FEBS 28772 FEBS Letters 575 (2004) 19–22

# Distribution of IRAG and cGKI-isoforms in murine tissues

A. Geiselhöringer, M. Gaisa, F. Hofmann, J. Schlossmann*

*Institut für Pharmakologie und Toxikologie der TU München, Biedersteiner Straße 29, 80802 München, Germany*

Received 5 July 2004; revised 16 August 2004; accepted 16 August 2004

Available online 26 August 2004

Edited by Amy McGough

**Abstract cGMP kinase I (cGKI) signaling modulates multiple physiological processes including smooth muscle relaxation. The expression of cGKI and its substrate IRAG (Inositol 1,4,5-trisphosphate receptor associated cGMP kinase substrate) was studied. IRAG and cGKI were colocalized in the smooth muscle of aorta and colon. IRAG was present in the thalamus and in most of the myenteric plexus in the absence of cGKI. Coexpression of IRAG and cGKIβ or cGKIα in COS-7 cells revealed that IRAG recruits cGKIβ but not cGKIα to the endoplasmic reticulum. These results suggest that IRAG may be involved in cGKI-dependent and -independent pathways.**


*Keywords:* Tissue distribution; Immunohistochemistry; cGMP-dependent protein kinase; In-situ hybridization; IRAG; Smooth muscle

## 1. Introduction

Signal transduction via NO/cGMP/cGKI is involved in a variety of physiological mechanisms including smooth muscle relaxation, platelet aggregation and synaptic plasticity [1]. Smooth muscle tone is mainly regulated by the rise and fall of intracellular calcium concentration. Activation of cGKI via cGMP lowers cytosolic $Ca^{2+}$ concentration and thus leads to smooth muscle relaxation [2,3]. The crucial role of cGKI for smooth muscle function became apparent in cGKI-deficient mice which showed hypertension and severe gastrointestinal dysfunction [4]. However, the molecular mechanisms of cGMP signaling distal to cGKI are not yet fully understood. The two existing isoforms of cGKI, cGKIα and cGKIβ, which differ only in their amino termini, are expressed in vascular and non-vascular smooth muscles [5]. These amino termini are involved in homodimerization and target protein recognition [6,1]. For the versatility of signaling, different cGKI substrates are needed. The amino terminus of cGKIα interacts specifically with the myosin-binding subunit of myosin phosphatase [7], whereas the amino terminus of cGKIβ interacts specifically with IRAG [8]. In contrast to this detailed information on the biochemistry of the cGKI isoforms, their specific cellular functions are unclear.

An important member of the cGKI-mediated intracellular calcium regulation is IRAG, a cGKIβ substrate, which was identified in tracheal smooth muscle membranes [9]. Phosphorylation of IRAG by cGKI inhibits $IP_3$-induced calcium release after coexpression of both proteins in COS-7 cells [8,9]. Furthermore, the inhibitory effect on calcium release from $IP_3$-sensitive stores was abolished in human colonic smooth muscle cells when IRAG expression was substantially diminished [10].

So far, the expression pattern of IRAG in murine tissues is widely unknown. Also, no systematical analysis of cGKI isoforms in murine tissues was performed yet. In the present study, we determined the expression profile of IRAG and cGKI to obtain additional insights in the functional role of IRAG and cGKI isoforms.

## 2. Material and methods

### 2.1. Western blot analysis

Protein was isolated from different mouse organs. The tissues were homogenized with extraction buffer (20 mM Tris–HCl; 100 mM NaCl, pH 8.0, with 2.5 mM DTT; 2.5 mM EDTA; 2.5 mM Benzamidine; 2.5 mM PMSF and 1 μg/μl Leupeptin; 4 °C) in an Ultra Turrax homogenizer at ~26 000 rpm. The homogenates were centrifuged at 13 000 × *g*, 4 °C for 10 min and the supernatants were stored at −80 °C. The proteins were separated by SDS–PAGE and blotted onto PVDF membranes. The blots were probed with selective antibodies for IRAG, cGKI (cGKIcommon which recognizes both isoforms, cGKIα and cGKIβ with identical affinity), cGKIα and cGKIβ (raised against the $NH_2$-terminal domain of the corresponding enzyme expressed in *E. coli*), and MAPK (Cell Signaling) at a dilution of 1:500, 1:200, 1:80, 1:1000 and 1:1000, respectively. Immunoblots were visualized by incubation with an anti-rabbit IgG antibody coupled to horseradish peroxidase (Dianova) followed by enhanced chemiluminescence (ECL system, Perkin Elmer).

