IRAG and novel PKG targeting in the cardiovascular system

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Schlossmann J, Desch M. IRAG and novel PKG targeting in the cardiovascular system. Am J Physiol Heart Circ Physiol 301: H672–H682, 2011. First published June 10, 2011; doi:10.1152/ajpheart.00198.2011.—Signaling by nitric oxide (NO) determines several cardiovascular functions including blood pressure regulation, cardiac and smooth muscle hypertrophy, and platelet function. NO stimulates the synthesis of cGMP by soluble guanylyl cyclases and thereby activates cGMP-dependent protein kinases (PKGs), mediating most of the cGMP functions. Hence, an elucidation of the PKG signaling cascade is essential for the understanding of the (patho)physiological aspects of NO. Several PKG signaling pathways were identified, meanwhile regulating the intracellular calcium concentration, mediating calcium desensitization or cytoskeletal rearrangement. During the last decade it emerged that the inositol triphosphate receptor-associated cGMP-kinase substrate (IRAG), an endoplasmic reticulum-anchored 125-kDa membrane protein, is a main signal transducer of PKG activity in the cardiovascular system. IRAG interacts specifically in a trimeric complex with the PKG1 isoform and the inositol 1,4,5-trisphosphate receptor I and, upon phosphorylation, reduces the intracellular calcium release from the intracellular stores. IRAG motifs for phosphorylation and for targeting to PKG1α and 1,4,5-trisphosphate receptor I were identified by several approaches. The (patho)physiological functions for the regulation of smooth muscle contractility and the inhibition of platelet activation were perceived. In this review, the IRAG recognition, targeting, and function are summarized compared with PKG and several PKG substrates in the cardiovascular system.

intrinsic triphosphate receptor-associated guanosine 3’,5’-cyclic monophosphate-kinase substrate; nitric oxide; guanosine 3’,5’-cyclic monophosphate-dependent protein kinase

Function of NO/cGMP/PKG Signaling Cascade

NO/cGMP signaling regulates several tasks in the cardiovascular system including vascular smooth muscle, heart muscle, platelet, and immune function as summarized in several reviews (6, 36, 40). Hence, the modulation of NO/cGMP signaling is an important pharmacological target pathway for the prevention of cardiovascular diseases such as angina pectoris, hypertension, heart insufficiency, and atherosclerosis (7, 57, 62). The knowledge of the details regarding NO/cGMP signal transduction is essential for the understanding of the physiological and pathophysiological regulation and the modulation at diverse steps in this signaling pathway (Fig. 1).

Synthesis of NO is mediated via endothelial, inducible, and neuronal NO synthases, which are present in diverse tissues including endothelial cells, immune cells, e.g., macrophages and nervous cells, e.g., in the central nervous system and in the gastrointestinal tract, respectively. NO activates soluble guanylyl cyclases to synthesize cGMP (55). cGMP is cellularly synthesized also by membrane-localized particular guanylyl cyclases that are stimulated by natriuretic peptide, e.g., in the cardiovascular system mainly by the atrial natriuretic peptide (ANP). Interestingly, it was recently observed in myocardial cells that NO soluble guanylyl cyclase elicited a negative-feedback regulation of cytosolic cGMP, whereas ANP activa-
tion led to a feed-forward activation, enhancing cGMP production in the subsarcolemmal pool (13).

Furthermore, the second messenger cGMP stimulates PKG [see review (34)]. Three different PKGs exist: PKG1α, PKG1β, and PKG2. PKG1α and PKG1β are inter alia expressed in the cardiovascular system. PKG1α and PKG1β are derived from the same gene and differ only in the NH₂-terminal region containing a LZ that is important for dimerization, localization, and target recognition. However, it is still a matter of debate which isoform is mainly responsible for NO- or ANP-mediated effects in the cardiovascular system. In this regard it was revealed that the mutation of the PKG1α leucine zipper suppressed the relaxation of large and resistance vessels and increased systemic blood pressure (54). However, the selective expression of PKG1α or PKG1β in smooth muscle did not show altered vascular smooth muscle relaxation (90). A further issue is whether the stimulation of guanylyl cyclases changes the intracellular localization and targeting of PKGs.

PKG signals through phosphorylation of specific substrate proteins [see summary (75)]. Meanwhile, various substrates of PKG were identified. PKG substrates regulate diverse functions comprising tissue contractility, cell motility, cell contact, cellular secretion, cell proliferation, and cell differentiation. On the molecular level, PKG1 substrates fulfill various cellular functions regulating, e.g., the intracellular calcium and potassium concentration, the calcium sensitivity, and the organization of the intracellular cytoskeleton. PKG2 substrates are involved, e.g., in chloride transport, sodium/proton transport, and transcriptional regulation.

