inhibitors. (b) Pretreatment with the MEK-inhibitor PD98059 prevents PDGF-induced upregulation of IK_{Ca}/$rK_{Ca}3.1$ function in A7r5 cells. (c) Total K_{Ca} currents normalized to cell capacitance after PDGF stimulation with or without pretreatment with PD98059. Charts: left ordinate: $\Delta\Delta C_t$ values ($\Delta\Delta C_t = \Delta C_{t_{w/o}} - \Delta C_{t_x}$) represent change in expression over control (unstimulated VSMC ($n = 19$)). Right ordinate: x-fold change ($2^{\Delta\Delta C_t}$) above control. Data are given as mean ± s.e.; $^*P < 0.01$ vs w/o; $^{\#}P < 0.01$ vs PDGF alone; ANOVA was used to compare respective ΔC_t values.

Akt pathway in these cells. As shown in Figure 3b applying a phospho-S473-Akt-specific antiserum, PDGF induced a robust phosphorylation of Akt in Western blots that was sensitive to inhibition with PI-3 kinase inhibition by wortmannin (Mizutani *et al*., 2002) or LY294002 (data not shown).

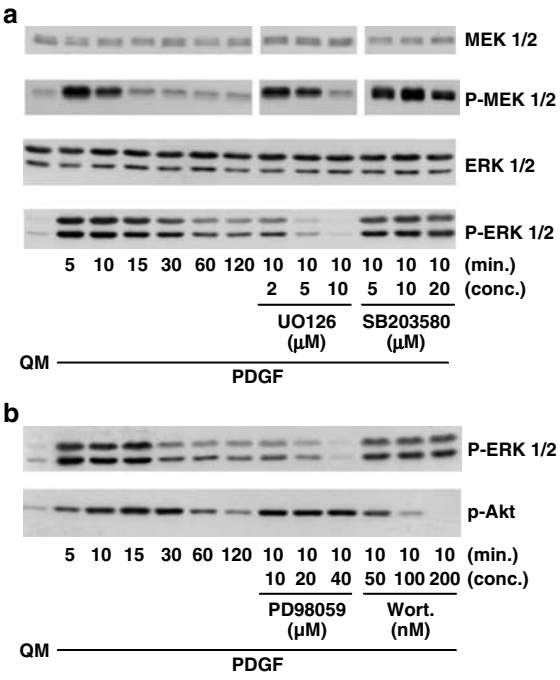

Figure 3 Effect of MAP-kinase inhibitors on the phosphorylation pattern of MEK1/2, ERK1/2 (a) and Akt (b) in PDGF-stimulated A7r5 cells. A7r5 cells were treated with different inhibitors as indicated. After 30 min, cells were stimulated with PDGF-BB (10 ng ml^{-1}) for the indicated times (in min). For each time point, aliquots of cell lysates were separated on SDS–PAGE and probed for the phosphorylated forms of MEK (phospho-S217/221), ERK1/2 (phospho T202/Y204) and Akt (phospho-S473). Equal loading is demonstrated with antibodies detecting the respective total proteins. The experiment shown is representative of three experiments showing similar results. QM = serum-free quiescent medium.

Calcium dependence of PDGF-induced upregulation of $rK_{Ca}3.1$ expression

Since PDGF-stimulation resulted in a Ca^{2+} release from internal stores in A7r5 (not shown) and cultured native VSMC (Reusch *et al*., 2001a, b), we tested whether inhibition of phospholipase C (PLC) or Ca^{2+} chelation/application of intracellular calcium antagonists modulate ERK phosphorylation and consequently $rK_{Ca}3.1$ expression. Chelation of intracellular Ca^{2+} with BAPTA-AM (10 μM) or application of the intracellular calcium antagonist, TMB-8 (100 μM) as well as pretreatment with the PLC-selective inhibitor U73122 (10 μM) prevented ERK phosphorylation in a dose-dependent manner (Figure 4) and pretreatment with BAPTA-AM (not shown) or TMB-8 prevented upregulation of $rK_{Ca}3.1$ mRNA levels (Figure 2a) following stimulation with PDGF.