### 2.2. Immunohistochemistry

After fixation (10% formalin, 4–12 h, 4 °C), the tissues were briefly rinsed in phosphate-buffered saline (PBS; pH 7.4), dissected according to anatomical borders and cleared of intraluminal material. After dehydration (graded series of ethanol, cleared with Roticlear [Roth]), the tissues were paraffin-embedded. Sections were cut at 6–10 μm on a sliding microtome (Microm), mounted on Superfrost Plus slides (Menzel-Gläser) and dried at 37 °C overnight. The sections were deparaffinized in Roticlear and rehydrated in a descending series of ethanol. *Immunoperoxidase staining:* After a rinse in PBS, the activity of endogenous peroxidase was blocked with 10% methanol and 3% hydrogen peroxide in PBS. The sections were rinsed in PBS and incubated with 10% normal goat serum (NGS; Vectastain, Vector Laboratories, Burlingame, CA) for 1 h at room temperature in a humid chamber. After a 5 min rinse in PBS, the incubation was continued overnight with the primary antibody (IRAG, cGKIcommon, cGKIα or β; raised in rabbit) at 4 °C, diluted in PBS containing 3% NGS (1:50,

* Corresponding author. Fax: +49-89-41403261.
*E-mail address:* schlossmann@ipt.med.tu-muenchen.de (J. Schlossmann).

*Abbreviations:* cGKI, cGMP kinase I; IRAG, inositol 1,4,5-trisphosphate receptor associated cGMP kinase substrate; $InsP_3R$, inositol 1,4,5-trisphosphate-receptor

0014-5793/$22.00 
doi:10.1016/j.febslet.2004.08.030

1:50, 1:50 or 1:100, respectively) and was terminated by three rinses of 5 min each. The following steps were performed as described in Vectastain Elite ABC Kit (Vector). Stained sections were rinsed in PBS, coverslipped in Aquatex (Merck) and photographed. *Immunofluorescence staining:* Colocalization of proteins was investigated by a sequential double immunostaining protocol. Following a 5 min rinse in TBST (Tris buffered saline with Tween), sections were incubated for 1 h in TBST containing 10% normal donkey serum (NDS, Dianova). All incubations, besides the overnight step with the cGKI antibody (4 °C), were performed at room temperature. Antibodies employed were diluted in TBST with 3% bovine serum albumin (Dianova). The rinsed sections were incubated with a specific antibody against IRAG (1:50; raised in guinea pig) for 3.5 h. After three rinses with TBST, each 5 min, the incubation was continued overnight with cGKIcommon antibody (1:50; raised in rabbit) and terminated by several rinses in TBST. Immunoreactivity was detected with a mixture of indocarbocyanin (Cy3)- and carbocyanin (Cy2)-conjugated secondary antibodies (donkey anti-guinea pig 1:50 and donkey anti-rabbit 1:500, respectively; 1 h; Dianova). The fluorescence-labeled sections were coverslipped with ProLong antifade kit (Molecular Probes). Controls included omission of the primary antibody, antibodies preadsorbed with the respective antigen or, in the case of cGKI, cGKI-deficient tissue.

### 2.3. Expression of IRAG-eGFP, cGKIα-dsRed and cGKIβ-dsRed in COS-7 cells

For construction of IRAG-eGFP and cGKI-dsRed fusions, appropriate PCR products were cloned into pEGFP-N3 (Clontech) and pDsRed1 (Clontech), respectively. COS-7 cells grown in DMEM (Gibco) were transiently transfected by using the calcium phosphate method. Te following transfection procedures were used: IRAG-eGFP, cGKIα-dsRed or cGKIβ-dsRed alone and IRAG-eGFP together with cGKIα-dsRed or cGKIβ-dsRed. After 48 h of incubation at 37 °C, expression of fusion proteins was visualized using a confocal laser scanning microscope LSM510 (Zeiss).