Specific substrates determine the physiological function of PKG through diverse signaling pathways (1, 28, 54, 75). Many of these substrates are regulated by both PKG1 isozymes. Some substrates are recognized by a specific PKG1 isozyme, namely, IRAG by PKG1β and the regulatory myosin phosphatase targeting subunit 1 (MYPT1) or the regulator of G protein signaling 2 (RGS2) by PKG1α. These specific pathways lead to different targeting and determine distinct functions of the PKG1 isozymes.

During the last decade it emerged that signaling via IRAG effects diverse cardiovascular functions including smooth muscle and platelets (74, 75). Hence, in this review we summarize the function of IRAG compared with other PKG substrates for PKG targeting and NO/cGMP function.

**IRAG as PKG Target: Signaling and Function**

**IRAG signaling.** IRAG is a 125-kDa membrane protein that resides in the endoplasmic reticulum (ER) membrane via a COOH-terminal transmembrane anchor (74, 80). Two NH₂-terminal variants of IRAG, a 911-amino acid (aa) IRAGa and a 859-aa IRAGb, deriving from alternative splicing of the same gene, are known. A functional difference of these splice variants was not revealed so far. IRAG targets PKG1β to the ER and additionally binds with its coiled-coil domain to the ino-
sitol 1,4,5-trisphosphate receptor I (IP$_3$RI) (Fig. 2). A targeting of PKG1β is mediated via a recognition motif residing in the NH$_2$-terminal part of IRAG that interacts with the LZ region of PKG1β (1). PKG1β stably associates with IRAG independently of cGMP (1, 12). Furthermore, the coiled-coil region of IRAG interacts with the IP$_3$R independently of PKG (28, 53). These stable interactions are prerequisite for the PKG-mediated phosphorylation of IRAG at Ser696, which suppresses the hormone-induced calcium release via the IP$_3$RI (1, 74). Upon collagen-related peptide (CRP) activation of human platelets, a differential enrichment of IRAG and IP$_3$R was observed in the PKG1β signaling complex. Furthermore, a CRP stimulation of platelets decreased the phosphorylation of IRAG at Ser696 by about 60% (51). IRAG mutant proteins with a targeted mutation of the NH$_2$-terminal part of the coiled-coil domain of IRAG lose the ability to interact with the IP$_3$RI (26, 53). This loss of interaction prevents the modulation of calcium release by PKG1β. However, the regulation of IP$_3$R in neuronal cells, e.g., the cerebellum, differs as the neuronal splice form of IP$_3$R is activated by cGMP-dependent protein kinase 1, thereby inducing calcium release (87). Interestingly, recent reports suggest that also IP$_3$RII- and IP$_3$RIII-mediated calcium release is attenuated by PKG1β/IRAG signaling. Moreover, upon the CRP stimulation of platelets, IP$_3$RI was enriched in the PKG1β/IRAG complex (51). Furthermore, cAMP-dependent protein kinase (PKA)-inhibited calcium release of IP$_3$R is also dominantly regulated by IRAG (53). However, the effects of cAMP-stimulation in murine IRAG-deficient knockout (IRAG-KO) or IRAG-mutant smooth muscle or platelets were not altered compared with wild-type mice (2, 20, 28, 73).

**IRAG function.** IRAG is NO/cGMP-dependently phosphorylated by PKG1β and thereby inhibits the calcium release via IP$_3$R in vascular and gastrointestinal smooth muscles and in platelets. The physiological function of IRAG was established in murine transgenic models with an IRAG-KO murine strain or with an IRAG mutant lacking exon 12 (IRAGΔ12 mice), which deleted interaction to the IP$_3$RI (21, 28). NO/cGMP-mediated vascular smooth muscle relaxation of hormone-induced contraction was severely affected in IRAG-KO and IRAGΔ12 aortic tissues. Furthermore, ANP-mediated relaxation was strongly reduced in IRAG-KO aortic tissues. Contrarily, NO/cGMP-dependent relaxation of depolarization-induced contraction (addition of potassium) was not affected in IRAG mutant smooth muscle tissues. Defects in smooth muscle relaxation by IRAG mutation or deletion were particularly apparent in the gastrointestinal tract. The gastrointestinal relaxation was also severely reduced in IRAG mutant mice, leading to a strong enlargement of the gastrointestinal tract including esophagus, stomach, colon, and caecum. IRAGΔ12 mice revealed strongly enhanced gastrointestinal passage times (28). Interestingly, a differential significance of IRAG for smooth muscle relaxation of intestinal tissues was observed: IRAG determined calcium-dependent relaxation in colon smooth muscle, whereas jejunum relaxation was dominantly affected by calcium desensitization via MYPT1 (25). Both IRAG mutant strains exhibited a significantly reduced life span. Basal blood pressure was not altered in IRAG-KO mice, and exogenously applied NO similarly reduced blood pressure in wild-type and IRAG-KO mice. Lipopolysaccharide-induced sepsis strongly enhancing endogenous NO synthesis mediated a blood pressure drop in wild-type mice, whereas blood pressure was nearly not changed in IRAG-KO mice (21).

