Role for G_{12}/G_{13} in Agonist-Induced Vascular Smooth Muscle Cell Contraction

Antje Gohla, Günter Schultz, Stefan Offermanns

Abstract—Receptor-induced vascular smooth muscle cell contraction is mediated by dual regulation of myosin light chain (MLC_{20}) phosphorylation through Ca^{2+}-dependent stimulation of myosin light chain kinase and Rho/Rho-kinase-mediated inhibition of myosin phosphatase. Although myosin light chain kinase regulation is initiated by the coupling of receptors to G proteins of the G_q family, G_s and G_{11}, it is not known how receptors regulate the Rho/Rho-kinase-mediated pathway. In vascular smooth muscle cells, receptor-mediated MLC_{20} phosphorylation and cell contraction was blocked by inhibitors of each of the pathways. Receptors of various vasocontractors were found to couple to G_{12}/G_{13}, and constitutively active forms of Go_{12} and Go_{13} induced a pronounced contraction of vascular smooth muscle cells that could be blocked by C3 exoenzyme, by inhibition of Rho-kinase, and by stable analogues of cGMP and cAMP. Receptor-mediated smooth muscle cell contraction was strongly inhibited by dominant-negative forms of Go_{12} and Go_{13}. These data indicate that a G_{12}/G_{13}-mediated Rho/Rho-kinase-dependent pathway operates in smooth muscle cells and that dual regulation of MLC_{20} phosphorylation by vasocontractors is initiated by the dual coupling of their receptors to G proteins of the G_q and G_{12} families. (Circ Res. 2000;87:221-227.)

Key Words: smooth muscle ■ vasocontractors ■ G proteins ■ Rho-kinase ■ myosin light chain phosphorylation

Contraction and relaxation of smooth muscle cells are primarily regulated by the phosphorylation and dephosphorylation of the regulatory light chain of myosin (MLC_{20}). The phosphorylation state of MLC_{20} is under dual control by myosin light chain (MLC) kinase (MLCK) and myosin phosphatase. The classical pathway through which contracting stimuli induce MLC_{20} phosphorylation is initiated by coupling of their receptors to members of the G_q family of heterotrimeric G proteins. This results via activation of β isoforms of phospholipase C (PLC) and formation of inositol-1,4,5-trisphosphate in an increase of the free cytosolic Ca^{2+} concentration. The complex of Ca^{2+} and calmodulin then activates MLCK, leading to increased MLC_{20} phosphorylation.

The role of Ca^{2+} in the regulation of smooth muscle contraction is well established, and it has long been known that various physiological stimuli can also induce smooth muscle contraction in the absence of an increase in the free cytosolic Ca^{2+} concentration. During recent years, it has become clear that this Ca^{2+}-independent regulation occurs through the inhibition of myosin phosphatase and involves the monomeric GTP-binding protein RhoA. Activation of RhoA leads to the stimulation of Rho-kinase. Rho-kinase, in turn, phosphorylates the regulatory myosin-binding subunit of myosin phosphatase, which results in the inhibition of the enzyme. This Ca^{2+}-independent pathway mediates, at least in part, the tonic contraction induced by various stimuli, and evidence has been provided that this Rho/Rho-kinase-mediated mechanism plays an important role in the maintenance of increased vascular tone under pathological conditions. However, it remains unclear how activated receptors regulate the Ca^{2+}-independent Rho-mediated pathway in smooth muscle cells.

In the present study, we used isolated vascular smooth muscle cells to gain insight into the mechanism by which receptors regulate smooth muscle cell contraction via the Ca^{2+}-independent Rho-mediated pathway. We demonstrate that G_{12}/G_{13} can induce vascular smooth muscle cell contraction through a Rho/Rho-kinase-mediated pathway, and we provide evidence that receptors couple to G proteins of the G_q as well as of the G_{12} family to efficiently induce cell contraction via dual regulation of MLC_{20} phosphorylation.

Materials and Methods

The Materials and Methods section is available online at http://www.circresaha.org.

