Gβγ mediate differentiation of vascular smooth muscle cells

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Running title: Gβγ-induced differentiation of smooth muscle cells
SUMMARY

Proliferation and subsequent dedifferentiation of vascular smooth muscle (VSM) cells contribute to the pathogenesis of atherosclerosis and postangioplasty restenosis. The dedifferentiation of VSM cells in vivo or in cell culture is characterized by a loss of contractile proteins such as smooth muscle-specific α-actin and myosin heavy chain (SM-MHC). Serum increased the expression of contractile proteins in neonatal rat VSM cells indicating a redifferentiation process. RNase protection assays defined thrombin as a serum component that increases the abundance of SM-MHC transcripts. Additionally, serum and thrombin transiently elevated cytosolic Ca\textsuperscript{2+} concentrations, led to a biphasic ERK phosphorylation, upregulated a transfected SM-MHC promoter construct, and induced expression of the contractile proteins SM-MHC and α-actin. Pertussis toxin, N17-Ras/Raf, and PD 98059 prevented both the serum- and thrombin-induced second phase ERK phosphorylation and SM-MHC promoter activation. Constitutively active Gα\textsubscript{q}, Gα\textsubscript{i}, Gα\textsubscript{12}, and Gα\textsubscript{13} failed to upregulate SM-MHC transcription whereas Gβγ concentration-dependently increased the SM-MHC promoter activity. Furthermore, the Gβγ-scavenger βARK1ct abolished the serum-mediated differentiation. We conclude that receptor-mediated differentiation of VSM cells requires Gβγ and an intact Ras/Raf/MEK/ERK signaling.
INTRODUCTION

Fully differentiated, contractile vascular smooth muscle (VSM) cells are major determinants of blood pressure and flow. In chronic vascular diseases such as hypertension and atherosclerosis, VSM cells proliferate and undergo a phenotypic modulation characterized by local matrix degradation and a loss of contractile function (1). In vivo, dedifferentiated VSM cells can gradually revert towards a more contractile phenotype (2). Interest in the underlying mechanisms and participating signal transduction pathways leading to altered phenotypes of VSM cells has led to extensive study of the VSM cell phenotype both in vivo and in vitro (for review see 3).

Differentiated VSM cells are characterized by high expression levels of contractile proteins such as smooth muscle α-actin (SM-α-actin) and smooth muscle myosin heavy chain (SM-MHC) (4). The expression of SM-MHC isoforms SM-1 and SM-2 is restricted to smooth muscle cells (5, 6) and is downregulated in proliferating cells (7). High expression levels of SM-1/2, therefore, are valuable markers for the differentiated phenotype of VSM cells. Similar to pathological proliferation during vascular disease, VSM cells downregulate SM-1/2 expression in primary culture. Although cultured VSM cells initially retain SM-1/2 expression when cultured on laminin or under serum-free conditions, they undergo morphological changes towards a dedifferentiated phenotype within a few days (8). Patterns of gene expression similar to those in cultured VSM cells from neonatal rats have been observed in neointimal cells within injured vessels (9). Neonatal VSM cells can, therefore, provide an in vitro model for studying phenotypic modulation processes in vascular disease. The mechanisms and signaling pathways that induce a phenotypic modulation towards the contractile phenotype of VSM cells are still largely elusive.

It has been shown that application of mechanical forces can actively change the VSM phenotype (10). Depending on extracellular matrix composition, cultured VSM cells can either proliferate or
differentiate in response to mechanical strain (11). These findings were recently corroborated by applying mechanical forces to cultured whole vessels (12). Interestingly, phenotypic modulation of VSM cells depends on the activation of mitogen activated protein kinases (MAPK) in both experimental settings.

In many cellular systems, the receptor-mediated proliferation and differentiation involves the extracellular signal regulated kinase (ERK) subfamily of MAPK (13, 14). ERKs are part of a multi-kinase module through which a variety of extracellular stimuli (growth factors, differentiation signals and cellular stress) are transmitted into the cell (15). Receptor tyrosine kinases (RTKs), upon autophosphorylation and activation of adaptor proteins, recruit Ras and subsequently engage the Raf/MEK/ERK cascade. Alternatively, G protein-coupled receptors (GPCRs) have been shown to stimulate ERKs via the G\textsubscript{i}-, G\textsubscript{q}- or G\textsubscript{12/13}-subfamilies of heterotrimeric G proteins. In addition, transactivation of receptor tyrosine kinases has been demonstrated to participate in signaling from GPCRs to ERKs (16-18).

Receptor-mediated signaling pathways which alter the phenotype of VSM cells are poorly defined. We, therefore, studied receptor-mediated pathways in neonatal rat VSM cells and in particular their participation during phenotypic modulation. Our findings clearly indicate that G\textsubscript{i}βγ subunits released from the G\textsubscript{i}-subfamily of heterotrimeric G proteins mediate enhanced expression of contractile proteins in VSM cells.
EXPERIMENTAL PROCEDURES

Materials
Culture media and trypsin were purchased from Life Technologies. Fetal calf serum and phosphate-buffered saline were obtained from Biochrom. Radiochemicals were from NEN Life Science Products. The Maxiscript and RPA II kits from Ambion were used for RNase protection assays. The anti-SM-1/SM-2 antiserum was kindly provided by Berlex Pharmaceuticals. Pertussis toxin (PTX), recombinant platelet derived growth factor (PDGF-BB), bisindolylmaleimide (BIM) and phorbol-12-myristate-13-acetate (PMA) were obtained from Calbiochem. Thrombin receptor-activating peptide (TRAP, SFLLRNPNKYEPF) was purchased from Tocris. All other reagents were obtained from Sigma.