NO/cGMP via PKG1 mediates the inhibition of agonists (thrombin, collagen, and thromboxane A$_2$)-induced platelet aggregation. IRAG mutant platelets revealed that IRAG is a dominant NO/cGMP-dependent mechanism inhibiting platelet aggregation (2). Furthermore, α-granule secretion (P-selectin) or dense granule secretion (ADP and serotonin) is reduced by NO/cGMP, mainly via PKG1β/IRAG (73). This might be responsible for the reduced bleeding time observed in IRAG-KO mice. Interestingly, IRAG mutant platelets showed hyperaggregability upon agonist activation. Polymorphisms of Mrv11, homologous to the human IRAG gene, were also associated with enhanced platelet aggregation in human patients (38). In vivo it was revealed that IRAG is a NO/cGMP/PKG1-dependent factor which thereby inhibited arterial thrombosis (2). IRAG signaling might be involved in further disease processes not related to cardiovascular or smooth muscle function. IRAG was described as a potential tumor suppressor gene in chronic myeloid leukemia (80), indicating a possible role of IRAG suppressing proliferative diseases. Recent reports suggested a function of IRAG in osteoclast attachment or acid secretion (97). Moreover, an overexpression of IRAG was observed in keratocytes from patients with keratoconus, suggesting a functional role in cellular growth and differentiation-inducing corneal thinning (48).

**Other Targets for PKG**

**MYPT1.** MYPT1, the regulatory myosin-binding subunit of phosphatase 1, determines the desensitization of smooth muscles (Table 1, and Fig. 1). MYPT1 is necessary for embryogenesis since the total deletion of MYPT1 in mice results in embryonic lethality before day 7.5 post coitum (59). PKG1 deletion reduced myosin phosphatase activity and thereby affected relaxation (36). Interestingly, desensitization has a distinct impact on relaxation in various smooth muscle types since the calcium-independent inhibition of contraction was more important for jejunum relaxation, whereas colon relaxation was strongly calcium dependent (25). MYPT1 is specifically phosphorylated by PKG1α in vascular smooth muscle cells (VSMCs) at Ser695, which results in enhanced phospha-

![Fig. 2. Scheme of the trimeric PKG1β macromplex. The PKG1β-isoform specifically forms a ternary complex with the IRAG protein and the IP$_3$RI. Activation of PKG1β with cGMP results in a reduced calcium release from intracellular stores.](http://ajpheart.physiology.org/10.1152/ajpheart.00203.2011)
Table 1. Important substrates of PKG1 in the cardiovascular system