Results

It is generally believed that vasocontractors lead to an increase in the free cytosolic Ca^{2+} concentration through the receptor-mediated activation of G proteins of the G_q family. To determine the G proteins potentially involved in stimul-
induced vasconstriction, we studied the coupling of various vasoconstrictor receptors to heterotrimeric G proteins in plasma membranes of aortic smooth muscle cells. Photolabeling of receptor-activated G proteins with the hydrolysissensitive GTP analogue GTP-azidoanilide and subsequent immunoprecipitation of individual G-protein α subunits confirmed that endothelin, vasopressin, and angiotensin II receptors couple to Gα12, Gα13, Gαq/11, and Gαi1-3. Precipitated proteins were subjected to SDS-PAGE. Shown are autoradiograms of dried gels.

**Figure 1.** Receptor-mediated activation of G proteins in membranes of aortic smooth muscle cells. Membranes from bovine aortic smooth muscle cells were photolabeled with [γ-32P]GTP azidoanilide in the absence (−) or presence of 1 μmol/L angiotensin II (AT II), 1 μmol/L vasopressin (VP), or 1 μmol/L ET-1. Membranes were solubilized, and G-protein α subunits were immunoprecipitated with antisera recognizing Gα12, Gα13, Gαq/11, and Gαi1-3. Precipitated proteins were subjected to SDS-PAGE. Shown are autoradiograms of dried gels.

Injection of the C3 exoenzyme of Clostridium botulinum, which ADP-ribosylates and inactivates Rho, completely blocked the effect of Gα12QL and Gα13QL (Figures 2B and 3). Similarly, incubation of cells with the Rho-kinase inhibitor Y-27632 as well as coexpression of the dominant-negative Rho-kinase mutant RB/PH(TT) blocked Gα12QL- and Gα13QL-induced cell contraction (Figure 2B). Incubation of cells with tyrosine kinase inhibitors, such as genistein or tyrphostin 25, was without effect (data not shown). The results demonstrate that constitutively active forms of Gα12 and Gα13 can induce vascular smooth muscle cell contraction in a Rho/Rho-kinase-dependent manner.

Smooth muscle cell contraction is primarily regulated by the phosphorylation state of MLC20. We tested various vasoconstrictors, namely, endothelin-1 (ET-1), angiotensin II, vasopressin, and the thromboxane A2 mimetic U46619, for their ability to induce MLC20 phosphorylation by using the anti–phospho-MLC antiserum pp2b. Incubation of cells with ET-1 produced the strongest phosphorylation of MLC20. Preincubation of cells with the PLC inhibitor U73122 markedly reduced the effect of ET-1, whereas an inactive analogue, U73343, was without effect (Figure 4A). Similarly, inhibition of MLCK by ML-7 blocked ET-1–induced MLC20 phosphorylation (Figure 4B). The Rho-kinase inhibitor Y-27632 and C3 exoenzyme were used to determine the contribution of the Rho/Rho-kinase pathway in receptor-dependent MLC20 phosphorylation. Both agents were without effect on ET-1–induced elevation of free cytosolic Ca2+ (data not shown). Treatment of the cells with Y-27632 markedly reduced basal phosphorylation of MLC20 and completely prevented ET-1–dependent MLC20 phosphorylation (Figure 4B). C3 exoenzyme did not affect basal phosphorylation of MLC20 but also blocked the effect of ET-1 (Figure 4C), whereas pretreatment of cells with pertussis toxin had no effect (Figure 4D). This indicates that both the Ca2+-dependent pathway involving PLC and MLCK as well as the Rho/Rho-kinase–mediated pathway are involved in receptor-dependent MLC20 phosphorylation in smooth muscle cells and that both pathways are required for efficient regulation of MLC20 phosphorylation through receptors.