Cell Culture, Transient Transfections and Reporter Assays
Primary cultures of VSM cells from newborn rats were established as previously described (19). Cells were grown in MEM supplemented with 10% fetal calf serum (complete medium, CM), 2% tryptose phosphate broth, penicillin (50 units/ml), and streptomycin (50 units/ml). In all experiments, cells from passages 10-15 were used. Growth arrest was induced in a serum-free quiescent medium (QM) containing 1% (w/v) BSA and 4 mg/ml transferrin instead of serum. Prior to agonist application, cells were maintained in QM for 48-72 h. Where indicated, cells were pretreated with PTX (200 ng/ml) for 12-18 h.

The transcriptional regulation of SM-1/SM-2 was assessed with a chloramphenicol acetyl transferase (CAT) reporter gene expressed under the control of the myosin heavy chain promoter (nt -1346 to +25, pCAT-1346) (20). For transient transfection assays, cells were seeded into 6-well plates at a density of 7.5 x 10⁴ cells/well (60-80% confluency) and growth-arrested in QM for 48 h prior to transfection. Transient transfections were performed in triplicates with 1 µg of
plasmid DNA and 10 µl/well Superfect transfection reagent (Quiagen) for 5 h. After 48 h, cell lysates were prepared using the CAT Enzyme Assay System (Promega). CAT activities were normalized to the protein concentration of each sample as measured by the BCA assay. Transfection of a promoterless CAT construct served as a base-line indicator, allowing all other promoter constructs to be expressed relative to promoterless activity. All CAT activities (means ± SEM) represent at least three independent transfection experiments with each setting tested in triplicate per experiment. Cotransfection of a viral promoter/β-Gal or LacZ construct to control for transfection efficiency was discontinued since variations in transfection efficiency among independent experimental samples are small (≤ 10%). Furthermore, it has been shown that such constructs interfere with SM-specific promoters, presumably due to competition for common transcription factors (21).

Immunostaining

VSM cells were grown to confluency on Nunc Chamber Slides (Nalge Nunc International). After fixation in 1% formaline in PBS and methanol, a monoclonal anti-proliferating cell nuclear antigen (anti-PCNA) antibody (1:100; DAKO) was incubated for 1 h at 20°C in PBS supplemented with 5% fetal calf serum. A secondary, biotinylated anti-mouse IgG (Sigma) and streptavidin-conjugated Texas red (Amersham Pharmacia) were applied for detection. After the PCNA staining, smooth muscle α-actin was detected by using a monoclonal antibody (1:150; Sigma) and a FITC-conjugated goat anti-mouse (1:40, Dianova). Representative visual fields were sequentially exposed by applying appropriate filter sets.

Immunoblotting procedures

VSM cells were directly lysed in 1x Laemmli buffer containing 10 mM dithiothreitol. Proteins were separated on polyacrylamide gels and electroblotted to nitrocellulose membranes. Smooth
muscle myosin isoforms were separated on 4% gels and detected with a polyclonal anti-SM1/SM2 antiserum (1:1000). This antiserum has been characterized previously (11). ERK1/2 were separated on 10% gels and probed with affinity-purified polyclonal anti-phospho-ERK1/2 or with anti-ERK1/2 antibodies (New England Biolabs) to confirm equal loading of the gels. Primary antibodies were detected with a horseradish peroxidase-coupled secondary antibody (1:2000, New England Biolabs) using a chemiluminescence substrate (Lumiglo, New England Biolabs).

**RNase protection assay**

RNA isolation, generation of DNA templates and hybridization conditions have been described previously (11). In brief, 10 µg of total RNA were hybridized with a radiolabeled probe covering the alternatively spliced C-terminal exons of SM-1 and SM-2 variants of rat SM-MHC. After overnight incubation at 42°C, non-hybridized fragments were digested with a diluted RNase A/T1 mixture. The remaining protected fragments (380 nt for SM-2 and 261 nt for SM-1, respectively) were separated by denaturing (8% urea) polyacrylamide gel electrophoresis and exposed to Amersham Hyperfilm at -80°C for 2-24 h. Bands were excised and counted in a liquid scintillation counter. Equal loading was controlled by hybridization of a second aliquot with a rat glutaraldehyde-phosphate-dehydrogenase (GAPDH)-radiolabeled probe.

**Single-cell [Ca^{2+}]_i Measurements**

Cells were seeded on 24 mm glass coverslips and grown for 24 h prior to loading with fura-2 (2-4 µM) in a buffer (HBS) containing 135 mM NaCl, 6 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5.5 mM glucose, 10 mM Hepes pH 7.4, and 0.2% BSA. Coverslips were mounted in a monochromator-equipped (TILL-Photonics) inverted microscope (Carl Zeiss). Fura-2 was alternately excited at 340 nm and 380 nm. Emitted light was filtered (505 nm longpass) and
recorded with a 12-bit CCD-camera. After correction for background signals, intracellular Ca\(^{2+}\) concentrations were calculated as described (22). \(R_{\text{max}}\), \(R_{\text{min}}\) and \(F_{380\text{min}} / F_{380\text{max}}\) were determined in fura 2-loaded cells equilibrated for 3 h in HBS supplemented with 1 \(\mu\)M ionomycin and either 10 mM Ca\(^{2+}\) or 10 mM EGTA, pH 7.8.
RESULTS

