**The EMBO Journal (2004) 23,** 4222–4231 | © 2004 European Molecular Biology Organization | All Rights Reserved 0261-4189/04
www.embojournal.org

THE EMBO JOURNAL

# IRAG is essential for relaxation of receptor-triggered smooth muscle contraction by cGMP kinase

**Angela Geiselhöringer[1], Matthias Werner[1,2], Katja Sigl, Petra Smital, René Wörner, Linda Acheo, Juliane Stieber, Pascal Weinmeister, Robert Feil, Susanne Feil, Jörg Wegener, Franz Hofmann and Jens Schlossmann***

Institut für Pharmakologie und Toxikologie, Technische Universität München, München, Germany

**Signalling by cGMP-dependent protein kinase type I (cGKI) relaxes various smooth muscles modulating thereby vascular tone and gastrointestinal motility. cGKI-dependent relaxation is possibly mediated by phosphorylation of the inositol 1,4,5-trisphosphate receptor I ($IP_3RI$)-associated protein (IRAG), which decreases hormone-induced $IP_3$-dependent $Ca^{2+}$ release. We show now that the targeted deletion of exon 12 of IRAG coding for the N-terminus of the coiled-coil domain disrupted *in vivo* the IRAG–$IP_3RI$ interaction and resulted in hypomorphic $IRAG^{\Delta12/\Delta12}$ mice. These mice had a dilated gastrointestinal tract and a disturbed gastrointestinal motility. Carbachol- and phenylephrine-contracted smooth muscle strips from colon and aorta, respectively, of $IRAG^{\Delta12/\Delta12}$ mice were not relaxed by cGMP, while cAMP-mediated relaxation was unperturbed. Norepinephrine-induced increases in $[Ca^{2+}]_i$ were not decreased by cGMP in aortic smooth muscle cells from $IRAG^{\Delta12/\Delta12}$ mice. In contrast, cGMP-induced relaxation of potassium-induced smooth muscle contraction was not abolished in $IRAG^{\Delta12/\Delta12}$ mice. We conclude that cGMP-dependent relaxation of hormone receptor-triggered smooth muscle contraction essentially depends on the interaction of cGKI–IRAG with $IP_3RI$.**

*The EMBO Journal* (2004) **23,** 4222–4231. doi:10.1038/sj.emboj.7600440; Published online 14 October 2004
*Subject Categories*: signal transduction; molecular biology of disease
*Keywords*: cGKI; $IP_3R$; IRAG; relaxation; smooth muscle

## Introduction

Nitric oxide (NO) is a powerful relaxant of smooth muscle that stimulates soluble guanylyl cyclases and increases cGMP in many cells (Friebe and Koesling, 2003). cGMP-dependent relaxation required activation of cGMP-dependent protein kinase type I (cGKI) (Pfeifer *et al*, 1998; Sausbier *et al*, 2000). Two splice variants of cGKI, cGKIα and cGKIβ, exist, which differ in their amino termini and might interact with different targets (Surks *et al*, 1999; Schlossmann *et al*, 2000). In $cGKI^{-/-}$ mice, both isoforms are inactivated (Pfeifer *et al*, 1998). The targets downstream of cGKI are unknown. $Ca^{2+}$-independent (Somlyo and Somlyo, 2000; Bonnevier *et al*, 2004) and $Ca^{2+}$-dependent (Hofmann *et al*, 2000; Feil *et al*, 2003; Schlossmann *et al*, 2003) signalling pathways may be influenced. $Ca^{2+}$-independent downstream effector systems include inhibition of Rho activation (Sauzeau *et al*, 2000), phosphorylation of HSP20 (Beall *et al*, 1999; Rembold *et al*, 2000), and phosphorylation and inhibition of myosin light chain phosphatase (Surks *et al*, 1999). The $Ca^{2+}$-dependent effector mechanisms comprise inhibition of inositol 1,4,5-trisphosphate ($IP_3$) synthesis (Ruth *et al*, 1993; Xia *et al*, 2001), increase of the open probability of $BK_{Ca}$ channel (Alioua *et al*, 1998; Fukao *et al*, 1999; Sausbier *et al*, 2000; Zhou *et al*, 2001) and inhibition of $Ca^{2+}$ release from unidentified intracellular stores (Cornwell and Lincoln, 1989; Eigenthaler *et al*, 1993; Pfeifer *et al*, 1998; Tertyshnikova *et al*, 1998; Feil *et al*, 2002) and from $IP_3$-sensitive stores (Schlossmann *et al*, 2000). The finding that cGMP-dependent inhibition of $Ca^{2+}$ release required the phosphorylation of inositol 1,4,5-trisphosphate receptor I ($IP_3RI$)-associated protein (IRAG; Schlossmann *et al*, 2000; Ammendola *et al*, 2001), a protein that copurifies in a macrocomplex together with the $IP_3$ receptor type I ($IP_3RI$) and the β-isoform of cGKI (cGKIβ) (Schlossmann *et al*, 2000), supported the hypothesis that cGKI decreases vascular tone by reducing $[Ca^{2+}]_i$. IRAG has been found together with cGKI in all smooth muscles, suggesting that it interacts also *in vivo* with the $IP_3R$ (Geiselhöringer *et al*, 2004). However, these observations did not establish the physiological significance of IRAG. It was reported that cGKI phosphorylated the $IP_3R$ directly *in vitro* (Komalavilas and Lincoln, 1994) and in cultured cells (Wagner *et al*, 2003; Soulsby *et al*, 2004). We now studied the physiological effects of IRAG on cGKI signalling in smooth muscle using a mouse line with a mutation in the IRAG–$IP_3RI$ interaction domain.

## Results

IRAG contains an amino-terminal site that interacts specifically with the amino terminus of cGKIβ (Ammendola *et al*, 2001) and a coiled-coil domain in the carboxy-terminal part of the protein (Schlossmann *et al*, 2000). The site of interaction with the $IP_3R$ is unknown, but we reasoned that the coiled-coil domain might interact with the $IP_3R$. Disruption of this interaction site should yield smooth muscles with an altered cGMP response. Exon 12 of the IRAG gene encodes

*Corresponding author. Institut für Pharmakologie und Toxikologie, Technische Universität München, Biedersteiner Straße 29, 80802 München, Germany. Tel.: +49 89 4140 3265; Fax: +49 89 4140 3261; E-mail: schlossmann@ipt.med.tu-muenchen.de
[1]These authors contributed equally to this work
[2]Present address: Department of Pharmacology, College of Medicine, University of Vermont, Burlington, VT 05405, USA

**Received: 27 July 2004; accepted: 16 September 2004; published online: 14 October 2004**

 

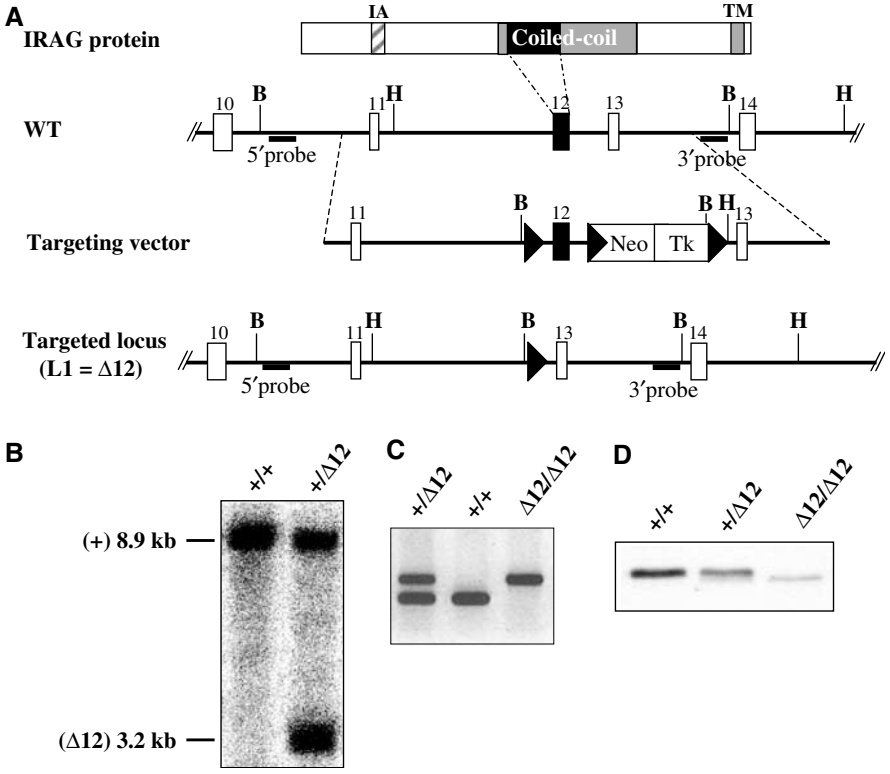


**Figure 1** Targeted exon 12 deletion of the murine IRAG gene. (**A**) Targeting strategy. Top: Structure of the IRAG protein. The fasciated box indicates the cGKIβ–IRAG interaction domain (IA), and the grey box the transmembrane domain (TM). Upper middle: Organisation of the WT IRAG target locus (numbers indicate exons; B: *Bgl*II, H: *Hin*dIII). Lower middle: The targeting vector contains exon 12, which is flanked by loxP sites (black arrowheads) followed by a *Neo/Tk* cassette and a third loxP site. Bottom: Exon 12 and the *Neo/Tk* cassette were deleted by Cre recombinase after recombination resulting in the targeted Δ12 locus. (**B**) Southern blot hybridisation of *Bgl*II-digested genomic ES cell DNA with the 3′ probe results in specific fragments of 3.2 kb (deleted exon: Δ12) and 8.9 kb (WT: +). (**C**) PCR analysis in mice using specific primers for WT (+) (lower band: 171 bp) and Δ12 (upper band: 213 bp). (**D**) Immunoblot of stomach proteins from WT (+/+), heterozygous (+/Δ12) and homozygous mutants (Δ12/Δ12) with IRAG-specific antibodies. The upper band represents WT IRAG (molecular weight ∼125 kDa), and the lower band the approximately 5 kDa smaller IRAGΔ12 protein.