In additional screening experiments, PKC inhibitors like chelerythrine (10 μM) or bisindolylmaleimide (data not shown), an inhibitor of CaMKII, KN-93 (30 μM), blockage of Src kinases by PP2 (1 μM) or of PI3 kinase by wortmannin (100 nM) did not prevent PDGF-induced augmentation of $rK_{Ca}3.1$ expression (Figure 2a). Inhibition of all these signaling molecules did not alter basal $rK_{Ca}3.1$ mRNA expression levels in unstimulated cells, except inhibition of MEK by PD98059, which resulted in a significant reduction of basal $rK_{Ca}3.1$ mRNA expression levels (not shown). A similar Ca^{2+} mobilization was observed after stimulation with the other

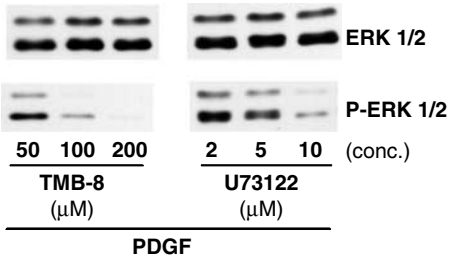

Figure 4 Calcium dependence of PDGF-induced ERK1/2 phosphorylation. A7r5 cells were treated with inhibitors as indicated. After 30 min, cells were stimulated with PDGF (10 ng ml^{-1}) for 5 min. Cellular lysates were probed with phospho-specific ERK1/2 antisera. Equal loading is demonstrated with antibodies detecting the respective total proteins.

mitogens, EGF (10 ng ml^{-1}) and thrombin (1 U ml^{-1}) (not shown).

Taken together, these results suggest that PDGF-induced *$rK_{Ca}3.1$* expression and augmented IK_{Ca} functions are mediated *via* activation of the Raf/MEK- and ERK-signaling cascade in a Ca^{2+}-dependent manner.

PDGF downregulates BK_{Ca} function and BK_{Ca}/$rK_{Ca}1.1$ expression in A7r5 cells

Our previous studies on K_{Ca} functions in proliferating neointimal VSMC *in situ* revealed that upregulation of *$rK_{Ca}3.1$* functions was paralleled by a loss of BK_{Ca} function and *$rK_{Ca}1.1$* expression (Kohler *et al*., 2003). We therefore hypothesized that this loss of BK_{Ca} function and expression may also be a consequence of mitogenic stimulation in VSMC.

Patch-clamp experiments were conducted in the presence of 100 nM TRAM-34 to block IK_{Ca} currents and unmask small BK_{Ca} currents in A7r5 cells. Following 48 h treatment with PDGF the current amplitude of BK_{Ca} present in unstimulated cells was reduced (Figure 5a, upper and lower panels). With respect to BK_{Ca}, parallel RT–PCR studies revealed that mRNA levels of r*Slo* encoding the pore-forming α-subunit of the BK_{Ca} channel complex were significantly decreased by half after PDGF stimulation for 2 h (Figure 5b). Similar to PDGF, FCS stimulation for 48 h significantly diminished BK_{Ca}/*$rK_{Ca}1.1$* expression levels by half (Figure 5b).

PDGF-induced downregulation of BK_{Ca}/$rK_{Ca}1.1$ expression does not require the activation of the Raf/MEK and ERK-signaling cascade

To characterize the signal transduction mechanisms underlying the PDGF-induced downregulation of BK_{Ca}/*$rK_{Ca}1.1$* expression we analyzed BK_{Ca}/*$rK_{Ca}1.1$* expression in the same sets of experiments as stated above. The modest downregulation of BK_{Ca}/*$rK_{Ca}1.1$* expression at 2 h following mitogenic stimulation was not prevented by inhibiting ERK1/2 phosphorylation with PD98059, p38 MAP kinase with SB203580, or of JNK with SP600125 (Figure 5b). Additionally, pretreatment of A7r5 cells with BAPTA-AM (not shown) and TMB-8, inhibition of PKC with chelerythrine, Src kinases with PP2, or PI3-K with wortmannin had no effects on PDGF-induced reduction of BK_{Ca}/*$rK_{Ca}1.1$* expression (Figure 5b), whereas blockade of CaMKII with KN-93 prevented downregulation of BK_{Ca}/*$rK_{Ca}1.1$* expression