Because the Rho/Rho-kinase–mediated pathway is involved in agonist-induced MLC phosphorylation and because G12 and G13, which are activated by vasoconstrictors, are able to induce Rho/Rho-kinase–mediated cell contraction, we next examined whether G12 and G13 are involved in agonist-induced smooth muscle cell contraction. ET-1 led to contraction of 90% of smooth muscle cells within 15 minutes (Figure 5). ET-1–induced smooth muscle cell contraction lasted for 30 minutes. Thereafter, the cells reexpanded, indicating that agonist-dependent contraction was a reversible process (data not shown). To inhibit G12 and G13 function, we expressed dominant-negative forms of Gα12 (Gα12Q228A) and Gα13 (Gα13Q225A) via intranuclear injection of respective expression plasmids in vascular smooth muscle cells. Both mutants have been shown to block receptor-induced G12/G13–mediated stress fiber formation in fibroblasts. Cells successfully injected were identified by fluorescence of coinjected fluoro-emerald–labeled dextran. In contrast to uninjected cells or to cells injected with a control plasmid,
90% of cells expressing a mixture of dominant-negative Gα12 and Gα13 did not show ET-1–induced contraction (Figures 5 and 7). Expression of Gα12 G228A and Gα13 G225A alone had no measurable effect on ET-1–induced smooth muscle cell contraction. Calyculin A, a PP1/2A-type phosphatase inhibitor, which inhibits myosin phosphatase, induced contraction of injected and uninjected smooth muscle cells, indicating that expression of dominant-negative Gα12 and Gα13 did not unspecifically prevent smooth muscle cell contraction (see Figure 5). These data show that ET-1–induced smooth muscle cell contraction involves G12/G13.

We evaluated the role of the Ca2+/dependent and the Rho/Rho-kinase–mediated pathway in ET-1–induced smooth muscle cell contraction analogously to the experiments shown in Figure 4. Inhibition of PLC by U73122 and inhibition of MLCK by ML-7 completely blocked the effects of ET-1 on cell contraction (Figures 6 and 7). Similarly, ET-1–induced cell contraction was prevented by injection of the cells with C3 exoenzyme and by expression of dominant-negative Rho-kinase (Figures 6 and 7), whereas pertussis toxin had no effect on cell contraction induced by ET-1 (Figure 7). Thus, receptor-mediated vascular smooth muscle contraction and MLC20 phosphorylation require a functional Ca2+/MLCK-mediated pathway as well as a Rho/Rho-kinase–dependent pathway.

Discussion
Vascular smooth muscle contraction by most physiological stimuli involves the activation of G protein–coupled receptors, resulting in the phosphorylation of MLC20. It is now well established that activated receptors can influence the phosphorylation state of MLC20.
phosphorylation through MLCK as well as the rate of dephosphorylation of MLC20 through myosin phosphatase. Stimulation of MLCK via Ca\textsuperscript{2+}/calmodulin is induced by the coupling of activated receptors to G proteins of the G\textsubscript{q} family. Inhibitory regulation of myosin phosphatase in contrast does not seem to require an elevated Ca\textsuperscript{2+} concentration but involves the Rho/Rho-kinase–mediated pathway. It is not known how activated receptors lead to stimulation of Rho/Rho-kinase–mediated MLC20 phosphorylation in smooth muscle cells.

Among various vasocontractors tested, ET-1 most effectively induced MLC20 phosphorylation in bovine aortic smooth muscle cells. The effect of ET-1 could be blocked by an inhibitor of PLC as well as by the MLCK inhibitor ML-7 (Figure 4). Receptor-induced phosphorylation of MLC20 was also inhibited by the preincubation of cells with C3 exoenzyme and the Rho-kinase inhibitor Y-27632. Inhibition of Rho-kinase by Y-27632 markedly reduced the basal levels of MLC20 phosphorylation, suggesting that myosin phosphatase was under tonic inhibition by Rho-kinase also in the absence of an exogenously added receptor agonist. ET-1–induced smooth muscle cell contraction exhibited a similar dependence on both G\textsubscript{q/11}/PLC-\beta–mediated Ca\textsuperscript{2+}-dependent MLCK regulation and Rho/Rho-kinase–mediated myosin phosphatase regulation (Figures 6 and 7). These data are consistent with the well-established role of Ca\textsuperscript{2+}-dependent MLCK activation in agonist-induced MLC20 phosphorylation and smooth muscle cell contraction. The data also agree with several reports showing that especially the tonic phase of receptor-mediated contraction of intact smooth muscle is strongly inhibited after the inactivation of Rho or the inhibition of Rho-kinase.\textsuperscript{10,11,15,23} The sensitivity of agonist-induced MLC20 phosphorylation and cell contraction to inhib-
bition of each pathway suggests that there is a considerable level of basal phosphorylation and dephosphorylation of MLC20 and that a coordinated induction of Ca\textsuperscript{2+}-dependent MLCK activation and of Rho/Rho-kinase–mediated inhibition of myosin phosphatase is required for agonist-induced MLC20 phosphorylation and tonic contraction of smooth muscle cells.

