IRAG determines nitric oxide- and atrial natriuretic peptide-mediated smooth muscle relaxation

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Aims
Nitric oxide (NO) and atrial natriuretic peptide (ANP) signalling via cGMP controls smooth muscle tone. One important signalling pathway of cGMP-dependent protein kinase type I (cGKI) is mediated by IRAG (IP3 receptor associated cGKI substrate) which is highly expressed in smooth muscle tissues. To elucidate the role of IRAG for NO- and ANP-mediated smooth muscle tone regulation, cGKI localization, and for its possible function in blood pressure adjustment, we generated IRAG-knockout mice by targeted deletion of exon 3.

Methods and results
IRAG deletion prevented stable interaction of IP3 receptor type I (IP3RI) with cGKIβ determined by cGMP affinity chromatography. Confocal microscopy in vascular smooth muscle cells (VSMCs) showed that localization of cGKIβ and cGKIα did not change in absence of IRAG. NO-, ANP-, and cGMP-dependent relaxation of hormone-contracted aortic vessels and colon was significantly affected in IRAG-knockout mice. The suppression of cGMP-induced relaxation was not rescued by selective expression of cGKIβ in smooth muscle from cGKIα-transgenic mice. NO-, ANP-, and cGMP-mediated inhibition of the hormone-induced increase in intracellular calcium concentration measured by Fura2 was suppressed in IRAG-deficient VSMC. Telemetric measurements revealed that IRAG-deficient animals exhibited normal basal tone, but were resistant to blood pressure reduction induced by lipopoly saccharide-treatment.

Conclusion
These findings indicate that signalling of cGKIβ via IRAG is an essential functional part for regulation of smooth muscle tone and of intracellular calcium by NO (exogenously applicated or endogenously synthesized) and by ANP. IRAG signalling does not modulate basal tone but might be important for blood pressure regulation under pathophysiological conditions.

Keywords
Inositol trisphosphate receptor-associated cGMP kinase I substrate • Nitric oxide • Atrial natriuretic peptide • cGMP-dependent protein kinase I • Smooth muscle relaxation

1. Introduction
Nitric oxide (NO)/cGMP signalling activates cGMP-dependent protein kinases (cGK) which modulate a variety of physiological functions including smooth muscle contractility.1–3 Signal transduction by cGK in smooth muscle is mediated by the isoforms cGKII and cGKIβ which are both highly expressed in these tissues e.g. in aorta and colon.4,5 For both isoforms, several substrates were identified including the inositol-trisphosphate receptor-associated cGMP kinase substrate (IRAG), myosin-binding subunit (MYPT-1), and regulator of G-protein signalling 2 (RGS2).6–8 cGKII interacts with and phosphorylates MYPT-1 which leads to calcium desensitization of myosin regulatory

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light-chain phosphorylation. Furthermore, cGKIβ regulates RGS2 and thereby modulates receptor-mediated signalling via $G_{0/11,12}$. cGKIβ interacts specifically with the IRAG protein, which associates with IP$_3$ receptor type I (IP$_3$R1) via its coiled-coil domain. Deletion of the IP$_3$R1-interaction site in mice prevented cGMP-dependent relaxation of hormone-induced smooth muscle contraction. In this report, we aimed to gain IRAG-deficient murine mutants to get an insight into the function of this substrate for intracellular localization of cGKI, for NO- and atrial natriuretic peptide (ANP)-mediated smooth muscle relaxation and intracellular calcium modulation, and for regulation of blood pressure.

2. Methods

2.1 IRAG-knockout mice

Construction of the targeting vector for deletion of exon 3 of the IRAG gene and generation and genotyping of IRAG-knockout mice are described in Supplementary Material online.

Smooth muscle-specific cGKIβ-transgenic mice (SMcGKIβ/cGKI$^{+/+}$) were bred and analysed as described earlier. cGKIβ-transgenic mice were intercrossed with heterozygous IRAG$^{+/−}$ mice to yield IRAG-deficient cGKIβ-transgenic mice (SMcGKIβ/cGKI$^{+/−}$/IRAG$^{−/−}$).