the amino-terminal part of the coiled-coil domain (Figure 1A). Deletion of exon 12 (Figures 1B and C) resulted in a 5 kDa smaller IRAG protein (IRAGΔ12; Figure 1D). Wild-type (WT), heterozygous IRAG$^{+/\Delta12}$ and homozygous IRAG$^{\Delta12/\Delta12}$ mice were born at normal Mendelian ratio, but 50% of the homozygous IRAG$^{\Delta12/\Delta12}$ mice died before 6 months of age. Necropsy of IRAG$^{\Delta12/\Delta12}$ mice revealed an extended stomach and large intestine (Figure 2A) with a 2.5-fold increased pylorus muscle region. The stomach weight was $1.5\pm0.1$ and $4.5\pm0.5$% of the body weight ($n=5$) for WT and IRAG$^{\Delta12/\Delta12}$, respectively. The passage of barium sulphate through the intestine was slowed in the IRAG$^{\Delta12/\Delta12}$ mice (Figure 2B) and was retained in the stomach even after 30 min. These results suggest a functionally significant pylorus stenosis and a severe intestinal movement disorder in the mice.

Mean arterial blood pressure measured by the tail cuff method was $93.2\pm3.2$ ($n=6$) and $80\pm1.2$ ($n=6$) mmHg in WT and IRAG$^{\Delta12/\Delta12}$ mice, respectively. Heart rate was $601.9\pm21.1$ ($n=6$) and $572.8\pm15.4$ ($n=6$) beats/min in WT and IRAG$^{\Delta12/\Delta12}$ mice. The slight difference in heart rate was also seen with long-term measurements using telemetric electrocardiography. No major difference in heart rate regulation was noticed between WT and IRAG$^{\Delta12/\Delta12}$ mice when the β-selective agonist isoproterenol (1 mg/kg) or the $\alpha_1$-selective agonist methoxamine (6 mg/kg) was injected.

Immunoblot analysis showed that IRAG and cGKI were coexpressed in the intestinal smooth muscle tissue of WT mice (Figure 3A). Deletion of exon 12 reduced the concentration of IRAG in the intestinal smooth muscle (Figure 3). IRAG expression was also reduced in aorta and other tissues (Figure 3A). IRAG concentration was already decreased in the aorta of heterozygous IRAG$^{+/\Delta12}$ mice (Figure 3A). Quantitative Western blot analysis of the colon from IRAG$^{\Delta12/\Delta12}$ mice showed that the mutant IRAG protein and cGKIβ were reduced by 75 and 40%, respectively, whereas the α-isoform of cGKI (cGKIα) was increased to 140% and the amount of IP$_3$RI was unchanged (Figure 3B). A similar decrease in the concentration of the mutant IRAG and cGKIβ was observed in the aorta. This finding suggested that expression of IRAG affected the concentrations of cGKIβ and cGKIα in opposite directions. Interestingly, deletion of cGKI decreased the expression of IRAG by 30% (Figure 3C).

The interaction of IRAG and IP$_3$R was studied in Cos-7 cells and in smooth muscle. Co-immunoprecipitations (CoIPs) of both heterologously expressed proteins revealed their direct interaction (Figure 4A). Furthermore, CoIP from cGKI$^{-/-}$ smooth muscle proteins showed that deletion of cGKI did not interfere with the coassembly of IRAG and IP$_3$RI in smooth muscle (Figure 4D). However, deletion of the N-terminal part of the coiled-coil domain abolished the

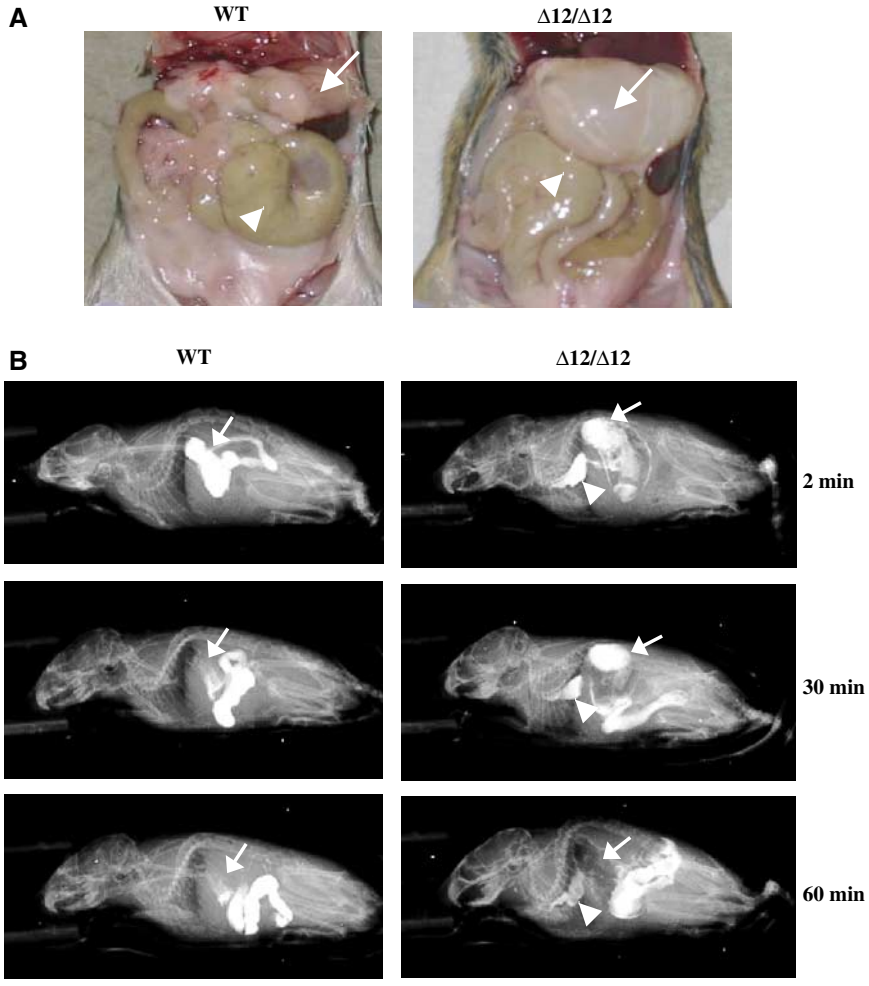


**Figure 2** Gastrointestinal phenotype of the IRAG$^{\Delta12/\Delta12}$ mice. (**A**) Abdominal situs from WT (left) and IRAG$^{\Delta12/\Delta12}$ (right) mice. The IRAG$^{\Delta12/\Delta12}$ mice have a strongly enlarged stomach (arrow) and a dilated caecum (arrowhead). Due to the enlarged stomach, the position of the caecum in IRAG$^{\Delta12/\Delta12}$ mice is reversed. (**B**) X-ray analysis of intestinal motility. Litter matched WT and IRAG$^{\Delta12/\Delta12}$ mice were fed with barium sulphate, and the X-rays were taken 2, 30 and 60 min after feeding. The positions of the stomach and the oesophagus are indicated by the arrow and the arrowhead, respectively. The experiment was performed with 12 animal pairs of various ages (9, 15 and 19 weeks) with similar results.

interaction of IRAG with the IP$_3$R in Cos-7 cells (Figure 4A). To study the formation of the cGKI macrocomplex upon disruption of the IRAG–IP$_3$RI interaction site, the IRAGΔ12 protein was coexpressed together with cGKIβ and IP$_3$RI in Cos-7 cells. CoIP of Cos-7 and smooth muscle cell extracts revealed that the assembly of the IP$_3$RI into the mutant IRAG–cGKI macrocomplex was abolished in the expression system (Figure 4B) and in smooth muscles from IRAG$^{\Delta12/\Delta12}$ mice (Figure 4C), whereas the interaction with cGKI was unperturbed. These results confirm that *in vivo* the coiled-coil domain is responsible for the interaction of the IP$_3$RI with IRAG and, consequently, is essential for assembly of the IP$_3$RI into the cGKI–IRAG macrocomplex.