Regulation of SM-MHC expression by serum components

The presence of serum is essential to grow VSM cells in primary cell culture. In addition to its mitogenic properties, we observed that fetal calf serum enhanced the expression of contractile proteins in neonatal rat VSM cells. Dual staining of proliferating cell nuclear antigen (PCNA) and smooth muscle-specific α-actin or SM-MHC revealed that after short-term (24 h) exposure to serum, PCNA expression was positive, whereas SM-α-actin was poorly detectable (Fig. 1A). Continuous culturing in the presence of serum for additional 4 days markedly enhanced the expression of SM-α-actin (Fig. 1B) and SM-MHC (data not shown). Consistently, the expression of the SM-1 and SM-2 splice-variants of smooth muscle-specific myosin heavy chain (SM-MHC) were upregulated by serum within 2-3 days as detected by immunoblotting of whole-cell lysates normalized for their protein content (Fig. 1C). Serum withdrawal reduced SM-α-actin and SM-1/SM-2 expression again within 48 h to about 20% of the expression levels in the presence of serum, demonstrating that alterations in contractile protein expression are bidirectional (data not shown). Thus, serum contains factors capable of inducing in vitro redifferentiation of rat neonatal VSM cells.

Since serum contains multiple growth factors, vasoactive peptides and other agonists mediating their responses through activation of several signaling pathways, we analyzed which receptor-coupled pathways are involved in increasing SM-MHC transcription. RNase protection assays revealed that SM-1/SM-2 transcripts are 22 ± 1.2-fold more abundant in serum-treated (CM) VSM cells as compared to serum-starved controls (QM). The relatively low expression levels of SM-1/SM-2 in serum-starved VSM cells allowed us to study the effect of single compounds on SM-1/SM-2 expression. Both, 1 µM angiotensin II and 10 ng/ml PDGF-BB did not alter the SM-
1/SM-2 expression significantly, whereas treatment with 10 ng/ml TGF-β resulted in a further reduction of SM-1/SM-2 steady-state expression (Fig. 2). In contrast, 1 U/ml thrombin increased SM-1/SM-2 expression by the 10 ± 0.9-fold. The substantial increase in SM-1/SM-2 steady-state expression most likely represents an upregulation of the transcriptional activity, although changes in RNA stability and/or turnover cannot be ruled out.

Since the inhomogeneous response to vasoactive peptides may rely on the presence or absence of the corresponding receptors in cultured VSM cell preparations, functional coupling of receptors in VSM cells was characterized by single-cell [Ca\textsuperscript{2+}]i analysis. Thrombin (0.1 U/ml) induced transient elevations of [Ca\textsuperscript{2+}]i in more than 95 % of VSM cells after a lag phase of 10 to 20 s (Fig. 3). To exclude unspecific, receptor-independent effects of the serine protease thrombin, we applied the thrombin receptor-activating peptide (TRAP), which corresponds to the intramolecularly tethered ligand of the PAR-1 receptor. Indeed, TRAP (80 µM) induced similar calcium transients without a protease typical lag phase (Fig. 3). Addition of lysophosphatidic acid (LPA) induced calcium responses in about 60% of the cells. Only few cells (less than 10%) were activated by angiotensin II (1 µM). Challenging VSM cells with PDGF-BB (10 ng/ml) elicited a delayed and more sustained elevation of [Ca\textsuperscript{2+}]i, characteristic for ligands binding to tyrosine kinase receptors (Fig. 3). Carbachol (100 µM) failed to raise [Ca\textsuperscript{2+}]i in VSM cells, indicating the absence of endothelial cell contaminations (data not shown). Using single-cell [Ca\textsuperscript{2+}]i analysis, we demonstrated that PAR receptors, Edg receptors and PDGF receptors are present and functional in the majority of VSM cells.

**Serum, thrombin and TRAP induce biphasic ERK phosphorylation**

Since both proliferative and differentiating signals can be transmitted via ERK1/2, depending on the cellular context and the transient or sustained character of their activation, we studied the
kinetics of ERK phosphorylation in VSM cells. A rapid and transient phosphorylation of ERK1/2 was elicited by serum, PDGF-BB, EGF, thrombin, TRAP, LPA, and - to a lower extent - by angiotensin II. The early ERK1/2 phosphorylation was maximal after 3-5 min for all agonists. Only serum, thrombin, TRAP and LPA elicited a delayed second phase ERK phosphorylation (Fig. 4A). The second phase ERK phosphorylation appeared approximately 45 min after agonist application and continuously rose for another 2 h. In contrast, a delayed ERK phosphorylation was absent in response to PDGF-BB, EGF, or angiotensin II. The weak and monophasic ERK1/2 activation by angiotensin II may rely on low AT1 receptor expression in our VSM cell preparation (Fig. 3). The early ERK1/2 phosphorylation, but not the late-phase ERK activation, closely correlated with the ability of agonists to raise 

\[ \text{Ca}^{2+} \]. Consistently, Ca\(^{2+}\)-ionophores (1 µM ionomycin or 1 µM A 23187) induced early but not delayed ERK1/2 phosphorylation (Fig. 4B). Permanent activation of protein kinases C by phorbol-12-myristate-13-acetate (PMA, 100 nM) resulted in a monophasic and sustained ERK1/2 activation (Fig. 4B). Thus, in VSM cells, three distinct temporal patterns of ERK1/2 phosphorylation are elicited by different receptor ligands, Ca\(^{2+}\)-ionophores or phorbol esters.

