The A-Site In The Pkg Iα Regulatory Domain Controls Both Cgmp- And Oxidative-Dependent Activation

Jessica Lynne Sheehe
University of Vermont

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THE A-SITE IN THE PKG Iα REGULATORY DOMAIN CONTROLS BOTH cGMP- AND OXIDATIVE-DEPENDENT ACTIVATION

A Dissertation Presented

by

Jessica Lynne Sheehe

to

The Faculty of the Graduate College

of

The University of Vermont

In Partial Fulfillment of the Requirements
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Dissertation Examination Committee:

Wolfgang R. Dostmann, Ph.D., Advisor
Bryan A. Ballif, Ph.D., Chairperson
Alan K. Howe, Ph.D.
Albert van der Vliet, Ph.D.
Cynthia J. Forehand, Ph.D. Dean of the Graduate College
Abstract

The type Iα cGMP-dependent protein kinase (PKG Iα) is an essential regulator of vascular tone and systemic blood pressure. Located in the smooth muscle of resistance vessels, PKG Iα stimulates vasodilation through the phosphorylation of multiple intracellular substrates. Its primary regulator is the small molecule, 3′,5′-cyclic guanosine monophosphate (cGMP); however, the Iα isoform can also be activated by oxidation. Despite the established physiological importance of PKG Iα, the structural underpinnings of these two activation mechanisms are largely unknown.

The work presented in this dissertation demonstrates the importance of the cGMP-binding domain A (CBD-A) in regulating both of these mechanisms of PKG Iα activation. Using a monomeric, N-terminally truncated form of PKG Iα (Δ53), Chapter 2 investigates the mechanism of inhibition through the autoinhibitory domain and the influence of dimerization on cooperative cGMP-dependent activation and cyclic nucleotide selectivity. We observed that autoinhibition occurs in cis, whereas cooperativity requires interprotomer contacts facilitated by the N-terminal dimerization domain. Furthermore, the loss of selectivity for cGMP over cAMP of this construct suggests the dimerization domain plays a critical role in preventing cross-reactivity with cAMP-dependent signaling. These observations culminate into an overarching model wherein binding of cGMP to CBD-A is necessary and sufficient for activation and cooperativity is driven by the dimerization domain.

Chapter 3 investigates the cysteine residues that mediate oxidation-dependent activation of PKG Iα. Using PKG Iα constructs with point mutations at specific cysteine residues, it was found that oxidation-dependent activation is driven by C117 in CBD-A. Furthermore, the interprotomer disulfide bond that forms in the dimerization domain at C42 does not contribute to this mechanism. Finally, we propose a model wherein the disulfide bond that forms between C117 and the adjacent cysteine at position 195 acts as a protective mechanism to prevent activation and higher oxidation states form contacts with nearby residues in the linker region of PKG Iα to disrupt binding of the adjacent autoinhibitory domain to the catalytic domain.

Finally, Chapter 4 provides a discussion of the results presented herein in context with previous studies and suggests future directions for the PKG field.
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For my daughter, Emily.
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Finally, I would like to thank my family and friends for their love and support. I would not have been able to pursue or complete graduate school without them. My parents, Michael and Beth, taught me perseverance. My family has always been willing to lend an ear and patiently listened to my descriptions of research and lab life. I am also deeply indebted to my friends and former roommates, Drs. Johanna Schleifenbaum and Laura Haynes, for their unwavering support and help with my daughter.
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Chapter 1

Introduction

1.1 Expression and sequence conservation of the type I cGMP-dependent protein kinase (PKG I)

The cGMP-dependent protein kinase (PKG) is a member of the AGC (PKA, PKG, and PKC) family of serine/threonine kinases. These enzymes are activated by the small molecule, cyclic 3’5’ guanosine monophosphate (cGMP) and phosphorylate intracellular substrates thereby controlling processes that range from vascular reactivity to insulin secretion (Hofmann et al., 2009). PKG is a ubiquitously-expressed enzyme found in diverse eukaryotic species, including apicomplexan parasites (e.g. *plasmodium falciparum* and *toxoplasmodia gondii*), insects (e.g. honey bees, mosquitoes, ants, and fruit flies), and vertebrates (e.g. humans, cows, rats, mice, rabbit, and puffer fish) (Fitzpatrick and Sokolowski, 2004; Billker et al., 2009). In mammalian tissues, two genes, *prkg1* and *prkg2*, encode for PKG types I and II, respectively. PKG I is expressed as two splice variants, PKG Iα and Iβ, of the *prkg1* gene (Hofmann et al., 2009). Due to the breadth of literature within the PKG field, this review will focus on the PKG type I isoforms, α and β.

1.1.1 Sequence conservation of PKG I isoforms

The sequences of PKG Iα and Iβ are highly conserved across placental mammals. As of Feb 19, 2018, the National Center for Biotechnology Information (NCBI) protein database lists PKG Iα and Iβ sequences from 25 species across 8
orders within the *mammalia placentalis* subclass. The highest representation of
sequences are within the primates and rodentia classifications (Figure 1.1).

![Alignment of PKG isoforms](image)

Figure 1.1: All currently available sequences (as of Feb. 19, 2018) of PKG I
isoforms expressed in placental mammals (*mammalia placentalis*) were obtained
from the National Center for Biotechnology Information (NCBI) database. Species
are organized by order and include the genus species information, common names,
and NCBI accession numbers for the Iα and Iβ isoforms. Sequences were aligned
in Jalview V2 using the Clustal OWS algorithm (Sievers et al., 2011; Waterhouse
et al., 2009). The full sequence alignment can be found in Appendix 2.

A comparison of PKG Iα and Iβ using BLOSUM62 scoring found that
the overall sequence identity across placental mammals is 88%, with the highest
variation occurring in the N-terminal dimerization (29% identity), autoinhibitory
domain (44% identity), and the two linker regions (6% identity) (Figure 1.2).
Their regulatory and catalytic domains are nearly 100% identical with only a
handful of species-specific residue differences between them (15/593 residues); of
these, most are conservative replacements (e.g. residue 227 in Iα is either an
Asp or a Glu). Thus, the only differences between PKG Iα and Iβ exist in their
N-termini.

1.2 Structural architecture of PKG I isoforms

PKG Iα and Iβ are homodimers with each protomer composed of an N-terminal dimerization domain, an autoinhibitory domain, a regulatory domain containing two cGMP binding sites, and a catalytic domain (Pinkse et al., 2009). As a result of alternative-splicing of the prkg1 gene, PKG Iα and Iβ are identical from the beginning of their regulatory domains to the C-terminus (residues 78-671 in Iα and 93-686 in Iβ) (Orstavik et al., 1992, 1997). The differences in their dimerization and autoinhibitory domains have been shown to impart isoform specific differences in cGMP-dependent activation and targeting to substrates (Casteel et al., 2010; Qin et al., 2015; Ruth et al., 1991).

Figure 1.2: PKG Iα and Iβ Domain Architecture. PKG I isoforms are homodimers with the same overall domain architecture, which consists of a dimerization domain, an autoinhibitory domain, a regulatory domain containing two in-tandem cGMP binding sites (A and B), and a catalytic domain. The regions between the dimerization, autoinhibitory, and regulatory domains are separated by linker regions 1 and 2. The residues that span the specific domains within PKG Iα and Iβ are indicated below the schematic.
1.2.1 Dimerization Domain

The N-terminal dimerization domain is a left-handed, coiled-coil, leucine zipper motif (Landgraf et al., 1991; Atkinson et al., 1991; Qin et al., 2015; Casteel et al., 2010). The structure is characterized by a heptad repeat of amino acids (abcdefg) where residues a and d are typically hydrophobic and residues e and g are typically hydrophilic. The leucine zipper of PKG Iα spans residues 2-47 (Qin et al., 2015; Casteel et al., 2010; Ruth et al., 1991). In addition to its leucine and isoleucine residues, there are also four non-leucines/isoleucines at the d positions that provide additional non-hydrophobic interhelical contacts (Phe7, Lys14, Lys28, Cys42) (Figure 1.3) (Qin et al., 2015). One of these residues, Cys42, is unique to the Iα isoform and forms an interprotomer disulfide bond in the presence of oxidizing agents, such as hydrogen peroxide (Burgoyne et al., 2007; Landgraf et al., 1991). The leucine zipper of PKG Iβ spans residues 4-53, and also contains four non-leucine/isoleucine residues at d positions (Lys13, Arg20, Lys34, and Tyr48) (Casteel et al., 2010). These non-canonical residues in both isoforms primarily form contacts with residues at the e position of the opposing helix. Though the majority of the non-canonical residues are basic, their differences in position within the helices likely prevent PKG I protomers from forming heterodimers both in vitro and in vivo.

The outer residues of the PKG Iα and Iβ leucine zippers impart isoform specific charge distributions across their surfaces that mediate substrate targeting and localization (Figure 1.3- top panel) (Schlossmann and Desch, 2009). Several reviews have focused on the substrate specificities of the Iα and Iβ isoforms (Hofmann et al., 2000, 2009; Hofmann and Wegener, 2013; Schlossmann and Desch, 2009). Most substrates are phosphorylated by a single isoform, while others, such as vasodilator stimulated phosphoprotein (VASP), can be phosphorylated by either Iα.
Figure 1.3: Dimerization domains of PKG Iα (PDB 4R4L) and Iβ (PDB 3NMD). Electrostatic surface models show the charge distribution patterns for each isoform (top) (basic = blue; acidic = red; white = neutral). The cartoon models show the residues within the helices (middle). The sequences show interprotomer contacts between homodimers (bottom). Canonical and non-canonical residues at the a or d positions are denoted with vertical lines, Xs, or brackets. Residues at the a position within the helical repeat (abcdefg) are highlighted with yellow boxes.

or Iβ. Thus far, more than 20 PKG I substrates have been identified (Hofmann et al., 2009; Hofmann and Wegener, 2013; Schlossmann and Desch, 2009). Even though the phosphorylation sites have been identified, only the specific binding interfaces between the PKG I dimerization domains and myosin-binding subunit (MBS) of myosin light-chain phosphatase (MYPT1) (Blanton et al., 2013; Lee et al., 2007; Sharma et al., 2008, 2017; Surks et al., 1999; Surks, 2007; Zhou, 2011), the Ras homolog gene family member A (RhoA) (Kato et al., 2012), the general transcription factor regulator, TFII-I, and the inositol 1,4,5-trisphosphate receptor-associated PKG substrate (IRAG) (Casteel et al., 2005) have been elucidated using biochemical or structural methods (PKG Iα-MBS and MYPT1; PKG Iβ-TFII-I and IRAG).
Substrate targeting has been shown to be integral in regulating PKG signaling (Blanton et al., 2013; Michael et al., 2008; Ramchandran et al., 2014). Mutation of the first four leucines/isoleucines within the dimerization domain of PKG Iα has been shown to be sufficient in disrupting phosphorylation of the substrates RhoA and MYPT1 in vascular smooth muscle cells. Despite this effect on targeting to that full-length substrate, the mutant construct was able to be activated in a cGMP-dependent manner and phosphorylated a peptide substrate. Introduction of these mutations into a mouse model (LZM) also disrupted PKG Iα signaling (Ramchandran et al., 2014). These experiments highlight the importance of distinguishing substrate targeting from activation defects.

1.2.2 Autoinhibitory Domain

The autoinhibitory domain is a critical component of PKG I activation and spans residues 51-68 and 66-83 in PKG Iα and Iβ, respectively. When PKG I is inactive, the autoinhibitory domain binds to the catalytic domain and acts as a pseudosubstrate (Aitken et al., 1984; Francis et al., 1996; Wolfe et al., 1987). Autoinhibition of the catalytic domain is mediated by Gly62 (PKG Iα) and Ala77 (PKG Iβ) within the conserved G/AISAEP sequence (Kemp et al., 1988; Francis et al., 1996). These residues are thought to occupy the phosphate acceptor site (P0) to prevent substrate phosphorylation in a mechanism similar to PKA holoenzyme formation (Francis et al., 1996). It is thought that cGMP induces structural reorganization of the regulatory domain and acts to relieve this autoinhibition (Alverdi et al., 2008; Zhao et al., 1997). The functional importance of this domain was confirmed by the generation of the truncation mutant PKG Iα Δ77. This construct can be generated endogenously through trypsin cleavage at Arg77 and was found to be constitutively active due to loss of its autoinhibitory sequence (Heil et al., 1987; Landgraf et al., 1990; Scholten et al., 2007). Fur-
thermore, activated PKG Iα has been shown to have increased exposure of the autoinhibitory domain and the putative substrate binding region of the catalytic domain, suggesting these two regions are likely in contact with each other in the inactive state (Alverdi et al., 2008).

Research has suggested the cyclic nucleotides, cAMP and cGMP, can both stimulate autophosphorylation of PKG I isoforms primarily within the autoinhibitory domain (Aitken et al., 1984; Hofmann and Flockerzi, 1983; Takio et al., 1983). Initial experiments using PKG Iα purified from bovine lung indicated cyclic nucleotide-dependent differences in the sites of autophosphorylation. Incubation with cAMP predominantly stimulated autophosphorylation at Ser50, Thr58, Ser72, and Ser84, whereas cGMP predominantly stimulated autophosphorylation at Thr58 (Aitken et al., 1984). A more recent examination using recombinant PKG Iα corroborated these results, and also noted autophosphorylation at Ser44 with cAMP (van de Waterbeemd et al., 2014). In PKG Iβ, the major sites of autophosphorylation were found to occur at Ser63 and Ser79 (Smith et al., 1996). No cyclic nucleotide-dependent differences in the sites were observed; however, autophosphorylation was slower with cGMP than cAMP. In addition, the basal activity of autophosphorylated PKG Iβ was increased up to 4-fold compared to the non-autophosphorylated form (Francis et al., 1996; Smith et al., 1996). This may be due to the location of these sites in the P+2 position within the pseudosubstrate region. In contrast, the autophosphorylation sites of PKG Iα flank the pseudosubstrate region; therefore, it is unknown whether this same increase in basal activity would occur.

The physiological significance of PKG I autophosphorylation is an understudied area of investigation. Autophosphorylation has been suggested to mediate ubiquitination and intracellular turnover for PKG Iα, but not PKG Iβ (Dey et al.,
On its surface, this model is plausible given that maximum autophosphorylation of PKG I isoforms requires 1-2 h. However, this would suggest PKG I\textsubscript{b} turnover occurs through a different mechanisms (Aitken et al., 1984; Hofmann and Flockerzi, 1983; Smith et al., 1996; van de Waterbeemd et al., 2014).

### 1.2.3 Regulatory Domain

The regulatory domains of PKG I\textsubscript{a} and I\textsubscript{b} are identical and contain two tandem cyclic nucleotide binding sites (A and B sites) per protomer. Despite this similarity, PKG I\textsubscript{a} and I\textsubscript{b} have distinct cGMP-dependent activation properties, with activation constants (K\textsubscript{a} values) of approximately 100 nM and 1-2 \(\mu\)M, respectively (Gill et al., 1976; Richie-Jannetta et al., 2003; Ruth et al., 1991, 1997; Moon et al., 2015). This 10-20-fold difference is attributed to their distinct N-termini. It was found that the leucine zipper domain, the autoinhibitory domain, and the linker region just before the start of the regulatory domain (Linker 2; Figure 1.2) are important for conferring these isoform-specific properties (Ruth et al., 1997). Furthermore, the leucine zipper and the autoinhibitory domains appear to work synergistically and have the greatest influence since swapping of either domain from PKG I\textsubscript{a} into PKG I\textsubscript{b} was sufficient to achieve a 4-fold reduction in the K\textsubscript{a} for cGMP (Ruth et al., 1997).