The physiological consequence of the disruption of the IRAG–IP$_3$RI interaction was tested using colon muscle strips, a phasic intestinal smooth muscle, and aortic rings, a tonic vascular smooth muscle. Neither the rhythm nor the amplitude of the spontaneous activity of the colon was affected by deletion of exon 12. In addition, the peak amplitude and the tonic tension of carbachol (CCh)-induced contractions were the same in muscles from WT and IRAG$^{\Delta12/\Delta12}$ mice (peak amplitude (N/g): WT ($n=74$) $0.55\pm0.05$, Δ12/Δ12 ($n=58$) $0.66\pm0.06$; tonic tension (N/g): WT ($n=74$) $0.14\pm0.015$, Δ12/Δ12 ($n=58$) $0.11\pm0.012$). Preincubation of WT longitudinal smooth muscle strips from the colon in the presence of 300 μM 8-Br-cGMP reduced the CCh-induced phasic contraction peak to 30% (Figures 5A and C). The tonic contraction induced by CCh was reduced to 10% of the maximal tension by 300 μM 8-Br-cGMP (Figures 5A and D). Similar results were obtained with colon from heterozygous IRAG$^{+/\Delta12}$ mice (not shown). In contrast, 8-Br-cGMP did not affect the phasic contraction peak of muscle strips from IRAG$^{\Delta12/\Delta12}$ colon (Figures 5B and C) and did not relax these muscle strips (Figures 5B and D). A similar defect on the phasic contraction response and relaxation was observed with colon muscle strips from cGKI-deficient mice (Figures 5C and D). The defect was specific for cGMP-induced relaxation, because the relaxation induced by the cAMP analogue cBIMPS was unaffected in IRAG$^{\Delta12/\Delta12}$ and cGKI-deficient muscle strips (Figure 5E), confirming previous findings (Pfeifer *et al*, 1998) that cGMP and cAMP signal by different mechanisms in smooth muscle.

Next we tested the ability of the mutant IRAG protein to reduce depolarisation-induced contraction. Depolarisation

 

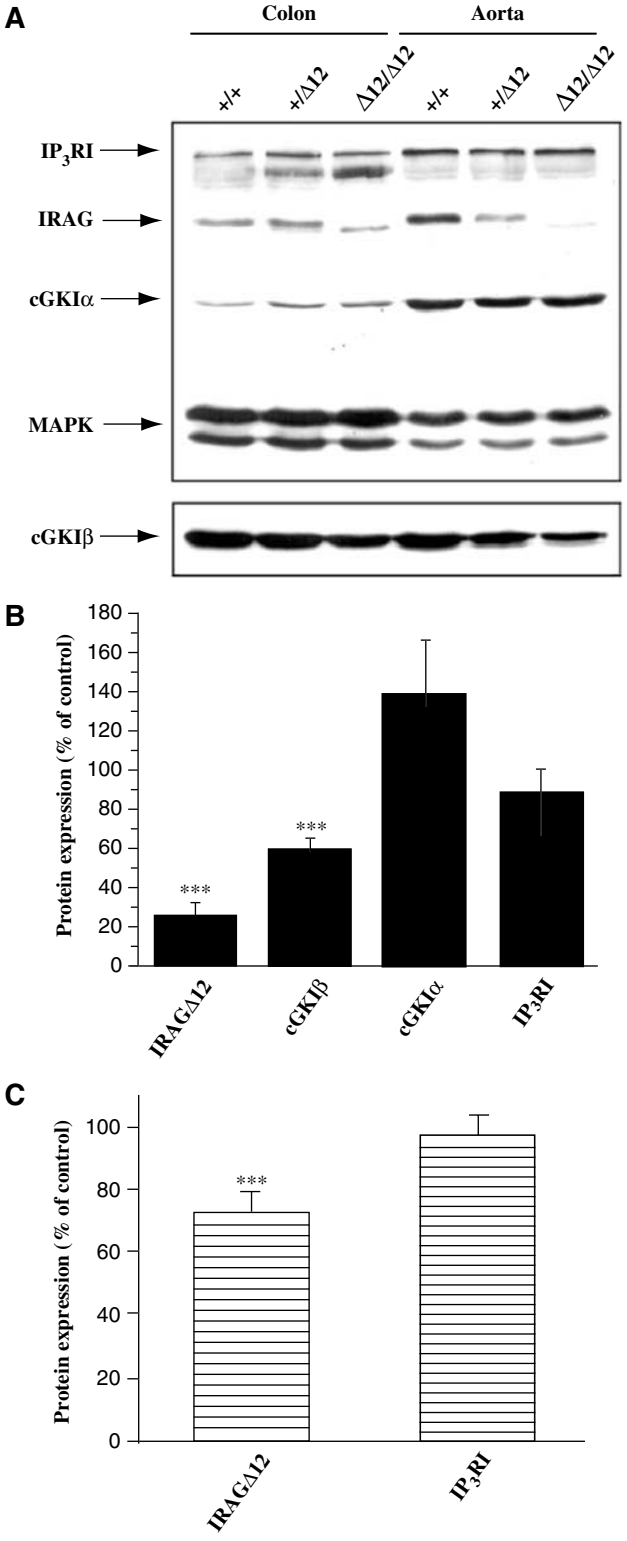


**Figure 3** Expression of IRAG, cGKI and IP$_3$RI protein in WT, IRAG$^{/\Delta12}$, IRAG$^{\Delta12/\Delta12}$ and cGKI$^{-/-}$ mice. (**A**) Western blot analysis of colon and aorta proteins (each 75 μg/lane) from WT (+/+), heterozygous (+/Δ12) and homozygous (Δ12/Δ12) animals. The expression of IP$_3$RI, IRAG and the cGKI isoforms, α and β, was studied with specific antibodies. As loading control, MAP-kinase (MAPK) was used. (**B, C**) Statistical analysis of protein expression in the colon of IRAG$^{\Delta12/\Delta12}$ (solid bars) (B) and cGKI$^{-/-}$ mice (striped bars) (C) in comparison to protein expression in WT mice (set to 100%). All values are the means±s.e.m. of 6–8 animals of each genotype. The asterisks indicate significant differences (***$P<0.001$).

opens L-type calcium channels (Moosmang *et al*, 2003; Wegener *et al*, 2004). Channel opening should not be affected by IRAG located on the endoplasmic reticulum (Schlossmann *et al*, 2000). However, it was possible that the IRAG mutation might affect the tonic phase following the initial depolarisation-induced contraction, since the tonic phase depends partially on $Ca^{2+}$-induced $Ca^{2+}$ release from intracellular stores and the influx of $Ca^{2+}$ through store-operated channels (Bolton *et al*, 1999). The depolarisation-induced contraction peak was not affected in colon strips from WT and IRAG$^{\Delta12/\Delta12}$ mice by preincubation with 8-Br-cGMP (not shown). The tonic phase was partially affected by the IRAG mutation, whereas it was abolished in cGKI$^{-/-}$ mice, suggesting that cGKI signals through an additional pathway (Figure 5F).

Identical results were obtained with vascular smooth muscle. 8-Br-cGMP relaxed phenylephrine (PE)-precontracted aortic segments from WT mice (residual tension 15% of control) (Figures 6A and C), but was poorly effective on segments from IRAG$^{\Delta12/\Delta12}$ and cGKI$^{-/-}$ mice (residual tension 83%) (Figures 6B and C). cGMP relaxed only partially (residual tension 53%) aortas from heterozygous IRAG$^{+/\Delta12}$ mice (Figure 6C) in agreement with the reduced IRAG concentration in aortas from these mice (see Figure 3A). Relaxation of $K^+$-contracted rings was not affected in IRAG$^{\Delta12/\Delta12}$ aortas (remaining tension 50–60% in WT and IRAG$^{\Delta12/\Delta12}$ aortas), whereas aortic rings from cGKI$^{-/-}$ mice showed no relaxation upon addition of 8-Br-cGMP (Figure 6D). These results confirmed those of the colon and suggested that cGMP-mediated relaxation of receptor-triggered smooth muscle contraction required IRAG and depended on the interaction between IRAG and IP$_3$RI. Furthermore, they suggested that cGMP-dependent relaxation of depolarisation-induced contraction depended on other mechanisms than IP$_3$R–IRAG signalling.

Previously, it was shown (Schlossmann *et al*, 2000; Ammendola *et al*, 2001) that cGMP-dependent phosphorylation of IRAG decreased the IP$_3$-induced release of $Ca^{2+}$ from intracellular stores. Therefore, we tested whether the defective smooth muscle relaxation in the IRAG$^{\Delta12/\Delta12}$ mice might result from an altered $[Ca^{2+}]_i$ regulation. Fura 2-AM-loaded primary aortic smooth muscle cells from WT and IRAG$^{\Delta12/\Delta12}$ mice were stimulated by norepinephrine (NE, 25 nM) in the presence or absence of 1 mM 8-Br-cGMP (Figures 7A and B). cGMP significantly decreased the $Ca^{2+}$ transients in WT smooth muscle but had no effect on the transients in IRAG$^{\Delta12/\Delta12}$ and, as shown previously (Pfeifer *et al*, 1998; Feil *et al*, 2002), in cGKI$^{-/-}$ cells (Figure 7C). Interestingly, the cAMP analogue cBIMPS did not show any effect on the noradrenaline-induced $Ca^{2+}$ transients of WT cells. Potassium-induced $Ca^{2+}$ transients were reduced by cGMP in WT and IRAG$^{\Delta12/\Delta12}$ aortic cells but were unaffected by cGMP in cGKI$^{-/-}$ cells (Figure 7D). These results indicated again that cGMP/cGKI affected hormone- and depolarisation-induced contraction by distinct pathways. Additional experiments indicated that mutation of IRAG did not affect the depletion-induced $Ca^{2+}$ influx (Figures 8A and B). cGMP did not affect the $Ca^{2+}$ transient induced after readdition of extracellular calcium (Figure 8A). Furthermore, inhibition of the influx pathway by $Gd^{3+}$ did not abolish cGMP-induced decrease in the calcium transient of WT cells (Figure 8B). cGMP had no significant effect on the $Ca^{2+}$ transient in cells

from IRAG$^{\Delta12/\Delta12}$ mice. These results support the notion that the interaction of IP$_3$RI with IRAG was essential for the cGKI-mediated reduction of hormone-induced increases in $[Ca^{2+}]_i$.