**Activation of Ras/Raf/MEK/ERK is a prerequisite for SM-MHC promoter activation by serum and thrombin**

Enhanced SM-1/SM-2 expression in response to thrombin (Fig. 2) is indicative of a G protein-mediated regulation of the SM-MHC promoter activity. To characterize signaling pathways that control transcription of contractile proteins, we studied the SM-MHC promoter activity by using a chloramphenicol acetyltransferase (CAT) reporter gene construct expressed under the control of the -1346 nt promoter region of the SM-MHC gene (pCAT-1346). In the absence of serum, CAT activities in VSM cells transfected with pCAT-1346 were approximately 4-6-fold higher as
compared to cells transfected with a promoterless pCAT-basic vector. Serum-treatment further increased the CAT activity by the 5.1 ± 0.5-fold (Fig. 5A). Expression of CAT driven by an SV40 promoter (pCAT-control) was about 4-fold higher as compared to the SM-MHC promoter in the presence of serum (data not shown). Transfection of pCAT-1346 in Swiss 3T3 fibroblasts did not significantly induce CAT activity irrespective of the absence or presence of serum (data not shown).

In serum-starved VSM cells transfected with pCAT-1346, the addition of PDGF-BB (10 ng/ml), EGF (10 ng/ml), LPA (10 µM), or thrombin (1 U/ml) resulted in a 1.1 ± 0.1, 1.2 ± 0.1, 2.1 ± 0.2, and 2.0 ± 0.1-fold increase in CAT activity over control cells incubated in serum-free quiescent medium (QM, Fig. 5A). These data confirm that increases in SM-1/SM-2 mRNA steady-state concentrations (Fig. 2) indeed result from transcriptional activation. Furthermore, the reporter gene assay allows for analysis of signaling cascades by applying genetically encoded modulators. To define participation of members of the Ras/Raf/MEK/ERK cascade, the reporter gene construct pCAT-1346 was cotransfected with expression plasmids encoding dominant negative N17-Ras or N17-Raf. In all cotransfection experiments the total amount of transfected plasmid cDNA was kept constant by adding cDNA encoding promoterless pCAT basic. The thrombin-stimulated CAT activity was abrogated by coexpression of dominant negative N17-Ras or N17-Raf in a concentration-dependent manner (Fig. 5B). Conversely, coexpression of the Raf-c-terminus increased CAT activity about 2-fold in the absence of agonists (data not shown). Additionally, the MEK inhibitor PD98059 largely reduced the thrombin-stimulated SM-MHC promoter activity (Fig. 5C). Since PD98059 was dissolved in DMSO, the effect of the solvent on the SM-MHC promoter activity was assessed in parallel. DMSO (up to 0.5%) further increased the thrombin-stimulated CAT activity almost 1.8-fold, an effect that was also blocked by PD98059. The observed half-maximal inhibitory concentration of PD98059 (3-5 µM) is well in
line with its described IC$_{50}$ to inhibit ERK1/2 phosphorylation (23). Correspondingly, in VSM cells the serum- or thrombin-mediated ERK1/2 phosphorylation was largely reduced by 5-20 µM PD98059 and abolished by 50 µM of the MEK inhibitor (data not shown). These higher concentrations, however, exhibited a toxic effect during long-term incubation of VSM, thereby precluding a subsequent determination of SM-MHC promoter activity. Both modulations, expression of dominant negative Ras/Raf and pretreatment of VSM cells with PD98059, also impaired the serum-mediated up-regulation of the SM-MHC promoter (data not shown). These data strongly suggest that the thrombin and serum-induced increase in SM-1/SM-2-expression depends on an intact Ras/Raf/MEK/ERK signaling cascade. Furthermore, the ability of different agonists to upregulate the SM-MHC promoter activity closely correlated with a biphasic and sustained ERK1/2 phosphorylation.

**Differentiation of VSM cells requires pertussis-toxin-sensitive G proteins**

The transient elevation of [Ca$^{2+}$]$_i$ and biphasic ERK1/2 phosphorylation induced by thrombin could be mimicked with the tethered ligand of the PAR-1 receptor, TRAP. PAR-1 receptors couple to the G$_i$, G$_q$, and G$_{12/13}$-subfamilies of heterotrimeric G proteins (24). The putative involvement of G$_{12/13}$ in the regulation of the SM-MHC promoter was tested by overexpressing constitutively active (GTPase-deficient) mutants of G$\alpha_{12}$ (pCIS/G$\alpha_{12}$ Q229L) and G$\alpha_{13}$ (pCIS/G$\alpha_{13}$ Q226L). Both constructs and their combination failed to significantly induce SM-MHC promoter activity over a wide range of transfected cDNA concentrations (data not shown). The biological activity of these constructs has been previously demonstrated by their ability to induce contraction of VSM cells (25). G$_q$ and G$_i$ proteins couple to phospholipases C $\beta$ to release [Ca$^{2+}$]$_i$ from inositol-1,4,5-trisphosphate-sensitive stores. The possible role of the G$_i$-class of heterotrimeric G proteins was assessed by pretreating cells with 200 ng/ml pertussis toxin (PTX)
for at least 18 h. Inactivation of G$_i$ proteins by this protocol was demonstrated by a more than 80% reduction of [Ca$^{2+}$]$_i$ signals in response to LPA (Fig. 6A). PTX reduced the peak [Ca$^{2+}$], after thrombin stimulation by about 40% (Fig. 6A). The partial block of thrombin-induced [Ca$^{2+}$], transients in PTX-pretreated VSM cells reflects coupling to both G$_i$ and G$_{q/11}$. To further test whether the G$_i$-subfamily also participates in the prolonged ERK1/2 activation, serum-starved VSM cells were pretreated with PTX. Subsequent addition of serum, thrombin, and LPA left early ERK1/2 phosphorylation almost unaltered, whereas the second phase of ERK1/2 activation was completely abrogated in PTX-pretreated cells (Fig. 6B).