### 2.4. Confocal laser scanning microscopy

Sections and COS-7 cells were analyzed by confocal laser scanning microscopy on an LSM510 (Zeiss). Fluorochromes were excited with 488 and 543 nm lines, respectively, by a Krypton–Argon laser and a Helium–Neon laser. Single optical sections and extended focus images were taken with 20× dry or 40× immersion objective lenses and various zoom factors. When controls were compared, care was taken to keep the pinhole and gain of the photomultiplier constant. Double staining (colocalization) was visualized with "multitrack" to eliminate emission crosstalk.

### 2.5. In situ hybridization

Adult C57BL/6 mice were killed by cervical dislocation, the brain was removed and frozen in isopentane cooled in a dry ice/ethanol bath. Cryostat sections (16 μm) were thaw-mounted onto poly-L-lysine coated slides, fixed with 4% paraformaldehyde and dehydrated in a graded series of ethanol. Sections were pretreated for hybridization as described previously [11] and then prehybridized for 2 h at 42 °C in hybridization buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 0.3 M NaCl, 50 mM DTT, 1× Denhardt' solution, 10% Dextran, and 50% deionized formamide). Hybridization was performed as described by Moosmang et al. [12]. Subsequently, the sections were dehydrated and exposed to Biomax MR film (Kodak) for 6–20 days. [$^{35}$S] UTP-labeled RNA probes were transcribed in vitro corresponding to amino acids 531–652 of IRAGb, 1–89 of cGKIα, 1–104 of cGKIβ, 507–674 of cGKIcommon and 320–580 of cGKII (Accession Nos. U63407, X16086, X54289, X16086 and L12460). In situ hybridizations were always performed including the corresponding sense cRNA probes on adjacent sections. These control hybridizations showed no signals.

## 3. Results

### 3.1. Expression of IRAG and cGKI in different tissues

The protein expression of IRAG in tissues has not been elucidated yet. As a first step into this issue, we determined the expression pattern of IRAG in different mouse tissues. Western blot analysis showed that IRAG is predominantly expressed in smooth muscle tissues, including aorta, stomach, colon, urinary bladder, uterus, lung and trachea (Fig. 1A). IRAG was additionally highly abundant in human platelets (Fig. 1A). cGKI was ubiquitary distributed in peripheral tissues and in distinct regions of the brain, e.g. cerebellum (Fig. 1B). The expression profile of the cGKI isoforms, Iα and Iβ, was investigated with specific antibodies (Fig. 1D). cGKIα showed strong signals in lung, heart and cerebellum, whereas cGKIβ was the dominate isoform in most smooth muscle tissues and platelets. Approximately equal amounts of both isoforms were found in small intestinal tissues (Fig. 1B).

We examined neuronal expression of IRAG and cGK in mouse brain with in situ hybridization. A strikingly restricted pattern was observed for IRAG transcripts. IRAG mRNA was detected only in thalamic relay nuclei (Fig. 2A). In contrast, cGKI was not found in thalamus, but in cerebellum, olfactory bulb, cortex and hippocampus (Fig. 2D). cGKI isoform specific probes revealed different expression profiles. cGKIα was strongly present in cerebellar cortex (Fig. 2E), whereas intense signals of cGKIβ were detected in olfactory bulb and hippocampus (Fig. 2F). Interestingly, the distribution of cGKII was partly similar to that of IRAG. We observed signals in cortex, olfactory bulb and thalamus (Fig. 2C). Western blot analysis confirmed the expression of IRAG and