## Discussion

The present paper shows that the IP$_3$RI specifically assembles *in vivo* via IRAG into an IP$_3$RI–IRAG–cGKI macrocomplex. The integration of IP$_3$RI into this complex was abolished when IRAG was replaced by the modified IRAG (IRAGΔ12) in which the amino-terminal part of the coiled-coil domain was deleted. The concentration of the IRAGΔ12 protein was downregulated in smooth muscle from IRAG$^{\Delta12/\Delta12}$ mice. Cos-7 cells expressed normal amounts of the IRAGΔ12 protein, showing that the mutant protein is stable *in vivo*. Therefore, the decrease was smooth muscle specific and most likely caused by an increased protease accessibility of the IRAGΔ12 protein due to a defective integration of the IP$_3$RI into the macrocomplex. Interestingly, the concentration of the IP$_3$RI was unchanged, whereas the IRAG mutation led to a specific reduction of cGKIβ protein that went along with an increased cGKIα expression. These results indicate a specific coregulation of the cGKIβ and IRAG protein level. This notion is further supported by the finding that the protein level of IRAG was reduced in cGKI-deficient mice.

The deletion of the IP$_3$RI–IRAG interaction domain in IRAG$^{\Delta12/\Delta12}$ mice led to the inability of cGMP to relax receptor-triggered phasic and tonic smooth muscle contraction. Obviously, interactions between IRAG and the IP$_3$RI are essential for this cGMP/cGKIβ response in intestinal and vascular smooth muscle. Interestingly, deletion of the IP$_3$RI–IRAG interaction cannot be compensated by other cGKI-mediated mechanisms, although the expression of cGKIα was enhanced in smooth muscles from IRAG$^{\Delta12/\Delta12}$ mice. These results suggest that the cGKIβ/IRAG signalling pathway is of major importance for NO/cGMP-mediated relaxation of receptor-triggered contraction.

cGMP-mediated relaxation of smooth muscle depends on calcium-dependent and calcium-independent mechanisms. The relative importance of these mechanisms is not clear. The results of this paper indicate that the specific inactivation of the calcium-dependent cGKIβ/IRAG signalling mechanism in the IRAG$^{\Delta12/\Delta12}$ mice abolished cGMP-dependent relaxation of receptor-triggered smooth muscle contraction. Mutation of IRAG had no effect on cAMP-dependent relaxation, supporting the notion that several independent pathways exist that modulate smooth muscle tone. These results do not exclude the possibility that calcium-independent

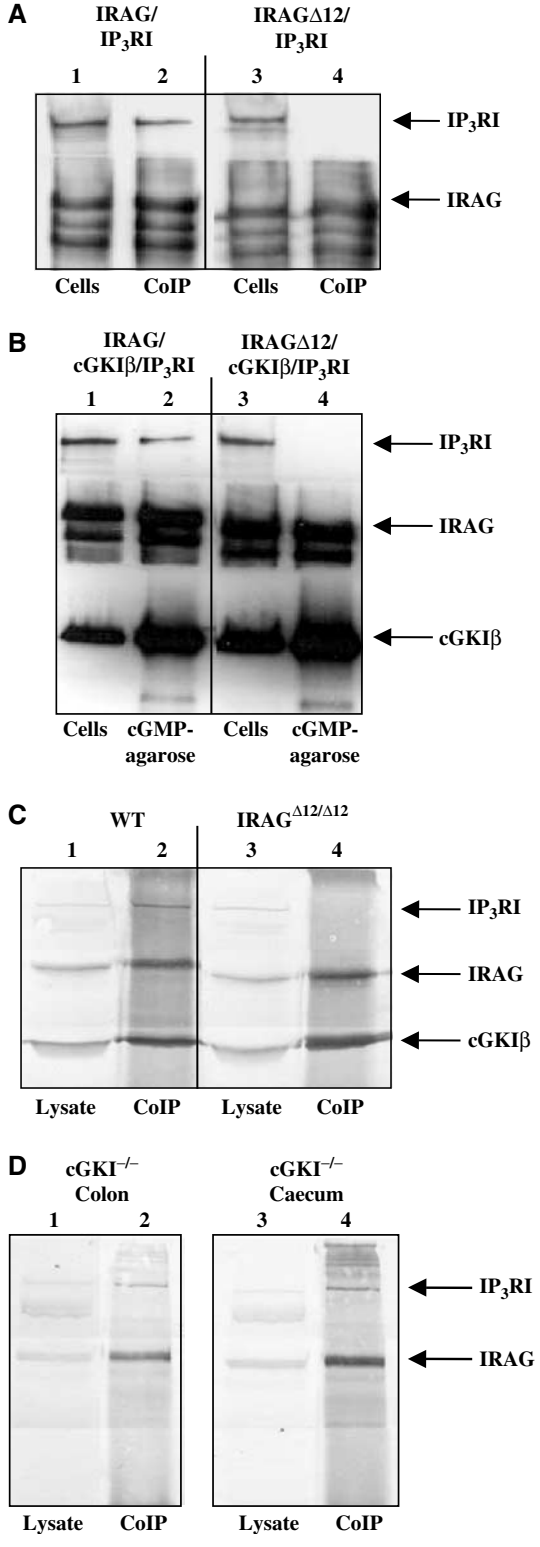


**Figure 4** Interaction of IRAG with IP$_3$RI and cGKIβ in Cos-7 cells and smooth muscle tissues. (**A**) IP$_3$RI and WT IRAG (lane 1, 20 μg total protein) or IRAG carrying the Δ12 mutation (IRAGΔ12) (lane 3, 20 μg total protein) were heterologously expressed in Cos-7 cells. CoIP was performed with the corresponding Cos-7 cells (200 μg total protein each) using IRAG-specific antibodies (lanes 2 and 4). Western blots were analysed with specific antibodies. (**B**) After heterologous expression in Cos-7 cells of the IP$_3$RI, cGKIβ, and WT IRAG (lane 1, 20 μg total protein) or IRAGΔ12 (lane 3, 20 μg total protein), respectively, protein complexes were isolated from these Cos-7 cells (200 μg total protein) by 8-AET-cGMP-agarose affinity chromatography (cGMP-agarose). The Western blot analysis showed that cGKIβ and IP$_3$RI associated together with WT IRAG (lane 2). In contrast, only cGKIβ but not IP$_3$RI was found in a stable complex with the IRAGΔ12 protein (lane 4). (**C**) CoIP with IRAG-specific antibodies of WT and IRAG$^{\Delta12/\Delta12}$ uterus proteins. Protein lysates from WT and IRAG$^{\Delta12/\Delta12}$ were analysed by immunoblotting (lanes 1 and 3; 50 μg/lane). CoIPs were performed with proteins from WT (1.5 mg) or IRAG$^{\Delta12/\Delta12}$ (3 mg) and were analysed by the immunoblots shown in lanes 2 and 4. (**D**) CoIPs with IRAG-specific antibodies of cGKI$^{-/-}$ colon and caecum proteins. Inputs of protein lysates were 40 μg/lane in lanes 1 and 3, and 1 mg for the CoIP analysed in lanes 2 and 4. The arrows represent the positions of IP$_3$RI, IRAG and cGKIβ in the immunoblots.