Binding of cGMP to the A site (slow dissociation, high affinity) before binding to the B site (fast dissociation, low affinity site) (Corbin and Doskeland, 1983; Zhao et al., 1997). The difference in binding affinities between the sites is surprising since the residues involved in cyclic nucleotide binding are mostly conserved between the A-site and the B-site (Table 1.1). It was suggested that a conserved threonine in both sitesThr\textsubscript{177/192} and Thr\textsubscript{301/316} (I\textsubscript{a} and I\textsubscript{b} numbering, respectively) acts as a selectivity filter for binding cGMP over cAMP (Kim et al., 2011). However, isothermal titration calorimetry (ITC) measurements of
Table 1.1: Predominant residues that bind to cGMP in the cyclic nucleotide binding domains of PKG I

<table>
<thead>
<tr>
<th>Cyclic Nucleotide Position</th>
<th>A-site (α/β)</th>
<th>Å</th>
<th>B-site (α/β)</th>
<th>Å</th>
<th>Binding Affinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanine moiety- O</td>
<td>L155/170</td>
<td>N/A</td>
<td>R280/295</td>
<td>3.0</td>
<td>Moderate</td>
</tr>
<tr>
<td>Guanine moiety- NH₂</td>
<td>K152/166</td>
<td>4.5</td>
<td>R268/283</td>
<td>4.3</td>
<td>Weak</td>
</tr>
<tr>
<td>Phosphate- O/OH</td>
<td>R176/191</td>
<td>2.8</td>
<td>R300/315</td>
<td>2.9</td>
<td>Moderate</td>
</tr>
<tr>
<td>Phosphate- O/OH</td>
<td>T177/192</td>
<td>2.5</td>
<td>T301/316</td>
<td>2.6</td>
<td>Moderate</td>
</tr>
<tr>
<td>Ribose- OH</td>
<td>E166/181</td>
<td>2.4</td>
<td>E290/305</td>
<td>2.4</td>
<td>Strong</td>
</tr>
</tbody>
</table>

Interacting residues were determined based on the cGMP-bound regulatory domain structure (PDB 4Z07; residues 106-212 and 121-227 in PKG Iα and Iβ, respectively (Kim et al., 2015)).

the isolated A-site indicated binding constants (K_D) of 12 and 27 nM for cGMP and cAMP, respectively, suggesting this threonine alone poorly discriminates between the two. From these data, it was postulated that the selectivity for cGMP of the full-length kinase is conferred through the B-site. To corroborate this, a neutron diffraction structure of the isolated cGMP-bound B-site identified a conserved arginine residue (Arg281/296) that forms contacts with O6 and N7 of the guanine moiety (Huang et al., 2014). In the A-site, the residue at the equivalent position within the structure is a leucine (Leu155/170) (Kim et al., 2011, 2016). The predominant contacts between residues in the A- and B-sites with cGMP are shown in Figure 1.4.

The crystal structures of PKG I that have been solved thus far have only included isolated dimerization and regulatory domain components (Campbell et al., 2017; Huang et al., 2014; Kim et al., 2011, 2016; Osborne et al., 2011). Since the N-terminal dimerization and autoinhibitory domains are known to influence the characteristics of the regulatory domain, these structures provide an incomplete picture of PKG I activation with cGMP. The crystal structure solved by Osborne et al. (2011) shows a helical segment at the C-terminal end of the B-site that was found to form interprotomer contacts with the hydrophobic pocket of another B
Figure 1.4: Alignment of the A and B cyclic nucleotide binding domains of PKG I bound to cGMP (PDB 4Z07): A. The A (green) and B (gray) cyclic nucleotide binding sites were selected and aligned in PyMOL using CE-align (A-site residues 121-217; B-site residues 240-340; RMSD = 1.585744). B. Structures of cAMP and cGMP. The red squares indicate the groups that are distinct between the two. C. Enlarged image of the cyclic nucleotide binding domain. The residues that bind to the guanine moiety are shown as sticks (α/β numbering: L155/170, R280/295, K152/K166, R268/283). Some of the residues that bind to the cyclic phosphate and ribose sugar are also shown (α/β numbering: T177/192, T301/316, E166/181, E290/305). The yellow and orange dashed lines indicate the interactions with the A-site and B-site residues, respectively.

site (Figure 1.5). This "switch helix" provided the structural basis for developing cGMP-independent, peptidic activators. The most potent peptide that mimics the helical segment observed in the crystal structure, S1.5, was found to only activate PKG Iα and had no effect on PKG Iβ (Moon et al., 2015). This result was unexpected since the regulatory domain sequences are identical between the two isoforms. Thus, a large gap exists in the PKG field regarding how the N-terminal
1.2.4 Catalytic Domain

PKG I contains a C-terminal catalytic domain that phosphorylates target substrates by transferring the $\gamma$-phosphate from ATP onto serine or threonine residues. This region is encompassed by residues 360-671 and 375-686 in PKG I$\alpha$ and $\beta$, respectively. Due to the lack of a crystal structure of this region, the PKG field has relied on biochemical and biophysical approaches to investigate this region as well as structures of the PKA catalytic domain. In the inactive state, the site of catalysis is occluded through binding to PKG I's autoinhibitory domain, and binding of cGMP induces a conformational change that relieves this inhibition (Alverdi et al., 2008; Chu et al., 1997; Heil et al., 1987; Landgraf et al., 1987).
A summary of the major evidences for this model have been described in the "Autoinhibitory Domain" and "Regulatory Domain" subsections of this review.

The catalytic domains of protein kinases are highly conserved in both their tertiary structure and the residues responsible for catalytic activity (Hanks et al., 1988; Hanks and Quinn, 1991). All catalytic domains have two lobes (N and C) with the interface between them forming the site of catalysis (Bastidas et al., 2013; Knighton et al., 1991; Taylor and Kornev, 2011; Zheng et al., 1993). The N-lobe contains the glycine-rich loop (G-X-G-X-X-G), which binds ATP, and the Mg\(^{2+}\) binding loop, which coordinates two Mg\(^{2+}\) ions essential for stabilizing the phosphate groups of ATP and transfer of the γ-phosphate. The N-lobe also contains other conserved residues that coordinate with C-lobe components to create the active site. The C-lobe contains the bulk of the residues required for catalysis, which are predominantly found in the catalytic loop, the activation loop, and the substrate binding segment. The catalytic loop contains an aspartate that acts as a catalytic base to deprotonate the serine/threonine hydroxyl oxygen on the substrate. The DFG motif of the activation loop, along with the N-lobe residues, helps position the Mg\(^{2+}\) ions. The activation loop also contains a phosphorylated threonine residue that coordinates the active site components by forming contacts with the catalytic loop, the activation loop, and the αC helix on the N-lobe (Bastidas et al., 2013; Steichen et al., 2012). In PKG I, mutation of this residue (T517) to alanine completely abolished catalytic activity (Feil et al., 1995).

The putative substrate binding segment of the catalytic domain (residues 611-671 and 626-686 in PKG Iα and Iβ, respectively) is also located on the C-lobe and contains acidic, basic, and hydrophobic residues that dictate substrate specificity (Alverdi et al., 2008; Hofmann et al., 1992; Dostmann et al., 1999).
Figure 1.6: Structure of the catalytic domain of PKA (PDB 1ATP). Since all kinase domains have the same overall architecture, the catalytic domain of PKA is used as a model for PKG. A. This domain is composed of an N- and C-lobe with the conserved loops important for catalysis shown in color (Glycine rich loop-yellow; Catalytic loop- red; Activation Loop- green; P+1 Loop- orange). B. ATP and Mn$^{2+}$ ions are shown coordinated in the active site by D166, K168, and N171. C. Phosphorylated T197 in the activation loop integrates the active site components through contacts with other activation loop residues (T195 and K189) as well as R165 in the catalytic loop and H87.

The consensus sequence for PKG I is X-R-R-K-S/T-X-X-X with two arginines positioned at the $P^{-3}$ and $P^{-2}$ positions, a lysine at the $P^{-1}$ position, a serine or threonine residue at the $P^{0}$ position, and typically hydrophobic or basic residues at the $P^{+1}$ position (Francis et al., 2010; Tegge et al., 1995). This sequence was determined using synthetic peptides that subsequently served as the foundation for developing peptidic inhibitors of PKG I (Dostmann et al., 1999, 2000, 2002; Nickl et al., 2010; Pinkse et al., 2009; Taylor et al., 2004; Tegge et al., 1995). The
autoinhibitory domains of PKG I\textalpha and I\textbeta mimic this consensus sequence and bind to the catalytic cleft in the inactive state. Interestingly, PKG I\textalpha contains only one arginine at the P-2/-3 positions while I\textbeta contains two (Hofmann et al., 1992; Francis et al., 2010). This notable difference provides one potential mechanism by which isoform-dependent differences in their kinetic profiles may be conferred.

1.2.5 Models of the spatial orientations of PKG domains

1.2.5.1 Models of PKG have been influenced by PKA

The cGMP-dependent protein kinase (PKG) was discovered in 1970, and its expression in mammalian tissues was determined shortly thereafter in 1972 (Hofmann and Sold, 1972; Kuo and Greengard, 1970). During the latter half of the 1970s and the early 1980s, most of the work on PKG focused on obtaining pure, homogenous preparations of PKG I\textalpha and I\textbeta from bovine lung and aorta for further characterization (Gill et al., 1976; Lincoln et al., 1977; Hofmann and Flockerzi, 1983; Corbin and Doskeland, 1983; Wolfe et al., 1987). This work coincided with the discovery and initial characterization of the cAMP-dependent protein kinase (PKA) (Glass and Krebs, 1979; Kuo et al., 1970). Thus, PKG and PKA have always been closely linked in the literature. The discovery that these kinases are highly homologous further solidified this association (Lincoln et al., 1977; Hashimoto et al., 1982; Takio et al., 1983, 1984). Since structures of PKA were the first to be solved by x-ray crystallography, PKA has served as a model for understanding PKG, particularly regarding the spatial organization of its domains (Huggins et al., 1994; Kim et al., 2007; Knighton et al., 1991).

The sequences of PKG and PKA are approximately 35% identical and have the same overall domain organization, which consists of an N-terminal dimerization domain, an autoinhibitory domain, a regulatory domain that binds cyclic nucleotides, and a C-terminal catalytic domain (Takio et al., 1984; Huang et al.,
Their dimerization domains mediate subcellular localization—PKA is tethered through its association with AKAPs (A-kinase anchoring proteins), whereas most literature in the field suggests PKG is targeted directly to its substrates (Taylor et al., 2004; Hofmann and Wegener, 2013; Schlossmann et al., 2003). In addition, the catalytic domains of both kinases are autoinhibited by peptidic regions that mimic their preferred substrate consensus sequences (Francis et al., 2010; Hofmann et al., 1992; Kreegipuu et al., 1998). Finally, each dimeric PKG and PKA regulatory domain binds four cyclic nucleotide molecules (two with high affinity and two with low affinity) that mediate cooperative activation of the kinases (Byeon et al., 2010; Dostmann et al., 1996; Kim et al., 2007; Richie-Jannetta et al., 2006; Su et al., 1995; Wu et al., 2004). Despite these similarities, a few notable differences exist between PKG and PKA. Each PKG protomer contains a regulatory and catalytic domain, whereas PKA regulatory and catalytic domains are expressed as separate protomers (Takio et al., 1984). Thus, dissociation between PKG’s regulatory and catalytic domains is constrained by the peptide backbone. Furthermore, the locations of the high and low affinity cyclic nucleotide binding sites are reversed in PKG and PKA (PKG: A=high, B=low; PKA: A=low, B=high) (Alverdi et al., 2008; Zhao et al., 1997). In light of these similarities and differences, several groups have proposed models that describe the overall domain organization of PKG as it relates to function. The major evidences describing the overall shape of PKG I and the two predominating models that have emerged in the field—the tweezer and the switch model—are discussed below.
1.2.5.2 The tweezer model of PKG domain organization—the protomers function independently

The overall dimensions of PKG I were first determined by size exclusion chromatography and small angle x-ray scattering (Chu et al., 1997; Zhao et al., 1997). PKG I\(\alpha\) was found to adopt an overall ellipsoidal shape, and that cGMP binding induces a change in conformation into a more extended, asymmetrical shape (Zhao et al., 1997). These reorientations were suggested to occur through domain reorganization, likely through flexible linker regions between domains, rather than through changes in secondary structure. Significant reorganization can occur through cGMP binding to the A-site alone; however, binding to both cGMP sites is required to induce the full shape change. Overall, it was proposed that the apo structure of PKG has three regions: an N-terminal dimerization domain and two regions each composed of a regulatory and catalytic domain. This model, though not explicitly stated, is the likely precursor of the tweezer model wherein each R:C complex can operate independently to perform catalytic function. This model would also suggest that cooperative cGMP-dependent activation is driven through crosstalk between the A- and B-sites of individual protomers.

Further investigation into the interdomain contacts formed in PKG I were deduced using truncated constructs of PKG I\(\alpha\) and I\(\beta\) without catalytic domains (PKG I\(\alpha\) 1-348; PKG I\(\beta\) 1-363). It was found that the Stokes radii were not appreciably different between full-length and truncated PKG I\(\alpha\) and I\(\beta\), even though the truncated constructs were half the masses of their full-length counterparts (Richie-Jannetta et al., 2006). These results suggested that the interface between the N-terminus (dimerization, autoinhibitory, and regulatory domains) and the catalytic domain is quite large and results in a highly compact R:C complex. To investigate the domain reorganization induced by cGMP binding, Alverdi et al. (2008) used hydrogen/deuterium exchange mass spectrometry. Their results in-
dicated that residues involved in cGMP binding domains become less solvent exposed upon activation, whereas the autoinhibitory region and substrate binding region of the catalytic domain become more solvent exposed. This provided further evidence of the autoinhibitory domain’s function in occluding the catalytic domain in the inactive state. Similar to the previous publication, this study depicts R:C protomers as independent, catalytic entities.

1.2.5.3 The switch model of PKG domain organization—crosstalk occurs between protomers

The first x-ray crystal structure of PKG I included only the regulatory domain (residues 78-356 using PKG Iα numbering) Osborne et al. (2011), and provided the first opportunity to connect the structures of the cGMP-binding domains with previous biochemical analyses. In addition, the structure showed interprotomer contacts between the B-sites of opposing protomers, wherein a helical segment at the end of the B-site docks into a hydrophobic pocket on the opposing site. Mutational disruption of the residues implicated in this interaction and SAXS analyses of 78-326 (without helical domain) and 78-356 (with helical domain) were used to show that this crosstalk between protomers occurs in solution (Osborne et al. (2011) and unpublished data). Further experiments were performed using mixed dimers of PKG Iα that were composed of protomers containing mutations at either E167A and T517A, which rendered the regulatory and catalytic domains, respectively, inactive. These mixed dimers retained catalytic activity, suggesting the regulatory domain of one protomer could control the activity of the opposing catalytic domain (unpublished data, Dostmann Lab). Thus, the second model of PKG I suggests activation involves crosstalk between protomers (in trans regulation) rather than independently functioning protomers.
1.3 Intracellular modulation of PKG I activity in the cardiovascular system

The primary intracellular modulator of PKG I is the small molecule, cGMP (Hofmann and Wegener, 2013; Surks, 2007). Cyclic GMP is produced from guanylyl cyclases in a nitric oxide- or natriuretic peptide-dependent manner (soluble and particulate GC, respectively) (Hofmann et al., 2009). The cGMP-dependent activation of PKG I was first determined to play an important role in vascular relaxation (Francis et al., 1988; Sausbier et al., 2000). This finding corroborated an earlier observation that the amount of PKG detected per dry weight of tissue correlated with the degree of vascularization (Hofmann et al., 1992).

1.3.1 cGMP-dependent activation of PKG in resistance vessels

Resistance vessels, which comprise the small arteries and arterioles, are the primary regulators of systemic blood pressure. They achieve this by controlling vessel diameter through the contraction and relaxation of smooth muscle (Bayliss, 1902). Vessels respond to both short-term and long-term effectors (Ayada et al., 2015; Cowley, 1992; Raven and Chapleau, 2014). Short term effectors include circulating factors found in the blood and neuronal stimulation (e.g. baroreceptor reflex), while longterm effectors primarily stem from the kidney to regulate blood volume (Dunn et al., 2007; Wadei and Textor, 2012). In addition to systemic factors, the diameter of resistance vessels is controlled locally through response to frictional forces from circulating blood and mechanical stretch from blood pulses (Hahn and Schwartz, 2009). The culmination of these global and local factors maintain constant downstream blood flow (Hahn and Schwartz, 2009; Tucker and Bhimji, 2018).

Resistance vessels are composed of three layers: the tunica intima, the tu-
nica media, and the tunica externa (Tucker and Bhimji, 2018). The innermost layer, the tunica intima, is composed of endothelial cells that are in direct contact with the lumen. The middle layer, the tunica media, is composed of smooth muscle, elastin, and collagen while the tunica externa is the outermost layer composed of collagen, connective tissue, and elastic fibers, which provide structural rigidity.

There are numerous factors that stimulate vasorelaxation by targeting surface receptors on endothelial cells (Vanhoutte et al., 2016). In response, endothelial cells release three primary molecules: endothelium-derived hyperpolarizing factor (EDHF), prostaglandins, and nitric oxide (Dautzenberg and Just, 2013). EDHF is produced within the first 30 s of a stimulus and its signal rapidly decays. In contrast, prostaglandins and nitric oxide initiate within 30-60 s of a stimulus and are considered to be the elements that contribute to prolonged responses by the vasculature.

Endothelial nitric oxide synthase (eNOS) produces nitric oxide as part of the urea cycle through the conversion of L-arginine to citrulline (Vanhoutte et al., 2016). Nitric oxide (a dissolved gas) passively diffuses through the plasma membrane into the smooth muscle layer where it activates soluble guanylyl cyclase (sGC) to produce cGMP (Friebe and Koesling, 2003). PKG I activated by cGMP phosphorylates multiple intracellular targets, which culminate in vasodilation by modulating calcium flux from both the extracellular space and intracellular, endoplasmic reticulum stores (Felbel et al., 1988; Harnett and Biancani, 2003; Lincoln et al., 1990; Morgado et al., 2012; Surks and Mendelsohn, 2003).