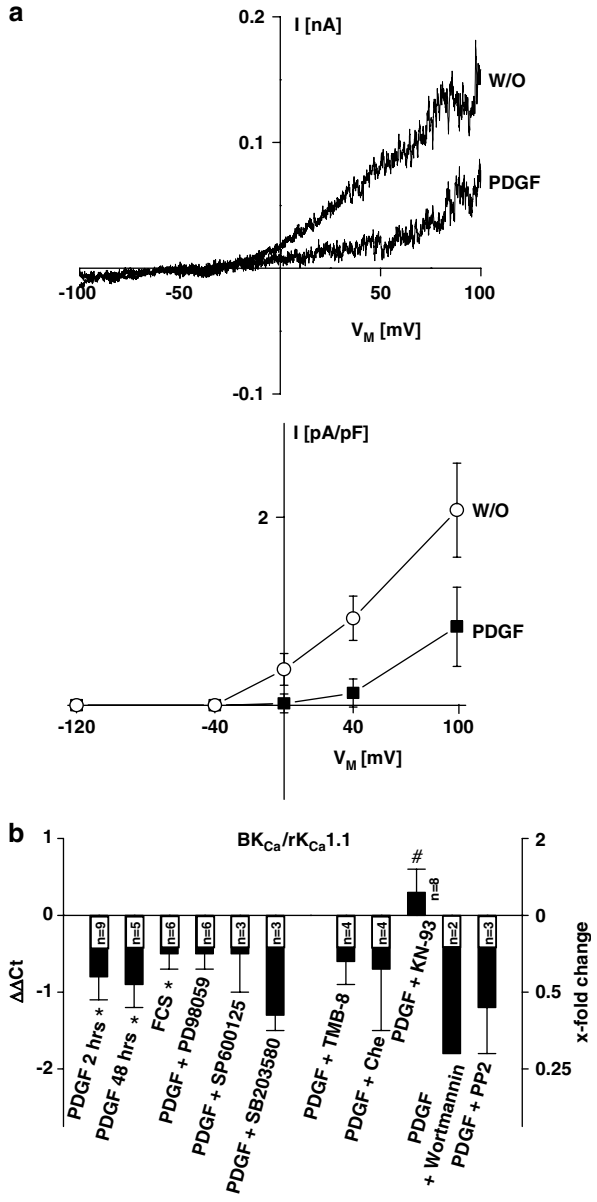

Figure 5 Mitogenic stimulation with PDGF for 48 h resulted in decreased functional expression of BK_{Ca}/*$rK_{Ca}1.1$* in A7r5 cells. (a) Upper panel, Representative whole-cell recordings of BK_{Ca} currents and, lower panel, mean BK_{Ca} currents (normalized to cell capacitance) in PDGF-stimulated and -unstimulated (w/o) A7r5 cells in the presence of the IK_{Ca}-blocker TRAM-34 (100 nM). Data points represent mean ± s.e., $*P<0.05$, Student's *t*-test. (b) Effects of MAPK blockers and other signal transduction modulators on mRNA expression levels of BK_{Ca}/*$rK_{Ca}1.1$* in mitogen-stimulated A7r5 cells. Semiquantitative RT–PCR analysis of BK_{Ca}/*$rK_{Ca}1.1$* mRNA expression levels following stimulation with PDGF for 2 h and with FCS (10%) for 48 h as well as following 2 h-PDGF stimulation after pretreatment with signal transduction inhibitors. Charts: left ordinate: $\Delta\Delta C_t$ values ($\Delta\Delta C_t = \Delta C_{t_{w/o}} - \Delta C_{t_x}$) represent change in expression over control (unstimulated VSMC ($n=13$)). Right ordinate: *x*-fold change ($2^{\Delta\Delta C_t}$) above control. Data are given as mean ± s.e.; $*P<0.01$ vs w/o; $^{\#}P<0.01$ vs PDGF alone (ANOVA).

(Figure 5b). Overall, these results suggest that, in contrast to *$rK_{Ca}3.1$*, the PDGF-induced downregulation of BK_{Ca}/*$rK_{Ca}1.1$* expression in A7r5 cells is most likely independent of the

activation of MAP kinases and Ca^{2+} mobilization, but may require CaMKII activity.

Knockdown of $rK_{Ca}3.1$ expression by siRNA inhibits mitogenesis in A7r5 cells in vitro

As described earlier, pharmacological inhibition of IK_{Ca} channel activity by TRAM-34 largely attenuated epidermal growth factor (EGF)-induced mitogenesis in VSMC *in vitro* and neointima formation *in vivo* (Kohler *et al*., 2003). To further support and specify the importance of IK_{Ca} channels for mitogenesis in A7r5 cells, a siRNA approach was chosen. For this purpose, we transfected A7r5 cells with either siRNA directed towards *$rK_{Ca}3.1$* mRNA or suitable siRNA controls of equal length with no known mRNA interference. Following siRNA transfection using the siPort *Amine*™ transfection reagent, cells were further cultivated in medium containing 10% FCS or 10 ng ml^{-1} PDGF and cell number as well as K_{Ca} functions were determined after 48 h.