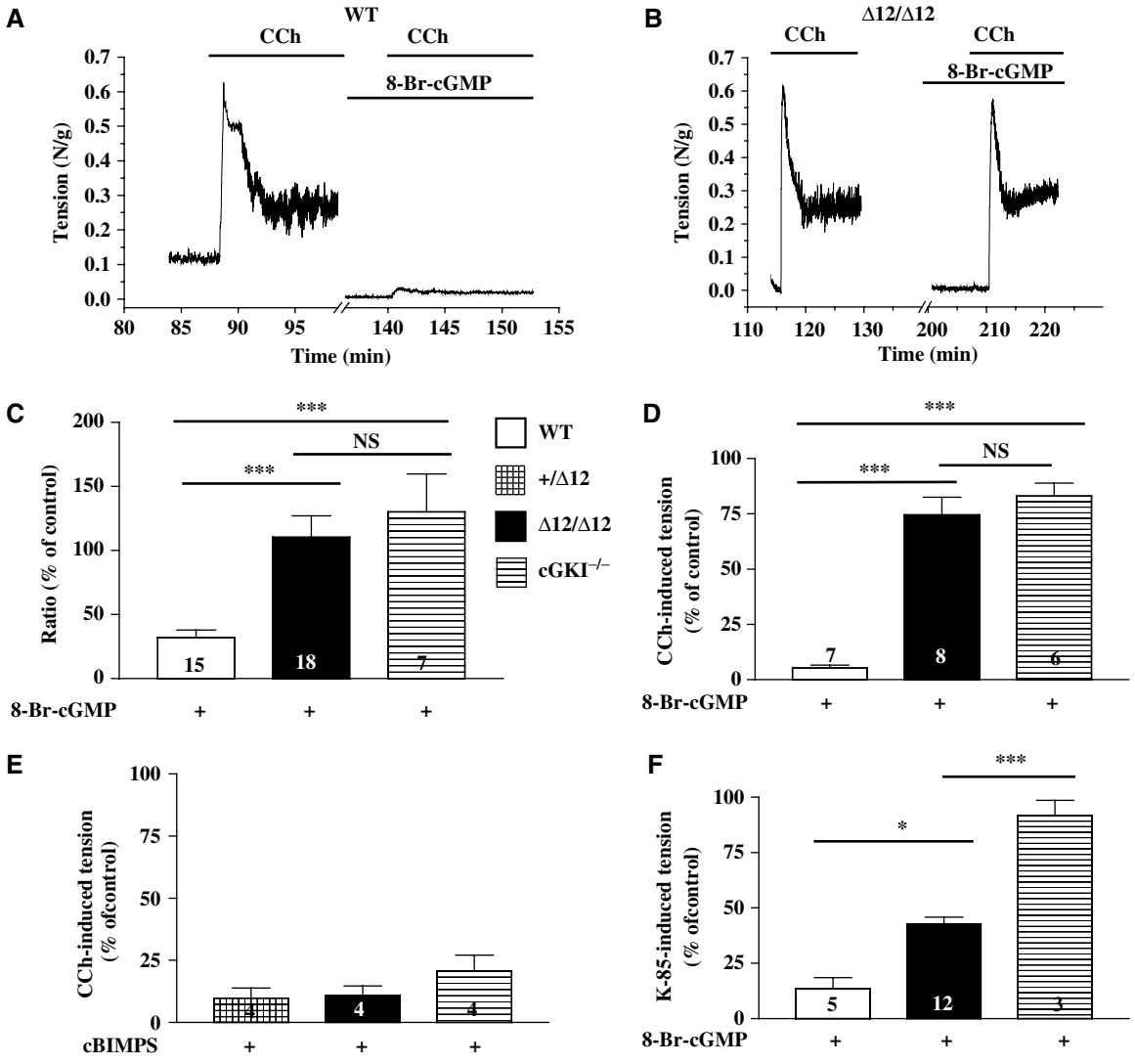


**Figure 5** Effect of 8-Br-cGMP on hormone- and depolarisation-induced contraction in colonic smooth muscle. (**A, B**) Original recordings from colon longitudinal smooth muscle of (A) WT and (B) IRAG$^{\Delta12/\Delta12}$ mice in N/g wet weight. Bars indicate the presence of CCh (10 μM) and 8-Br-cGMP (300 μM). 8-Br-cGMP was applied 10–15 min before the second stimulation. (**C**) Analysis of peak tension induced by CCh. Ratios of two subsequent peak amplitudes (second in relation to the first) were calculated without and with 8-Br-cGMP (300 μM), which was applied 10–15 min before the second stimulation. Ratios obtained without 8-Br-cGMP were taken as control (100%). Columns correspond to WT (open bars), IRAG$^{\Delta12/\Delta12}$ (solid bars) and cGKI$^{-/-}$ (striped bars) (see bar legend), and represent means ± s.e.m. ***$P<0.001$; NS, not statistically significant. (**D**) Analysis of tonic tension induced by CCh. Values reflect tension in the presence of 300 μM 8-Br-cGMP in relation to steady-state conditions (100%). Columns represent means ± s.e.m. ***$P<0.001$; NS, not statistically significant. (**E**) Effect of 300 μM cBIMPS on CCh-induced tonic tension in colon smooth muscle of IRAG$^{+/\Delta12}$ (crossed bars), IRAG$^{\Delta12/\Delta12}$ and cGKI$^{-/-}$ mice. (**F**) Analysis of depolarisation-induced tonic tension. Depolarisation was induced by exchange of 85 mM $Na^+$ with $K^+$. Values reflect tension in the presence of 300 μM 8-Br-cGMP in relation to steady-state conditions (100%). Columns represent means ± s.e.m. ***$P<0.001$; *$P<0.05$; NS, not statistically significant.

mechanisms are involved in the relaxation of noradrenaline- and CCh-induced smooth muscle contraction.

cGKIβ regulates smooth muscle tone by modulating $[Ca^{2+}]_i$, because the cGMP-mediated inhibition of receptor-induced intracellular calcium release was reduced in smooth muscles from IRAG$^{\Delta12/\Delta12}$ mice. However, this mechanism is not exclusive and universal. As might be expected, the cGMP-induced relaxation of depolarisation-induced tonic contraction was only slightly affected or unaffected by the IRAG mutation in the colon or the aorta, respectively, whereas deletion of cGKI completely blocked the cGMP-dependent relaxation in colon and aorta. Identical results were obtained when the effect of cGMP on potassium-induced $[Ca^{2+}]_i$ transients was studied. These differential effects of the IRAG mutation are further evidence that cGKIβ/IRAG signalling acts specifically on intracellular calcium release channels. They also suggest that other cGKI targets exist that need to be involved in the regulation of depolarisation-induced contractility and might be phosphorylated by cGKIα (Feil *et al*, 2002). Up to now, the deletion of the proven cGKI *in vivo* substrate phospholamban (a subunit of the ER-localised $Ca^{2+}$-ATPase SERCA II) (Lalli *et al*, 1999) or the vasodilator-stimulated phosphoprotein VASP (Aszodi *et al*, 1999; Hauser *et al*, 1999) did not result in major defects of smooth muscle contractility, whereas the deletion of the $BK_{Ca}$ channel did affect smooth muscle contractility (Meredith *et al*, 2004;

Sausbier *et al*, 2004). The involvement of the $BK_{Ca}$ channel in the studied regulation is unlikely, since the muscles were depolarised by 85 mM $K^+$. Alternative possible cGKI targets could be the L-type calcium channel (Ruiz-Velasco *et al*, 1998) and a TRP-like cation channel (Kwan *et al*, 2004). cGKI-dependent phosphorylation of these targets could explain the difference in the effects of the IRAG mutation and cGKI deletion on depolarisation-induced smooth muscle contraction and $[Ca^{2+}]_i$. Opening of the L-type calcium channel has been reported to be an essential part of the receptor-triggered smooth muscle contraction (Moosmang *et al*, 2003; Wegener *et al*, 2004). However, we anticipate that also other pathways such as HSP20 might be involved in this regulation. The physiological significance of the regulation of vascular smooth muscle contraction by depolarisation is not obvious. Physiologically, vascular smooth muscle contraction is triggered by activation of G-protein-coupled hormone receptors and not by depolarisation. Therefore, cGKIβ signalling via IRAG/IP$_3$RI is an important pathway regulating smooth muscle tone.

## Materials and methods

### *Generation of IRAG$^{\Delta 12/\Delta 12}$ mice*

Using a genomic 129/Sv bacterial artificial chromosome (BAC) library (Genome Systems, St Louis, MO), the targeting vector was constructed such that exon 12 was flanked by a loxP-*Neo/Tk*-loxP cassette and a single loxP site. The construct was electroporated into R1 ES cells and G418-resistant clones were screened by Southern blot. Five positive clones were transfected with a Cre-expressing plasmid to excise the *Neo/Tk* cassette yielding +/L2 clones with two loxP sites flanking exon 12. Three of these clones were injected in C57BL/6 blastocysts. Chimaeras were bred with C57BL/6 mice and the resulting heterozygotes were crossed with 'Cre-deleter' CMV-Cre mouse, which expresses the Cre recombinase as transgene (Dupe *et al*, 1997). The CMV-Cre mouse was kindly provided by Pierre Chambon (Institut de Genetique et de Biologie Moleculaire et Cellulaire, Universite Louis Pasteur, Strasbourg, France). The resulting heterozygous chimaeras with a deleted exon 12 (+/L1) were crossed several generations with WT mice to get rid of the Cre recombinase transgene. Then, the heterozygous chimaeras IRAG$^{+/\Delta 12}$ were intercrossed to yield IRAG$^{\Delta 12/\Delta 12}$ mice. Only mice on a 129/Sv background were used. Mice were bred and maintained in the animal facility of the Institut für Pharmakologie und Toxikologie, TU München. Genotyping was performed by PCR using three different primers (A: CTG CTT ACA CAG CTC TGG CCT; B: CCT TGA TGG TGA AAC ACA CCA; C: GCC AGG CTA TAA AGA GAG GTT). The primer pair A and B amplifies the WT (+) allele (173 bp), whereas primer pair A and C amplification comprises the single loxP site of the Δ12 allele (213 bp). Mice were analysed at an age older than 9 weeks.

### *X-ray studies of the gastrointestinal motility*

X-ray studies were performed similar to Pfeifer *et al* (1998). In summary, WT mice and their litter matched IRAG$^{\Delta 12/\Delta 12}$ siblings (in total 12 pairs of mice) were fed with barium sulphate suspension (0.3 ml; Micropaque, Guerbet, Sulzbach, Germany) via stainless feeding needles (Ejay International, Glendora, CA) for about 20 s. Then, the animals that were awake were put into a restrainer bag and X-rays were taken 2, 15, 30 and 60 min after feeding.