Vascular smooth muscle cells express both isoforms of PKG I–α and β (Keilbach et al., 1992). Calcium flux from the extracellular space is controlled by calcium channels expressed on the plasma membrane (Harnett and Biancani, 2003; Vanhoutte et al., 2016; Tayo and Bevan, 1987). The Iα isoform directly tar-
Figure 1.7: Regulation of vascular tone by endothelium-derived nitric oxide (NO). Top: Increased release of endothelium-derived nitric oxide (NO) by endothelial nitric oxide synthase (eNOS) is caused by both physical factors (increases in shear stress and temperature lowering) and neurohumoral mediators (through activation of specific endothelial cell membrane receptors). Bottom: Vascular smooth muscle cell contraction is caused by an increase in cytoplasmic Ca$^{2+}$ concentration either from endoplasmic reticulum stores or influx through Ca$^{2+}$ channels at the plasma membrane. Calcium ions bind to calmodulin (CaM), and the calcium-calmodulin complex activates myosin light-chain kinase (MLCK). MLCK phosphorylates myosin light chain, which leads to cross-bridge formation between the myosin heads and the actin filaments and subsequent smooth muscle contraction. This process is counterbalanced by relaxation mechanisms stimulated by endothelial NO. NO activates soluble guanylyl cyclase (sGC), which produces cGMP from GTP. Cyclic GMP-dependent activation of PKG I initiates phosphorylation of multiple intracellular targets that collectively result in decreased cytosolic Ca$^{2+}$ and decreased actin-myosin cross-bridges to cause relaxation. (Vanhoupt et al., 2016)
of voltage-dependent calcium channels (VDCC) (Fukao et al., 1999; Kyle et al., 2013; Robertson et al., 1993; Sausbier et al., 2000; Schubert and Nelson, 2001). Calcium flux through the endoplasmic reticulum is controlled by the sarcoendoplasmic reticulum calcium transport ATPase (SERCA) pump and the inositol triphosphate receptor (IP$_3$R), respectively (Harnett and Biancani, 2003; Tayo and Bevan, 1987; Vanhoutte et al., 2016). This system is targeted by both PKG I$\alpha$ and I$\beta$. PKG I$\alpha$-dependent phosphorylation of regulator of G-protein signaling 2 (RGS2) induces RGS2 translocation to the plasma membrane. Here, it inhibits G$_q$-dependent signaling of contractile agonists, such as angiotensin II, endothelin-1, and norepinephrine, to suppress inositol triphosphate (IP$_3$) production (Hofmann et al., 2006; Tang et al., 2003; Sun et al., 2005; Wu et al., 2003). PKG I$\beta$ phosphorylates the inositol trisphosphate receptor-associated cGMP-kinase substrate (IRAG) (Geiselhoringer et al., 2004; Schlossmann et al., 2000). These two processes block IP$_3$R-mediated calcium efflux from the endoplasmic reticulum. Furthermore, phosphorylation of the SERCA pump modulator, phospholamban, by PKG I$\beta$ leads to increased calcium uptake into the endoplasmic reticulum (Hofmann et al., 1992; Lalli et al., 1999; Koller et al., 2003). Through these mechanisms, PKG I signaling leads to an overall decrease in free, cytosolic calcium. Finally, PKG I also acts directly on the cytoskeletal contractile machinery. Smooth muscle contraction and relaxation is dependent on the balance of phosphorylated and unphosphorylated myosin light chain (MLC), which are controlled by the activities of myosin light chain kinase (MLCK) and myosin light chain phosphatase (MLCP), respectively (Vanhoutte et al., 2016). PKG I$\alpha$ directly phosphorylates the myosin phosphatase targeting subunit 1 (MYPT1) to increase the activity of MLCP (Yuen et al., 2011). PKG I$\alpha$ also phosphorylates RhoA, thereby inhibiting its effect on MYPT1 through Rho kinase (ROCK) (Jin and Burnett, 2006; Kato et al., 2012; Sharma et al., 2017; Surks et al., 1999; Surks and Mendelsohn, 20
These two mechanisms lead to an increase in MLCP activity to increase levels of unphosphorylated MLC. Furthermore, the decrease in global intracellular calcium described above disrupts calmodulin-dependent activity of MLCK (Wu et al., 1998). The increase in the unphosphorylated form disrupts actin binding and polymerization, culminating in smooth muscle relaxation (Chen et al., 2008).

Thus, PKG I isoforms are central regulators of vasodilation.

Based on the current list of known PKG I substrates, there is a strikingly disproportionate number of proteins that are phosphorylated by PKG Iα compared with Iβ (Schlossmann and Desch, 2009; Hofmann et al., 2014). A previous study using murine models determined that PKG I in smooth muscle is essential, and rescue through knock-in of either isoform was sufficient to restore normal vascular function (Weber et al., 2007). This would suggest that either PKG Iβ’s effect on blocking calcium release from the endoplasmic reticulum is sufficient for vasorelaxation or that there are other targets of PKG Iβ that have yet to be identified.

1.3.2 Oxidation-dependent activation of PKG Iα

In addition to activation via a cGMP-dependent mechanism, PKG Iα becomes constitutively active in the presence of oxidizing agents, such as heavy metals, hydrogen peroxide, and ambient oxygen (Burgoyne et al., 2007; Landgraf et al., 1991). This effect can be blocked with the cysteine-modifying agents, maleimide or iodoacetimide. In the first study that described this mechanism of activation, three disulfide bonds were suggested to form in oxidized PKG Iα (Landgraf et al., 1991). The first was an interprotomer disulfide bond between C42 and C42’ in the N-terminal dimerization domain. This covalent attachment between 75 kDa protomers was observed as a 150 kDa band by denaturing, non-reducing SDS-PAGE. The second and third disulfide bonds were intraprotomer
and suggested to form between C117 and C195 in the A site and C312 and C195 in the B-site and catalytic domain, respectively. These sites were identified by HPLC and Edman degradation; however, it was unclear whether one or both of the disulfide bonds actually formed. Of the three sites, only the first two have been confirmed in crystal structures (Osborne et al., 2011; Qin et al., 2015).

The observation that oxidative activation only occurs with PKG Iα, and not PKG Iβ, is important to note, because it has heavily influenced the recent literature (between 2007-2017) (Burgoyne et al., 2007, 2012, 2013a; Burgoyne and Eaton, 2013a,b; Burgoyne et al., 2013b,c; Kalyanaraman et al., 2017; Khavandi et al., 2016; Nakamura et al., 2015; Prsyazhna et al., 2012, 2016; Rudyk et al., 2012). Since PKG Iα and Iβ are splice variants of the same gene (prkg1), they only differ in their N-terminal dimerization and autoinhibitory domains (approximately residues 1-100). C42 is the only cysteine residue unique to PKG Iα; thus, it has been suggested that the interprotomer disulfide bond between C42 and C42’ must be the primary player in oxidative activation. This conclusion is further supported by the observation that hydrogen peroxide only decreased cytosolic Ca\(^{2+}\) in cells expressing PKG Iα, but not Iβ (Muller et al., 2012). However, this hypothesis has resulted in a shortcoming within the field since analyses of the other disulfide bonds presented in the Landgraf et al. (1991) study were never performed (Burgoyne et al., 2007).

Oxidation of PKG Iα appears to affect activation and substrate targeting. Both are required for PKG Iα phosphorylation of intracellular substrates, and disruption of either is sufficient to disrupt PKG Iα signaling (Feil et al., 1995; Michael et al., 2008; Ramchandran et al., 2014). C42 in PKG Iα appears to be invariant across PKG I amino acid sequences of placental mammals obtained from the NCBI database (Appendix 2), suggesting this residue may have an important
intracellular function. Since C42 is located at the end of the dimerization do-
main—the region that mediates substrate targeting—it has been suggested that
the disulfide bond between C42 and C42’ may also alter substrate binding. In-
direct binding measurements of oxidized PKG I\(\alpha\) have only been demonstrated
for a few substrates: the BK\(\text{Ca}\) channel, RhoA, MYPT1, and RGS2 (Burgoyne
et al., 2007; Nakamura et al., 2015); enhanced binding was observed for the BK\(\text{Ca}\)
channel, RhoA, and MYPT1, but not RGS2. Thus, PKG I\(\alpha\) activated by oxida-
tion may only target a subset of intracellular proteins. This hypothesis is further
supported by previous intracellular studies using cardiomyocytes that expressed
fluorescently-labeled wild-type PKG I\(\alpha\) or C42S (Nakamura et al., 2015). This re-
port found that incubation with hydrogen peroxide induced translocation of both
PKG I\(\alpha\) constructs to the plasma membrane within 10 minutes. After 1-2 h, PKG
I\(\alpha\) redistributed throughout the cytosol; however, C42S remained tethered to the
plasma membrane. Thus, the interprotomer disulfide bond may regulate PKG I\(\alpha\)
localization by altering its affinities for different substrates.

This has been further suggested by mutational studies that have disrupted
either substrate targeting through the dimerization domain or catalytic function
(Blanton et al., 2013; Casteel et al., 2005; Feil et al., 1995; Surks et al., 1999).
As most studies on oxidized PKG I\(\alpha\) have been performed under intracellular or
physiological conditions, it remains unclear how substrate targeting and activity
are independently affected by this mechanism, and whether both are controlled
by the interprotomer disulfide bond at C42. The role of C42 in the oxidative
activation of PKG I\(\alpha\) has been questioned in a recent paper by Kalyanaraman
et al. (2017), which found that the mutant, C42S, could still be activated by
hydrogen peroxide. This is in contrast to previous studies using a PKG I\(\alpha\) C42S
knock-in mouse model, which observed a hypertensive phenotype caused by a
sustained increased in blood pressure (Prysyzhna et al., 2012)

More recently, other potential effects on PKG Iα resulting from oxidizing intracellular conditions have been investigated. It has been suggested that 8-nitro-cGMP, a derivative of cGMP formed in the presence of high concentrations of reactive oxygen species or nitric oxide, can adduct to C42 and C195 in PKG Iα (Akashi et al., 2016). Moreover, S-guanylation of C195 correlated with constitutive activation. Since the protein coverage of PKG Iα peptides by mass spectrometry was only 26%, it is possible that other sites could be observed with increased coverage. It would be interesting to determine whether S-guanylation at C117, the residue previously suggested to form a disulfide bond with C195, also results in PKG Iα activation. The model of the A-site presented in the manuscript indicates a distance of approximately 22 Å from C8 of the guanine moiety to C195. This model seems unlikely given that the optimum distance for disulfide bond formation is approximately 6 Å. More plausibly, 8-nitro-cGMP binds to the cyclic nucleotide binding pocket in the A-site, and a second molecule adducts to C195. Regardless, these results highlight the importance of the A-site in regulating PKG Iα activity.

Hydrogen sulfide, a purported endothelium-derived hyperpolarizing factor (EDHF), has also been reported to induce oxidative activation of PKG Iα (Baragatti et al., 2013; Stubbert et al., 2014). Since the pKₐ of H₂S is approximately 6.8, it will be predominantly deprotonated at physiological pH and reactive with oxidants. The authors suggest the HS-S-SH intermediate could result in sulfhydration of cysteine residues in PKG Iα, which can be resolved by proximal cysteine residues within the kinase to form inter- or intraprotomer disulfide bonds. However, this mechanism would still require oxidizing agents, which could activate PKG Iα without an intermediary player. Thus, it remains unclear whether hydrogen sulfide plays an integral role in PKG Iα activation.
1.3.3 Oxidized PKG Iα signaling in the cardiovascular system

There are many intracellular sources of hydrogen peroxide, including NADPH oxidases (NOX), superoxide dismutases (SOD), and in the mitochondrial electron transport chain (Trachootham et al., 2008). Depending on the isoform, NOX complexes can either produce superoxide (O$_2^•^-$) or hydrogen peroxide (H$_2$O$_2$) (Burtenshaw et al., 2017). Superoxide can be converted to hydrogen peroxide by SOD. Since hydrogen peroxide is membrane permeable, both endothelial and smooth muscle cells are potential sources in the vasculature (Burtenshaw et al., 2017). Hydrogen peroxide itself is a weak oxidizing agent compared to other reactive oxygen species since it does not contain unpaired electrons. However, it can also react with iron to form hydroxyl radicals (•OH), which have greater oxidizing potential (Ardanaz and Pagano, 2006). The interconversion of reactive oxygen species makes studying their effects in cells particularly challenging.

NOX isoforms have been shown to have different effects in the vasculature (Burtenshaw et al., 2017; Sag et al., 2017). In general, NOX1 and 2 are associated with conditions characterized by endothelial and smooth muscle cell dysfunction, such as atherosclerosis, hypertension, and peripheral vascular disease. These processes occur through decoupling of eNOS-dependent NO synthesis, conversion of NO to peroxynitrite (ONOO$^-$), and subsequent transition of endothelial and smooth muscle cells to migratory cells. Furthermore, NOX4—the only NOX family member that directly produces hydrogen peroxide—has been associated with cardioprotection by increasing NO bioavailability and preventing the transition of endothelial and smooth muscle cells to migratory cells (Gray et al., 2016; Rashdan and Lloyd, 2015; Ray et al., 2011). Thus, pathogenic and non-pathogenic conditions appear dependent on the types of ROS produced, with strong, fast oxidizers associated with pathogenic conditions and weak, slow oxidizers associated with
non-pathogenic conditions. PKG I\(\alpha\) is broadly distributed throughout the cytosol of smooth muscle cells. Thus, oxidized PKG I\(\alpha\) is likely spatially and temporally limited by ROS production.

1.4 Aims of the present study

The work presented in this dissertation demonstrates the importance of the cGMP-binding domain A (CBD-A) in regulating both the cGMP- and oxidation-dependent mechanisms of PKG I\(\alpha\) activation. Using a monomeric, N-terminally truncated form of PKG I\(\alpha\) (\(\Delta53\)), Chapter 2 investigates the mechanism of inhibition through the autoinhibitory domain and the influence of dimerization on cooperative cGMP-dependent activation and cyclic nucleotide selectivity. We observed that autoinhibition occurs \textit{in cis}, whereas cooperativity requires interprotomer contacts facilitated by the N-terminal dimerization domain. Furthermore, the loss of selectivity for cGMP over cAMP of this construct suggests the dimerization domain plays a critical role in preventing cross-reactivity with cAMP-dependent signaling. These observations culminate into an overarching model wherein binding of cGMP to CBD-A is necessary and sufficient for activation and cooperativity is driven by the dimerization domain.

Chapter 3 investigates the cysteine residues that mediate oxidation-dependent activation of PKG I\(\alpha\). Using PKG I\(\alpha\) constructs with point mutations at specific cysteine residues, it was found that oxidation-dependent activation is driven by C117 in CBD-A. Furthermore, the interprotomer disulfide bond that forms in the dimerization domain at C42 does not contribute to this mechanism. Finally, we propose a model wherein the disulfide bond that forms between C117 and the adjacent cysteine at position 195 acts as a protective mechanism to prevent activation and higher oxidation states form contacts with nearby residues in the
linker region of PKG Iα to disrupt binding of the adjacent autoinhibitory domain to the catalytic domain. Finally, Chapter 4 provides a discussion of the results presented herein in context with previous studies and suggests future directions for the PKG field.
Chapter 2

An N-terminally truncated form of cyclic GMP-dependent protein kinase Iα (PKG Iα) is monomeric, autoinhibited, and provides a model for activation

Thomas M. Moon 1,4,6, Jessica L. Sheehe 1,6, Praveena Nukareddy 2, Lydia W. Nausch 1,5, Jessica Wohlfahrt 1, Dwight E. Matthews 2, Donald K. Blumenthal 3, Wolfgang R. Dostmann 1

The Department of Pharmacology1, Larner College of Medicine and the Department of Chemistry2, The University of Vermont, 89 Beaumont Ave, Burlington, VT 05405, USA, and the Department of Pharmacology and Toxicology3, The University of Utah, Salt Lake City, UT 84112, USA.
Present Address4: The University of Arizona, Department of Chemistry and Biochemistry, Tucson, AZ 85721, USA.
Present Address5: The Nuremberg Hospital Medical School PMU, Department of Physiology, Prof. Ernst-Nathan-Str.1 Nuremberg 90419, Germany.
These authors contributed equally to this work.6

To whom correspondence should be addressed: Wolfgang R. Dostmann, wolfgang.dostmann@uvm.edu and Thomas M. Moon, thomasmoon@email.arizona.edu

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29
2.1 Abstract

The type I cGMP-dependent protein kinases serve essential physiological functions, including smooth muscle relaxation, cardiac remodeling, and platelet aggregation. These enzymes form homodimers through their N-terminal dimerization domains, a feature implicated in regulating their cooperative activation. Previous investigations into the activation mechanisms of PKG I isoforms have been largely influenced by structures of the cAMP-dependent protein kinase (PKA). Here, we examined PKG Iα activation by cGMP and cAMP by engineering a monomeric form that lacks N-terminal residues 1-53 (Δ53). We found that the construct exists as a monomer as assessed by whole-protein mass spectrometry, size-exclusion chromatography, and small-angle X-ray scattering (SAXS). Reconstruction of the SAXS 3D envelope indicates that Δ53 has a similar shape to the heterodimeric RIα:C complex of PKA. Moreover, we found that the Δ53 construct is autoinhibited in its cGMP-free state and can bind to and be activated by cGMP in a manner similar to full-length as assessed by surface plasmon resonance spectroscopy (SPR). However we found that the Δ53 variant does not exhibit cooperative activation, and its cyclic nucleotide selectivity is diminished. These findings support a model in which, despite structural similarities, PKG Iα activation is distinct from PKA, and its cooperativity is driven by in trans interactions between protomers.