In permeabilized smooth muscle preparations, evidence has been provided that agonists differ in their efficacy to induce Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}-independent pathways,\textsuperscript{1,6} suggesting that signaling pathways induced by activated receptors bifurcate at a level relatively upstream in the signaling cascade. However, at present, it is not clear at which level the agonist-induced signaling pathways diverge. G proteins of the G\textsubscript{12} family have been shown to regulate Rho/Rho-kinase–dependent signaling processes.\textsuperscript{17,18,24–26} Therefore, we decided to study the hypothesis that agonist-induced vasocontraction of smooth muscle cells via dual regulation of MLC20 phosphorylation is initiated by the coupling of receptors to G proteins of the G\textsubscript{q} and G\textsubscript{12} families. Many G protein–coupled receptors that are able to activate G\textsubscript{q} family also couple to G\textsubscript{12} and G\textsubscript{13}.\textsuperscript{19,27} In membranes of aortic smooth muscle cells, we could demonstrate that receptors for ET-1, angiotensin II, and vasopressin couple to G\textsubscript{q}/11 as well as to G\textsubscript{12} and G\textsubscript{13}, supporting the notion that G\textsubscript{12}/G\textsubscript{13} is involved in the responses of smooth muscle cells to vasocontractors. We also observed an activation of Gi-type G proteins by these stimuli. Activation of G\textsubscript{i} results in the inhibition of adenylyl cyclase but may also contribute to receptor-mediated activation of PLC\_\beta isoforms and other effectors through \beta\gamma subunits released from the activated heterotrimer.\textsuperscript{28} Inactivation of G\textsubscript{i}-type G proteins by pretreatment of cells with pertussis toxin did not affect receptor-induced MLC20 phosphorylation and cell contraction (Figures 4 and 7), suggesting that G\textsubscript{i} is not involved in these acute responses of smooth muscle cells.

Although receptor-mediated smooth muscle cell contraction required activation of the Rho/Rho-kinase as well as the Ca\textsuperscript{2+}-dependent pathway, expression of the constitutively
active mutants of $\alpha_{G12}$ and $\alpha_{G13}$ alone resulted in a pronounced contraction of isolated vascular smooth muscle cells (Figure 2A). This effect appeared to be specific, inasmuch as cells injected with a control plasmid or with plasmids carrying active forms of $\alpha_{Gq}$ and $\alpha_{G12}$ showed no morphological change. Cell contraction induced by activated $\alpha_{G12}$ and $\alpha_{G13}$ was blocked by C3 exoenzyme, Y-27632, and by dominant-negative Rho-kinase (Figures 2B and 3). These data demonstrate that a pathway involving $\alpha_{G12}/\alpha_{G13}$, Rho, and Rho-kinase operates in smooth muscle cells and that activation of this pathway by constitutively active $\alpha_{G12}/\alpha_{G13}$ results in smooth muscle contraction. Analogues of cyclic nucleotides cAMP and cGMP blocked $\alpha_{G12}/\alpha_{G13}$-induced cell contraction (Figures 2B and 3), indicating that the $\alpha_{G12}/\alpha_{G13}$-induced signaling pathway is subject to inhibitory regulation by cGMP- and cAMP-dependent processes. This is in line with data showing that cAMP inhibits Rho/Rho-kinase–mediated processes in various cells. Although the exact mechanism for cAMP-dependent inhibition of the pathway is currently unclear, there is evidence that cGMP can accelerate MLC20 dephosphorylation by myosin phosphatase and that this effect involves a direct interaction of cGMP-dependent protein kinase with the regulatory subunit of myosin phosphatase and/or a telokin-mediated mechanism.