Since sustained ERK activation may be required for the regulation of transcriptional activity, we tested whether PTX-pretreatment affects the ligand-induced SM-MHC promoter activity. In addition to the modulation of ERK1/2 signaling, PTX-treatment abolished the thrombin-induced upregulation of the SM-MHC promoter activity (Fig. 7A). Moreover, even the strong induction of the SM-MHC promoter by serum was completely reverted in PTX-pretreated VSM cells. This indicates that all serum components that are involved in the upregulation of the SM-MHC promoter depend on the presence of functional G$_i$ proteins.

Since either the α or the βγ subunits may transmit the signal that results in SM-MHC expression, we coexpressed the constitutively active Gα$_i$ (Q205L) together with pCAT-1346. Gα$_i$ (Q205L), however, even reduced the SM-MHC promoter activity below baseline values (Fig. 7B). On the contrary, coexpression of Gβ$_1$ and Gγ$_2$ mimicked the receptor-mediated upregulation of the SM-MHC promoter in a concentration-dependent fashion. Neither expression of Gβ$_1$ nor Gγ$_2$ alone was sufficient to increase the activity of the cotransfected CAT reporter. Consistent with an essential role of Gβγ, coexpression of the Gβγ-scavenging C-terminal peptide of the β-adrenergic
receptor kinase 1 (βARK1ct) concentration-dependently reverted the serum-induced activation of
the SM-MHC promoter (Fig. 7C).

Finally, the G\textsubscript{i} protein-dependent redifferentiation in response to thrombin and serum was
confirmed by analyzing the expression of contractile proteins in untransfected cells. In whole cell
lysates from VSM cells stimulated with thrombin or serum and normalized for protein content, an
increased expression of SM-α-actin and of SM-MHC was detected (Fig. 8). When incubated in
the continuous presence of PTX, thrombin and serum failed to increase the expression of both
contractile proteins (Fig. 8). Hence, these data demonstrate, that G\textsubscript{βγ} released from G\textsubscript{i} proteins
link proximal signaling to the Ras/Raf/MEK/ERK-cascade to mediate the \textit{in vitro}
redifferentiation of vascular smooth muscle cells shown in Fig. 1.
DISCUSSION

In this study we describe a receptor-mediated signaling pathway leading to differentiation of vascular smooth muscle cells. The thrombin-induced SM-MHC expression is transmitted via the Ras/Raf signaling cascade and leads to a biphasic temporal pattern of ERK1/2 phosphorylation. Pertussis toxin abrogated both the second phase ERK1/2 phosphorylation and the upregulation of contractile proteins in response to serum, thrombin and LPA. Since coexpression of Gβγ-subunits mimicked and βARK1ct abrogated the activation of the SM-MHC promoter in response to serum components, we conclude that Gβγ mediate the agonist-induced differentiation of VSM cells.

A limited number of reports describe a phenotypic modulation of mature VSM cells towards a more contractile phenotype. Vasoconstrictors such as angiotensin II or vasopressin have been shown to increase levels of steady-state mRNA and SM-α-actin protein expression (26-29). In both cases, CC(A/T)₆GG cis-elements (CArG-boxes) within the SM-α-actin promoter are essential for the ligand-induced promoter regulation. In addition, the same serum response factor (SRF)-binding cis-elements positively regulate the SM-MHC promoter activity in VSM cells (20, 30). The upstream signaling pathways that regulate the receptor-induced expression of contractile proteins in VSM cells are poorly defined. The findings presented herein demonstrate that serum components activate the Ras/Raf/MEK/ERK cascade in VSM cells. Since dominant negative N17-Ras, N17-Raf or the MEK-inhibitor PD98059 prevented the SM-MHC promoter regulation by serum and thrombin, the entire Ras/Raf/MEK/ERK cascade appears to be required. Activated ERKs may then translocate into the nucleus to phosphorylate Elk-1 or related transcription factors (31). This signaling cascade may therefore provide a comprehensible model for activation of SRFs in response to G protein-coupled receptors in VSM cells. Alternatively, Garat et al. (32) described that the vasopressin-mediated transcriptional regulation of SM-α-actin is completely
blocked by pharmacological inhibition of p38 and partially sensitive to overexpressed dominant negative JNKs. The upstream signaling molecules that link the vasopressin-induced receptor activation to the MAPK branches p38 and JNK, however, remain unresolved. Moreover, RhoA-mediated cytoskeletal rearrangements have been implicated in the regulation of the transcriptional regulation of SM22α and smooth-muscle α-actin (33). Since the authors observed an activation of these promoters by either pharmacological disruption or polymerization of actin stress fibers, RhoA-promoted cytoskeletal assembly cannot serve as the only mechanism for regulation of contractile protein expression in VSM cells. Nonetheless, these findings emphasize the importance of cytoskeletal structures for the maintenance of SM-α-actin expression in cultured VSM cells. Whether receptor-dependent signaling utilizes cytoskeletal dynamics to regulate the expression of contractile proteins, remains to be clarified. Our data outline that the serum-induced expression of SM-MHC is transmitted by the Ras/Raf/MEK/ERK cascade. ERKs therefore, either exclusively or in concert with other MAPKs, promote the expression of contractile proteins. Since thrombin stimulation activated ERKs, but neither p38 nor JNKs in VSM cells (Reusch, unpublished data), activation of ERKs appears to be both sufficient and necessary for receptor-mediated differentiation of VSM cells. The ERK pathway regulates two mutually opposing processes, cellular proliferation and differentiation, depending on the duration of activation and the cellular context (15, 34, 35). Our data indicate that a sustained rather than a short-lived ERK phosphorylation is a requirement for the differentiation of VSM cells. Several other cellular models including megakaryocytes (35), thymocytes (37) and PC-12 cells (38) support the idea that the short versus long-term ERK phosphorylation determines the proliferative or differentiating outcome, respectively.