2.2 Introduction

Cyclic GMP-dependent protein kinases (PKG) are members of the AGC kinase family (PKA/PKG/PKC). They are conserved in eukaryotes, and ubiquitously expressed throughout the human body (Hofmann et al., 2009; Keilbach et al., 1992). These kinases regulate critical processes such as vascular tone, car-
vascular remodeling, and platelet aggregation (Bian and Murad, 2007; Hofmann et al., 2009; Kemp-Harper and Schmidt, 2009; Potter et al., 2009). The primary activator of PKG isoforms is the second messenger cyclic-3,5-guanosine monophosphate (cGMP) (Hofmann et al., 2009). In vascular smooth muscle, activated PKG I phosphorylates intracellular targets including myosin phosphatase target subunit 1 (MYPT1), regulator of G-protein signaling 2 (RGS2), inositol trisphosphate receptor-associated cGMP-kinase (IRAG), and the large conductance calcium-activated potassium channel (K_{Ca1.1} or BK) resulting in vasodilation (Ammendola et al., 2001; Antl et al., 2007; Robertson et al., 1993; Sausbier et al., 2000; Schmidtko et al., 2008; Schubert and Nelson, 2001).

PKG I is expressed as two splice variants that form homodimers (α/β) (Keilbach et al., 1992; Orstavik et al., 1997; Wernet et al., 1989). In both isoforms, each protomer is composed of a dimerization domain formed by a leucine zipper (LZ), followed by an autoinhibitory domain segment, a regulatory domain containing two cGMP-binding sites (A and B), and a catalytic domain (Figure 1a) (Pfeifer et al., 1999). PKG Iα and Iβ have low sequence conservation in their dimerization and autoinhibitory domains, and this difference is thought to both control the activity and response of the kinase to cGMP as well as mediate its targeting to specific substrates (Ruth et al., 1997; Ammendola et al., 2001; Blanton et al., 2013; Casteel et al., 2005, 2010; Kato et al., 2012; Lee et al., 2007). How the regulatory and catalytic domains interact in the inactive state and communicate to control cooperative activation have remained unsettled questions for the field (Dostmann et al., 1996; Richie-Jannetta et al., 2006). In the inactive state, it has been hypothesized that the autoinhibitory domain of PKG I occupies the catalytic cleft formed between the N and C-terminal lobes and acts as a pseudo-substrate in a manner similar to the cAMP-dependent protein kinase (PKA) (Alverdi et al., 2008).
PKG isoforms share sequence homology with PKA (28% and 41% identity in its regulatory domain and catalytic domain, respectively). Consequently, current models of the relationship between PKG I structure and function have been overwhelmingly influenced by studies of PKA. However, PKA is expressed as separate catalytic and regulatory domains and PKG exists as a single polypeptide chain (Huang et al., 2014; Osborne et al., 2011; Zhao et al., 1998). Structural and biochemical studies of PKA have demonstrated an in cis mechanism for PKA activation (Byeon et al., 2010; Su et al., 1995; Wu et al., 2004). The sequential binding of cAMP to two coupled binding sites (A and B) within the regulatory subunit constitutes the physical basis for cooperativity in both cAMP binding and kinase activation. While it is known that full-length PKG I dimers also exhibit cooperative activation, it is unknown whether cooperativity is driven by the regulatory domain (in a fashion similar to PKA) or whether the dimeric state influences this effect (Moon et al., 2015; Ruth et al., 1997; Osborne et al., 2011).

It has been suggested that the N-terminal domains in the hinge region between the leucine zipper and autoinhibitory domains in PKG I are highly flexible, and structural models of PKG I have depicted the N-terminus protruding from the holoenzyme (regulatory and catalytic domain complex) (Alverdi et al., 2008; Francis and Corbin, 1994; Pfeifer et al., 1999). While it has been widely suggested that dimerization is essential for catalytic function, it has never been experimentally tested in the Iα variant. Informed by previous models, we hypothesized that PKG Iα could be truncated to generate a functional monomer by removing the flexible N-terminus (zipper and hinge). Moreover, we surmised that this construct would retain cGMP-dependent activation due to the inclusion of the autoinhibitory domain. In this study, we provide the biochemical and biophysical characterization of PKG Iα Δ53 (subsequently referred to as Δ53) by demonstrating its monomeric architecture, phosphorylation state, cGMP-binding properties,
and the kinetic characteristics of its cGMP-dependent activation. Through the use of the ∆53 construct, we address the following: 1) whether PKG Iα is inhibited in cis or in trans; 2) how cyclic nucleotide binding and selectivity of the A-site is linked to activation; and 3) a putative mechanism by which cGMP-mediated cooperativity is derived in PKG. Our data indicate that PKG Iα can form an in cis autoinhibited complex. Furthermore, cooperative activation of PKG Iα by cGMP—in contrast to PKA—relies on interactions within the homodimeric regulatory domain. We conclude that cooperativity is driven in part by the N-terminal dimerization domain by localizing PKG monomers within close proximity during cGMP binding to form in trans interprotomer interactions.

2.3 Results
2.3.1 Design, Expression, and Purification of ∆53

The design of a monomeric form of PKG Iα was accomplished using a bioinformatic approach by comparing to a previously-solved structure of the homolog, PKA, in a heterodimeric complex with its regulatory domain (Figure 3.1a-b). The complex of R1α:C (PDBID: 2QCS) was chosen due to the high sequence conservation of its autoinhibitory segment with PKG Iα and the availability of its three-dimensional coordinates (Figure 3.1c). A multiple sequence alignment of the PKG Iα autoinhibitory fragment with that of known isoforms of PKA was performed. The distance from the first residue (Lys92) to the start of the autoinhibitory sequence in the R1α:C structure was used to determine that the residue equidistant from the autoinhibitory sequence in PKG Iα was Pro56 (Figure 3.1b). We hypothesized that, in a similar fashion to R1α:C, the autoinhibitory segment preceding the canonical A/GISAE pseudo-substrate segment would confine the P+1 loop of the catalytic domain and make further contacts with the C-terminal tail, glycine-rich loop, and the F-helix from the large lobe (Kim et al., 2007,
As a consequence, and as to not exclude residues that may be important for autoinhibition, \( \Delta 53 \) was engineered to begin at Ile54.

Following expression and purification of \( \Delta 53 \) (Figure 3.2a), we examined the kinase by size exclusion chromatography (Figure 3.2b). Both the apo and cGMP-bound forms exhibited reproducible elution profiles centered at 14.8 mL and 14.7 mL, respectively, indicative of a 70 kDa species according to molecular weight standards (Figure S1). The addition of cGMP appeared to have a negligible effect on the elution volume of the protein, suggesting that dimer for-
mation was not present in the apo or cGMP-saturated samples. ∆53 was further examined by time-of-flight (TOF) mass spectrometry to determine its mass and phosphorylation state (Figure 3.2c). A mass of 70,511 Da, corresponding to a mono-phosphorylated, monomeric species was observed. We also discovered the presence of a doubly phosphorylated species comprising 20% of the total ion content (70,591 Da). Tandem mass spectrometry analyses of trypsin-digested samples were used to confirm the primary phosphorylation site at T517 (full-length enzyme numbering scheme) located in the activation loop of the catalytic domain (Table 2.1). This phosphorylation site has been previously identified in full-length PKG Iα, and is required for catalytic activity (Feil et al., 1995). To confirm this, we also examined preparations of full-length PKG Iα and found a single phosphorylation site corresponding to T517 (Table 2.3). Based on previous studies, the minor phosphorylation site observed for ∆53 was suspected to reside in the autoinhibitory domain (Aitken et al., 1984; van de Waterbeemd et al., 2014). However, the peaks corresponding to the peptide containing the autophosphorylated pseudosubstrate sequence, (R)AQGISAEPQTYR(S) (residues 61-72), were of high intensity and suggested no phosphorylations were present.

2.3.2 SAXS Analysis

The results presented thus far indicate that ∆53 exists as a monomer in solution. In an effort to characterize the low-resolution solution structure of the autoinhibited complex, we utilized size exclusion chromatography coupled to small-angle X-ray scattering wherein during analysis single species associated with ∆53 was observed (SEC-SAXS, Table 2.5,2.6,2.7). Concurrent Guinier calculation during data collection indicated a constant $R_g$ across the sample peak (Figure 3.3a). The averaged scattering intensity curve ($I_q$ vs $q$) for ∆53, which was obtained by averaging frames 410-459 of the SEC-SAXS peak, exhibited a linear Guinier plot.
Figure 2.2: **Biophysical characterization of ∆53.** A) Coomassie-stained 12% SDS-PAGE of PKG Iα full-length and ∆53 under denaturing and reducing conditions. B) Size exclusion chromatography traces of ∆53 in its apo (black) and cGMP-saturated (red) forms. C) A raw m/z trace (inset) and the deconvoluted TOF-MS displaying the two predominant masses observed for ∆53 corresponding to singly and doubly phosphorylated species. The mass correlated to the location of an unphosphorylated monomer is also denoted.

(Figure 3.3b and inset, Table 2.5, 2.6, 2.7). The Guinier indicated the presence of a monodisperse system with no evidence of aggregation. A Kratky plot of the data suggested the presence of a well-folded species (Figure 3.3c). The P(r)
curve calculated from the scattering intensity contained a single peak at \( \sim 30 \) Å that smoothly decayed to a maximum linear dimension (\( D_{\text{max}} \)) of 97 Å (Figure S2). When compared to the \( P(r) \) curve of the homologous PKA heterodimer upon which we based our construct, RIA:C (green), both showed a single peak at 34 Å, but the RIA:C heterodimer had an extended \( D_{\text{max}} \) of \( \sim 130 \) Å. Comparison of the \( \Delta 53 \) \( P(r) \) to that observed for a different PKA heterodimer, RII\( \beta \):C (red), demonstrated that they have nearly identical dimensions (Table 2.4) (Vigil et al., 2005).

Predicted X-ray scattering from the crystal structures of the PKA RIA:C, PKA RII\( \beta \):C, and PfPKG from \( P. falciparum \) (a monomeric species containing 4 cGMP binding sites and a catalytic domain) was conducted using both CRYSOL and FoXS (Table 2.5, 2.6, 2.7) (Schneidman-Duhovny et al., 2013; Schneidman-Duhovny et al.). The best fit of the experimental SAXS data to the predicted scattering was obtained from RIA:C using CRYSOL with constant subtraction (\( \chi^2 = 0.82 \), Figure 3.3d, Table 2.5, 2.6, 2.7). Using the the RII\( \beta \):C heterodimer in the same analysis resulted in a poorer fit to the experimental data (\( \chi^2 = 1.25 \)). The PfPKG fits were poor using CRYSOL (\( \chi^2 = 9.21 \)), but acceptable with the FoXS server (\( \chi^2 = 1.29 \), Table 2.5, 2.6, 2.7, Figure 2.11). We also found that the \( R_g \) values calculated using the predicted scattering curves for both the PKA RIA:C and RII\( \beta \):C heterodimer crystal structures were in closer agreement with the measured and calculated values for \( \Delta 53 \) (Table 2.5, 2.6, 2.7). Next, DAMMIF and DAMMIN modeling was implemented. Using SUPCOMB, the resulting averaged and filtered DAMMIF/DAMMIN \( ab \) \textit{initio} three-dimensional envelope accommodated both crystal structure models of the RIA:C and RII\( \beta \):C heterodimers with little protrusion outside of the envelope (Figure 3.3e-f, Table 2.5, 2.6, 2.7). Among the 3D models examined, these data cumulatively suggest \( \Delta 53 \) adopts a shape most similar to PKA RIA:C.
Figure 2.3: **SEC-SAXS analysis of PKG Iα Δ53 in the autoinhibited state.**  

A) SEC-SAXS trace of Δ53 depicting scattering intensity (red circles, right axis) and $R_g$ values (black circles, left axis) estimated from Guinier plots of each detector exposure frame.  

B) The intensity profile ($I_q$ vs $q$) for Δ53 was derived from the averaging of frames 410-459 from panel (a).  

C) Kratky plot of the scattering profile of Δ53 determined using the averaged scattering data from panel (B)  

D) The resulting fit and residual plot of the intensity profile ($I_q$ vs $q$) for Δ53 by CRYSOL (allowing constant subtraction) for R1α:C (PDBID: 2QCS, green) and R1β:C (PDBID: 4WBB, red). The 3D envelope of the scattering curve derived from Δ53 (grey balls) fit to the crystal structures of (E) R1α:C (NSD=1.06) and (F) R1β:C (NSD=1.09) heterodimers.
2.3.3 Binding and Activation of Full-length PKG Iα and Δ53 with cNMP

Finally, we studied the activation kinetics of Δ53. No significant differences in basal activity were observed between PKG Iα full-length and Δ53, indicating that the monomeric construct was autoinhibited at levels comparable to previous reports (Moon et al., 2015; Ruth et al., 1997). Moreover, the cGMP-dependent activation of Δ53 and full-length PKG Iα were analogous with respect to their maximum velocities of 4.0 and 3.9 µmol/min*mg, respectively (Figure 3.4Ba). These results indicate that the cGMP-dependent fold-stimulation of Δ53 is indistinguishable from full-length. However, differences between the two constructs were observed with respect to their activation constants and degree of cooperativity. We observed $K_\text{a}$ values and Hill coefficients corresponding to 182 nM ($n_H=1.6$) for PKG Iα full-length and 250 nM ($n_H=1.0$) for Δ53 ($p<0.0001$ for both measurements). When we tested the activation profiles of full-length and Δ53 PKG Iα with cAMP, we observed that the full-length enzyme exhibited cooperative activation, while again Δ53 was non-cooperative. Comparisons of the fold-difference in activation by cGMP and cAMP for both enzymes demonstrated that the removal of the N-terminus of PKG Iα reduced the selectivity for cyclic nucleotide from 58-fold (full-length) to 3.5-fold (Δ53), representing an overall 16-fold decrease in selectivity for cGMP over cAMP.

To probe how binding of cyclic nucleotide correlates with the activities of the enzymes, we measured cGMP and cAMP binding to PKGIα constructs by surface plasmon resonance (SPR) spectroscopy. Binding of cyclic nucleotides was fit using a two-site binding model since the regulatory domain of PKGIα contains two cGMP sites per monomer. To determine cooperativity of the binding, we also fit the data using a one-site model with Hill coefficient. PKG Iα full-length exhibited an almost 3-fold weaker affinity for cGMP than Δ53 ($^{\text{FL}}K_D=7.9$ µM
Figure 2.4: **Biochemical characterization of Δ53.**  
**A)** Domain diagram depicting the constructs used for the activation and binding studies.  
**B)** Activation of PKG constructs. Data are represented as the mean ± SD.  
**Ba)** Activation of PKG Iα full-length (black) and Δ53 (red) with cGMP. Significant differences in activation by individual cGMP concentrations were confirmed by two-way ANOVA (p<0.005), and denoted by an asterisk.  
**Bb)** Normalized activation of PKG Iα (black) and Δ53 (red) with cGMP (solid lines) and cAMP (dotted lines). Shaded areas depict the mean K_D ± SD of the high affinity cyclic nucleotide binding site for cGMP (solid) and cAMP (dashed).  
**C)** cNMP binding curves associated with PKG constructs in (A) as measured by SPR spectroscopy. Data are represented as the mean ± SD and fit with a two-site binding model.  
**Ca)** Binding curves showing cGMP (solid lines) and cAMP (dotted lines) association with full-length PKG Iα full-length (black), and Δ53 (red).  
**Cb)** Binding curves denoting PKG Iα:1-326 (blue) and PKG I:78-326 (green) with cGMP (solid lines) and cAMP (dotted lines).
and $\Delta^{53}K_D=2.9 \mu M$), when fit with a one-site model (Table 2.2). However, when fit with a two-site binding model, the ratio dropped to 1.6-fold. Moreover, both constructs displayed negative cooperativity in binding cGMP ($^{F1}n_H=0.60$ and $\Delta^{53}n_H=0.74$). When cAMP binding was measured for the two constructs, the $K_D$ values for their high affinity sites increased by over thirty-fold. However, cAMP binding remained negatively cooperative in both enzymes. In binding to either cyclic nucleotide, we found that the low-affinity site demonstrated much weaker binding than the high affinity site.