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The experimental protocols were approved by the local authorities for animal research (Regierung der Oberpfalz, Bayern, Germany; #54-2532.1-31/08) and were conducted according to the German law for animal care.

2.2 Cyclic GMP determination

Dissected aortae of WT and IRAG-KO mice were incubated in buffer A (30 min, 37°C) prior to stimulation with/without 1 μM DEA-NO for 1 min. After homogenization of the tissue with 10% trichloroacetic acid, supernatant was extracted with diethyl ether and assayed for cGMP content using a cGMP enzyme immunoassay (EIA) system kit.

cGMP was determined in tissue homogenates using reversed phase ion-pair chromatography adapting a published method. For detailed experimental procedures, see Supplementary Methods.

2.3 Myography

Segments of thoracic aorta and colon were dissected from mice and placed in buffer solution at 37°C (in mM: NaCl 137, KCl 5.4, CaCl$_2$ 1.8, MgCl$_2$ 1, NaH$_2$PO$_4$ 0.42, NaHCO$_3$ 12, glucose 5.6; aerated with 95% O$_2$ and 5% CO$_2$). Segments of colon were mounted longitudinally into organ baths (Myograph 601, Danish Myo Technology). Resting tension was set to 10 mN (longitudinal colon segments). Colon segments were contracted by 10 μM carbachol. Depending on the experiment, endothelium was destroyed by blowing air through the aorta and contracted with 3 μM phenylephrine in the presence of 100 μM L-NAME. Tension was recorded isometrically.

Hormone-induced contraction was measured after establishment of steady-state conditions (tonic tension) and the effects of submaximal relaxation doses of DEA-NO/ANP/Acetylcholine and 8-Br-cGMP on tension were determined.

2.4 Long-term radiotelemetric blood pressure analysis

Mean arterial blood pressure (MAP), heart rate (b.p.m.), and locomotor activity were analysed in conscious male control (WT) and IRAG-KO mice (litter- and age-matched; age 8–12 weeks) using the system of Data Sciences International (DSI) and the transmitter-type TA11PA-C10. Basal MAP, b.p.m., and activity were recorded at days 6, 7, and 8 after surgery, when mice had regained normal locomotor activity. Radiotelemetric signals were sampled for 2 min at 1 min intervals. Lipopolysaccharide (LPS) was dissolved in sterile 0.9% NaCl (Braun AG, Melsungen, Germany) and administered intraperitoneally (i.p.).

2.5 Preparation and analysis of COS-7 cells and vascular smooth muscle cells

Cell culture and lystate preparation of COS-7 cells were performed as described previously. COS-7 cells were transfected with WT bovine IRAGa or IRAGΔint (=ΔAA 152-184)-pcDNA3.1 together with cGKIβ cloned in pcDNA3.1 using the Fugene-Transfection reagent (Roche).

Aortae were dissected from 8- to 12-week-old mice (wild type and IRAG-knockout) and vascular smooth muscle cells (VSMCs) were prepared from aorta as described. Cultured cells were stimulated with/without 8-Br-cGMP or 8-pCPT-cGMP and then analysed with primary antibodies against IP$_3$R1, IRAG, cGKIα, cGKIβ, cGKIcommon, or α-Actin and appropriate secondary antibodies as described in Supplementary methods. Photomicrographs were taken with a confocal microscope (Zeiss LSM 510) at ×400 magnification.

Calcium measurements using Fura2-AM in VSMCs were performed as described in Supplementary methods.

2.6 Biochemical methods

Proteins from different mouse organs were isolated by homogenization in extraction buffer (in mM: Tris–HCl 20, NaCl 100, DTT 2.5, EDTA 2.5, benzamidine 2.5, PMSF 2.5, and protease inhibitor cocktail (Roche), 4°C) followed by centrifugation at 13000 g, 4°C for 10 min. The supernatants containing the tissue proteins were stored at −80°C. Western blot analysis of tissue proteins was performed with selective primary antibodies and secondary antibodies coupled to horseradish peroxidase. Antibodies were used as described previously or purchased from ABR (IP$_3$R1), Calbiochem (VASP), Santa Cruz (RGS 2, Phospholamban), or UBS (MYPT-1). As internal standard of the expression, the β-actin was used.