Because receptors of various vasococontractors are able to couple to $\alpha_{G12}/\alpha_{G13}$ and because active forms of $\alpha_{G12}/\alpha_{G13}$ induce smooth muscle cell contraction in a manner depending on Rho and Rho-kinase, we tested whether dominant-negative active forms of $\alpha_{Gq}$ and $\alpha_{G12}$ can interfere with receptor-mediated smooth muscle cell contraction. Co-expression of $\alpha_{G12}$G228A and $\alpha_{G13}$G225A blocked agonist-induced smooth muscle contraction (Figures 5 and 7). This inhibitory effect was comparable to that observed after inhibition of Rho or Rho-kinase (Figures 6 and 7). Our data clearly indicate that G proteins of the $G_i$ family are involved in receptor-dependent smooth muscle contraction. We propose a model for agonist-induced phosphorylation of MLC20 in which the dual regulation of MLC20 phosphorylation through Ca$^{2+}$-dependent MLCK activation and Rho/Rho-kinase–mediated myosin phosphatase inhibition is initiated by the dual coupling of receptors to G proteins of the $G_q$ and $G_{12}$ families (Figure 8). A very similar scenario has been described for stimulus-induced MLC20 phosphorylation in platelets. The mechanism by which $\alpha_{G12}/\alpha_{G13}$ activates Rho in smooth muscle cells remains to be clarified. Tyrosine kinases have been involved in $\alpha_{G12}/\alpha_{G13}$-induced Rho activation in fibroblasts and neuronal cells. However, $\alpha_{G12}/\alpha_{G13}$-induced Rho/Rho-kinase–mediated smooth muscle cell contraction was insensitive to tyrosine kinase inhibitors (data not shown), indicating that similar to the situation in platelets, this pathway apparently does not involve tyrosine kinases. Regulation of Rho by $\alpha_{G12}/\alpha_{G13}$ may be mediated by a Rho-specific guanine nucleotide exchange factor (GEF). Genetic studies in Drosophila have demonstrated that the Drosophila RhoGEF protein DRhogeF is under control of the concertina gene product, a homologue of $\alpha_{G12}/\alpha_{G13}$. Related mammalian Rho-GEF proteins, such as p115RhoGEF and PDZ-RhoGEF, have recently been shown to interact with $G_{12}$ and $G_{13}$.

In vascular smooth muscle cells, we show that receptors of vasococontractors couple to $G_{12}/G_{13}$, that $G_{12}/G_{13}$ is able to induce cell contraction in a Rho/Rho-kinase–dependent manner, and that efficient agonist-induced cell contraction involves $G_{12}/G_{13}$. These data clearly indicate that the contractile response of smooth muscle cells to stimuli depends not only on a $G_{12}/G_{13}$-mediated pathway (resulting in MLCK activation) but also on a $G_{12}/G_{13}$-mediated pathway (leading to Rho/Rho-kinase–dependent inhibition of myosin phosphatase). Thus, dual regulation of MLC20 phosphorylation through MLCK and myosin phosphatase by receptor agonists appears to be induced by the dual coupling of activated receptors to $G_q/G_{11}$ and $G_{12}/G_{13}$. Our data also suggest that differences in the efficacy of vasococontractors to induce Ca$^{2+}$-dependent and Rho-Rho-kinase–mediated signaling pathways may be due to different abilities of their receptors to activate G proteins of the $G_q$ and $G_{12}$ family, respectively.

Acknowledgments
This study was supported by the Deutsche Forschungsgemeinschaft and Fonds der Chemischen Industrie.

References


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Circ Res. 2000;87:221-227
doi: 10.1161/01.RES.87.3.221

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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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