To distinguish the cyclic nucleotides sites responsible for activation in the full-length PKG Ia kinase, we mutated the glutamic acid residues in the Phe-Gly-Glu (FGE) motif of the phosphate binding cassettes within the A-site (E168G) and the B-site (E292A). Since the glutamate residue is involved in binding to the 2'-hydroxyl from the ribose, mutation of this site in PKA has been shown to disrupt cyclic nucleotide binding (Diller et al., 2001; Ogreid et al., 1988; Steinberg et al., 1987; Su et al., 1995; Zawadzki and Taylor, 2004). Mutation of the A-site ($^{E168G}PKGI_a$) completely abolished cGMP-dependent activation of the full-length kinase (Figure S4). These results demonstrate that a functional A-site is necessary for kinase activity. Moreover, when we examined the cGMP-dependent activation of $^{E292A}PKGI_a$, we observed cooperative ($n_H=1.45$) stimulation of the kinase with a $K_a$ of 95 nM.

In an effort to further examine the contribution of the N-terminus and the catalytic domain on cyclic nucleotide binding selectivity, we purified two constructs of the regulatory domain of PKG Ia. The first construct, $^{1-326}PKGI_a$, contains the N-terminus and cGMP-binding regulatory domain, whereas the second construct, $^{78-326}PKGI$, contains only the regulatory domain. When we examined binding of cGMP to $^{1-326}PKGI_a$ and $^{78-326}PKGI$, we found that the one-site model $K_D$ shifted significantly compared to values observed for full-length PKG
Table 2.1: Phosphopeptides of ∆53 PKG Iα.

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<tr>
<th>Precursor MH⁺ (Da)</th>
<th>z</th>
<th>Start</th>
<th>End</th>
<th>Sequence</th>
<th>Modifications</th>
<th>Retention Time (min)</th>
<th>Intensity (×10³)</th>
</tr>
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<tr>
<td>1320.665</td>
<td>2.9</td>
<td>61</td>
<td>72</td>
<td>(R)AQGISAEPQTYR(S)</td>
<td></td>
<td>19.74</td>
<td>9.523</td>
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<tr>
<td>2262.055</td>
<td>2.7</td>
<td>515</td>
<td>533</td>
<td>(K)TWTFCTPEYVAPEILNK(G)</td>
<td>PO₄-T517</td>
<td>44.89</td>
<td>1.591</td>
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<tr>
<td>2182.082</td>
<td>2.7</td>
<td>515</td>
<td>533</td>
<td>(K)TWTFCTPEYVAPEILNK(G)42.80</td>
<td>123</td>
<td>260</td>
<td></td>
</tr>
<tr>
<td>2290.130</td>
<td>3.0</td>
<td>514</td>
<td>533</td>
<td>(K)KTWTFCGTPEYVAPEILNK(G) PO₄-T517</td>
<td>41.01</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>2310.173</td>
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<td>514</td>
<td>533</td>
<td>(K)KTWTFCGTPEYVAPEILNK(G)</td>
<td></td>
<td>39.02</td>
<td>10</td>
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</table>

Iα and ∆53 (Figure S5). For 1-326PKG Iα, the two-site binding model showed that the $K_D$ value for cGMP binding to the A-site increased by 8-fold compared to the full-length enzyme (Table 2.2). Furthermore, binding of cAMP showed a drastic decrease in selectivity for cGMP over cAMP. The B-site showed a clear selectivity for cGMP over cAMP (4-fold). However, when the N-terminus was excluded in 78-326PKG I, we observed no difference in the affinities for cGMP and cAMP in the high affinity A-site. The B-site retained a clear selectivity for cGMP over cAMP (10-fold).

2.4 Discussion

PKG activation has been extensively studied by traditional biochemical methods and, more recently, using modern biophysical approaches (Casteel et al., 2010; Huang et al., 2014; Kim et al., 2011; Osborne et al., 2011; Wall et al., 2003). These investigations sought to determine the architecture of the homodimeric kinase in its basal and activated states, the order and selectivity of cGMP binding, and, by extension, the origin of its cooperativity. The generation of a functional, monomeric form of PKG Iα is a useful tool for investigating the molecular basis of these long-observed biochemical phenotypes.

PKG Iα ∆53 purified from Sf9 cell extracts exists as a mixture of mono- and diphosphorylated forms; no unphosphorylated form was detected. The primary phosphorylation site, T₅₁₇, was found in high abundance by time-of-flight mass spectrometry (TOF-MS) (Figure 3.2c, Table 2.1). Phosphorylation of T₅₁₇,
Table 2: Small angle X-ray scattering data collection and analysis

(a) Sample details

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<td>Source (Catalogue No. or reference)</td>
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<tr>
<td>UniProt sequence ID (residues in construct)</td>
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(b) SEC-SAS

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<td>Flow rate (ml/min&lt;sup&gt;-1&lt;/sup&gt;)</td>
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<td>Average C in combined data frames (mg/ml&lt;sup&gt;l&lt;/sup&gt;)</td>
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<td>Solvent (solvent blanks taken from SEC flowthrough prior to elution of protein)</td>
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(c) SAS data collection parameters

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<tr>
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<td>Method for monitoring radiation damage, X-ray dose where relevant</td>
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<tr>
<td>Exposure time, number of exposures</td>
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<td>Sample configuration including path length and flow rate where relevant</td>
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(c) Software employed for SAS data reduction, analysis and interpretation

| SAS data reduction to sample–solvent scattering, and extrapolation, merging, desmearing etc. as relevant | Sastool |

Figure 2.5
Calculation of \( \epsilon \) from sequence

Protparam (Gasteiger et al, 2005).

Calculation of \( \Delta P \) and \( \Sigma \) values from chemical composition

Primus from ATSAS v2.8.3

Basic analyses: Guinier, \( P(r) \), scattering particle volume

DAMMIF (Frank \& Svergun, 2009) and DAMMIN (Svergun, 1999) via ATSAS online (https://www.embl-hamburg.de/bioscs/atsas-online/)

FoXS (Schneider-Havrylov et al, 2013) via web server (https://modbase.compbio.ucsf.edu/foxs/) CRYSTOL from PRIMUSqt in ATSAS 2.8.1 (Svergun et al., 1995)

PyMOL v1.7 Mac

Shape/bead modelling

Atomic structure modelling (homology, rigid body, ensemble)

Molecular graphics

\((d)\) Structural parameters

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<td>( R(0) ) (cm(^{-1}))</td>
<td>321.37 ± 1.23</td>
</tr>
<tr>
<td>( R_B ) (Å)</td>
<td>29.71 ± 0.58</td>
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<tr>
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<td>( qR_B ) max</td>
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<td>( M ) from ( I(0) ) (ratio to expected value)</td>
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\((e)\) Porod volume (Å\(^3\)) (ratio \( P_B/\pi a^3 \))

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\((e)\) Shape modeling results (a complete panel for each method)

\( \text{DAMMIF (default parameters, 20 calculations)} \)

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Figure 2.6
Resolution (Å) (from SASRES) 35 ± 3
M estimate as 0.5 x volume of models (Da) 73473 (1.04)
(ratio to expected)

DAMMIN (default parameters)

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(f) Atomistic modelling

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<th>PDB: 4WBB</th>
<th>PDB: 5DYK</th>
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<td>PKA RIIβ-C</td>
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<tr>
<td>heterodimer</td>
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CRYSOL (with default parameters)

<table>
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<tr>
<th>q-range for fitting</th>
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<tr>
<td>χ² value, P-value</td>
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<td>Predicted Rf (Å)</td>
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<td>Vol (Å³), Ra (Å), Dro (e Å⁻³)</td>
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CRYSOL (with default parameters)

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FoolS (with default parameters)

(g) Data and model deposition IDs

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</table>

Figure 2.7

45
which is located in the activation loop of the catalytic domain, is essential for catalytic activity (Feil et al., 1995). In the PKA catalytic domain, the analogous phosphorylation at T197 forms contacts with other activation loop residues, the catalytic loop, and the αC-helix to integrate the active site components (Steichen et al., 2012; Zheng et al., 1993). It has been hypothesized that phosphorylated T517 in PKG serves a similar function since the lack of phosphorylation or mutation of this residue renders PKG inactive (Feil et al., 1995). The presence of this phosphorylation site in Δ53 corroborates the cGMP-dependent activation observed by our phosphotransferase assays. In regard to the second, less abundant phosphorylation site, we had hypothesized that this residue would be located in the autoinhibitory domain based on previous published data (Aitken et al., 1984; Takio et al., 1983; van de Waterbeem et al., 2014). Analysis of autoinhibitory domain residues suggested S64 and T70 were not phosphorylated (parent peptide Ala61-Arg72; Table 2.1). Another potential site, T58, has previously been identified as the major site that is autophosphorylated most rapidly in the presence of cGMP in vitro or cAMP in native PKG I preparations isolated from bovine lung (Aitken et al., 1984; Takio et al., 1983). Since Sf9 cells endogenously express adenylyl cyclases, we hypothesized that T58 could be the minor phosphorylation site (Kawabe et al., 1996; Lee et al., 2000). However, our LC-MS/MS data could not confirm the precise location. These results are in agreement with previously published results that observed the phosphorylation at T517, but were also unsuccessful in identifying the second site (Alverdi et al., 2008). Furthermore, these results suggest that neither its dimerization through the N-terminal dimerization domain nor exposure to cGMP are required for activation loop phosphorylation.

Despite the presence of these two phosphorylation states, we consistently observe a compact, monodisperse, monomeric species in solution by size exclusion chromatography (SEC) (Figure 3.2b). In addition, the elution profile of
Δ53 did not change with the addition of cGMP, suggesting that it maintains its monomeric state even during long-lived exposure. In an effort to reconstruct the three-dimensional shape of inactive Δ53 and confirm its monomeric character, we collected data by SEC-SAXS and compared the reconstructed 3D-envelope to the crystal structures and SAXS data from two PKA heterodimers (PDBID: 2QCS and 4WBB) and PfPKG (PDBID: 5DYK). We found that Δ53 adopts a similar shape to that of the PKA RIα:C heterodimer (2QCS) based on the best fit of the atomistic modeling in CRYSOL to the scattering data ($\chi^2 = 0.82$) (Figure 3.3d, Table 2.5, 2.6, 2.7). In addition, the crystal structure is readily accommodated by the 3D envelope calculated from the Δ53 SAXS data (NSD = 1.06, Figure 3.4f). These data concurred with previously published results for the apo state of monomeric PKG Iβ Δ1-52 (Table 2.4) (Wall et al., 2003). PKG Iβ Δ1-52 contains 18 additional residues upstream of its autoinhibitory domain compared to PKG Iα Δ53. Considering the high overlap between these two truncated constructs in the SAXS analyses, this suggests that the linker in Iβ Δ1-52 adopts a compact conformation relative to the regulatory-catalytic domain complex. Based upon these data, we propose a model wherein each monomer within the dimeric PKG Iα complex can be autoinhibited by its own regulatory domain and autoinhibitory sequence (Fig-

<table>
<thead>
<tr>
<th>PKG Iα Construct</th>
<th>Binding (SPR)</th>
<th>Activation (^32PO4-assay)</th>
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<tr>
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<td></td>
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<td>$K_D$ (µM)</td>
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<tr>
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<tr>
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</tr>
<tr>
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<td>cGMP</td>
<td>30.0 ± 1.1</td>
</tr>
<tr>
<td></td>
<td>cAMP</td>
<td>340 ± 10</td>
</tr>
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The data are represented as the mean ± SD. -, not measured.
Finally, we report that $\Delta 53$ is activated by cGMP at concentrations similar to the full-length kinase (Figure 3.4 Ba). A previous study by Richie-Jannetta et al and a more recent study by Kim et al expressed truncated forms of PKG I$\beta$ ($\Delta 1$-53 and $\Delta 1$-55) to investigate the influence of the dimeric interface on cGMP-dependent activation (Richie-Jannetta et al., 2003; Kim et al., 2016). Both studies described significant shifts in the activation constants of the truncated I$\beta$ constructs. These results contrast with our observations of PKG I$\alpha$. $\Delta 53$ does not exhibit an appreciable shift in the activation constant compared to the full-length enzyme. These results support the longstanding conclusion that PKG I$\alpha$ and I$\beta$ are enzymatically distinct due to differences in their N-termini (Hofmann et al., 2009; Lee et al., 2000, 2007; Moon et al., 2015; Ruth et al., 1997).

Interestingly, $\Delta 53$ does not exhibit cooperative cGMP-dependent activation (Figure 3.4 Ba). The loss of cooperativity has been previously reported for PKG I$\beta$, wherein full length PKG I$\beta$ ($n_H = 2.1$) becomes noncooperative when the N-terminus is removed ($\Delta 1$-52 PKG I$\beta$) (Moon et al., 2015; Kim et al., 2016). In addition, we observed that cAMP also cooperatively activates the full-length kinase ($n_H = 1.75$), but not $\Delta 53$ ($n_H = 0.81$). These results suggest that the cooperative activation is mediated by the N-terminus through the facilitation homodimer formation. The N-terminal dimerization domains of PKG I$\alpha$ and I$\beta$ are left-handed, coiled-coil, leucine zipper (LZ) motifs. The LZ is characterized by a heptad repeat of amino acids ($abcdefg$) where residues $a$ and $d$ are typically hydrophobic and residues $e$ and $g$ are typically hydrophilic. The leucine zipper of PKG I$\alpha$ spans residues 1-47. In addition to its leucine and isoleucine residues, there are also four non-leucine/isoleucine residues at the $d$ positions that provide additional non-hydrophobic interhelical contacts (Phe7, Lys14, Lys28, Cys42) (Qin et al., 2015). One of these residues, C42, is unique to the I$\alpha$ isoform and forms an
interprotomer disulfide bond in the presence of oxidizing agents, such as hydrogen peroxide (Burgoyne et al., 2007; Landgraf et al., 1991). The leucine zipper of PKG Iβ spans residues 4-53, and also contains four non-leucine/ isoleucine residues at the d positions (Lys13, Arg20, Lys34, and Tyr48) (Casteel et al., 2010). Although the majority of the non-canonical residues are basic, their differences in position within the helices and the overall discrepancy in length of the helices are reasons why PKG I protomers have not been shown to form heterodimers either in vitro or in vivo.

It is well-characterized that the cGMP A-site in the regulatory domain is the high affinity binding site, and, thus, is the first to bind cyclic nucleotide (Huang et al., 2014; Kim et al., 2011; Zhao et al., 1998). Our data of PKG Iα full-length and Δ53 agree with these previously published results and indicate a clear selectivity for cGMP over cAMP in the A-site (Table 2.2). To further test the contributions of the A- and B-sites on PKG Iα activity, we measured the activities of E167G PKG Iα and E292A PKG Iα. We found that disruption of cyclic nucleotide binding to the B-site did not have an appreciable effect on activity (Figure S4). In contrast, disruption of cyclic nucleotide binding to the A-site completely abolished activity. These data collectively support the importance of the A-site in regulating activation of PKG Iα. However, previous studies using isolated regulatory domain constructs have suggested that the A-site is non-selective for cyclic nucleotide (Kim et al., 2011; Osborne et al., 2011). These results suggest selectivity for cGMP binding to the A-site must be controlled through regions outside of the regulatory domain.

To investigate the influence of regions outside of the regulatory domain on the cyclic nucleotide-dependent characteristics of PKG Iα, we expressed and purified two additional constructs: 1-326 PKG Iα and 78-326 PKG Iα. PKG Iα without its
Figure 2.8: **Putative model of activation of FL and Δ53 PKG Iα** The model depicted hypothesizes that one cyclic nucleotide bound to the high-affinity A-site is sufficient to activate both the full-length and Δ53 PKG Iα constructs. Cooperativity arises the *in trans* interaction of protomers at the knob/nest site and not from cooperative cyclic nucleotide binding. In the Δ53 construct, cooperativity is not observed and cannot be facilitated due to the lack of dimerization. The organization of the full-length enzyme must also lend itself to generate a cGMP selectivity filter for the A-site based upon our full-length and truncated data.
catalytic domain \((1-326)\text{PKG I}_a\) showed a significant decrease in binding affinity for cGMP and loss of selectivity for cGMP over cAMP in the A-site compared to the full-length construct (Table 2.2). Selectivity was only slightly reduced in the B-site, and the values agree with those measured previously for the isolated B-domain (Kim et al., 2011). These results differ from the binding and nucleotide selectivity results observed for PKG I\(_a\) full-length and \(\Delta53\). Thus, these characteristics seem to be integrated into the architecture of the inactive holoenzyme.