Statistical calculations were performed with Origin 7.1. Protein complexes were isolated with 8-AET-cGMP-agarose (Biolog) or by co-immunoprecipitation with specific antibodies as described.

2.7 Statistics

All data are expressed as mean ± SEM. For the calculation of statistical differences between two means, the T-test was used, and for three means, the ANOVA was used. The significance of the P-value was indicated by asterisks ($^* P < 0.05$, $^{**} P < 0.01$, $^{***} P < 0.001$; n.s., not statistically significant). N indicates the number of experiments.

3. Results

3.1 Generation of IRAG-knockout and IRAG-KO × cGKIβ transgenic hybrid mice

The cGKIβ interaction site of IRAG is encoded in exon 4. Therefore, to obtain IRAG-knockout mice, we deleted exon 3 of the IRAG gene by homologous recombination using LoxP-flanking sites. Deletion of exon 3 resulted in a frame shift leading to an IRAG-knockout mutant (Figure 1A). IRAG-knockout was verified on the genomic level by Southern blot (Figure 1B) and PCR analysis (Figure 1C), on protein level by immunoblotting with IRAG-specific antibodies (Figure 1D). IRAG-knockout mice showed a slightly reduced life expectancy (Supplementary material online, Figure S1). IRAG deficiency resulted in an enlarged gastrointestinal tract indicating a physiological defect of these mice in regulating smooth muscle contractility (Supplementary material online, Figure S2). IP$_3$R1, IRAG, and cGKI form a complex in smooth muscle. The protein expression
level of these three proteins in aorta and colon of IRAG-KO mice was analysed (Figure 2A). IRAG was absent from all smooth muscle tissues in IRAG-KO. The expression level of IP₃RI was unchanged and that of cGKI was reduced. The reduction was caused by a lower expression of cGKIβ isozyme (Figure 2A). It was possible that affected physiological functions of IRAG-KO mice could be caused by reduction of cGKIβ expression and not by the absence of IRAG protein. Therefore, IRAG-KO mice were crossed with cGKIβ-transgenic mice (SMIβ) that express cGKIβ in all smooth muscles.¹⁷ As shown in Figure 2A, the hybrid mice expressed cGKIβ protein approximately to the same level as wild-type mice.

In contrast to IRAG and cGKIβ, expression of most cGKI substrates (IP₃RI, RGS2, and VASP) was not changed in vascular and colonic smooth muscle of IRAG-KO mice (Figure 2A and B). Expression of MYPT-1—a substrate for cGKIα—was also unchanged in vascular smooth muscle, but was upregulated in colonic smooth muscle (Figure 2C). Furthermore, expression of eNOS and sGCβ1 was not altered (Figure 2B). NO-induced cGMP synthesis was not affected by the IRAG deletion as determined by HPLC analysis and EIA analysis (Supplementary material online, Figure S3). These results supported the notion that deletion of IRAG did not affect significantly the production of cGMP in smooth muscle.

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**Figure 1** Generation of IRAG-deficient mice. (A, top panel) Structure of the IRAG protein. Location of sites for cGKIβ interaction or IP₃RI interaction (coiled-coil domain) and the transmembrane domain (TM) is schematically drawn; targeting strategy: exon 3 was deleted resulting in a frameshift mutation and thereby generating an IRAG-knockout mutant. (B) Southern blot analysis of wild-type (+/+) heterozygous (+/−) and IRAG-deficient (−/−) genomic DNA using the 5’-probe. (C) PCR strategy with Primers a, b, and c for the analysis of wild-type and IRAG-deficient mice. The lower band represents WT IRAG allele (+), the upper band the IRAG exon 3 deleted allele (−). (D) Immunoblot of aorta or colon proteins from wild type (+/+), heterozygous (+/−), and IRAG-deficient (−/−) mice with IRAG antibodies. As loading control, the presence of GAPDH or β-actin was tested.
The cGKI complex was previously identified in tracheal smooth muscle. It assembles a stable ternary macrocomplex by specific interactions with cGKI and IP3RI. Smooth muscle lysates from WT mice contained stably associated IP3RI, IRAG, and cGKI complex. The interaction of cGKI and IP3RI was destroyed by IRAG deletion supporting the notion that the IP3RI lacks a stable interaction site for cGKI (Figure 2D).