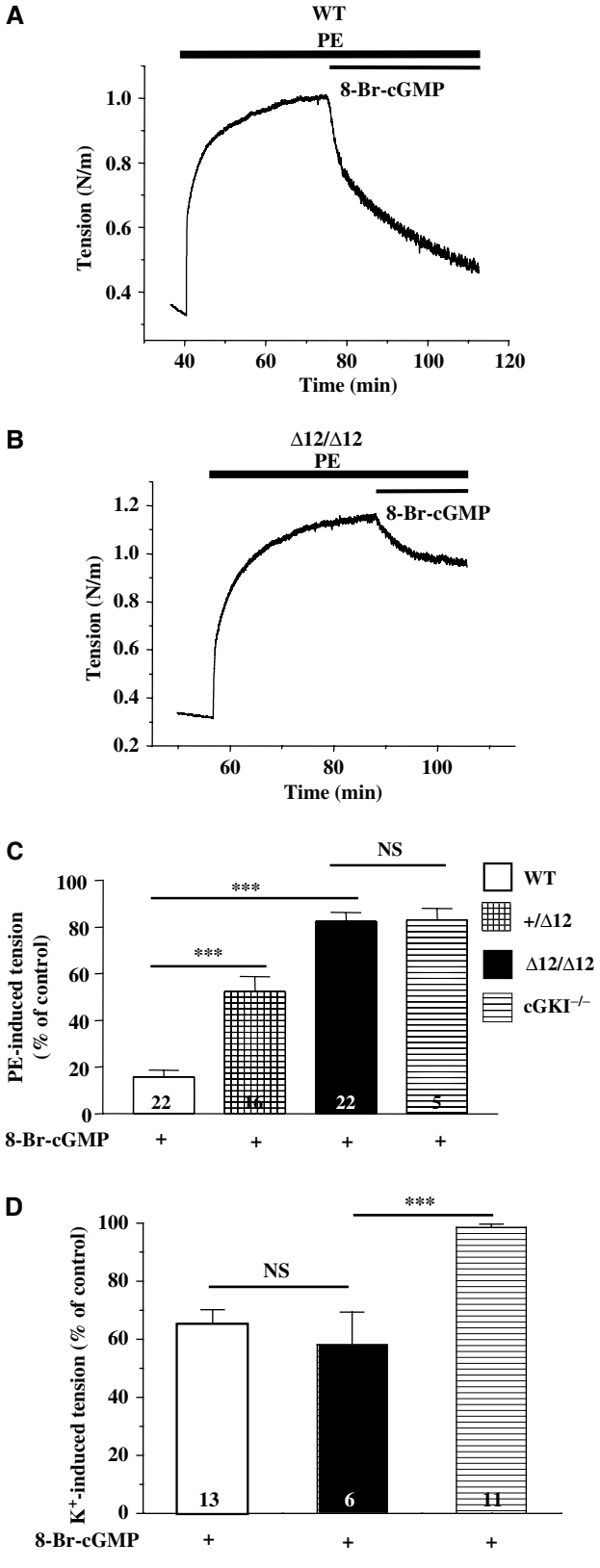


**Figure 6** Effect of 8-Br-cGMP on hormone- and depolarisation-induced contraction in aortic smooth muscle. (**A, B**) Original recordings from aortic rings of (A) WT and (B) IRAG$^{\Delta 12/\Delta 12}$ mice in N/m. Bars indicate the presence of PE (3 μM) and 8-Br-cGMP (300 μM). 8-Br-cGMP was applied after establishment of steady-state conditions. (**C**) Analysis of PE-induced tension. Values reflect tension in the presence of 300 μM 8-Br-cGMP in relation to steady-state conditions (100%). Columns correspond to WT (open bars), IRAG$^{+/\Delta 12}$ (crossed bars), IRAG$^{\Delta 12/\Delta 12}$ (solid bars) and cGKI$^{-/-}$ (striped bars) (see bar legend) and represent means±s.e.m. ***$P<0.001$; NS, not statistically significant. (**D**) Analysis of depolarisation-induced tension. Depolarisation was induced by exchange of 85 mM $Na^+$ with $K^+$. Values reflect tension in the presence of 300 μM 8-Br-cGMP in relation to steady-state conditions (100%). Columns represent means±s.e.m. ***$P<0.001$; NS, not statistically significant.

### *Measurement of blood pressure and heart rate*

Tail-cuff plethysmography (Softron BP-98A, Tokyo) from mice (3–4 months of age), which were trained at least 5 days to the experimental conditions, was used to measure the mean arterial pressure. Heart rate was recorded from the arterial pulse wave at the same time. Electrocardiography measurements in conscious, freely ambulatory mice were made using implantable, minituarised radio frequency transmitters (TA10EA-F20, Data Sciences International) (Ludwig *et al*, 2003).

### *Tension recordings*

Segments of thoracic aorta and colon were taken from WT, heterozygous $IRAG^{+/\Delta12}$, homozygous $IRAG^{\Delta12/\Delta12}$ or cGKI-deficient ($cGKI^{-/-}$) mice (Wegener *et al*, 2002) and placed in buffer solution at 37°C (in mM: NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1, $NaH_2PO_4$ 0.42, $NaHCO_3$ 12, glucose 5.6; aerated with 95% $O_2$ and 5% $CO_2$). Connective tissue and adhering fat were removed. Segments of *Taenia coli* were tied with silk ligatures and mounted longitudinally into organ baths (Myograph 601, Danish Myo Technology). Aortic rings were cleaned off from the endothelium by gently rubbing with a manicure painting brush and mounted into the organ baths. Tension was recorded isometrically. Resting tension was set to 5 mN (aortic rings) and 10 mN (longitudinal colonic segments). Hormone-induced contraction was measured at the maximum (peak tension) and after establishment of steady-state conditions (tonic tension).

### *Cell culture and $[Ca^{2+}]_i$ measurement*

Cell culture and lysate preparation of Cos-7 cells were performed as described previously (Ammendola *et al*, 2001). Cos-7 cells were transfected with WT bovine IRAGa- or IRAGaΔ12(=ΔAA530–576)-pcDNA3.1, cGKIβ cloned in pcDNA3.1, and $InsP_3RI$ in pCMVI-9 or pcDNA3 (a gift from Dr Ilya Bezprozvanny (University of Texas Southwestern Medical Center, Dallas, TX, USA)) using the PolyFect transfection reagent (Qiagen).

Vascular smooth muscle cells were isolated from the aorta of WT or $IRAG^{\Delta12/\Delta12}$ mice and grown on coverslips in DMEM (PAA Laboratories) supplemented with 10% FCS as described previously (Feil *et al*, 2002). Prior to the measurements, cells from primary culture were maintained in serum-free medium for 2 days. Then, the cells were loaded with Fura 2-AM (3 μM) in tyrode solution (in mM: NaCl 140, KCl 5, $CaCl_2$ 2, $MgSO_4$ 1.2, glucose 10, HEPES 5, pH 7.4) and 1% BSA for about 60 min and washed with tyrode solution. The ratio of the emission at 350 nm (F1) and 380 nm (F2) excitation wavelength was measured as an indicator of $[Ca^{2+}]_i$ in single cells with the dual-wavelength microfluorescence technique using a digital system (TILL Photonics) and an Axiovert-35 microscope (Zeiss). Identical or similar wavelengths have been used by Lee *et al* (2000) and Rose *et al* (2003). Fura 2-loaded vascular smooth muscle cells were stimulated with NE (25 nM) or by depolarisation with a tyrode solution (in mM: NaCl 60, KCl 85, $CaCl_2$ 2, $MgSO_4$ 1.2, glucose 10, HEPES 5, pH 7.4).