The initial comparison of the activation curves for full-length and \(\Delta53\) to the one-site binding model indicates a large disparity between the concentration of cGMP required for full saturation of the A and B-sites and the minimum concentration required for full activation. Early measurements of cyclic nucleotide binding with \(^3\)H-cGMP observed a significant difference in the \(K_D\) values for cGMP binding at 4 and 30 (Landgraf and Hofmann, 1989). Our measurements for cGMP are consistent with these early experiments. When a two-site model is applied to the binding data, we can directly correlate the \(K_D\) for the A-site with the \(V_{\text{max}}\) for activation. This model is reasonable since there are two cGMP binding sites with distinct binding affinities. Furthermore, this effect is observed for both cGMP and cAMP, equally (Figure 3.4). This implies that full-length PKG I\(_a\) only requires that half of the A-sites are occupied to stimulate full activation. These data, which also suggest the B-site is non-essential for kinase activity, are further supported by the fact that the \(K_D\) for the B-site (\(~90\ \mu\)M) is higher than estimated intracellular concentrations of cGMP (\(~10\ \text{nM} - 10\ \mu\)M) (Francis et al., 2005; Nausch et al., 2008; Trivedi and Kramer, 1998). Moreover, we observe that mutation of the nucleotide binding cassette in the B-site does not appreciably affect activation of the full-length kinase (Figure S4). Thus, we can also conclude that binding studies that are either directed toward the isolated PKG regulatory domains or employ equilibrium-exchange of radiolabeled cyclic nucleotides are limited; and
future studies should examine these effects in the context of the intact holoenzymes.

These SPR measurements also indicate that binding of cGMP to both full-length and Δ53 is negatively cooperative ($n_H < 1$), which indicates that binding of cGMP to subsequent sites becomes more difficult with increasing concentrations. These results suggest a lack of avidity between cyclic nucleotide binding domains A and B, and this has been corroborated by recent structural evidence Kim et al. (2016). These observations stand in contrast to PKA where the origins of cooperativity has been directly linked to the regulatory domain, wherein binding of cAMP into the B-site enhances binding for the A-site through intra-subunit contacts (Su et al., 1995; Byeon et al., 2010; Kim et al., 2007). It has been suggested that the cooperativity observed for PKG activation arises from a similar mechanism (Alverdi et al., 2008; Huang et al., 2014; Kim et al., 2011). However, the loss of cooperative activation in PKG Iα through disruption of the dimer via two mechanisms: 1) by removing residues that allow for N-terminal dimerization (this study) and 2) via mutation of residues forming the knob-nest interface - suggests that this is not the case (Osborne et al., 2011).

Finally, we propose a model based on our findings wherein autoinhibition of PKG Iα is driven in cis. The high affinity A-site provides cyclic nucleotide selectivity through contacts outside of the regulatory domain and cooperative activation of PKG Iα is driven in trans, facilitated by the N-terminus - which ensures monomers are close enough to form necessary interprotomer contacts (Figure 5). These data reinforce our earlier hypothesis that cooperative activation of the kinase is not driven through cooperative binding of cyclic nucleotides to the regulatory domain within the same protomer. Rather, it is driven through an inducible dimeric interface mediated by the switch helix (Moon et al., 2013, 2015). Therefore, we conclude that the mechanism driving cooperativity in PKG Iα lies in the
interface between protomers and not between cGMP binding sites within the same protomer.

2.5 Experimental Procedures

2.5.1 Sequence Alignments

Sequences for PKG Iα and PKA regulatory domain isoforms were downloaded from the NCBI and Uniprot repositories (ncbi.nlm.nih.gov; UniProt.org) and uploaded to JalView Desktop (Waterhouse et al., 2009). The following accession numbers were used: NP_001091982 (PKG Iα Homo sapiens), NP_776861 (PKG Iα Bos taurus), NP_001013855 (PKG Iα Mus musculus), NP_001099201 (PKG Iα Rattus norvegicus), NP_037313 (PKA RIIα Rattus norvegicus), NP_997637 (PKA RIIα Homo sapiens), NP_001068669 (PKA RIIβ Bos taurus), NP_001240819 (PKA RIIβ Mus musculus) NP_062137 (PKA RIIα Rattus norvegicus), P12367 (PKA RIIα Mus musculus), NP_001178296 (PKA RIIα Bos taurus), NP_004148 (PKA RIIα Homo sapiens), P31324 (PKA RIIβ Mus musculus), NP_001025191 (PKA RIIβ Rattus norvegicus), NP_002727 (PKA RIIβ Homo sapiens), P31322 (PKA RIIβ Bos taurus). A multiple sequence alignment using CLUSTAL-O with the default parameters was accomplished using the JalView Desktop interface (Sievers et al., 2011).

2.5.2 Maintenance of Sf9 cells

Sf9 cells were purchased from Life Technologies. Frozen cell stocks were prepared according to the Growth and Maintenance of Insect Cell Lines Manual (Life Technologies). Typically, one vial containing Sf9 cells (1 x 10⁶ cells/vial in freezing medium (80% supplemented Grace’s Insect Medium (Gibco), 10% fetal bovine serum (FBS; Sigma), and 10% DMSO) was suspended in 15 mL Grace’s medium (Life Technologies) supplemented with 5% fetal bovine serum (Sigma)
and 10 µg/mL gentamicin (Sigma). Cells were grown in a 75 cm² flask until cells reached 80% confluency. Cells were detached from the flask and suspended in Sf900 III medium (Life Technologies) supplemented with 1x lipids (Sigma), 1% poloxamer 81 (Sigma), and 10 µg/mL gentamicin (Sigma). Sf9 cells were maintained in suspension at 27, 80 rpm and passaged every 3.5 days to 1.2 x 10⁶ cells/mL.

2.5.3 Expression of PKG Iα constructs in Sf9 cells

Full-length PKG Iα (Bos taurus) was amplified and cloned into pFAST Bac-HTA (Invitrogen) as previously described (Dostmann et al., 2000). PKG Iα 54-671 (Δ53) was amplified by PCR from bovine PKG Iα 1-671 using forward and reverse primers containing NcoI and XhoI cut sites, respectively: 5'-GGC GCC ATG GAT ATC GGC CCC CGG ACC ACC-3' (sense); 5'-ATG CCT CGA GAC CTA TTA GAA GTC TAT GTC CCA TCC TGA-3' (antisense) (Integrated DNA Technologies). The resulting product was cloned into pFastBac-HTA (Invitrogen) using NcoI, XhoI, and DNA ligase (New England Biolabs). Site-directed mutagenesis was performed on the full-length PKG Iα construct in pFAST Bac-HTA to produce E167G and E292A using the following primers: 5-AAG GTG TTT GGA GGG TTG GCT A-3 (E167G- sense); 5-ATA GCC AAC CCT CCA AAC ACT T-3 (E167G- antisense); 5-GGA AAA GGA GAT TGG TTT GGA GCG AAA GCC TTG-3 (E292A- sense); 5-TTC CCC CTG CAA GGC TTT CGC TCC AAA CC-3 (E292A- antisense). Transposition of the full-length PKG Iα, mutant, and Δ53 genes into bacmid using DH10 Bac E. coli, confirmation of its insertion by PCR, and transfection into Sf9 cells to produce baculovirus was performed following the Bac-to-Bac Baculovirus Expression System Manual (Life Technologies). Third amplification viruses were used at dilution ratios of approximately 1:500 to express both constructs (virus:Sf9 cells). At 72 hours post-infection,
cultures were harvested by centrifugation at 500 × g for 10 min. Pellets were re-suspended in lysis buffer (10 mM TES, 300 mM NaCl, 5 mM imidazole, pH 7) at a volume of 20 mL per liter of Sf9 cells, flash frozen in liquid nitrogen, and stored at -80°C.

2.5.4 Expression of PKG I regulatory domain constructs in *E. coli*

The PKG I regulatory domain constructs (78-326PKG I and 1-326PKG I) were amplified by PCR from the full-length PKG Iα gene using forward and reverse primers containing BamHI and EcoRI cut sites, respectively: PKG I-78-f 5’-CGG GAT CCA TGC AGG CAT TCC GGA AGT TC-3’ (sense), PKGI-1-f 5’-TGG GGA TCC AGC GAG CTG GAG-3’ (sense), PKG I-326-r 5’-TCG AAT TCC ATG CTA TTA TAA TCC TCC AAT CAA ATG-3’ (antisense). The respective fragments were ligated into BamHI/EcoRI-digested pRSET-A using T4 ligase (NEB). Both constructs were expressed in Rosetta2 *E. coli* (Novagen). Overnight cultures were used to inoculate 1L of TB supplemented with 50 µg/mL ampicillin and 25 µg/mL chloramphenicol and subsequently grown at 37, 220 rpm to an OD₆₀₀ = 0.8. Expression was induced by the addition of IPTG (1 mM final), and the induced cultures were incubated overnight at 25 at 220 rpm. *E. coli* were harvested by centrifugation at 1500 × g for 30 min, and the resulting bacterial pellets were flash frozen in liquid nitrogen and stored at -80°C.

2.5.5 Purification of PKG Iα constructs

Protease inhibitors (Roche) were dissolved in 50 mL of lysis buffer and added to 1L of pelleted Sf9 cells containing either PKG Iα full-length, E167G, E292A, or ∆53 (*B. taurus*). Sf9 cell pellets were thawed on ice for 40 min followed by gentle mixing. Resuspended cell pellets were lysed using a French pressure cell (2 passes at >1,500 bar; SLM-AMINCO) or by passing cells through a 22.5 gauge
needle (3 passes).

Bacterial pellets containing \textsuperscript{78-326}PKG I were resuspended by vortexing in 50 mL of lysis buffer supplemented with protease inhibitors (Roche) and were lysed using a French pressure cell (5 passes at \(>1,500\) bar; SLM-AMINCO).

All cell lysates were clarified by centrifugation at 30,000 \(\times g\) for 30 min at 4 and passed through a 0.22 \(\mu\)m PES syringe filter (Millipore). The resulting clarified, filtered lysates were loaded onto a 5 mL prepacked Ni-IMAC column (Bio-Rad) using a P-1 peristaltic pump (GE) and washed with 5 column volumes of lysis buffer and 6 column volumes of mid-wash buffer (lysis buffer supplemented with 30 mM imidazole). Proteins were eluted from the column with elution buffer (lysis buffer supplemented with 250 mM imidazole) using a Profinia FPLC (Bio-Rad). Peak fractions containing PKG I\(\alpha\) were pooled and analyzed by SDS-PAGE. PKG I\(\alpha\) was dialyzed against 4 L of 50 mM MES, 300 mM NaCl, 1 mM TCEP, pH 6.9 using 12-14 kDa MWCO dialysis tubing and 1 L against 50 mM MES, 300 mM NaCl, 1 mM TCEP, 10\% glycerol, pH 6.9. Aliquots of PKG I\(\alpha\) were flash frozen in liquid nitrogen and stored at -80 \(^\circ\)C.

2.5.6 Analytical size exclusion chromatography (SEC)

Samples containing 0.5 mg of PKG I\(\alpha\) \(\Delta53\) (apo or preincubated with 5 \(\mu\)M cGMP) were loaded sequentially onto a Superdex 200 10/300 column (GE) connected to an kta PURE FPLC (GE) and eluted isocratically in 50 mM MES, 300 mM NaCl, 1 mM TCEP, pH 6.9 (apo) or buffer supplemented with 5 \(\mu\)M cGMP (bound). Peak fractions were examined for the presence of PKG I\(\alpha\) \(\Delta53\) by SDS-PAGE. Peaks were analyzed with Unicorn 6.4 (GE) and plotted using DataGraph (Visual Data Tools).
2.5.7 Phosphotransferase assays

Activation of PKG Iα constructs by cyclic 3',5'-guanosine monophosphate (cGMP; BioLog) was assessed by measuring phosphorylation of a synthetic peptide substrate (W15, TQAKRKKSLAMA) with γ-32P-ATP similar to the method described previously (Dostmann et al., 2000). Each 100 µL reaction contained 20 µL of 5x MES Mix (250 mM MES, 5 mM MgOAc, 50 mM NaCl, pH 6.9), 10 µL of 100 mM DTT, 10 µL of 10 mg/mL BSA (Cohn’s fraction V; Sigma), 10 µL of 100 µM W15, 10 µL of cGMP (0-5 µM), 20 µL of 5 nM PKG Iα (in 50 mM MES, 1 mM TCEP, pH 6.9), 10 µL of ATP mix (γ-32P-ATP in 1 mM ATP; approximate specific activity = 200 cpm/min), and 10 µL of H2O. Reactions were performed at 30°C and initiated with the addition of either PKG Iα or ATP mix. Blank reactions were performed by substituting the PKG I and cGMP with H2O. Reactions were quenched after 1.5 or 3 min by spotting onto P81 phosphocellulose (Whatman and Reaction Biology Corp.) and washing with 0.8% phosphoric acid. Determination of 32P incorporation into W15 substrate was measured by liquid scintillation. Data were analyzed using Excel (Microsoft) and Prism v7 (GraphPad) and then plotted using DataGraph (Visual Data Tools).

2.5.8 Measurement of cGMP binding by surface plasmon resonance (SPR) spectroscopy

SPR measurements were conducted on a SR7500 dual-channel surface plasmon resonance spectrometer connected to an SR7100 autosampler utilizing a standard, dual-channel flow cell (Reichert Technologies). All steps were performed at 25 °C. Gold sensorchips coated with a 1500 nm linear polycarboxylate hydrogel (HC1500M, Xantec) were installed on the instrument and pre-equilibrated with SPR buffer (50 mM MES, 150 mM NaCl, 1 mM EDTA, 0.05% Tween20, 1 mM TCEP, pH 6.9) at 20 µL/min. Sensorchips were washed sequentially with a so-
olutions of 2 M NaCl and 10 mM NaOH for 5 min each, followed by 2 min with SPR buffer. The sensorchip was activated for 5 min with a solution of freshly prepared, degassed 100 µM N-hydroxysuccinimide (NHS) and 200 µM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) dissolved in 1 mL of 500 mM MES pH 5.5. PKG constructs were diluted to 1 µM in 20 mM NaOAc pH 5.0 (500 µL) and injected into the left channel for 10 min until 10,000-15,000 µRIU was observed in the difference channel. Excess protein was washed from the surface for 5 min. Any remaining reactive groups on the sensorchips were deactivated by injecting 1 M ethanolamine pH 8.5 into both channels for 5 min and then washing with SPR buffer for 1 min. Solutions of cyclic nucleotides were prepared using the same SPR buffer used during the PKG I coupling steps. During cNMP binding experiments, solutions were injected at 50 µL/min for 1 min (association) and then washed with SPR buffer for 3 min (dissociation). All injected solutions were prepared in clear or amber borosilicate screw-top glass vials with robotic screw-top vial closures (Fisher Scientific). Data were reduced using Scrubber (BioLogic). Data were fit to one-site (with Hill coefficient) and two-site (specific binding) models using GraphPad Prism (GraphPad Software) and plotted using DataGraph (Visual Data Tools).

2.5.9 Mass Spectrometry

All LC-MS measurements were performed using a Xevo G2-XS quadrupole time-of-flight (QTOF) mass spectrometer connected to an Acquity ultra performance liquid chromatography system (UPLC) (Waters Corp.) in positive electrospray ionization (ESI) mode. Mass spectrometry of intact protein was achieved using a Jupiter 5-µm 300-Å, 1-mm x 150-mm C4 column (Phenomenex). Conditions: LC flow = 80 µL/min; A = water with 0.1% formic acid; B = acetonitrile; isocratic flow = 0-1 min with 90% A and 10% B, 1-4 min ramp to 10% A and
90% B; Scan range = 500-2000 m/z. Approximately 2 pmol of PKG Δ53 was injected per run. The resulting spectra were processed using MassLynx v4.1 and the deconvolution software, MaxEnt (Waters Corp.).

LC-MS/MS was performed to determine specific phosphorylation sites as follows: Two 150-pmol aliquots of dried PKG Δ53 or PKG Iα full-length enzyme were resuspended in 25 µL of 50 mM ammonium bicarbonate buffer (pH 8.5) and 30 µL of 0.02% ProteaseMAX (Promega). A 5-µL aliquot of sequencing grade trypsin (Promega) was added at a 1:20 ratio (enzyme to substrate). Samples were incubated overnight at 37°C and acidified with formic acid to obtain a final concentration of 0.5%. The samples were evaporated and resuspended in 10 µL of 98% water, 2% acetonitrile, 0.1% formic acid prior LC-MS analyses. UPLC separation of the tryptic peptides was performed on an Acquity HSS T3 1.8-µm, 1.0 x 150 mm column (Waters Corp.). UPLC conditions: LC flow = 50 µL/min; column temperature = 45°C; A = water and 0.1% formic acid; B = acetonitrile and 0.1% formic acid; linear gradient from 98% A and 2% B to 65% A and 35% B over 50 min. Approximately 30 pmol of digested PKG Δ53 was injected. All samples were analyzed by data independent acquisition between 100-2000 m/z using the MSE mode with alternating low (4 V) and high energy (15-40 V) acquisitions. The MSE data were processed using ProteinLynx Global Server v3.0.1 (Waters Corp.) that searched against a consolidated database that included human cGMP-dependent protein kinase 1 (Q13976) and included possible modifications, including phosphorylations.