Multiple upstream signaling pathways link receptor activation to phosphorylation of ERK1/2. In VSM cells, the biphasic kinetic pattern of ERK-phosphorylation in response to serum, thrombin,
or LPA suggests that at least two independent pathways control the early and delayed phases of ERK phosphorylation. Considering that strong Ca\textsuperscript{2+}-signals result from thrombin stimulation of VSM cells, a Ca\textsuperscript{2+}- and PKC-dependent formation of Ras/Raf-1 complexes (39, 40) may engage ERKs. Alternatively, Ca\textsuperscript{2+}/calmodulin-dependent activation of Pyk2 (41) and subsequent Src-activation may target Ras either including (42-44) or bypassing transactivated receptor tyrosine kinases (45, 46). Since Ca\textsuperscript{2+}-ionophores evoked large [Ca\textsuperscript{2+}]\textsubscript{i} signals, but failed to induce a long-lived ERK phosphorylation in VSM cells, an isolate Ca\textsuperscript{2+}-elevation was not sufficient to mimick the effects of serum components. In PTX-pretreated VSM-cells stimulated with serum, thrombin, or LPA, the remaining activation of G\textsubscript{q/11} and G\textsubscript{12/13} induced an early ERK activation, but failed to generate a sustained phospho-ERK signal. Since PTX pretreatment also abolished the contractile protein expression in response to serum and thrombin, we focussed on signaling pathways that are initiated by either G\textsubscript{\alpha\textsubscript{i}} or G\textsubscript{\beta\gamma} subunits released from activated G\textsubscript{i} proteins. The inhibition of adenylyl cyclases by G\textsubscript{\alpha\textsubscript{i}} lowers cAMP concentrations and subsequently protein kinase A activity. Signaling via the Ras/Raf/MEK/ERK cascade is counterregulated by protein kinase A-dependent phosphorylation and inactivation of Raf-1 (47, 48). Indeed, forskolin-treatment further reduced the basal ERK-phosphorylation in serum-starved VSM cells (Reusch, unpublished data). Thus, a disinhibition of Ras-Raf-signaling by further reducing the cAMP concentrations in quiescent cells might result in an increased activity of the c-Raf kinase as has been shown for another cell system (49). However, expression of constitutively active G\textsubscript{\alpha\textsubscript{i}} (Q205L) failed to increase the SM-MHC promoter activity. Most strikingly, coexpression of G\textsubscript{\beta\gamma} subunits mimicked while the G\textsubscript{\beta\gamma}-scavenger \textbeta\textsubscript{ARK1ct} attenuated the effects of thrombin or LPA. Within the multiple G\textsubscript{\beta\gamma} effector systems, G\textsubscript{\beta\gamma}-sensitive PLC-isoforms, phosphoinositide-3-kinases, or further unknown effectors bear the potential to feed into the Ras/Raf/MEK/ERK cascade. Although the molecular
mechanisms are currently poorly defined, a growing body of evidence points to a role of Gβγ in initiating the assembly of a multi-protein-complex including β-arrestin and c-Src in clathrin-coated pits (50, 51). Within these microdomains, a ligand-independent transactivation of receptor tyrosine kinases such as the EGF-receptor may link Gβγ signaling to Ras. Other concepts favour a direct association of Gβγ with Raf-1 (52), or the activation of a Ras-GEF other than Sos1 (52). Our preliminary data demonstrating that the tyrphostin AG 1478 prevents the thrombin-induced ERK phosphorylation in VSM cells, point to a crucial role of an EGF-receptor transactivation.

Receptors for endogenous vasoconstrictors such as endothelin-1, angiotensin II, and vasopressin or serum components like thrombin or LPA activate the Gαq-, Gαγ, and G12/13-classes of heterotrimeric G proteins. The Gαq-dependent pathway, via phospholipase C β-catalyzed formation of inositol-1,4,5-trisphosphate, increases [Ca^{2+}]i to activate the calmodulin-dependent myosin light chain kinase. In parallel, activated G12/13, via RhoA and Rho-kinase, inhibits a myosin phosphatase (25). Both pathways synergistically control the contraction of VSM cells by increasing the myosin light chain (MLC20) phosphorylation. The additional coupling to G1i proteins does not contribute to the acute regulation of contraction (25). It is, therefore, tempting to speculate that Gβγ released from G1i proteins function to maintain the contractile phenotype by enhancing the expression of contractile proteins.