<table>
<thead>
<tr>
<th>Substrate</th>
<th>kDa</th>
<th>PKG Isoform</th>
<th>Function (of Phosphorylation)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>BKCa</td>
<td>130</td>
<td>PKG1α</td>
<td>α- and β2-Subunit involved in PKG1-mediated activation of BKCa, increased open probability and membrane hyperpolarization</td>
<td>43, 70, 100</td>
</tr>
<tr>
<td>CRP4</td>
<td>22.5</td>
<td>PKG1</td>
<td>Enhanced cGMP/PKG-mediated smooth muscle-specific gene expression, altered VSMC phenotype via PKG</td>
<td>14, 99</td>
</tr>
<tr>
<td>FHOD-1</td>
<td>130</td>
<td>PKG1</td>
<td>Inhibition of stress fiber formation and migration in VSMC</td>
<td>89</td>
</tr>
<tr>
<td>IP3RI</td>
<td>230</td>
<td>PKG1</td>
<td>Phosphorylation of IP3RI-S1 isoform leads to enhanced calcium release from IP3-sensitive stores. The IP3,RI-S2 isoform is insensitive to PKG1 phosphorylation</td>
<td>31, 87</td>
</tr>
<tr>
<td>RGS2</td>
<td>125</td>
<td>PKG1β</td>
<td>Reduced calcium release from IP3-sensitive stores. Deficiency/ mutation-impaired smooth muscle relaxation and reduced inhibition of platelet activation</td>
<td>2, 21, 73, 74</td>
</tr>
<tr>
<td>MYPT1</td>
<td>130</td>
<td>PKG1α</td>
<td>Activation of MLCP leading to calcium desensitization</td>
<td>94</td>
</tr>
<tr>
<td>PDE5</td>
<td>100</td>
<td>PKG1</td>
<td>Activation and thereby increased cGMP degradation</td>
<td>68</td>
</tr>
<tr>
<td>Phospholamban</td>
<td>6</td>
<td>PKG1</td>
<td>Enhanced calcium uptake by the Ca2+-ATPase SERCA into the intracellular stores</td>
<td>44</td>
</tr>
<tr>
<td>Rap1Gap2</td>
<td>90</td>
<td>PKG1</td>
<td>Inhibition of 14-3-3 protein binding to Rap1Gap in platelets→reduced cell adhesion</td>
<td>33, 78</td>
</tr>
<tr>
<td>RGS2/4</td>
<td>24</td>
<td>PKG1α</td>
<td>In heart, RGS4 mediates antihypertrophic effects of locally secreted natriuretic peptides</td>
<td>30, 82, 84</td>
</tr>
<tr>
<td>RhoA</td>
<td>22</td>
<td>PKG1</td>
<td>Reduced MLC phosphorylation, reduced vesicle trafficking, suppressed cardiac hypertrophy and fibrosis</td>
<td>23, 72</td>
</tr>
<tr>
<td>SMTLN/CHASM</td>
<td>60</td>
<td>PKG1</td>
<td>Calcium desensitization via inhibition of MLCK</td>
<td>93</td>
</tr>
<tr>
<td>Telokin</td>
<td>17</td>
<td>PKG1</td>
<td>Inhibition of MLCK activity</td>
<td>88</td>
</tr>
<tr>
<td>Thromboxane receptor α</td>
<td>55</td>
<td>cGKI</td>
<td>Receptor desensitization</td>
<td>91</td>
</tr>
<tr>
<td>TRIM 39R</td>
<td>46</td>
<td>cGKI</td>
<td>Unknown function/cell homeostasis?</td>
<td>65</td>
</tr>
<tr>
<td>Tropinin T</td>
<td>33</td>
<td>cGKI</td>
<td>Regulation of muscle contraction in the heart</td>
<td>98</td>
</tr>
<tr>
<td>VASP</td>
<td>46/50</td>
<td>PKG1</td>
<td>Remodeling of actin cytoskeleton, regulation of vesicle trafficking</td>
<td>15, 16, 49</td>
</tr>
</tbody>
</table>

Overview of established substrates, their molecular masses, the activating cGMP-dependent protein kinase (PKG1) isoform, and the main (cellular/physiological) function after phosphorylation are shown. For details, see text. BKCa, calcium-activated maxi-K+ channel; CRP4, cysteine-rich LIM-only protein 4; VSMC, vascular smooth muscle (VSM) cell; FHOD-1, forming homology domain protein 1; IP3RI, 1,4,5-trisphosphate (IP3) receptor I; IRAG, inositol trisphosphate receptor-associated cGMP-kinase substrate; MYPT1, myosin phosphatase targeting subunit 1; MLCP, myosin light chain (MLC) phosphatase; PDE5, phosphodiesterase 5; SERCA, sarco(endo)plasmic reticulum calcium-ATPase; Rap1Gap2, Rap1-specific GTPase-activating protein 2; RGS, regulator of G protein signaling; GPCR, G protein-coupled receptor; SMTLN: smoothelin-like protein 1; CHASM, calponin homology activated in smooth muscle; MLCK, MLC kinase; TRIM, tripartite motif protein; VASP, Vasodepressor-stimulated phosphoprotein.

PKG1 interacts with the LZ domain of PKG1α (see also Targeting by PKG-substrate interaction).

RGS2 and RGS4. Signaling by G protein-coupled receptors is modulated by RGS proteins, which bind directly to Goq and/or Goi, inducing GTP hydrolysis and thereby affecting G protein signaling. Among the 30 known RGS proteins, RGS2 and RGS4 are main RGS proteins in the cardiovascular system (Table 1). RGS2 proteins are expressed in a variety of tissues including smooth muscle, heart, bone, immune system, olfactory epithelium, and brain [see review (5)]. RGS2 deletion in mice resulted in cardiovascular phenotypes including hypertension, cardiac hypertrophy, and developed renovascular abnormalities with increased responsiveness to vasopressin (5, 25, 101). RGS2 might play a role in the development of genetic cardiovascular diseases since RGS2 expression was enhanced in Bartter’s/Gitelman’s syndrome patients suffering from hypertension, diminished angiotensin-II signaling, and vasomotor tone (9).

NO determined the reduction of blood pressure via vascular RGS2 during the day (inactive phases of mice) (58, 82, 84, 85). RGS2 are cGMP-dependently phosphorylated by PKG1α at Ser46 and Ser64, increasing Goq, GTP hydrolysis and thereby inhibiting signaling via G protein-coupled receptors (Table 1, and Fig. 1). RGS4 might play a role for ANP-mediated protection against cardiac hypertrophy (86). ANP signaling enhanced the binding of RGS4 to PKG1α. Correspondingly, RGS4 phosphorylation was diminished in the hearts of GC-A knockout mice, which displayed cardiac hypertrophy. Vice versa, the overexpression of RGS4 attenuated cardiac hypertrophy of GC-A-knockout mice. Therefore, the observed up-regulation of RGS4 in failing heart due to cardiac myopathy might be compensatory (61).