2.5.10 Size exclusion chromatography- small angle x-ray scattering (SEC-SAXS)

SAXS data pertaining to Δ53 was collected at the Stanford Synchrotron Radiation Lightsource BL4-2 using an MX225-HE detector (Rayonix). A 35 µL
sample of 6 mg/mL PKG IαΔ53 was injected onto a Superdex 200 3.2/30 column (GE) and eluted isocratically using an Ettan FPLC (GE) at 0.05 mL/min in 50 mM MES, 300 mM NaCl, 1mM TCEP, pH 6.9 supplemented with 5 mM DTT as a radical scavenger. The eluant stream was connected in-line to a 1.5 mm quartz capillary positioned 1.7 m from the detector. Data were collected using a wavelength of 1.127 Å with a 1 second/frame exposure rate at 22°C. A total of 600 frames were collected with a measurement range of 0.0087 - 0.5126 Å⁻¹. Frames 410-459 containing the peak corresponding to Δ53 PKG were averaged and subtracted from the background (45 averaged frames of buffer alone). The data was truncated to exclude the range of 0.0087-0.016 Å⁻¹ due to radiation-induced aggregation and above 0.19 Å⁻¹ for all subsequent analyses. The scattering curve, Guinier and P(r) functions, and Porod volume were calculated using the PRIMUS program suite (Konarev et al., 2003). DAMMIF/DAMMIN/DAMMAVER/DAMFILT was used to generate a total of 20 models, of which 19 were used to calculate the final 3-D envelope. Models were excluded based upon their NSD to the mean using DAMSEL. The excluded model was determined to have a NSD that was two times the standard deviation from the mean of all NSD scores using a cross-correlation matrix created. SUPCOMB was further used to fit the averaged 3-D envelope to existing heterodimeric structures of PKA R1α:C (PDBID: 2QCS), R1β:C (PDBID: 4WBB), and PfPKG (PDBID:5DYK) (Franke and Svergun, 2009; Volkov and Svergun; Kozin and Svergun, 2001). Radii of gyration were calculated from the predicted scattering curves of the R1α:C and R1β:C heterodimers (using PDBID: 2QCS and 4WBB, respectively) using the FoXS and ATSAS servers (https://modbase.compbio.ucsf.edu/foxs/ and https://www.embl-hamburg.de/biosaxs/atsas-online) (Schneidman-Duhovny et al., 2013; Schneidman-Duhovny et al.). Data were deposited in the SASBDB using the identifier SAS-DDS4 (Valentini et al., 2015).
2.6 Acknowledgments

We would like to thank the beamline scientists, Tsutomu Mitsui and Thomas Weiss, at SSRL BL4-2 for their assistance with SEC-SAXS data collection. Use of the Stanford Synchrotron Radiation Lightsource, SLAC National Accelerator Laboratory, is supported by the U.S. Department of Energy, Office of Science, Office of Basic Energy Sciences under Contract No. DE-AC02-76SF00515. The SSRL Structural Molecular Biology Program is supported by the DOE Office of Biological and Environmental Research, and by the National Institute of General Medical Sciences at the National Institutes of Health (NIH) (including P41GM103393). The contents of this publication are solely the responsibility of the authors and do not necessarily represent the official views of NIGMS or NIH. Mass spectrometry measurements were assisted by Bruce O'Rourke and supported by NIH grants, S10-OD018126 and P30-GM118228. This research was supported by grants from the National Institutes of Health (NIH) with direct support to T.M.M. (5T32 HL007647) and J.L.S. (5T32 HL007594-30) and additional support from the Tostman Trust for Biomedical Research. We would like to thank Werner Tegge at the Helmholtz Centre for Infection Research (HZI) in Braunschweig, Germany for synthesizing the W15 peptide substrate used in the phosphotransferase assays. We would also like to thank Wolfgang Peti at the University of Arizona for the generous use of a Superdex 200 10/300 analytical column and Äkta PURE FPLC system, and Ross Buchan for helpful discussions regarding this manuscript.

2.7 Conflicts of Interest

The authors declare that there are no outstanding conflicts of interest.
2.8 Author Contributions


2.9 Supplemental Information

Table 2.3: Phosphopeptides of full-length PKG Ia.

<table>
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<th>Precursor MH⁺ (Da)</th>
<th>z</th>
<th>Start</th>
<th>End</th>
<th>Sequence</th>
<th>Modifications</th>
<th>Retention Time (min)</th>
<th>Intensity (x10⁴)</th>
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<tr>
<td>2,390.15</td>
<td>2.95</td>
<td>514</td>
<td>533</td>
<td>(K)KTWTFCGTPYVAPEILNKGK</td>
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<td>533</td>
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Table 2.4: Small Angle X-ray Scattering Results.

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<tr>
<th>Construct</th>
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<th>Derived</th>
<th>Calculated</th>
<th>D_max (Å)</th>
<th>V (Å³ x 10³)</th>
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<tr>
<td>PKG Iα Δ53</td>
<td>29.71 ± 0.58</td>
<td>30.23 ± 0.25</td>
<td>96.77</td>
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<tr>
<td>PKG Iβ Δ1-52*</td>
<td>29.4 ± 0.1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>PKA: RIα_AB/C †</td>
<td>36.4 ± 0.6</td>
<td>-</td>
<td>120</td>
<td>96</td>
<td></td>
</tr>
<tr>
<td>PKA: RIα_AB/C (calc.) †</td>
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<td>29.62</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>PKA: RIIβ_AB/C †</td>
<td>29.1 ± 1.0</td>
<td>-</td>
<td>90</td>
<td>99</td>
<td></td>
</tr>
<tr>
<td>PKA: RIIβ_AB/C (calc.) †</td>
<td>-</td>
<td>29.11</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

* Wall et al. (2003)
† Vigil et al. (2005)
‡ Calculated using the FoXS server; https://modbase.compbio.ucsf.edu/foxs/index.html
Figure 2.9: Molecular weight standard curve corresponding to elution on Superdex S200 10/300 column. The standards 1) Thyroglobulin (670 kDa), 2) γ-globulin (158 kDa), 3) Ovalbumin (44 kDa), 4) Myoglobin (17 kDa), and 5) Vitamin B12 (1.3 kDa) are displayed. The dashed red line corresponds to the predicted molecular weight and elution volume (14.6 mL) for ∆53 PKG Iα. The observed elution volume for ∆53 is 14.8 mL.

Figure 2.10: P(r) profiles comparing the experimentally measured scattering profiles of ∆53 (black, left axis) from this study, and RIα heterodimer (green, right axis), and RIIβ heterodimer (red, right axis) from Vigil et al.
Figure 2.11: Predicted versus experimental scattering for ∆53 with residual plots. Predicted scattering of crystal structures are shown for PKA RIα:C (green) heterodimer, PKA RIβ:C heterodimer (red), and PfPKG (blue). A) CRYSOL fit with constant subtraction. B) CRYSOL fit without constant subtraction C) FoXS fit using default parameters. D) The 3D envelope of the DAMMIF ab initio model based on the SAXS data obtained for ∆53 (grey balls) fit to the crystal structure of PfPKG (PDBID: 5DYK, NSD=1.09). E) View of PfPKG structure without overlay of 3D envelope.
Figure 2.12: Activation data for A-site (E167G, green) and B-site (E292A, violet) mutants compared to full-length (black) and ∆53 PKG Iα (red). The A-site mutant is unable to activate in a cGMP-dependent manner while the B-site mutant is active and displays a similar activation curve ($K_a = 95$ nM) and cooperativity ($n_H = 1.45$) to full-length ($K_a = 180$ nM, $n_H = 1.58$).

Figure 2.13: One-site models with Hill coefficient fits for A) cGMP and B) cAMP binding to full-length (black), ∆53 (red), 1-326 (blue) and 78-326 (green).


Chapter 3

Intraprotomer disulfide bond formation stimulates constitutive activation of the type Iα cGMP-dependent protein kinase

3.1 Abstract

The type I cGMP-dependent protein kinase (PKG I) is an essential regulator of vascular tone. It has been demonstrated that the type Iα isoform can be constitutively-activated by oxidizing conditions. However, the amino acid residues implicated in this phenomenon are not fully elucidated. To investigate the molecular basis for this mechanism, we studied the effects of oxidation using recombinant wild-type, truncated, and mutant constructs of PKG I. Using an in vitro assay, we observed that oxidation with hydrogen peroxide (H$_2$O$_2$) resulted in constitutive, cGMP-independent activation of PKG Iα. PKG Iα C42S and a truncation construct that does not contain C42 (∆53) were both constitutively activated by H$_2$O$_2$. In contrast, oxidation of PKG Iα C117S maintained its cGMP-dependent activation characteristics, though oxidized PKG Iα C195S did not. To corroborate these results, we also tested the effects of our constructs on the PKG Iα-specific substrate, the large conductance potassium channel (K$_{Ca}$1.1). Application of wild-type PKG Iα activated by either cGMP or H$_2$O$_2$ increased the open probabilities of the channel. Neither cGMP- or H$_2$O$_2$-activation of PKG Iα C42S significantly increased channel open probabilities. Moreover, cGMP-stimulated PKG Iα C117S increased K$_{Ca}$1.1 activity, but this effect was not observed under oxidizing conditions. Finally, we observed that PKG Iα C42S caused channel flickers, indicating dramatically altered K$_{Ca}$1.1 channel characteristics compared channels exposed to wild-type PKG Iα. Cumulatively, these results indicate that constitutive activation of PKG Iα proceeds through oxidation of C117 and further suggests that the formation of a sulfur acid is necessary for this phenotype.

3.2 Introduction

Blood pressure is maintained in part through constriction and relaxation of smooth muscle cells in the peripheral vasculature. During vasorelaxation,
endothelium-derived nitric oxide or circulating natriuretic peptides activate guanylyl cyclases, which produce 3,5-cyclic guanosine monophosphate (cGMP) (Friebe and Koesling, 2003). This second messenger binds to and activates the cyclic GMP-dependent protein kinase I (PKG), a homodimeric enzyme that is expressed as two isoforms in vascular smooth muscle cells (VSMCs), PKG Iα and Iβ (Hofmann et al., 2009). PKG Iα acts as a central regulator of vasorelaxation by modulating global and local Ca²⁺ levels within the cell. Its phosphorylation targets include the large conductance calcium-activated potassium channel (BKCa, KCa1.1), which causes membrane hyperpolarization and inhibition of voltage-dependent calcium channels (VDCCs) (Fukao et al., 1999; Sausbier et al., 2005; Schubert and Nelson, 2001). PKG Iα also phosphorylates the myosin phosphatase targeting subunit 1 (MYPT1), the small GTP-binding protein (RhoA), and the regulator of G-protein signaling 2 (RGS2), which mediate the downstream cytoskeletal rearrangement necessary for vascular relaxation (Kato et al., 2012; Sun et al., 2005; Yuen et al., 2011, 2014).

This signaling cascade and physiological effects associated with cGMP-dependent activation of PKG Iα are well-established (Hofmann et al., 2009). Under oxidizing conditions, PKG Iα is known to be constitutively active (Burgoyne et al., 2007; Landgraf et al., 1991; Muller et al., 2012). Several studies have suggested that a residue unique to the N-terminus of PKG Iα, C42, may be the source of this constitutive activation (Burgoyne et al., 2007; Khavandi et al., 2016; Prysyazhna et al., 2012; Rudyk et al., 2012). The proposed mechanism involves the formation of an intramolecular disulfide bond between C42 and C42 from the opposing protomer in the homodimeric holoenzyme. Since this disulfide bond is located in the substrate targeting region of the kinase, it has been hypothesized that oxidation at this site also modulates targeting to substrates by altering the geometry of this domain (Burgoyne et al., 2007; Landgraf et al., 1991). These observations have been consequential toward understanding the role of oxidizing agents, such as hydrogen peroxide, in both physiological and pathophysiological processes within the vasculature (Chan and Baumbach, 2013a,b; Gray et al., 2016; Kim et al., 2017; Patel et al., 2017; Rashdan and Lloyd, 2015; Schiffrin, 2012).

Both activation and substrate targeting are required for PKG I-dependent signaling, and disruption of either of these mechanisms is sufficient to alter normal physiological processes (Blanton et al., 2013; Casteel et al., 2005; Feil et al., 1995; Surks et al., 1999). In this study, we sought to isolate these two facets of PKG I signaling by studying the effects of oxidation using recombinant wild-type, truncated, and mutant constructs of PKG I. We found that both PKG Iα and Iβ are activated by oxidation, though its effect on PKG Iα is more pronounced. Furthermore, both PKG Iα C42S and Δ53 PKG Iα (a truncation mutant that does not contain C42) are activated by oxidation. Using PKG Iα C117S and C195S to test
the influence of a previously observed disulfide bond, we observed that oxidation at C117 is necessary and sufficient for oxidative activation of PKG Iα. Finally, we corroborated these in vitro results by measuring the PKG Iα-dependent activation of the K<sub>Ca</sub>1.1 channel. Taken together, these data support the hypothesis that oxidative disruption of PKG localization and constitutive activation are separate phenomena, although they have significant effects on PKG signaling in the vascular smooth muscle.

### 3.3 Results

**3.3.1 Oxidation of PKG Iα abrogates cGMP-dependent activation**

Under oxidizing conditions, there are two confirmed sites where disulfide bonds form in the PKG Iα holoenzyme. They are an interprotomer disulfide bond between Cys42 and Cys42 and an intraprotomer disulfide bond between Cys117 and Cys195 (Figure 1A) (Landgraf et al., 1991). These disulfide bonds have also been identified in recent structural studies of the isolated domains (Figure 1B, C) (Landgraf et al., 1991; Osborne et al., 2011; Qin et al., 2015). The first disulfide bond between C42-C42 forms at the C-terminal region of the dimerization domain (Figure 1B). Cys42 is one of four non-leucine/isoleucine residues at d positions within the coiled-coil and is unique to this isoform (Figure S1A). The second disulfide bond in cGMP-A forms between C117 and C195 and is proximal to the cGMP binding pocket (Figure 1C).

To characterize the oxidative phenotype, recombinant PKG Iα from Sf9 cells was used for in vitro phosphotransferase assays, wherein changes in kinase activity could be monitored. First, we confirmed that PKG Iα activity under reducing conditions (WT<sub>red</sub>) were consistent with previous studies (Moon et al., 2015, 2018). Exposure to increasing concentrations of cGMP activated the kinase in a cooperative manner (n<sub>H</sub> = 1.7) by 20-fold at a half maximal concentration of 200 nM (Figure 2A, Table 1). We next examined how increasing H<sub>2</sub>O<sub>2</sub> concentrations affected PKG Iα activity. WT<sub>red</sub> was exposed to H<sub>2</sub>O<sub>2</sub> and kinase activity was monitored under apo and saturating cGMP conditions (Figure 2B). A maximum response was observed at 500 µM, wherein we observed a reduction in the maximal velocity paired with a peak in the minimum velocity. At 1 mM H<sub>2</sub>O<sub>2</sub>, we observed a relative loss of activity the presence and absence of cGMP, suggesting that exposure of the enzyme to H<sub>2</sub>O<sub>2</sub> had compromised the kinases catalytic function. To expand our understanding of the conditions in which to measure the oxidative phenotype, the fold-activation of PKG Iα was monitored over a timed exposure to 500 µM H<sub>2</sub>O<sub>2</sub> (Figure 2C). After 30 minutes of exposure, we observed the greatest effect on the cGMP-dependent activation of the kinase.
wherein the fold-activation was reduced by approximately 90% to 2.5 (Table 1). These conditions were used for all subsequent measurements of the oxidation phenotype relating to cGMP-dependent activity.

Next, we tested the cGMP-dependent activation of oxidized PKG Iα. Oxidation resulted in a 5-6-fold increase in basal activity in the absence of cGMP (1.13 μmol/min×mg; p<0.001 compared to WTred; Figure 2A). In contrast, the addition of up to 4 μM cGMP increased the activity by only 2.5-fold (2.77 μmol/min×mg). Moreover, the activation of WTox with cGMP indicated a decreased sensitivity for the cyclic nucleotide. In agreement with previous studies, analysis of wild-type PKG Iα by non-reducing SDS-PAGE showed that increasing concentrations of H₂O₂ resulted in formation of an interprotomer disulfide bond. (Figure 2D). In determining whether our PKG I constructs formed the interprotomer disulfide bond using non-reducing, SDS-PAGE electrophoresis, preliminary experiments indicated that removal of the reducing agent from the storage buffer would expose the enzymes to ambient air; prior studies have used this method to activate PKG Iα by oxidation (Fig S1) [11, 35]. We sought to avoid the contribution from ambient air by retaining the reducing agent, tris-carboxyethyl phosphine (TCEP), in the buffer and adding H₂O₂ to levels which would overcome its reducing capacity. Since TCEP is degraded by H₂O₂ through oxidation to a phosphine oxide at a 1:1 ratio, the excess H₂O₂ could be calculated (Han et al., 1996; Tan et al.). Both the added (black) and final (H₂O₂ minus TCEP; red) H₂O₂ concentrations are shown.