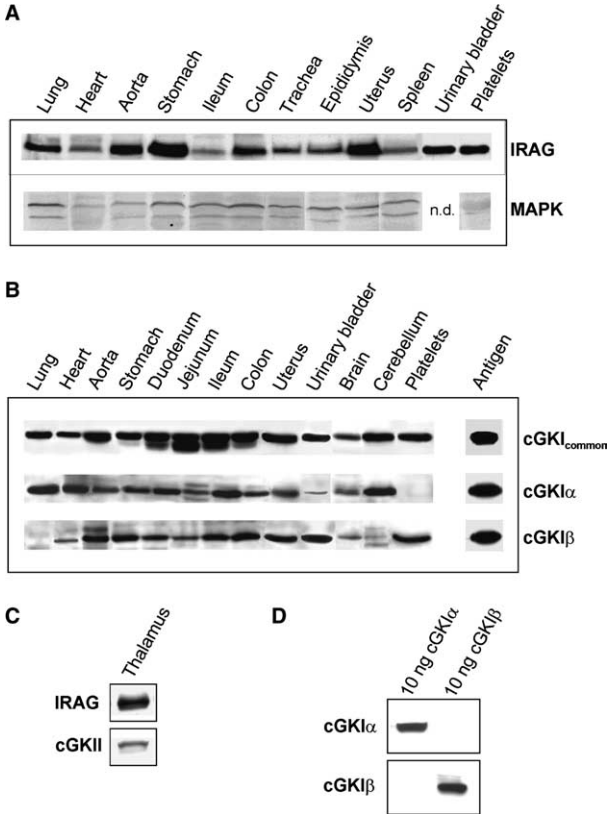


Fig. 1. Protein expression of IRAG and cGKI in different tissues. Western blot of different murine tissues (50 μg each lane) and human platelet membranes (20 μg) probed with specific antibodies for IRAG (A), cGKIcommon, cGKIα or cGKIβ, respectively (B). As control for (A) the tissue expression of the mitogen-activated protein kinase (MAPK) is shown. n.d.: not detected. (C) IRAG and cGKII expression in the thalamus region. (D) Specificity of cGKIα and cGKIβ isoform selective antibodies.

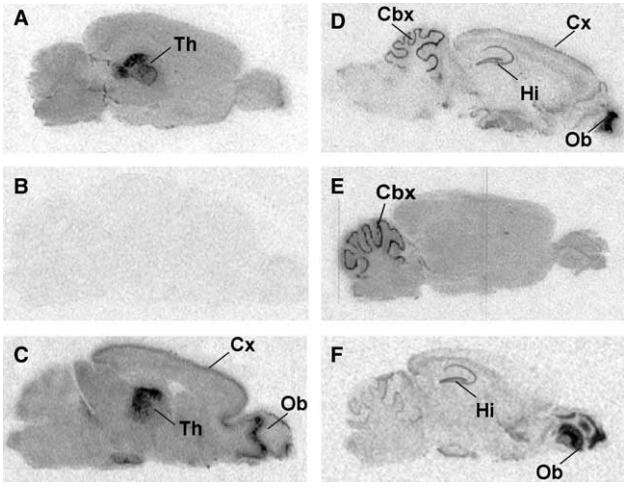


Fig. 2. CNS distribution of IRAG and cGK mRNAs in mouse brain. Autoradiographic film images of sagittal sections hybridized with antisense riboprobes specific for IRAG (A), cGKII (C), cGKIcommon (D), cGKIα (E) and cGKIβ (F). (B) Corresponding sense riboprobe for IRAG. Cbx, cerebellar cortex; Cx, cortex; Hi, hippocampus; Ob, olfactory bulb; Th, thalamus.

cGKII in the thalamus region (Fig. 1C). A similar distribution of the cGKI and II has been reported for rat [13,14].

### 3.2. Tissue localization of IRAG and cGKI

The immunohistochemical localization of IRAG, cGKIα and cGKIβ was examined in different peripheral tissues. Staining of aortic sections with specific antibodies against IRAG, cGKIcommon, cGKIα and cGKIβ revealed strong signals in myocytes of the media (Fig. 3A). Furthermore, IRAG and cGKI were colocalized in aortic smooth muscle cells (Fig. 4A). Immunohistochemical analysis of the gastrointestinal tract revealed ubiquitary expression of cGKIα and cGKIβ in contractile cells with strong staining for cGKIβ. cGKI was found in colonic muscularis mucosae, which represents the boundary between the mucosa and the submucosa. Furthermore, cGKI was abundant in the longitudinal and circular muscle layer (Fig. 3B) of the muscularis. IRAG was also expressed in the muscularis (longitudinal plus circular muscle) and muscularis mucosae. As shown by confocal laser scanning microscopy, IRAG and cGKI were colocalized in these smooth muscle layers (Fig. 4B). In addition, IRAG showed clear signals in the myenteric plexus (Fig. 3B), which is a part of the enteric nervous system. However, colocalization with cGKI in this region was rare and weak (Fig. 4B).