Calcium-activated maxi-K+ channel. The calcium-activated maxi-K+ channel (BKCa) is involved in the membrane potential regulation (Table 1). In the vasculature, BKCa, regulates basal blood pressure since the deletion of the pore-forming BKCa α-subunit in mice leads to a significant basal blood pressure elevation (69). However, knockout mice of the accessory calcium-sensitive β2-subunit were not hypertensive (96). Interestingly, the BKCa α-subunit deletion enhanced the expression of cGMP signaling proteins (PKG1 and IRAG) in tracheal smooth muscle and led to cholinergic bronchoconstriction in the airways (71), cGMP enhanced BKCa activity via PKG1 in vascular smooth muscle and thereby mediated smooth muscle hyperpolarization (70). PKG or PKA phosphorylate the α-subunit of BKCa at different sites, at Ser1134 or Ser922, respectively. Phosphorylation by protein kinase C at Ser1151 and Ser154 determines whether the BKCa channel is activated by PKG. A prevention of protein kinase C phosphorylation at these sites either by dephosphorylation, mutation, or by a splice variant lacking these sites switches to activation by PKA (100).
PKG1-induced phosphorylation of the BKαβ₁-subunit might be involved in the activation of BKα (43).

**Vasodilator-stimulated phosphoprotein.** Vasodilator-stimulated phosphoprotein (VASP) is an established PKG1 substrate in a variety of cells. Phosphorylation of VASP at Ser239 serves as a general marker for PKG-dependent activity. In the cardiovascular system VASP is involved in the remodeling of the actin cytoskeleton and enhancement of angiogenesis (15, 16). Phosphorylation of VASP by PKG at Ser239 in VSMCs reduced the binding of VASP to actin and interfered with VSMC invasion and contraction of collagen matrices (19). However, its murine deletion did not change cyclic nucleotide-mediated aortic smooth muscle relaxation (3). In platelets, VASP is involved in the regulation of the activation of the fibrinogen receptor. The VASP deletion induced megakaryocyte hyperplasia and enhanced agonist-induced platelet activation (32, 52). Cyclic nucleotide-mediated suppression of platelet activation is not primarily dependent on VASP since cAMP- and cGMP-induced inhibition of agonist-induced platelet aggregation was only slightly affected in VASP-knockout mice (3, 32).

**RhoA.** RhoA represents a 22-kDa protein that is involved in smooth muscle contraction, stress fiber formation, proliferation, and gene expression. RhoA signaling is associated with cardiac hypertrophy and fibrosis (67). RhoA is activated upon GTP binding. RhoA then translocates to the plasma membrane and stimulates RhoA kinase, which phosphorylates MYPT1, inhibiting myosin phosphatase. NO/natriuretic peptide/cGMP signaling counteracts vascular remodeling and fibrosis. An important mechanism in this antifibrotic process is PKG1 phosphorylation of RhoA at Ser188 (56) (see also Intracellular PKG targeting).

**Further PKG substrates.** There are a variety of substrate proteins that were previously described (summarized in Table 1). Only a few of them will be further discussed because of their interest in the targeting or macromolecular assembly of PKG1. Phospholamban was identified in the PKG1 macrocomplex in tracheal smooth muscle. PKG1 phosphorylates phospholamban, thereby reducing the inhibition of sarco(endo)plasmic reticulum calcium-ATPase (Table 1). Interestingly, murine phospholamban deletion only slightly suppressed NO/cGMP-dependent vascular smooth muscle relaxation and did not change blood pressure (44). Cysteine-rich LIM-only protein 4 (CRP4) is a transcriptional regulator that contains LIM domains including four zinc fingers (Table 1). CRP4 is phosphorylated by the PKG1 isoforms (37, 76, 99). The third zinc finger mediates binding to PKG1. CRP4 enhances cGMP/PKG1-mediated transcriptional activation of smooth muscle-specific proteins in a complex with the serum response factor. Phosphodiesterase 5 (PDE5) is regulated by PKG1, which phosphorylates PDE5 at Ser92 and thereby increases its phosphodiesterase activity and cGMP-binding affinity (68) (Table 1, and Fig. 1). An intracellular pool of PDE5 was found compartmented in a macrocomplex with PKG1 in human platelets, suggesting a spatial and temporal regulation of cGMP signaling in these cells (92). The low molecular-weight GTPase Rap1 is involved in cell adhesion via Rap1-specific GTPase-activating protein 2 (Rap1GAP2), which interacts with 14-3-3 proteins. Phosphorylation of Rap1GAP2 at Ser7 disrupts binding to 14-3-3 proteins, inhibiting cell adhesion (18, 33) (Table 1).