IK_{Ca} functions were absent in all *$rK_{Ca}3.1$*-siRNA-transfected cells ($n=11$) which were investigated by patch clamping. In contrast, Ctrl-siRNA-transfected cells ($n=14$) exhibited normal IK_{Ca} functions similar to the increased IK_{Ca} functions observed in PDGF- or FCS-stimulated cells. Original current traces and mean K_{Ca} currents normalized to cell capacitance are shown in Figure 6a and b.

In contrast to the effect on IK_{Ca} functions, transfection with *$rK_{Ca}3.1$*-siRNA had no impact on BK_{Ca} functions (Figure 6a and b) which were similar to the small BK_{Ca} functions observed in mitogen-stimulated cells (Figure 5a). This indicates that the IK_{Ca} currents in A7r5 cells are indeed encoded by the *$rK_{Ca}3.1$* gene and that the *$rK_{Ca}3.1$*-siRNA did not interfere with BK_{Ca}/*$rK_{Ca}1.1$* mRNA. Moreover, the deficiency in IK_{Ca} functions does not seem to be compensated by an upregulation of BK_{Ca} functions.

The proliferation studies revealed that Ctrl-siRNA-transfected cells were still able to proliferate in response to FCS and PDGF stimulation, whereas a significant proliferation of *$rK_{Ca}3.1$*-siRNA-transfected cells was not observed (Figure 6c). A similar suppression of proliferation was observed in the presence of the Ca^{2+}-chelator TMB-8 or the MEK-inhibitor PD98059 (not shown) as also described previously (Iwasaki *et al*., 1998). These results suggest that the presence of IK_{Ca} channels and upregulation of *$rK_{Ca}3.1$* gene expression are required for mitogen-induced VSMC proliferation as has been reported in neointimal VSMC (Kohler *et al*., 2003), and mitogenesis of cultured VSMC, fibroblasts, and lymphocytes *in vitro*.

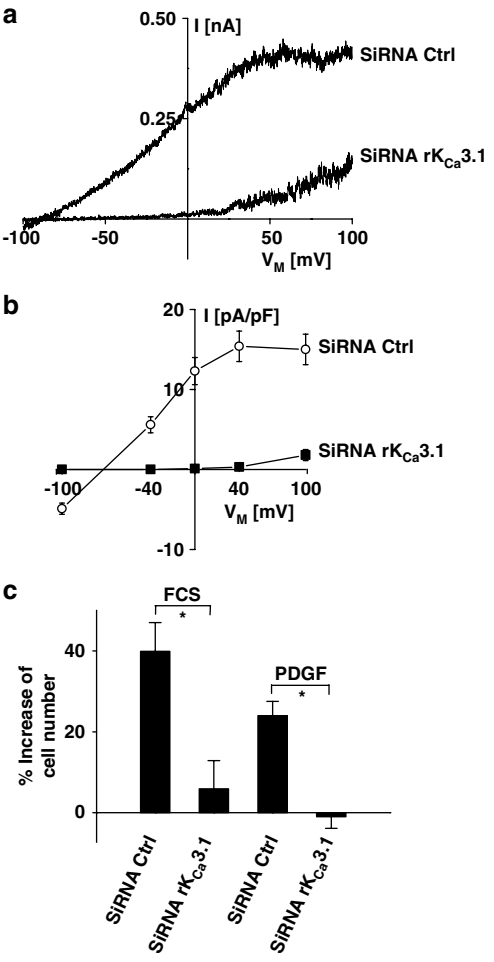

Figure 6 SiRNA against *$rK_{Ca}3.1$* abolishes functional expression of IK_{Ca}/*$rK_{Ca}3.1$* and mitogenesis in A7r5 cells. (a) Original whole-cell recording showing absence of functional IK_{Ca}/*$rK_{Ca}3.1$* in *$rK_{Ca}3.1$*-siRNA-treated A7r5 cells ($n=11$; from three independent experiments), but not in Ctrl-siRNA-treated cells ($n=14$). Note that BK_{Ca}/*$rK_{Ca}1.1$* currents were not affected by this treatment. (b) Mean K_{Ca} currents normalized to cell capacitance in rIK_{Ca}1-siRNA- and Ctrl-siRNA-transfected A7r5 cells. (c) Knockdown of *$rK_{Ca}3.1$* expression by *$IK_{Ca}3.1$*-selective siRNA but not Ctrl-siRNA abolishes A7r5 cell proliferation (% increase of Cell No.) induced by FCS (10%) or PDGF (10 ng ml^{-1}). Values are given as mean ± s.e.; $^*P<0.01$; Student's *t*-test.