3.2 Localization of cGKI isozymes and IRAG in COS cells and VSMCs

The cGKI isozymes interact with different substrates through their N-terminal leucine zipper domain. cGKÎ±a binds specifically to RGS 2 and MYPT-1, whereas cGKÎ± specific interaction with IRAG was shown. It has been reported that differential interaction of cGKI isozymes with their substrate proteins determines their intracellular localization, i.e. that IRAG restricts the intracellular localization of cGKIβ, cGKIβ heterologously expressed in Baby hamster kidney cells entered the nucleus after stimulation with cGMP in absence of IRAG, but stayed in the cytosol in the presence of IRAG. To test this hypothesis, we heterologously expressed cGKIβ with IRAG in COS cells. In the presence of the IRAG protein, cGKIβ was found in the cytosol where it colocalized with IRAG in perinuclear regions. In the absence of IRAG, cGKIβ was localized in the nucleus (Figure 3). The specificity of IRAG-cGKIβ interaction was revealed by deletion of the cGKIβ interaction site of IRAG which allowed entry of heterologously expressed cGKIβ into the nucleus of COS cells (Figure 3). We used VSMCs to study the
3.3 Defective NO/cGMP- and ANP/cGMP-dependent smooth muscle relaxation in IRAG-knockout tissues

The effect of IRAG signalling on smooth muscle relaxation was studied in colon and aorta (Figure 5; Supplementary material online, Figures S5 and S6). Wild-type and IRAG-KO colon generated the same tension in the presence of carbachol (Figure 5A). NO and 8-Br-cGMP relaxed carbachol-pre-contracted wild-type colon (Figure 5B and Supplementary material online, Figure S5A). Deletion of IRAG suppressed NO- and cGMP-mediated relaxation of the colon smooth muscle (Figure 5B and Supplementary material online, Figure S5B and C). These results indicated an important role of the IRAG protein in the intestinal relaxation induced by cGMP signalling.

In the next set of experiments, we analysed the functional effect of the IRAG deletion in aorta (Figure 5). Phenylephrine (3 μM) contracted aortas were relaxed by addition of the cGMP-analogue 8-Br-cGMP (300 μM) in wild-type aortas, but relaxation was abolished in IRAG-knockout mutants (Figure 5C, Supplementary material online, Figure S6A and Table S1). The NO-induced relaxation was slightly, but significantly reduced in IRAG-KO aortae (Figure 5C, Supplementary material online, Figure S6B and Table S1).

In the intact vascular system, endogenous NO-synthesis is mediated by ACh-stimulated endothelium. Therefore, we analysed whether physiological localization of cGKI in a native environment (Figure 4, Supplementary material online, Figure S4). cGKα was perinuclearly localized in WT and IRAG-KO VSMCs. cGKIβ and IP3RI colocalized with IRAG in reticular structures of native VSMCs (Figure 4, Supplementary material online, Figure S4A and C). However, cGKα and IRAG did not colocalize together (Figure 4, Supplementary material online, Figure S4B). In IRAG-knockout VSMC, cGKIβ remained in reticular structures even in presence of 8-pCPT-cGMP (1 mM, 60 min) (Figure 4). This localization indicates that additional proteins interact with cGKIβ and determines its localization in native smooth muscle cells.
Figure 4 Localization of cGKinas and signalling proteins in vascular smooth muscle cells (VSMC) from wild-type and IRAG-knockout mice. Confocal Images of primary murine VSMC are shown. (A) The analysed signalling proteins are indicated. Upper panels represent wild type VSMC (WT), lower panels IRAG-knockout VSMC (KO). (B) Stimulation with 8-pCPTcGMP (1 mM, 60 min, 37°C). The analysed signalling proteins are indicated. Left panels represent wild type VSMC, right panels IRAG-knockout VSMC.
ACh-induced relaxation is affected in IRAG-knockout aorta. Contraction was induced by phenylephrine (1 μM) followed by addition of ACh (1 μM) (Figure 5C, Supplementary material online, Figure S6C and Table SI). Relaxation was almost completely suppressed in the IRAG-KO aorta indicating that IRAG is an essential component for physiological NO-mediated relaxation of vascular smooth muscle.