**Targeting of PKG**

PKG is arranged as a protein that contains an NH₂-terminal LZ domain, an autoinhibitory domain, two cGMP-binding sites (cGMP-A and cGMP-B), and a catalytic domain including an ATP-binding site and a substrate peptide-binding pocket (see Fig. 3A). Recently, the crystal structure of cGMP-A from human PKG1β revealed that cGMP binds in syn configuration with a conserved threonine T193 anchoring the cyclic phosphate and the guanine (41). Cleavage of Arg77 of the PKG1α isoform removed dimerization and autoinhibition and produced constitutively active 65-kDa monomeric PKG1 [see summary (35)]. In conclusion, the targeting of PKG1 is mainly mediated by the NH₂-terminal LZ domains of PKG1α and PKG1β (Fig. 3, B and C). These domains lead to different activation constants of PKG1 isoforms, induce distinct autoinhibition mechanisms, mediate dimerization, and cause different recognition of substrates by PKG1α and PKG1β. These various aspects were studied by a mutational analysis of the LZ domain and substrate recognition sites, e.g., IRAG or MYPT1, by structural NMR and X-ray studies of the single leucine/isoleucine domains, by transgenic mice expressing solely a single isoform in smooth muscle cells, or by analyzing redox regulation (34). In this report the various aspects regarding intramolecular targeting of PKG1, the targeting by PKG1-substrate interaction, and the intracellular PKG1 targeting will be discussed.

**Intramolecular PKG targeting.** In mammalian cells two different isoforms of PKG1 are expressed: PKG1α and PKG1β. These isoforms are encoded by the same gene but differ in the NH₂-terminal ~100 aa where the autoinhibition and dimerization domains reside (34) (Fig. 3, A–C). Homodimerization increased sensitivity for cGMP activation of the enzyme (64). Oxidation of Cys residue 43 acts as a redox sensor in PKG1α and is a determinant for dimerization of the enzyme regulating its activity by hydrogen peroxide or hydrogen sulfide (8, 45). However, further Cys residues (Cys117, Cys195, Cys312, and Cys518) outside of the NH₂-terminal domain might form disulfide bonds that enhance PKG1 activity (46).

The NH₂-terminal structural of the PKG1 isoform was studied by X-ray, circular dichroism, NMR, or site-directed mutagenesis. Both isoforms form homodimers through their NH₂-terminal LZ domains (Fig. 3, B and C). The leucine/isoleucine residues in these domains are essential for the stability of the dimers.

Eight leucine/isoleucine heptad repeats were essential for PKG1β homodimerization. Recent reports described the molecular details of the PKG1β dimerization/docking domain upon crystallization of the LZ domain. The structure revealed the topology of this domain to expose specific hydrophobic and ionic interactions mediating dimerization (11). The hydrophilic residues were arranged in a “knobs into holes” fashion. This might explain the dimeric organization of the PKG1 isoforms since hydrophilic residues in the core of coiled coils support the formation of dimers.

The NH₂-terminal 39-aa residues of PKG1α formed a stable α-helical conformation (45). NMR studies showed a parallel monomeric association of the PKG1α coiled-coil domain (4, 77). The dimerization of the PKG1α probably occurs through a hydrophobic interaction of leucines/isoleucines on one side.
Fig. 3. Structural requirements/postulations for protein-protein interaction. A: overview of functional domains of PKG1. The regulatory domain consists of a LZ domain that mediates dimerization and docking to substrates. The inhibitory sequence (IS) inactivates the kinase when cGMP is absent. Each kinase monomer binds 2 cGMP molecules that lead to activation of the kinase. The catalytic domain consists of an ATP-binding and a substrate-binding domain. PKG1α/H9251 and PKG1β/H9252 are derived from the same gene and differ only in the NH2-terminal 100 amino acids regions containing the LZ domains.

B: overview of the NH2-terminal 45 amino acids of the PKG1α LZ that are postulated to interact as homodimer (11). The amino acids are arranged in a heptad repeat and nearly every a-position has a leucine or isoleucine residue (cyan marked); the majority of d-positions have charged or hydrophilic residues. Core-forming amino acids are green boxed; the red crosses mark interhelical salt bridges stabilizing the homodimer. The orange-boxed amino acids have positive charges, which seem to be relevant for docking to substrate proteins.

C: NH2-terminal 51 amino acids LZ domain of PKG1β/H9252. The orange-boxed amino acids have negative charges (corresponding to the positive-charged amino acids of PKG1α) that form a negatively charged surface responsible for substrate docking (11). The other marked residues correspond to the functions described in B.