### 3.3. Intracellular localization of coexpressed IRAG and cGKI in COS-7 cells

It is well established that IRAG is a membrane protein, whereas cGKIβ is a soluble enzyme and these proteins are assembled in a ternary complex with $InsP_3RI$ [9]. As identified by yeast two-hybrid system, IRAG interacts with the leucine zipper of cGKIβ but not of cGKIα [8]. The question whether the interaction between IRAG and cGKIβ causes an intracellular translocation of cGKIβ was studied with fusion proteins. COS-7 cells which expressed IRAG-eGFP, cGKIβ-dsRed or cGKIα-dsRed were analyzed by confocal laser scanning microscopy. cGKIα and cGKIβ revealed a distinct cytosolic distribution, whereas IRAG was found at the

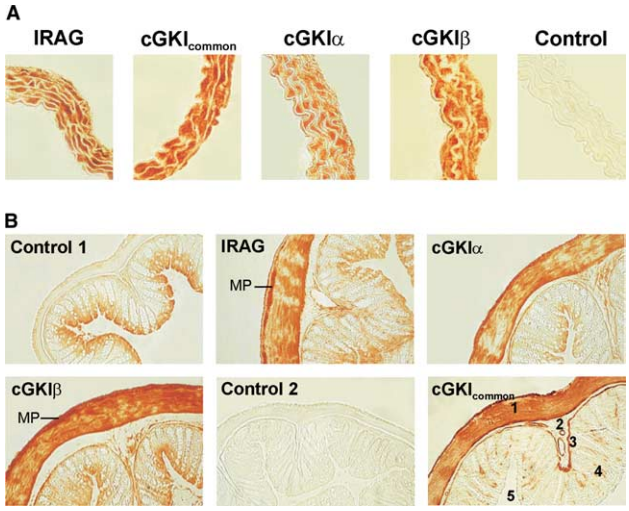


Fig. 3. Localization of IRAG and cGKI in aorta and colon. Paraffin-sections of mouse aorta (A) and colon (B). (A) Both IRAG and cGKI are expressed in myocytes of the media. No staining was observed at the control section ($cGKI^{-/-}$ aorta). (B) IRAG and cGKI were present in the muscularis, muscularis mucosae and in the myenteric plexus (MP). Control sections showed no signal besides unspecific staining of the epithelium (Control 1). Cross-sections with a magnification of 400× (A) and 100× (B) were 6 μm or 10 μm thick, respectively. 1, muscularis; 2, submucosa; 3, muscularis mucosae; 4, mucosa; 5, lumen. Control 1: preadsorbed IRAG antibody; Control 2: $cGKI^{-/-}$ colon stained with cGKI antibody.

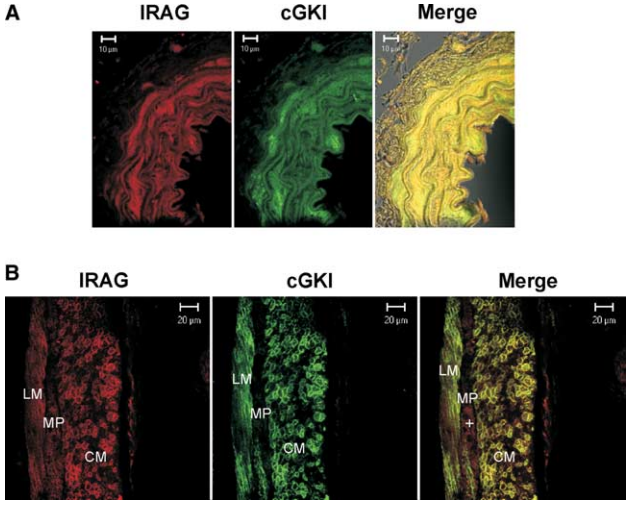


Fig. 4. Colocalization of IRAG and cGKI in aorta and colon. Confocal laser scanning images of aortic cross-sections (6 μm) (A) and colonic longitudinal sections (10 μm) (B) showed colocalization of IRAG and cGKI in smooth muscle cells of the aortic media (A) and the colonic muscularis (B). (B) Additionally, IRAG was expressed in the myenteric plexus (MP) between longitudinal and circular muscle-layer (LM, CM). A weak colocalization of IRAG with cGKI was observed in MP, marked with +. Scale bar: 10 or 20 μm, as indicated.

network of the endoplasmic reticulum (Fig. 5A). Coexpression of cGKIα with IRAG showed an unchanged cytosolic localization of cGKIα (Fig. 5B). In contrast, when cGKIβ was coexpressed with IRAG, both proteins were present at the network of the endoplasmic reticulum and a strong colocalization was observed (Fig. 5B). This effect confirms the specific in vivo interaction of IRAG with cGKIβ.