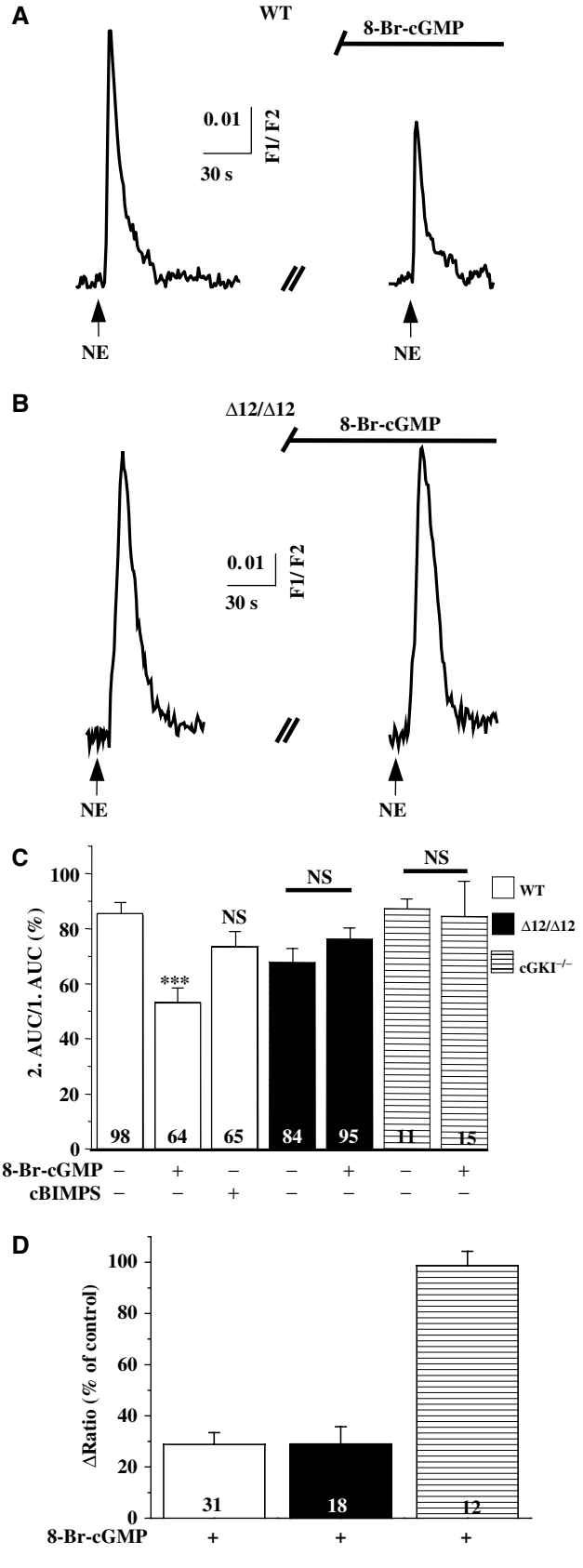


**Figure 7** Effect of 8-Br-cGMP on NE-induced calcium transients in vascular smooth muscle cells. Fura 2-loaded vascular smooth muscle cells were stimulated with NE (25 nM) and then superfused with tyrode solution for 5 min (gap), followed by a 5 min incubation with or without 1 mM 8-Br-cGMP or 0.1 mM cBIMPS and a second stimulation with NE. (**A, B**) Representative original $[Ca^{2+}]_i$ traces in single cells. The arrows mark the application of NE. F1/F2: Fluorescence ratio of the emission at 350 nm (F1) and 380 nm (F2). (A) WT and (B) $IRAG^{\Delta12/\Delta12}$ cells preincubated with 1 mM 8-Br-cGMP before the second stimulation. (**C**) Statistics of the effects of 8-Br-cGMP and cBIMPS on NE-induced $Ca^{2+}$ transients in WT cells (open bars), $IRAG^{\Delta12/\Delta12}$ cells (solid bars) and $cGKI^{-/-}$ cells (striped bars; adapted from Feil *et al*, 2002) (see bar legend) in the absence and presence of 8-Br-cGMP. Data are expressed as the ratio of the area under the curve (AUC) of the second over the first transient. (**D**) Statistics of the effects of 8-Br-cGMP on depolarisation-induced $Ca^{2+}$ transients in WT, $IRAG^{\Delta12/\Delta12}$ and $cGKI^{-/-}$ cells. Fura 2-loaded vascular smooth muscle cells were depolarised with tyrode solution containing 85 mM KCl. At steady-state conditions of the stimulated calcium transient, 8-Br-cGMP (1 mM) was applied. The ratio of the steady state after 8-Br-cGMP application in comparison to the steady state before 8-Br-cGMP application was calculated. Columns represent means±s.e.m. $***P<0.001$; NS: not statistically significant. The number of cells ($n$) measured under each condition is given inside each bar.

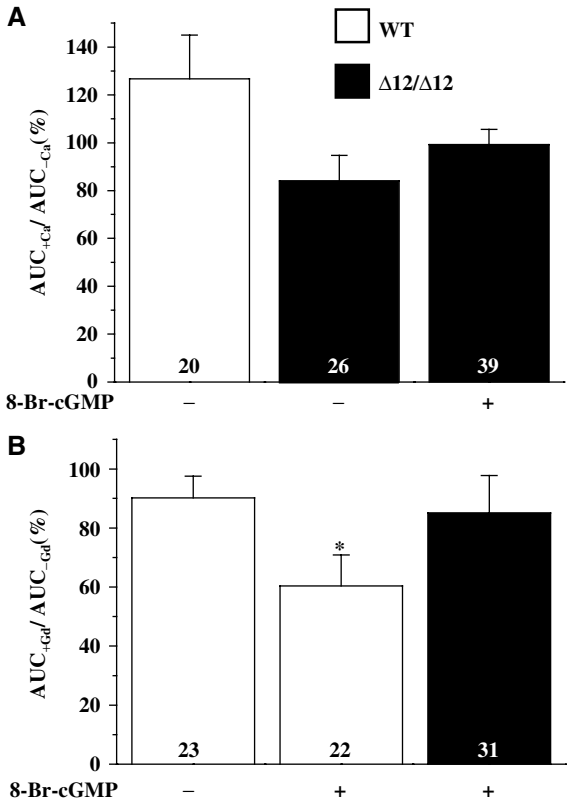


**Figure 8** Statistics of the effect of external $Ca^{2+}$ or $Gd^{3+}$ on NE-induced calcium transients in vascular smooth muscle cells in the absence or presence of 8-Br-cGMP. (**A**) Fura 2-loaded WT (open bar) or $IRAG^{\Delta12/\Delta12}$ cells (solid bar, middle) (see bar legend) were washed with $Ca^{2+}$-free buffer (tyrode solution without $CaCl_2$) and then stimulated with NE (25 nM). Subsequently, the cells were superfused with tyrode solution (containing 2 mM $CaCl_2$) for 5 min, followed by a 5 min resting time and a second stimulation with NE. The same experiment was performed with $IRAG^{\Delta12/\Delta12}$ cells in the presence of 1 mM 8-Br-cGMP (right bar). (**B**) Fura 2-loaded WT or $IRAG^{\Delta12/\Delta12}$ cells were stimulated with NE and then superfused with tyrode solution for 5 min, followed by a 3 min resting time and 2 min incubation of 100 μM $GdCl_3$ and a second stimulation with NE. Before the second stimulation, cells were incubated in the absence or presence of 8-Br-cGMP (1 mM, 5 min). Data are expressed as the ratio of the AUC of the second over the first transient. Columns represent means ± s.e.m. values. $^*P<0.025$. The number of cells ($n$) measured under each condition is given inside each bar.

### *Immunochemical methods*

Proteins from different mouse organs were isolated by homogenisation with an Ultra Turrax (IKA) in extraction buffer (in mM: Tris–HCl 20, NaCl 100, DTT 2.5, EDTA 2.5, benzamidine 2.5, PMSF 2.5, and 1 μg/μl leupeptin, pH 8.0, 4°C) followed by centrifugation (at 13 000 *g*, 4°C for 10 min). The supernatants containing the tissue proteins were stored at −80°C. Western blot analysis of the tissue proteins was performed with selective primary antibodies and with secondary antibodies coupled to horseradish peroxidase or alkaline phosphatase. Antibodies were as described by Ammendola *et al* (2001) and Geiselhöringer *et al* (2004). For quantification of proteins, Western blots were scanned and signals analysed by using AIDA software, version 2.11 (Raytest, Straubenhardt, Germany). As internal standard of protein expression, the signal of the MAPK was used. The statistical measurements were performed with the program Origin 6.1. Isolation of protein complexes with 8-AET-cGMP-agarose affinity chromatography from transfected Cos-7 cells or CoIP with specific antibodies and protein A-Sepharose from isolated tissue proteins was essentially performed as described (Schlossmann *et al*, 2000; Ammendola *et al*, 2001). In brief, the Cos-7 cell or tissue lysate proteins were solubilised in Lubrol buffer (2% Lubrol-PX; in mM: MOPS 20, NaCl 150, benzamidine 1, PMSF 0.3, and 0.5 μg/ml leupeptin, pH 8.0) for 20 min at 4°C and then centrifuged (14 000 r.p.m., 10 min, 4°C). The supernatant was added to 8-AET-cGMP-agarose beads (Biolog) or for immunoprecipitation to protein A-Sepharose beads (Sigma) prebound with IRAG-specific antibodies. After incubation for 2 h at 4°C in Lubrol buffer, the beads were washed. Proteins were eluted with Laemmli buffer and analysed by SDS–PAGE and Western blot.

### *Calculation and statistics*

All data are expressed as mean ± s.e.m. For the calculation of statistical differences between two means, the Student's *t*-test was used. The significance of the *P*-value was indicated by asterisks ($^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$; NS: not statistically significant). $n$ indicates the number of experiments.

## Acknowledgements

We thank Christine Wolf for her excellent technical assistance. Dr Christian Jorda (LMU München, Veterinary Medicine) is acknowledged for his help in the X-ray studies. This project was supported by the DFG, the SFB391, the Sanderstiftung and the GRK438.

## References

Alioua A, Tanaka Y, Wallner M, Hofmann F, Ruth P, Meera P, Toro L (1998) The large conductance, voltage-dependent, and calcium-sensitive $K^+$ channel, Hslo, is a target of cGMP-dependent protein kinase phosphorylation *in vivo*. *J Biol Chem* **273:** 32950–32956

Ammendola A, Geiselhöringer A, Hofmann F, Schlossmann J (2001) Molecular determinants of the interaction between the inositol 1,4,5-trisphosphate receptor-associated cGMP kinase substrate (IRAG) and cGMP kinase Ibeta. *J Biol Chem* **276:** 24153–24159

Aszodi A, Pfeifer A, Ahmad M, Glauner M, Zhou XH, Ny L, Andersson KE, Kehrel B, Offermanns S, Fassler R (1999) The vasodilator-stimulated phosphoprotein (VASP) is involved in cGMP- and cAMP-mediated inhibition of agonist-induced platelet aggregation, but is dispensable for smooth muscle function. *EMBO J* **18:** 37–48

Beall A, Bagwell D, Woodrum D, Stoming TA, Kato K, Suzuki A, Rasmussen H, Brophy CM (1999) The small heat shock-related protein, HSP20, is phosphorylated on serine 16 during cyclic nucleotide-dependent relaxation. *J Biol Chem* **274:** 11344–11351

Bolton TB, Prestwich SA, Zholos AV, Gordienko DV (1999) Excitation–contraction coupling in gastrointestinal and other smooth muscles. *Annu Rev Physiol* **61:** 85–115

Bonnevier J, Fassler R, Somlyo AP, Somlyo AV, Arner A (2004) Modulation of $Ca^{2+}$ sensitivity by cyclic nucleotides in smooth muscle from protein kinase G-deficient mice. *J Biol Chem* **279:** 5146–51451

Cornwell TL, Lincoln TM (1989) Regulation of intracellular $Ca^{2+}$ levels in cultured vascular smooth muscle cells. Reduction of $Ca^{2+}$ by atriopeptin and 8-bromo-cyclic GMP is mediated by cyclic GMP-dependent protein kinase. *J Biol Chem* **264:** 1146–1155