D: interaction of PKG1β/H9252 and IRAG. Orange-boxed amino acids carry a negative charge and are essential for binding to IRAG. Yellow-boxed amino acids have a positive charge and are essential for PKG1β binding. Yellow-typed amino acids are important for binding, but mutation only reduce binding affinity to PKG1β (10).

E: binding of the COOH-terminal region of MYPT1 to the PKG1α LZ. When the LZ of MYPT1 is present, PKG1α forms a homodimer with MYPT1. When the LZ of MYPT1 is lacking, PKG1α forms a heterotetramer with the MYPT1 N1-N2-coiled coil domain (29, 47, 79).
of the helical structure and supporting electrostatic interactions between flanking side chains.

**Targeting by PKG-substrate interaction.** Structural PKG requirements. The LZ domain mediated substrate-PKG interaction and thereby induced targeting of PKG inside the cell as an important regulatory mechanism for intracellular specificity of kinase-specific substrate activation. PKG1β contains an acidic, highly electronegative patch (D26, E29, and E31) in the NH2-terminal docking/dimerization domain, which presents a stable docking surface, interacting with positively charged residues within IRAG and transcription factor II-I (TFII-I) (10, 11) (Fig. 3, A, C, and D).

The in vivo role of the LZ domain of PKG1α was tested by a murine knockin mutant with a leucine to alanine mutation within this domain in mice (LZM mice) (54). On the molecular level, the binding of LZM mutant to glutathione-S-transferase fused to the myosin-binding subunit was reduced as well RhoA activation was enhanced in mutant VSMCs. Stress fiber formation was enhanced in LZM-VSMCs, suggesting a constitutive RhoA activation. The mutated LZ domain led to an abnormal relaxation of large and resistance blood vessels and an increase of systemic blood pressure.

**STRUCTURAL SUBSTRATE REQUIREMENTS.** Both PKG1 isozymes PKG1β and PKG1α are specifically targeted to different substrate proteins through their LZ domains. PKG1β binds specifically to the IRAG protein (Fig. 3D) and the transcriptional regulator TFII-I (10). A helix-loop-helix motif in TFII-I (aa 491–628) interacted specifically with PKG1β. An electrostatic interface mediates the interaction of an NH2-terminal located 32-aa IRAG domain with PKG1β (1, 10). The mutation of two arginines to alanine in this IRAG interaction domain (R124A and R125A of IRAGb) disrupted binding to PKG1β. The interaction of IRAG to PKG1β was disrupted by mutations of acidic aa E29K and D26K/E31R in the PKG1β coiled-coil domain (10). A stable macrocomplex of IRAG, IP3RI, and PKG1β at the ER was observed in heterologous expression systems, in smooth muscle cells, and in platelets using inter alia coimmunoprecipitation and immunocytochemistry (1, 2, 21, 53, 74) (Fig. 2). However, in osteoclasts it was observed that IRAG dissociated from IP3RI in the presence of NO donors or cGMP activators. Furthermore, IRAG complexes after the depletion of IP3RI contained VASP, migfilin, and actin. In these cells IRAG targets additionally to endosomal, nuclear, and plasma membrane compartments (97).

PKG1α interacts with MYPT1 (Fig. 3E), RGS2, and RhoA. Two diverse MYPT1 isoforms are expressed in vascular smooth muscles, depending on the developmental stage: a leucine zipper isoform (LZ+) containing a COOH-terminal leucine zipper motif that binds to PKG1α and sensitizes cGMP-dependent relaxation, a leucine zipper-deficient isoform that is cGMP insensitive (63). The LZ-deficient form of MYPT1 binds via its N1-N2 coiled-coil domain to PKG1α, forming a heterotrimer (29, 47) (Fig. 3E). Interestingly, NO tolerance is associated with an increased degradation of the LZ+-isoform of MYPT1 (22, 50). Furthermore, congestive heart failure was associated with a decreased expression of the LZ+ MYPT1 transcript and protein and reduced sensitivity to cGMP-mediated vascular smooth muscle relaxation (39).

The NH2-terminal 79 aa of RGS2 interact with the PKG1α-LZ domain (84). The NH2-terminal domain of RGS2 contains an amphipathic α-helical membrane-targeting domain. Ser46 and Ser64 phosphorylation stabilizes this domain and enables its membrane targeting (60). RhoA is phosphorylated at Ser188 by PKG1α, and thereby RhoA-GTP is inactivated, probably through binding to guanine exchange factors (23).

**Intracellular PKG targeting.** Cellular activity of PKG is regulated by diverse mechanisms comprising an intracellular localization of the enzyme, by targeting of the substrate, by diverse cGMP pools, or by changes of protein stability.