As suggested by the western blot (Figure 2), less cGKIβ was present in IRAG-KO than wild-type smooth muscle. To exclude the possibility that reduction of the cGKIβ isozyme could have resulted in defective cGMP-mediated vascular smooth muscle relaxation, expression of cGKIβ in IRAG-deficient mice was enhanced by breeding these mice into the cGKIβ transgenic/KO aorta. Statistics depicted by histogram analysis. Values are expressed as mean ± SEM. (×P < 0.05; **P < 0.01; ***P < 0.001, student’s t-test and ANOVA).

**Figure 5** (A) The tension induced by Carbachol (10 μM) is not affected in colonic KO tissue in comparison to WT. (B) Relaxation potency (in percentage) of 8-Br-cGMP (300 μM) and DEA-NO (30 μM) on colonic smooth muscle strips of WT and KO animals. (C) Relaxation potency (in percentage) of different NO/ANP/cGMP/cGK activating compounds in WT, IRAG-KO, and SMJb-transgenic/KO aorta. Statistics depicted by histogram analysis. Values are expressed as mean ± SEM. (×P < 0.05; **P < 0.01; ***P < 0.001, student’s t-test and ANOVA).

3.4 Defective NO- and ANP-dependent calcium regulation in IRAG-knockout VSMCs

IRAG was implicated to mediate the cGMP-dependent inhibition of hormone-induced calcium release.6,16 Calcium measurements using Fura2-AM showed that cGMP-dependent inhibition of noradrenaline-induced intracellular calcium release was absent in the IRAG-KO smooth muscle cells (Supplementary material online, Figure S7). Likewise, the inhibitory effect of NO and ANP on the hormone-stimulated intracellular calcium rise was significantly affected in IRAG-KO VSMC (Supplementary material online, Table SII). These results supported the notion that IRAG was necessary for NO/ANP/cGMP-mediated smooth muscle relaxation.

3.5 Role of IRAG for blood pressure regulation

So far, we showed only that IRAG was an important component of the NO/ANP signalling cascade in vascular and colon smooth muscle in *vivo*. The intestinal in *situ* situation revealed that IRAG-KO mice had an enlarged intestinal tract (Supplementary material online, Figure S2) suggesting that IRAG is important for physiological function of the intestine. We also tested the function of the vascular system. Telemetric measurements of the MAP showed no difference in MAP under basal conditions (WT: 109.1 ± 3.9 mmHg, n = 5; KO: 103.9 ± 3.1 mmHg, n = 5). Heart frequency (b.p.m.) and locomotor activity of the IRAG-KO was not changed (Figures 5 and 6; Supplementary material online, Table SI).

cGMP-mediated relaxation of aortic segments is not only activated by the NO-receptor soluble guanylyl cyclase but also stimulated by particular guanylyl cyclases upon addition of ANP. Therefore, we tested whether IRAG signalling might integrate both cGMP signalling pathways. The ANP (500 pm)-induced relaxation was strongly impaired by the IRAG deficiency (Figure 5C, Supplementary material online, Figure S6D and Table SI). These observations suggested a central role of IRAG signalling for vascular NO/cGMP- and ANP/cGMP-mediated smooth muscle relaxation.
activity were also unchanged. In conclusion, signalling via IRAG was not essential for maintaining the basal blood pressure.

We next tested whether IRAG has a role in blood pressure regulation under pathophysiological situations that increase NO synthesis. During sepsis, the activity of iNOS is strongly induced and thereby blood pressure is reduced. A sepsis-like situation was induced by intraperitoneal administration of E. coli LPS (10 mg/kg body weight) and the LPS-induced effect on blood pressure was measured by telemetry (Figure 6). In wild-type animals, the application of LPS decreased MAP by more than 30 mmHg. However, MAP of IRAG-KO animals stayed constant over 6 h after LPS application. This result strongly supports the notion that IRAG has an important role in the regulation of blood pressure under pathophysiological conditions.