Discussion

The results of the present study demonstrate that binding of PDGF-BB to its respective receptors leads to an upregulation of *$rK_{Ca}3.1$* mRNA expression and of IK_{Ca} functions in the rat cell line A7r5 which was dependent on the activation of the Raf/MEK and ERK signaling cascade and most likely required $[Ca^{2+}]_i$. In contrast to IK_{Ca}, low levels of BK_{Ca}/*$rK_{Ca}1.1$* mRNA expression and function in unstimulated A7r5 cells further decreased following mitogenic stimulation. Evaluation of participating signaling pathways revealed, that neither an activation of the Raf/MEK and ERK MAPK signaling or $[Ca^{2+}]_i$ transients were required for this events. The upregulation of IK_{Ca} seems to be an important mechanism in the control of mitogen-induced A7r5 cell proliferation since deficiency in *$rK_{Ca}3.1$* abolished A7r5 cell proliferation similar to the mitogenesis-suppressing effects of selective and non-selective IK_{Ca} blockers in cultured VSMC as well as in neointima formation *in vivo* (Kohler *et al*., 2003).

There is growing evidence that several ion channels are of importance in the control of cell proliferation (Ghanshani *et al*., 2000; Pena *et al*., 2000; Nilius & Droogmans, 2001; Kohler *et al*., 2003; Jager *et al*., 2004; Ouadid-Ahidouch *et al*., 2004; Grgic *et al*., 2005). Among the large family of K^+ channels, especially the IK_{Ca} channel has been shown to regulate cell cycle progression and mitogenesis in T lymphocytes, fibroblasts, and several tumor cell lines *in vitro* (Ghanshani *et al*., 2000; Jager *et al*., 2004; Ouadid-Ahidouch

et al., 2004; Grgic *et al*., 2005). Upregulation of this channel has also been found in tumor tissues and in proliferating, EGF-stimulated VSMC *in vitro* and in neointimal VSMC *in vivo* (Kohler *et al*., 2003). IK_{Ca} seems to participate in cell proliferation by increasing the ability of the cell to hyperpolarize and thereby to promote Ca^{2+} influx. Increased intracellular Ca^{2+} has been demonstrated to be required for progression through the Ca^{2+}-dependent cell cycle control point, that is from G1 to S (Berridge, 2001; Jager *et al*., 2004; Ouadid-Ahidouch *et al*., 2004). In addition, K^+ efflux through IK_{Ca} channels and the concomitant loss of water may mediate regulatory volume decrease (RVD) to counteract cell swelling which often occurs in the early phase of proliferation (Jakab *et al*., 2002). Such a role in RVD during cell proliferation has also been proposed for volume-regulated chloride channels (Jager *et al*., 2004). Correspondingly, blockers of both, K^+ and Cl^- channels have been shown to induce growth arrest in several cell types (Eggermont *et al*., 2001).

Thus far, little is know about the underlying signal transduction mechanism leading to an upregulation of this channel in VSMC. In T lymphocytes, studies revealed that stimulation of PKC resulted in an upregulation of *$K_{Ca}3.1$* mRNA expression in an AP-1 transcription factor-dependent manner (Ghanshani *et al*., 2000), and in fibroblasts, transforming growth factor-β-induced upregulation of IK_{Ca} function was sensitive to MEK inhibitors (Pena *et al*., 2000). Interestingly, in pancreatic cancer cell lines with 'overactive' Ras/Raf/MEK/ERK activity, IK_{Ca} functions and *$K_{Ca}3.1$* mRNA expression levels were dramatically increased (Jager *et al*., 2004). Together these studies suggest that the activation of Ras/Raf/MEK/ERK is critically linked to the regulation *$K_{Ca}3.1$* mRNA expression following stimulation with growth factors and activation of their respective receptors.