Dupe V, Davenne M, Brocard J, Dolle P, Mark M, Dierich A, Chambon P, Rijli FM (1997) *In vivo* functional analysis of the Hoxa-1 3′ retinoic acid response element (3′RARE). *Development* **124:** 399–410

 

Eigenthaler M, Ullrich H, Geiger J, Horstrup K, Honig-Liedl P, Wiebecke D, Walter U (1993) Defective nitrovasodilator-stimulated protein phosphorylation and calcium regulation in cGMP-dependent protein kinase-deficient human platelets of chronic myelocytic leukemia. *J Biol Chem* **268:** 13526–13531

Feil R, Gappa N, Rutz M, Schlossmann J, Rose CR, Konnerth A, Brummer S, Kuhbandner S, Hofmann F (2002) Functional reconstitution of vascular smooth muscle cells with cGMP-dependent protein kinase I isoforms. *Circ Res* **90:** 1080–1086

Feil R, Lohmann SM, de Jonge H, Walter U, Hofmann F (2003) Cyclic GMP-dependent protein kinases and the cardiovascular system: insights from genetically modified mice. *Circ Res* **93:** 907–916

Friebe A, Koesling D (2003) Regulation of nitric oxide-sensitive guanylyl cyclase. *Circ Res* **93:** 96–105

Fukao M, Mason HS, Britton FC, Kenyon JL, Horowitz B, Keef KD (1999) Cyclic GMP-dependent protein kinase activates cloned BKCa channels expressed in mammalian cells by direct phosphorylation at serine 1072. *J Biol Chem* **274:** 10927–10935

Geiselhöringer A, Gaisa M, Hofmann F, Schlossmann J (2004) Distribution of IRAG and cGKI-isoforms in murine tissues. *FEBS Lett* **575:** 19–22

Hauser W, Knobeloch KP, Eigenthaler M, Gambaryan S, Krenn V, Geiger J, Glazova M, Rohde E, Horak I, Walter U, Zimmer M (1999) Megakaryocyte hyperplasia and enhanced agonist-induced platelet activation in vasodilator-stimulated phosphoprotein knockout mice. *Proc Natl Acad Sci USA* **96:** 8120–8125

Hofmann F, Ammendola A, Schlossmann J (2000) Rising behind NO: cGMP-dependent protein kinases. *J Cell Sci* **113:** 1671–1676

Komalavilas P, Lincoln TM (1994) Phosphorylation of the inositol 1,4,5-trisphosphate receptor by cyclic GMP-dependent protein kinase. *J Biol Chem* **269:** 8701–8707

Kwan HY, Huang Y, Yao X (2004) Regulation of canonical transient receptor potential isoform 3 (TRPC3) channel by protein kinase G. *Proc Natl Acad Sci USA* **101:** 2625–2630

Lalli MJ, Shimizu S, Sutliff RL, Kranias EG, Paul RJ (1999) $[Ca^{2+}]_i$ homeostasis and cyclic nucleotide relaxation in aorta of phospholamban-deficient mice. *Am J Physiol* **277:** H963–H970

Lee SH, Rosenmund C, Schwaller B, Neher E (2000) Differences in $Ca^{2+}$ buffering properties between excitatory and inhibitory hippocampal neurons from the rat. *J Physiol* **525:** 405–418

Ludwig A, Budde T, Stieber J, Moosmang S, Wahl C, Holthoff K, Langebartels A, Wotjak C, Munsch T, Zong X, Feil S, Feil R, Lancel M, Chien KR, Konnerth A, Pape HC, Biel M, Hofmann F (2003) Absence epilepsy and sinus dysrhythmia in mice lacking the pacemaker channel HCN2. *EMBO J* **22:** 216–224

Meredith AL, Thorneloe KS, Werner ME, Nelson MT, Aldrich RW (2004) Overactive bladder and incontinence in the absence of the BK large conductance $Ca^{2+}$-activated $K^+$ channel. *J Biol Chem* **279:** 36746–36752

Moosmang S, Schulla V, Welling A, Feil R, Feil S, Wegener JW, Hofmann F, Klugbauer N (2003) Dominant role of smooth muscle L-type calcium channel $Ca_v1.2$ for blood pressure regulation. *EMBO J* **22:** 6027–6034

Pfeifer A, Klatt P, Massberg S, Ny L, Sausbier M, Hirneiß C, Wang G, Korth M, Aszódi A, Andersson E, Krombach F, Mayerhofer A, Ruth P, Fässler R, Hofmann F (1998) Defective smooth muscle regulation in cGMP kinase I-deficient mice. *EMBO J* **17:** 3045–3051

Rembold CM, Foster DB, Strauss JD, Wingard CJ, Eyk JE (2000) cGMP-mediated phosphorylation of heat shock protein 20 may cause smooth muscle relaxation without myosin light chain dephosphorylation in swine carotid artery. *J Physiol* **524:** 865–878

Rose CR, Blum R, Pichler B, Lepier A, Kafitz KW, Konnerth A (2003) Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells. *Nature* **426:** 74–78

Ruiz-Velasco V, Zhong J, Hume JR, Keef KD (1998) Modulation of $Ca^{2+}$ channels by cyclic nucleotide cross activation of opposing protein kinases in rabbit portal vein. *Circ Res* **82:** 557–565

Ruth P, Wang GX, Boekhoff I, May B, Pfeifer A, Penner R, Korth M, Breer H, Hofmann F (1993) Transfected cGMP-dependent protein kinase suppresses calcium transients by inhibition of inositol 1,4,5-trisphosphate production. *Proc Natl Acad Sci USA* **90:** 2623–2627

Sausbier M, Hu H, Arntz C, Feil S, Kamm S, Adelsberger H, Sausbier U, Sailer CA, Feil R, Hofmann F, Korth M, Shipston MJ, Knaus HG, Wolfer DP, Pedroarena CM, Storm JF, Ruth P (2004) Cerebellar ataxia and Purkinje cell dysfunction caused by $Ca^{2+}$-activated $K^+$ channel deficiency. *Proc Natl Acad Sci USA* **101:** 9474–9478

Sausbier M, Schubert R, Voigt V, Hirneiss C, Pfeifer A, Korth M, Kleppisch T, Ruth P, Hofmann F (2000) Mechanisms of NO/cGMP-dependent vasorelaxation. *Circ Res* **87:** 825–830

Sauzeau V, Le Jeune H, Cario-Toumaniantz C, Smolenski A, Lohmann SM, Bertoglio J, Chardin P, Pacaud P, Loirand G (2000) Cyclic GMP-dependent protein kinase signaling pathway inhibits RhoA-induced $Ca^{2+}$ sensitization of contraction in vascular smooth muscle. *J Biol Chem* **275:** 21722–21729

Schlossmann J, Ammendola A, Ashman K, Zong X, Huber A, Neubauer G, Wang GX, Allescher HD, Korth M, Wilm M, Hofmann F, Ruth P (2000) Regulation of intracellular calcium by a signalling complex of IRAG, IP3 receptor and cGMP kinase Ibeta. *Nature* **404:** 197–201

Schlossmann J, Feil R, Hofmann F (2003) Signaling through NO and cGMP-dependent protein kinases. *Ann Med* **35:** 21–27

Somlyo AP, Somlyo AV (2000) Signal transduction by G-proteins, rho-kinase and protein phosphatase to smooth muscle and non-muscle myosin II. *J Physiol* **522:** 177–185

Soulsby MD, Alzayady K, Xu Q, Wojcikiewicz RJ (2004) The contribution of serine residues 1588 and 1755 to phosphorylation of the type I inositol 1,4,5-trisphosphate receptor by PKA and PKG. *FEBS Lett* **557:** 181–184

Surks HK, Mochizuki N, Kasai Y, Georgescu SP, Tang KM, Ito M, Lincoln TM, Mendelsohn ME (1999) Regulation of myosin phosphatase by a specific interaction with cGMP-dependent protein kinase Ialpha. *Science* **286:** 1583–1587

Tertyshnikova S, Yan X, Fein A (1998) cGMP inhibits IP3-induced $Ca^{2+}$ release in intact rat megakaryocytes via cGMP- and cAMP-dependent protein kinases. *J Physiol* **512** (Part 1)**:** 89–96

Wagner II LE, Li WH, Yule DI (2003) Phosphorylation of type-1 inositol 1,4,5-trisphosphate receptors by cyclic nucleotide-dependent protein kinases: a mutational analysis of the functionally important sites in the S2+ and S2− splice variants. *J Biol Chem* **278:** 45811–45817

Wegener JW, Nawrath H, Wolfsgruber W, Kuhbandner S, Werner C, Hofmann F, Feil R (2002) cGMP-dependent protein kinase I mediates the negative inotropic effect of cGMP in the murine myocardium. *Circ Res* **90:** 18–20

Wegener JW, Schulla V, Lee T-S, Koller A, Feil S, Feil R, Kleppisch T, Klugbauer N, Moosmang S, Welling A, Hofmann F (2004) An essential role of $Ca_v1.2$ L-type calcium channel for urinary bladder function. *FASEB J* **18:** 1159–1161

Xia C, Bao Z, Yue C, Sanborn BM, Liu M (2001) Phosphorylation and regulation of G-protein-activated phospholipase C-beta 3 by cGMP-dependent protein kinases. *J Biol Chem* **276:** 19770–19777

Zhou XB, Arntz C, Kamm S, Motejlek K, Sausbier U, Wang GX, Ruth P, Korth M (2001) A molecular switch for specific stimulation of the BKCa channel by cGMP and cAMP kinase. *J Biol Chem* **276:** 43239–43245