4. Discussion

For the analysis of IRAG function in smooth muscle, an IRAG-deficient mouse line was generated, which is reported here for the first time. Thereby, several novel findings were revealed: IRAG signalling via cGKIβ was essential for smooth muscle relaxation and for intracellular calcium regulation by NO (exogenously applied or endogenously synthesized) and by ANP. In contrast to heterologous expression systems in BHK cells or COS-7 cells (see Results), the localization of cGKIβ in native VSMCs was not altered by IRAG-deficiency. Basal mean arterial pressure was not affected by IRAG-deletion. In contrast to wild-type mice, IRAG-deficient animals were resistant to blood pressure reduction upon LPS treatment.

4.1 Effect of IRAG on localization and function of cGKI isozymes in vascular smooth muscle cells

The cGMP kinase macrocomplex consisting of ternary core components cGKIβ, IRAG, and IP3RI was previously identified in tracheal smooth muscle membranes. Further studies showed that the cGMP kinase macrocomplex is generally assembled in smooth muscle tissues. The identification of interaction sites of IP3RI or cGKI in the core complex showed that IRAG is essential for integrity of this complex. Deletion of the interaction sites disturbed the association of the complex. The lacking IP3RI interaction site in IRAG prevented the association of IP3RI to the macrocomplex indicating that IP3RI was not stably interacting with cGKIβ or cGKia. This was further substantiated by lack of IP3RI association with cGK in IRAG-deficient smooth muscle. Targeting of cGKIβ to intracellular endoplasmic reticular compartments was dependent on IRAG expression in heterologous expression systems. However, we observed that intracellular distribution of cGKIβ did not change in IRAG-deficient VSMCs. This indicated that cGKIβ was stably associated with further components in smooth muscle and targeting of cGKIβ to intracellular compartments in native smooth muscle cells was not strictly dependent on the presence of IRAG. In this respect, it was shown that also additional components, e.g. phospholamban or PDE5a, are found in the cGMP kinase macrocomplex in minor amounts indicating a less stable association of these components in this complex. Elucidation of further stably associated components to cGKIβ will be of high impact for understanding the intracellular distribution of cGKIβ and will further substantiate the physiological function of cGKIβ. It must be emphasized that mechanisms regulating cGKia distribution are also not known. It might be that elucidation of the signalling pathway leading to integrin activation via Rho and its role in adhesion and differentiation will lead to a further substantiate view.

cGMP-dependent nuclear translocation of cGKI isozymes was found in some studies which could be important for cGKI-dependent gene regulation. A fragment of cGKI, which was named cGKih, was described, which was transferred into the nucleus upon stimulation. The translocation of cGKIβ into the nucleus was shown in heterologous expression systems using BHK cells. Similarly, nuclear localization of cGKIβ was observed in our studies in COS7-cells. In the heterologous expression system, localization of cGKIβ in the nucleus could be suppressed by expression of IRAG. This suppression of nuclear localization was dependent on the identified cGKIβ–IRAG interaction site as upon deletion of this site nuclear localization of cGKIβ was found. However, deficiency of IRAG did not lead to an altered distribution of cGKIβ in VSMCs indicating that there are also other interaction partners of cGKIβ with unknown functions in native smooth muscle.

Regulation of intracellular calcium by cGKI in VSMCs was shown previously. Upon transfection of cGKI-deficient VSMC with cGKia rather than cGKIβ, the cGMP-dependent inhibition of intracellular calcium transient could be restored. In coronary VSMCs, studies with siRNA indicated that the cGKia isozyme rather than the cGKIβ isozyme mediated NO/cGMP-dependent suppression of thrombin-receptor stimulated intracellular calcium rises. In contrast, smooth muscle-specific rescue of cGKIβ or cGKia in genetic models restored the cGMP-inhibited effect on calcium release in VSMC. In VSMC, in which an IRAG mutant protein is expressed lacking the interaction site with the IP3RI, the cGMP-dependent calcium regulation was affected. However, in these cells, the interaction of mutated IRAG with the cGKIβ was still present. Now we could show that also in absence of IRAG expression leading to freely available cGKIβ, the NO/cGMP-dependent inhibition of the calcium transient was suppressed. This suggests that targeting of cGKIβ to other substrate proteins than IRAG is not associated with calcium signalling and indicates that these other substrates of cGKIβ are associated with further cGKI-dependent functions in smooth muscle which might be associated with differentiation, proliferation, or migration. In this respect, it is important to note that particularly signalling of cGKIβ was associated with cell proliferation and it was reported that cGKIβ was involved in the prevention of metastasis of colon adenocarcinomas.