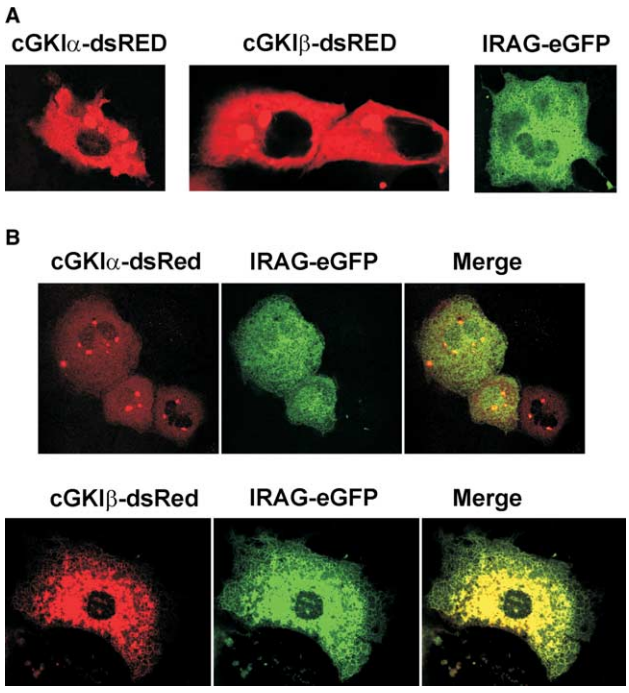


Fig. 5. Localization of IRAG-eGFP, cGKIα-dsRed and cGKIβ-dsRed in COS-7 cells. (A) cGKI fusion proteins (cGKIα- and cGKIβ-dsRed) are distributed cytosolically (left and middle image), whereas IRAG-eGFP is located at the endoplasmic reticulum (right image). (B) Co-expression of IRAG-eGFP with cGKIα-dsRed showed no local change of cGKIα. In contrast, cGKIβ was recruited through IRAG to the endoplasmic reticulum. Magnification 400×.

## 4. Discussion

This manuscript provides convincing evidence that IRAG is a major smooth muscle protein. Immunoblot analysis and immunohistochemistry suggest that cGKIβ and IRAG colocalize together in most smooth muscle tissues. Furthermore, cell expression studies in COS-7 cells revealed that both proteins are colocalized at the endoplasmic reticulum supporting the previous finding that the two proteins form a macrocomplex with the $IP_3RI$ [9]. In contrast to cGKIβ, cGKIα was unable to colocalize with IRAG in the cell expression experiments. With the exception of lung, the distribution of cGKIα did not accord much with that of IRAG in the Western blot analysis. Human platelets express exclusively IRAG and cGKIβ and the concentration of cGKIβ exceeds that of cGKIα in most smooth muscle rich tissues such as aorta and colon. However, it is possible that IRAG colocalizes in some cells with other proteins, since we found IRAG expression without the corresponding cGKIβ protein in the thalamus and some cells of the myenteric plexus. On the other hand, cGKIβ can activate substrates distinct from IRAG, since cGKIβ is expressed in the hippocampus and the olfactory bulb without IRAG. These findings support the notion that IRAG is the substrate for cGKIβ in smooth muscle. Previous experiments showed that cGMP-dependent phosphorylation of IRAG decreased the $IP_3$ induced $Ca^{2+}$ release in COS cells [8,9]. Reconstitution of cGKI isozymes into *cGKI*$^{-/-}$ aortic smooth muscle cells suggested that the $Ca^{2+}$ lowering effect of cGMP was only reconstituted by cGKIα [15]. The reasons for this discrepancy are not clear. The mechanism by which cGKIα lowered the $Ca^{2+}$ transients is unknown and may not involve the IRAG protein but inhibition of $IP_3$ synthesis as observed previously [16].