PKG-regulated gene expression inter alia in smooth muscle cells was described. However, the basic mechanisms are not completely understood. PKG substrates including CRP4 (Table 1) and TFII-1 mediated nuclear and transcriptional activity of PKG1 (99).

Recent reports suggested that PKG affected phosphorylation by E26-like protein 1, increasing E26-like protein-1 sumoylation and thereby increasing myocardin/serum response factor activity on smooth muscle cell-specific gene expression (17).

The nuclear translocation of PKG1 upon cGMP stimulation was observed in heterologous expression systems. The nuclear transfer can be prevented by the association of PKG1 with interacting proteins. IRAG association was sufficient to inhibit the nuclear entry of PKG1β but not of PKG1α (12, 21). In the absence of IRAG or in the presence of an IRAG mutant deficient in PKG1β-binding, a nuclear localization of PKG1β was induced upon a cGMP addition in baby hamster kidney cells mediating transcriptional activity. cGMP-dependent phosphorylation of IRAG did not affect the interaction with PKG1β and was hence not sufficient to induce a translocation of PKG1β into the nucleus. However, in a homologous system, namely, VSMCs, cGMP-dependent translocation of PKG1β into the nucleus was not observed even in the absence of IRAG (21), which suggests that additional factors to IRAG are involved to prevent nuclear targeting of PKG1β.

It was described that a proteolytic processing of PKG1 induced the presence of a carboxyterminal catalytically active fragment (named PKG1γ), which was found in the golgi apparatus and in the nucleolasm. The remaining NH2-termina leucine zipper domain-containing fragment was identified in the cytosol and the ER. The active PKG1γ entering the nucleus probably by an exposed nuclear localization signal was proposed to mediate nuclear cGMP signaling (81).

Targeting of PKG1β to the ER is mediated via an interaction with IRAG, which is anchored by a COOH-terminal domain in the ER membrane. In heterologous expression systems, PKG1β alone was localized in the cytosol, whereas the expression with IRAG induced the ER localization of PKG1β (27). However, in IRAG-deficient VSMCs, PKG1β was still found at the ER, indicating that other proteins are also involved in the ER tethering of PKG1β in VSMCs (21).

PKG1α phosphorylation of RGS2 transferred both proteins to the plasma membrane (83). The plasma membrane translocation of PKG1α was more transient in RGS2-deficient cells, indicating that RGS2 is involved in the plasma membrane targeting of PKG1α but that other factors are also integrated in the translocation process.

Upon activation, RhoA-GTP translocated to the plasma membrane. PKG1 phosphorylation at RhoA-Ser188 inactivated RhoA-GTP and thereby induced cytosolic RhoA localization (72).
Furthermore, the compartmentalization of cGMP pools were found to restrict cellular PKG activity in cardiomyocytes (13). cGMP is synthesized by natriuretic peptide activation of particulate guanylyl cyclases or by NO activation of soluble guanylyl cyclases. Interestingly, upon ANP activation, PKG elicited a feed-forward mechanism enhancing cGMP production, whereas upon NO activation, PKG lead to a negative-feedback mechanism restricting cGMP synthesis. These mechanisms might be involved in the local antihypertrophic function of ANP (42, 72). Hence, PKG controls both soluble and particulate cGMP pools in cardiomyocytes (13). However, it is not clear whether both these cGMP pools are regulated by different PKG isozymes and/or by a different localization of PKG upon cGMP activation (24).

PKG signaling capacity is also regulated by protein stability. RGS degradation in the proteasome was inhibited by PKG1 (60). RhoA phosphorylation at Ser188 protected RhoA from ubiquitin/proteasome-mediated degradation and enhanced RhoA expression (66). IRAG is probably important for the stability of PKG1β since IRAG deletion or mutation reduced the expression of PKG1β in VSMCs and platelets (2, 21, 73).

Conclusion

The identification of PKG substrate pathways has led to a refined view of PKG isozyme function and targeting in the cardiovascular system. Furthermore, the deletion of several substrates in mice, including IRAG, elucidated a differential functional network of cGMP signaling pathways. The interaction of some of these proteins with PKG1 were identified so far. However, the three-dimensional structure of the PKG1 holoenzyme and of PKG1 interacting with its substrates was not revealed. Furthermore, the mechanisms and substrates for intracellular targeting of PKG and its impact for cellular activation need further analysis. Further aspects are the accessibility of the targeted PKG1 isozymes for global or local cGMP. Furthermore, the crossactivation of cGMP/PKG by or with other intracellular cyclic nucleotide pathways might be involved in the pattern of PKG function (20, 95). The elucidation of these mechanisms will yield to a more refined view of PKG function and targeting in various organs and its physiological and pathophysiological consequences.

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