4.2 Mechanisms of cGMP-mediated smooth muscle relaxation

Our analysis showed that relaxation of aortic and colonic smooth muscle by exogenous addition of NO donors or cGMP analogues was affected in IRAG-deficient smooth muscle tissues. Furthermore, relaxation upon stimulation of endogenous synthesis of NO was suppressed in vascular smooth muscle from IRAG-deficient mice. In addition, ANP-mediated relaxation was abolished in IRAG-deficient aortae. These observations indicate that cGKIβ signalling via IRAG is essential for NO/cGMP- and ANP/cGMP-dependent relaxation of vascular and gastrointestinal smooth muscles. cGKia rescue mice lacking the expression of cGKIβ did not show any altered cGMP-dependent relaxation of smooth muscles. Therefore, it might be that cGKia expression is able to rescue cGKIβ signalling. However, expression of cGKia in IRAG-knockout tissue was not...
changed and did not restore cGMP-dependent relaxation. Furthermore, rescued expression of cGKIβ using the SMIB-transgene were not able to overcome the effect of IRAG deficiency suggesting that downstream signalling of cGKIβ is inhibited by IRAG deletion. These results might also indicate that IRAG is more generally involved in cGKI signalling of both isoforms.

There are other conflicting results to our observations that cGMP signalling via IRAG is essential for cGMP-mediated smooth muscle relaxation. At least two substrate proteins of cGKIs, MYPT-1 and RGS 2, were associated with cGMP-dependent smooth muscle function.7–9,12,33 The importance of these diverse substrate proteins for smooth muscle relaxation in comparison to the IRAG protein is still a matter of debate. The mutation of the cGKIs leucine zipper disrupting interaction to cGKIs-specific substrate proteins suppressed cGMP-dependent vascular relaxation and lead to hypertension.34 A possible explanation was that the leucine zipper mutation altered targeting and distribution of the cGKI substrate proteins which hampered smooth muscle relaxation. Furthermore, deletion of RGS2 reduced NO-dependent smooth muscle relaxation and affected NO-dependent blood pressure regulation.12,23 Upon IRAG deletion, the expression level of the RGS2 protein was not altered in diverse smooth muscle tissues. MYPT-1 expression did also not change in IRAG-deficient vascular smooth muscle. Interestingly, enhanced expression of MYPT-1 observed in colonic smooth muscle from IRAG-knockout mice did not compensate the defect of NO/cGMP-mediated smooth muscle relaxation in IRAG-deficient tissues. It might be that different physiological conditions determine the importance of cGKI signalling pathways in smooth muscle. In this respect, it was shown that under oxidative conditions, interaction of RGS2 and MYPT-1 with cGKIs was strongly enhanced.35

Deficiency of IRAG did not alter the basal blood pressure of mice. This is not surprising as smooth muscle selective expression of cGKIβ or cGKIs also did not change basal blood pressure.27 However, it might be that under conditions with enhanced endogenous NO- and thereby cGMP-synthesis one or both isoforms are needed for regulation of blood pressure. During sepsis, the endogenous NO-synthesis is enhanced by inducible NOS (iNOS) which thereby mediates the reduction of blood pressure.22,26 It was shown that iNOS-deficient mice are resistant to LPS-mediated long-term blood pressure reduction.37 Interestingly, IRAG deficiency suppressed the blood pressure lowering LPS effect. This indicates that NO/cGMP signalling via IRAG might be important for blood pressure regulation under pathophysiological conditions.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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References