There is excellent evidence that cGKI regulates the tonus of various smooth muscles [4,17,18]. The mechanisms behind this regulation are not solved at the present time. Relaxation may be the result of multiple regulatory pathways. Furthermore, different mechanisms may prevail in different smooth muscle. However, the strong expression of cGKIβ and IRAG makes it very likely that interaction between both proteins constitutes one important mechanism present in all smooth muscles leading to relaxation.

*Acknowledgements:* We thank Christine Wolf for her excellent technical assistance. This project was supported by the DFG, the SFB391, the Sanderstiftung and the GRK438.

## References

[1] Hofmann, F., Ammendola, A. and Schlossmann, J. (2000) J. Cell Sci. 113 (Pt 10), 1671–1676.
[2] Somlyo, A.P. and Somlyo, A.V. (1994) Nature 372, 231–236.
[3] Cornwell, T.L. and Lincoln, T.M. (1989) J. Biol. Chem. 264, 1146–1155.
[4] Pfeifer, A., Klatt, P., Massberg, S., Ny, L., Sausbier, M., Hirneiss, C., Wang, G.X., Korth, M., Aszodi, A., Andersson, K.E., Krombach, F., Mayerhofer, A., Ruth, P., Fassler, R. and Hofmann, F. (1998) Embo J. 17, 3045–3051.
[5] Keilbach, A., Ruth, P. and Hofmann, F. (1992) Eur. J. Biochem. 208, 467–473.
[6] Pfeifer, A., Ruth, P., Dostmann, W., Sausbier, M., Klatt, P. and Hofmann, F. (1999) Rev. Physiol. Biochem. Pharmacol. 135, 105–149.
[7] Surks, H.K., Mochizuki, N., Kasai, Y., Georgescu, S.P., Tang, K.M., Ito, M., Lincoln, T.M. and Mendelsohn, M.E. (1999) Science 286, 1583–1587.
[8] Ammendola, A., Geiselhöringer, A., Hofmann, F. and Schlossmann, J. (2001) J. Biol. Chem. 276, 24153–24159.
[9] Schlossmann, J., Ammendola, A., Ashman, K., Zong, X., Huber, A., Neubauer, G., Wang, G.X., Allescher, H.D., Korth, M., Wilm, M., Hofmann, F. and Ruth, P. (2000) Nature 404, 197–201.
[10] Fritsch, R., Saur, D., Kurjak, M., Oesterle, D., Schlossmann, J., Geiselhöringer, A., Hofmann, F. and Allescher, H.D. (2004) J. Biol. Chem. 279, 12551–12559.
[11] Ludwig, A., Flockerzi, V. and Hofmann, F. (1997) J. Neurosci. 17, 1339–1349.
[12] Moosmang, S., Biel, M., Hofmann, F. and Ludwig, A. (1999) Biol. Chem. 380, 975–980.
[13] El-Husseini, A.E.-D., Williams, J., Reiner, P.B., Pelech, S. and Vincent, S.R. (1999) J. Chem. Neuroanat. 17, 45–55.
[14] De Vente, J., Asan, E., Gambaryan, S., Markerink-van Ittersum, M., Axer, H., Gallatz, K., Lohmann, S.M. and Palkovits, M. (2001) Neuroscience 108, 27–49.
[15] Feil, R., Gappa, N., Rutz, M., Schlossmann, J., Rose, C.R., Konnerth, A., Brummer, S., Kuhbandner, S. and Hofmann, F. (2002) Circ. Res. 90, 1080–1086.
[16] Ruth, P., Wang, G.-X., Boekhoff, I., May, B., Pfeifer, A., Penner, R., Korth, M., Breer, H. and Hofmann, F. (1993) Proc. Natl. Acad. Sci. USA 90, 2623–2627.
[17] Sausbier, M., Schubert, R., Voigt, V., Hirneiss, C., Pfeifer, A., Korth, M., Kleppisch, T., Ruth, P. and Hofmann, F. (2000) Circ. Res. 87, 825–830.
[18] Bonnevier, J., Fassler, R., Somlyo, A.P., Somlyo, A.V., Arner, A., Bonnevier, J., Fassler, R., Somlyo, A.P., Somlyo, A.V. and Arner, A. (2004) J. Biol. Chem. 279, 146–151.