In summary, we have defined the G1-component of multiply coupling receptors as a pivotal step in the receptor-mediated expression of contractile proteins. Our data clearly indicate that Gβγ-subunits induce a sustained ERK phosphorylation which is critical for the differentiation of VSM cells. In the past, substantial data have been accumulated regarding the serum-factor-dependent promoter regulation of contractile proteins. The addition of our data, demonstrating how receptor-mediated differentiation signals may be transmitted to the nucleus, may converge to a more
clearly defined step-by-step model describing the regulation of contractile protein expression in vascular smooth muscle cells.

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FOOTNOTES:

The abbreviations used are: CAT, chloramphenicol acetyltransferase; $[\text{Ca}^{2+}]_i$, cytosolic Ca$^{2+}$ concentration; ERK, extracellular signal regulated kinase; MAPK, mitogen activated protein kinase; MEK, ERK kinase; PDGF, platelet derived growth factor; PTX, pertussis toxin; SM-$\alpha$-actin, smooth muscle $\alpha$-actin; SM-MHC, smooth muscle myosin heavy chain; VSM, vascular smooth muscle, $\beta$ARK1ct, $\beta$-adrenergic receptor kinase 1 C-terminal peptide.
FIGURE LEGENDS

Figure 1: Effects of serum on the expression of contractile proteins in vascular smooth muscle cells.

Rat neonatal vascular smooth muscle (VSM) cells were maintained in serum-free quiescent medium (QM) for 48 h and then reexposed to serum for 24 h (A) or 120 h (B). Filamentous smooth muscle-specific α-actin (green) and proliferating cell nuclear antigen (red) reflecting replicating nuclei were identified with immunofluorescence applying appropriate first and secondary antibodies (see Materials and Methods). Epifluorescence microphotographs were sequentially taken and then superimposed. The images represent typical viewing fields of six independent SM-α-actin staining experiments showing similar results. White bars depict a 50-µm-scale. (C) Cell lysates were prepared from VSM cells cultured without serum (QM) and then re-exposed to serum (10%) for the indicated number of days. Whole-cell lysates (20 µg protein/lane) were separated on a 4 % SDS-PAGE, electroblotted and probed with a polyclonal antiserum detecting both SM-1 (204 kDa) and SM-2 (200 kDa) isoforms of smooth muscle-specific myosin heavy chain.

Figure 2: Serum and receptor ligands upregulate transcription of the SM-1/SM-2 genes in VSM cells.

VSM cells were cultured either in serum-containing complete medium (CM), in serum-free quiescent medium (QM), or in QM supplemented with PDGF-BB (10 ng/ml), TGF-β (1 ng/ml), thrombin (1 U/ml), and angiotensin II (Ang-II, 1 µM) for the indicated times. RNase protection assays were performed with 10 µg total RNA/lane. The lengths of protected fragments correspond to the expected sizes for the SM-1 and SM-2 splice-variants of smooth muscle myosin heavy chain (P, full-length probe). Abundance of a control transcript was unaltered as demonstrated by reprobing equal aliquots of total RNA with a GAPDH probe (data not shown).
Figure 3: Agonist-induced transient elevation of \([Ca^{2+}]_i\) in single VSM cells.

VSM cells were subcultured on glass coverslips, loaded with the fluorescent \(Ca^{2+}\)-indicator fura-2 and washed in a Hepes-buffered medium containing 1 mM \(Ca^{2+}\). Coverslips were mounted in a monochromator-equipped fluorescence imaging system built around an inverted microscope, and fura-2 was alternatively excited at 340 and 380 nm every 0.5 s. Emitted light was recorded with a cooled CCD-camera. Cells were stimulated with 0.1 U/ml thrombin, 80 µM thrombin receptor-activating peptide (TRAP), 10 µM lysophosphatidic acid (LPA), 1 µM angiotensin II (Ang-II), or 10 ng/ml platelet-derived growth factor (PDGF-BB) as indicated. \([Ca^{2+}]_i\) was calibrated as described (22). Time-courses of \([Ca^{2+}]_i\) in individual single cells (grey lines) are shown to indicate the number of cells responding to the respective agonist. Mean \([Ca^{2+}]_i\) (bold black lines) was calculated from all cells selected in the experiment.

Figure 4: Time-course of receptor-mediated ERK1/2 phosphorylation

(A) Serum-starved VSM cells were stimulated with 10 % fetal calf serum (serum), 10 ng/ml PDGF-BB (PDGF), 10 ng/ml EGF, 1 µM angiotensin II (Ang-II), 1 U/ml thrombin (Thr.), 80 µg/ml thrombin receptor-activating peptide (TRAP), and 1 µM lysophosphatidic acid (LPA) for the indicated times. Whole cell lysates were subjected to 10% SDS-PAGE and electroblotted. Activated ERK was detected with a phospho-specific anti-ERK1/2 antiserum. Exposure times were optimized to maximize differences in band intensity within one experiment. Shown are representative experiments of three independent experiments with similar results. Each blot was reprobed with antibodies detecting total ERK1/2 demonstrating equal loading of the lanes (data not shown). (B) The effect of calcium elevation and of protein kinases C were assessed by 1 µM ionomycin, 1 µM calcimycin (A23187), or 100 ng/ml phorbol-12-myristate-13-acetate (PMA).
Figure 5: Receptor-mediated SM-MHC promoter activation requires the Ras/Raf/MEK/ERK signaling cascade.