In A7r5 cells which we used in the present study as well as other VSMC (Reusch *et al*., 2001b), stimulation of the PDGF receptors resulted in ERK1/2 phosphorylation and an increase in $[Ca^{2+}]_i$ transients. As expected, ERK phosphorylation was suppressed by the use of MEK inhibitors. Additionally, a concomitant increase in $[Ca^{2+}]_i$ seemed to be required for ERK phosphorylation since suppression of $[Ca^{2+}]_i$ release from internal stores by either blocking PLC activity or manipulations of $[Ca^{2+}]_i$ abolished ERK phosphorylation. These results correlate well with our findings regarding the regulation of *$rK_{Ca}3.1$* mRNA expression. Minimizing $[Ca^{2+}]_i$ and inhibition of ERK phosphorylation completely abolished the early upregulation of *$K_{Ca}3.1$* mRNA expression and inhibition of ERK phosphorylation prevented the increase in IK_{Ca} functions following PDGF stimulation. Further MAP kinases do not seem to be involved in the regulation of *$K_{Ca}3.1$* mRNA expression, since neither inhibitors of JNK nor p38 MAP kinase revealed any effects on mRNA expression. Similar to PDGF, a stimulation with thrombin ($1\,U\,ml^{-1}$) induced an increase in $[Ca^{2+}]_i$ transients and ERK phosphorylation, although the degree of ERK phosphorylation appeared to be much weaker and more transient in nature than following PDGF stimulation. However, thrombin failed to upregulate *$rK_{Ca}3.1$* mRNA expression and IK_{Ca} function. Thus, activation of the Ras/Raf/MEK/ERK-signaling cascade is required but not sufficient to upregulate *$rK_{Ca}3.1$* mRNA expression.

In contrast to $K_{Ca}3.1$, BK_{Ca}/*$rK_{Ca}1.1$* mRNA expression and BK_{Ca} functions tended to decrease following mitogenic stimulation of A7r5 cells. Such a diminished or even absent BK_{Ca}/*$rK_{Ca}1.1$* mRNA expression and BK_{Ca} function has also been observed in proliferating neointimal VSMC *in situ* and in cultured proliferating VSMC *in vitro* (Kohler *et al*., 2003). In differentiated VSMC, BK_{Ca} channels mediate repolarization and the spontaneous transient outward currents resulting in a more negative membrane potential attenuate the depolarization-mediated activation of voltage-gated Ca^{2+}-channels. Therefore, the loss of BK_{Ca} functions together with an upregulation of $K_{Ca}3.1$ may be indicative of dedifferentiation and a change in functional plasticity as the cells switch from a contractile to a proliferating phenotype. In contrast to $K_{Ca}3.1$, the downregulation of BK_{Ca}/*$rK_{Ca}1.1$* mRNA expression in A7r5 cells appeared to occur independently of a participation of the Raf/MEK- and ERK- and JNK-, or p38-signaling cascade. With respect to upstream modulators of MAPK signaling, neither inhibition of PKC, Src kinases, Ca^{2+} signaling, nor PI-3 kinase was effective in preventing the observed downregulation of BK_{Ca}/*$rK_{Ca}1.1$* mRNA expression.

IK_{Ca} channels have been proposed to participate in the control of mitogen-induced proliferation of several cell types such as fibroblast, VSMC *in vitro*, cancer cell lines (Grgic *et al*., 2005), and activated T lymphocytes. Correspondingly, during initial experiments, inhibition of IK_{Ca} channels inhibited cell proliferation *in vitro* as well as neointima formation following angioplasty *in vivo*. To further support this pivotal role of IK_{Ca} channels for mitogenesis in VSMC, we used a siRNA strategy in which a deficiency of IK_{Ca} channels should inhibit VSMC proliferation. The results show that A7r5 cells, in which the IK_{Ca} channel functions were successfully eliminated, do not proliferate. This finding underlines the importance of IK_{Ca} channels for the observed A7r5 cell proliferation and demonstrates that IK_{Ca} currents in A7r5 cells are indeed encoded by the *$rK_{Ca}3.1$* gene. Since *$rK_{Ca}3.1$*-siRNA-transfected cells are devoid of any voltage-independent K_{Ca} currents, a deficiency in IK_{Ca} channels does not seem to be rapidly compensated by an increased functional expression of closely related K_{Ca} channels such as SK_{Ca} or by the low functional expression of BK_{Ca}/*$rK_{Ca}1.1$* channels present in these cells.

In summary, the present study demonstrated that stimulation of A7r5 cells with PDGF-BB results in a significant and long-lasting upregulation of IK_{Ca} channels. This upregulation is mediated *via* an activation of the Raf/MEK- and ERK-signaling cascade and most likely requires calcium fluxes. Deficient IK_{Ca} channels dramatically affected growth factor-induced A7r5 cells proliferation *in vitro*. These findings together with results of our earlier studies indicate that IK_{Ca} channels may participate during the pathogenic processes underlying cardiovascular disease states such as hypertension, arteriosclerosis, or neointima formation following angioplasty characterized by abnormal VSMC proliferation and vascular remodeling.

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