(A) VSM cells, serum-starved for 48 h, were transfected with a -1346 nt SM-MHC promoter-CAT fusion construct (pCAT-1346) and then incubated in the presence of serum free medium (QM), or in QM supplemented with PDGF (10 ng/ml), EGF (10 ng/ml), lysophosphatidic acid (LPA, 10 µM), thrombin (Thr., 1 U/ml), or serum (10%) for another 48 h. Cells were lysed and assayed for CAT activity. Depicted CAT activities were normalized for protein concentrations and compared to the CAT activity of cells transfected with a reporter gene construct lacking the SM-MHC promoter. Bars represent means ± SEM of at least 5 independent transfection experiments. (B) To test for participation of Ras/Raf in the thrombin-mediated SM-MHC promoter induction, VSM cells were cotransfected with pCAT-1346 (0.5 µg/well), and the indicated amounts (in µg) of dominant negative N17-Ras or N17-Raf expression constructs. The total amount of plasmid DNA was kept constant (1 µg/well) with promoterless pCAT-basic. (C) VSM cells transfected with pCAT-1346 (1 µg/ml) were preincubated for 30 min with different concentrations of the MEK-inhibitor PD98059 (black bars) and then stimulated with thrombin (1 U/ml). Since DMSO (the solvent of PD98059) further enhanced thrombin-stimulated SM-MHC promoter activities, controls were incubated in equivalent concentrations of the solvent (open bars). All values were normalized to the thrombin-mediated CAT activity without PD98059.

Figure 6: Pertussis-toxin blocks late-phase ERK phosphorylation and SM-MHC promoter activation.

(A) Time-courses of thrombin- (0.1 U/ml) and lysophosphatidic acid (LPA, 1 µM)-induced [Ca^{2+}]_i transients in controls and in pertussis toxin-pretreated VSM cells (+PTX, 200 ng/ml for 18 h). Mean ± SEM [Ca^{2+}]_i was computed from n = 3 independent experiments, each comprising
at least 100 single cells. (B) Effects of serum (10%), thrombin (1 U/ml), and LPA (1 µM) on ERK1/2 phosphorylation in control and pertussis toxin-pretreated (+PTX) VSM cells. Phosphorylated ERK1/2 was detected as described in figure 4. Blots were reprobed with anti-total ERK1/2 demonstrating equal loading of the lanes (data not shown).

**Figure 7: Gβγ released from Gi proteins induce the SM-MHC promoter.**

(A) Transfected VSM cells (pCAT-1346) were either left untreated or were pretreated with PTX (200 ng/ml for 18 h) and then incubated in serum-free medium (QM) or stimulated with thrombin (1 U/ml) or serum (10%) in QM for 48 h. PTX was either present or absent throughout the whole experiment. (B) CAT activities were determined in unstimulated cells transfected with pCAT-1346 (0.5 µg/well) and the indicated amounts (in µg) of expression plasmids encoding either constitutively active Gαi (Q205L-mutant) or equal amounts of wild-type Gβ1 and Gγ2. Total plasmid DNA concentrations were adjusted to 1 µg/well with pCAT-basic. (C) Cells were transfected with pCAT-1346 (0.5 µg/well), an expression plasmid encoding the Gβγ-scavenger βARK1ct as indicated, and adjusted to 1 µg/well with pCAT-basic. CAT activities were assayed in either unstimulated (open bar) or in serum-stimulated (black bars) VSM cells. Mean CAT activities and SEM were calculated from at least six independent transfection experiments.

**Figure 8: Pertussis toxin-sensitive induction of contractile protein expression by thrombin and serum.**

VSM cells were pretreated with (+PTX, 200 ng/ml for 18 h) or without pertussis toxin and then cultured in serum-free medium (QM). Thrombin (1 U/ml) or serum (10%) was added to the medium for the indicated number of days in the continuous presence or absence of PTX. Medium was changed every 24 h. Whole cell lysates were normalized for their protein content (10 µg/lane), subjected to SDS-PAGE, and probed with monoclonal antibodies detecting either SM-α-actin or SM-MHC.
Reusch et al.: Figure 4

A

serum

PDGF

EGF

Ang-II

Thr.

TRAP

LPA

QM  1'  3'  5'  10'  15'  30'  45'  60'  80'  100'  120'

B

QM  1'  5'  15'  50'  100'  QM  1'  5'  15'  50'  100'

ionomycin

A 23187

PMA

QM  1'  3'  5'  10'  15'  30'  45'  60'  80'  100'  120'
Reusch et al.: Figure 5

A

B

C

CAT activity (fold over basal)

QM  PDGF  EGF  LPA  Thr.  serum

N17-Ras

N17-Raf

CAT activity (fold over basal)

thrombin:

0  +  0.1  0.5

-  +  0.1  0.5

QM PDGF EGF LPA Thr. serum

CAT activity (% of thrombin-stimulated)

PD 98059: 0  1 µM  3 µM  5 µM  10 µM  20 µM
Reusch et al.: Figure 7
Reusch et al.: Figure 8

α-actin

SM-MHC

+PTX:

QM 1d 2d 3d

thrombin

1d 2d 3d

serum
Gbetalγ mediate differentiation of vascular smooth muscle cells
H. Peter Reusch, Michael Schaefer, Claudia Plum, Günter Schultz and Martin Paul

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