### 3.3.2 Cysteine mutants of PKG Iα probe oxidation-induced constitutive activation

Next, we examined whether the oxidized phenotype in PKG Iα is regulated by formation of the interprotomer disulfide bond between C42 and C42. To achieve this, we measured the activities of reduced and oxidized PKG Iα C42S (C42Sred and C42Sox) (Figure 3A, Table 1). When the mutant was measured under reducing conditions, we observed a similar basal activity to WTred (0.32 μmol/min×mg). However, when activated by cGMP, the maximal velocity was reduced by approximately 50% reduction compared to WTred (2.31 μmol/min×mg), resulting in a 7-fold stimulation of activity. Moreover, C42Sred showed a 3-fold shift in the activation constant (K_a = 580 nM, p<0.001 compared to WTred) and a loss of cooperative, cGMP-dependent activation (n_H=0.8). For C42Sox, we observed a significant increase in basal activity (1.103 μmol/min×mg; p<0.001 compared to C42Sred); however, the maximal activity was unchanged (2.357 μmol/min×mg) and displayed similar kinetic characteristics to WTox. Examination of C42Sox by non-reducing, denaturing SDS-PAGE did not show evidence of an intramolecular
disulfide bond between protomers (Figure 3E).

Due to the similar increases in basal activity between WT\textsubscript{ox} and C42S\textsubscript{ox} in response to H\textsubscript{2}O\textsubscript{2}, we next utilized \( \Delta 53 \) PKG I\( \alpha \)—a truncation construct of PKG I\( \alpha \) that lacks the N-terminus (and C42) but retains cGMP-dependent activation (Moon et al., 2018). \( \Delta 53\textsubscript{red} \) activity in the absence of cGMP was similar to wild-type PKG I\( \alpha \) (Figure 3B). In the presence of increasing concentrations of cGMP, \( \Delta 53 \) activity increased by 20-fold with a \( K_a \) for cGMP that was similar to wild-type PKG I\( \alpha \) (216 nM). When oxidized with H\textsubscript{2}O\textsubscript{2}, \( \Delta 53\textsubscript{ox} \) exhibited kinetic features similar to the WT\textsubscript{ox} and C42S\textsubscript{ox} constructs. The 7-fold increase in basal activity of \( \Delta 53\textsubscript{ox} \) to 1.380 \( \mu \text{mol/min} \times \text{mg} \) decreased its cGMP-dependent fold-activation to 1.1. Thus, \( \Delta 53 \) displayed the highest sensitivity to oxidation. Like C42S, SDS-PAGE analyses showed that \( \Delta 53 \) does not form an interprotomer disulfide bond in the presence of H\textsubscript{2}O\textsubscript{2} (Figure 3E).

Since PKG I\( \alpha \) and I\( \beta \) are splice variants of the prkg1 gene, within the same species they are completely identical in their cGMP-binding and catalytic domains (with 98% sequence identity across placental mammals). However, their N-terminal dimerization, autoinhibitory, and linker regions only retain 26% sequence identity (Figure S1). Thus, C42 in the N-terminus of I\( \alpha \) is not conserved in PKG I\( \beta \). Upon ruling out the contribution of C42 on the oxidation-dependent activation phenotype of PKG I\( \alpha \), we hypothesized that any effect of oxidation may originate within the sequence-conserved regulatory domain. To test this, we first examined reduced and oxidized PKG I\( \beta \). PKG I\( \beta \) stimulated by cGMP under reducing conditions displayed a cooperative, 86-fold stimulation of activity with a \( K_a \) of 1.21 \( \mu \text{M} \) (Table 1, Figure S3). When PKG I\( \beta \) was oxidized with H\textsubscript{2}O\textsubscript{2}, a 10-fold increase in basal activity was observed (0.42 \( \mu \text{mol/min} \times \text{mg} \)) paired with a 1.3-fold decrease in maximal velocity (2.780 \( \mu \text{mol/min} \times \text{mg} \), resulting in a reduction of the cGMP-stimulated fold activation to 6.6. In addition, under oxidizing condition, the activation constant for PKG I\( \beta \) decreased by 1.4-fold (\( K_a = 0.86 \mu \text{M} \)) and cooperativity was reduced to 1.2.

In addition to the interprotomer disulfide bond between C42 and C42', Landgraf \textit{et al.} also confirmed the presence of an intraprotomer disulfide bond between C117 and C195. This disulfide bond has been observed in the crystal structure of the PKG I regulatory domain (PDB 3SHR) (Osborne et al., 2011). Due to its location in the first cGMP-binding domain (CNB-A), we hypothesized that this disulfide bond may control oxidative activation of PKG I\( \alpha \). To test this, we expressed and purified the PKG I\( \alpha \) C117S mutant. Under reducing conditions, C117S\textsubscript{red} had a low basal activity that was similar to PKG I\( \alpha \) wild-type (Figure 3C). Furthermore, activation of C117S\textsubscript{red} with cGMP increased the activity by 26-fold with positive cooperativity (\( n_H = 1.4 \)), resulting in a maximum velocity of 4.09 \( \mu \text{mol/min} \times \text{mg} \) and a \( K_a \) of 130 nM. In contrast to PKG I\( \alpha \) wild-type,
stimulation of C117S<sub>ox</sub> by cGMP resulted in only a slight increase in basal activity and no significant change in its maximal velocity compared to C117S<sub>red</sub> (3.81 µmol/min×mg). However the K<sub>a</sub> for cGMP was slightly increased and the cooperativity was slightly decreased (n<sub>H</sub> = 1.2) compared to C117S<sub>red</sub>. These data suggest that the response to H<sub>2</sub>O<sub>2</sub> is highly attenuated by introduction of the C117S mutation.

To determine whether oxidative activation is dependent on disulfide bond formation with C195, we next examined the C195S construct. Under reducing conditions, the overall kinetic profile of C195S<sub>red</sub> was not significantly different to WT<sub>red</sub>; however, the basal and maximal activities were slightly higher, resulting in an overall fold activation of 10.8 after stimulation with cGMP. (Figure 3D, Table 3.1). Under oxidizing conditions, we observed kinetics consistent with the WT<sub>ox</sub> phenotype. This included a significant increase in basal activity and a decrease in the maximal velocity, suggesting oxidation at C195 is not necessary for oxidation-dependent activation.

3.3.3 Analysis of K<sub>Ca</sub>1.1 channel activity during exposure to PKG I<sub>α</sub>

Phosphorylation of the K<sub>Ca</sub>1.1 channel by PKG I<sub>α</sub> has been shown to increase its open probability, resulting in increased K<sup>+</sup> flux to the extracellular space (Alioua et al., 1995; Kyle et al., 2013; Schubert and Nelson, 2001). To examine the effects of both substrate targeting and activation of the channel, we measured K<sub>Ca</sub>1.1 channel currents by inside-out patch clamp configuration using exogenous application of reduced or oxidized PKG I<sub>α</sub> constructs (Figure 4A). Under reducing and cGMP-stimulating conditions, wild-type PKG I<sub>α</sub> increased the open probability of the K<sub>Ca</sub>1.1 channels by 3.2-fold compared to control patches (p<0.001). PKG I<sub>α</sub> that was pretreated with H<sub>2</sub>O<sub>2</sub> and then added to the extracellular face of the patch in the absence of cGMP also increased the open probability by 3.1-fold (p<0.02).

Application of C42S<sub>red</sub> under cGMP-saturating conditions did not result in a significant increase in channel open probability compared to control (NP<sub>exp</sub>/NP<sub>control</sub> = 1.4), whereas C42S<sub>ox</sub> resulted in a slightly higher increase in the open probability of the channels to 2.1-fold over baseline. Furthermore, we observed that the channel kinetics in the presence of C42S were altered under these conditions (Figure 4Ba). Examination of the channel dwell times show that K<sub>Ca</sub>1.1 patches exposed to cGMP-activated C42S<sub>red</sub> had a shortened mean open dwell time of 1.0 ms compared to baseline (5.8 ms) (Figure 4Bb, S4). However, when wild-type PKG I<sub>α</sub><sub>red</sub> was applied to the patch, we saw a significant but smaller decrease in mean open dwell time (baseline = 4.6 ms and PKG I<sub>α</sub> = 3.7 ms) (Figure 4Bb, S4).
The comparison of these differences by non-parametric Hodges-Lehmann analysis indicated that the change in dwell time in response to kinase addition was greater for C42S than wild-type PKG Iα (Figure 4Bb). To determine whether the change in KCa1.1 currents caused by C42S was dependent on activity, channel recordings in the presence of C42Sred was also measured under symmetrical K+ in the absence of cGMP and ATP (Figure S5). The short channel openings (flickers) were observed at both +40 and -40 mV, indicating that the effect was independent from C42S activity and membrane polarity.

Finally, application of C117Sred under cGMP-saturating conditions significantly increased the open probability of the channel compared to the control condition (Figure 4A, p<0.02). However, C117S that was pretreated with H2O2 (C117Sox) had no effect on KCa1.1 channel open probability (p=0.2413). As a control, application of 500 nM hydrogen peroxide directly to the bath solution did not significantly alter channel open probabilities. As a control, no effect on channel activity was observed with the addition of 500 nM H2O2—the concentration that equaled the maximum final concentration of H2O2 in the bath solution after addition of PKG Iαox constructs.

Table 3.1: cGMP-Dependent Phosphotransferase Activity

<table>
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<tr>
<th>Constructs</th>
<th>Reduced</th>
<th>Oxidized</th>
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<tbody>
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<td>PKG Iα</td>
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<td></td>
</tr>
<tr>
<td>WT</td>
<td>0.22 ± 0.00</td>
<td>0.13 ± 0.03</td>
</tr>
<tr>
<td>C42S</td>
<td>0.32 ± 0.06</td>
<td>0.16 ± 0.03</td>
</tr>
<tr>
<td>C53</td>
<td>0.18 ± 0.16</td>
<td>0.12 ± 0.08</td>
</tr>
<tr>
<td>C117S</td>
<td>0.16 ± 0.10</td>
<td>0.06 ± 0.04</td>
</tr>
<tr>
<td>C195S</td>
<td>0.43 ± 0.02</td>
<td>0.12 ± 0.04</td>
</tr>
<tr>
<td>PKG Iβ</td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>0.04 ± 0.04</td>
<td>0.04 ± 0.03</td>
</tr>
</tbody>
</table>

† The velocity values indicate the µmol of peptide substrate (W15) phosphorylated per min per mg of PKG (µmol/min*mg) and are represented as the mean ± the standard deviation.
* Values were obtained from non-linear regression fit analyses and are represented as the means ± standard deviations. Not applicable (N/A) values indicate the data for that condition could not be fit to a non-linear regression.

3.4 Discussion

PKG Iα is an essential signaling molecule in the vasculature and can be activated by cGMP or oxidation (Burgoyne et al., 2007; Dostmann et al., 2000; Francis et al., 1996; Heil et al., 1987; Ruth et al., 1991). The first study to describe that PKG Iα can be constitutively activated by oxidation used cupric cations as the oxidizing agent (Landgraf et al., 1991). This activity was found to be blocked
Figure 3.1: **Putative sites of oxidative activation in PKG Iα**

A) Linear domain diagram depicting the homodimeric PKG Iα and the sites of cysteine residues hypothesized to be involved in the oxidative phenotype. Structures denoting the location of disulfide bonds identified in the N-terminal dimerization domain (B) and the cGMP A-site (C) from PKG Iα (PDBIDs: 4R4L and 3SHR, respectively).

by iodoacetimide, which indicated involvement of cysteine residues in this mechanism. Moreover, three possible disulfide bonds were identified by non-reducing SDS-PAGE and Edmann degradation: an interprotomer disulfide bond between C42 and C42, an intraprotomer disulfide bond between C117 and C195, and an intraprotomer disulfide bond between C312 and C518. Of the three, the last disulfide bond could not be definitively confirmed.

Previous analyses of PKG I isoforms determined that PKG Iα, but not PKG Iβ, exhibits sensitivity to oxidation (Burgoyne et al., 2007; Muller et al., 2012). Thus, it was concluded that C42—the only cysteine residue unique to PKG Iα—must be the primary regulator of oxidation-dependent activation (Burgoyne et al., 2007). Moreover, formation of this interprotomer disulfide bond
Figure 3.2: Initial characterization of the PKG Iα oxidative phenotype. 

A) cGMP-dependent activation under reducing (solid line, N=12) and oxidizing (500 µM H₂O₂, 30 min, dashed line, N=16) conditions.  

B) PKG Iα activity plotted against increasing concentrations of H₂O₂. Activity was measured in the absence (black, N=4) and presence of cGMP (red, 4 µM, N=4). The concentration of H₂O₂ (500 µM) that resulted in the greatest increase in basal activity is highlighted in the boxed region.  

C) A time course examining PKG Iα oxidation with 500 µM cGMP wherein the fold-activity (V_max/V_min) was monitored (N=4).  

D) Representative non-reducing, denaturing SDS-PAGE of PKG Iα exposed to increasing concentrations of H₂O₂. The dashed line indicates the point at which the concentration of hydrogen peroxide overcomes the concentration of TCEP in the buffer. H₂O₂ values corrected for the presence of TCEP are shown in red. Where shown, data are represented as the mean ± SD.
Figure 3.3: **In vitro** phosphotransferase assays of PKG Iα under reducing and oxidizing conditions. cGMP-dependent activation of (A) PKG Iα wild-type (black circles) and PKG Iα C42S (red squares), (B) ∆53 PKG Iα (purple squares), (C) PKG Iα C117S (green triangles) and (D) PKG Iα C195S (blue triangles) under reducing (1 mM TCEP, solid lines) and oxidizing (500 µM H₂O₂, 30 min, dashed lines) conditions. Data are represented as the mean ± SD. (E) Non-reducing, denaturing SDS-PAGE of PKG Iα constructs treated with increasing concentrations of H₂O₂ (0-2 mM). The dashed line indicates the point at which the concentration of hydrogen peroxide overcomes the concentration of TCEP in the buffer. H₂O₂ values corrected for the presence of TCEP are shown in red.
Figure 3.4: Stimulation of $K_{Ca}1.1$ channels with PKG Iα constructs under reducing and oxidizing conditions

A) The combined results of single $K_{Ca}1.1$ recordings exposed to PKG Iα constructs under reducing and oxidizing conditions. Individual measurements are represented as a scatter plot overlaid with mean ± SEM. P values represent the comparison against PKG Iα under reduced, unstimulated conditions (first lane).

B a) Representative control (top) and experimental traces of single $K_{Ca}1.1$ channel openings in excised membrane patches from mouse cerebral artery myocytes under control conditions and when exposed to cGMP activated PKG Iα WT and C42S constructs. All measurements were recorded under reducing conditions.

B b) Analysis of channel open dwell times of $K_{Ca}1.1$ channels from representative patches under control, and exposure to PKG Iα WT and C42S under reducing conditions. Whisker boxes are represented as the medians bounded by the interquartile ranges (IQR). A non-parametric Hodges-Lehmann analysis containing the HL-coefficient and error are shown. *p<0.02; ***p<0.0001
Cysteine residues can be oxidized to multiple states, which proceed through a sulfenic acid intermediate. When in proximity to a free thiol, sulfenic acids can be condensed to a disulfide. Further oxidation of sulfenic acids results in the irreversible sulfenic and sulfonic acid forms. The sulfonic acid form can also be reached via a thiosulfinate intermediate. The sulfur acid forms hypothesized to mediate oxidation-dependent activation of PKG Iα are boxed. B) Sequence alignment of mammalian PKG Iα (green) and Iβ (gold) isoforms denoting the autoinhibitory (AI) domain and linker region. The segment within the linker region that is in close proximity to C117 is boxed in red. C) A structural alignment of the PKG Iα (green; PDB 3SHR) and the Iβ (yellow; PDB 3OD0) A-sites superimposed with the RIα:C holoenzyme of PKA (RIα-blue; C-gray; PDB 2QCS). The boxed region indicates the location of the linker region of PKG Iα and Iβ with their corresponding residues within close proximity to C117.
between C42 and C42’ of the opposing protomer was found to correlate with a vasodilatory response in isolated perfused rat hearts (Burgoyne et al., 2007). These observations helped to cement oxidation as an additional mechanism by which PKG Iα can be activated. Despite an abundance of data ascribing physiological importance to the formation of the interprotomer disulfide bond between C42 and C42, the structural and biochemical underpinnings of how this disulfide bond would activate PKG Iα remain unclear. Thus, we sought to expand upon these initial observations to more thoroughly understand the mechanism of oxidation-dependent activation in PKG Iα.

Under reducing conditions, the cGMP-dependent activation characteristics of wild-type PKG Iα were consistent with previously reported values (Figure 2C) (Moon et al., 2015, 2018). Furthermore, preincubation of wild-type PKG Iα with H₂O₂ induced interprotomer disulfide bond formation (Figure 2D) (Burgoyne et al., 2007; Kalyanaraman et al., 2017; Landgraf et al., 1991). We observed that exposure of wild-type enzyme to 500 µM H₂O₂ for 30 min at 24°C resulted in the greatest amount of constitutive activation, determined by increase in basal activity and corresponding decrease in cGMP-dependent activation (Figure 2B). Although this concentration of H₂O₂ is significantly higher than what has been implemented in prior studies, we sought to measure the H₂O₂ concentration dependence on PKG Iα activity in a similar fashion as Landgraf et al. (Burgoyne et al., 2007; Landgraf et al., 1991; Kalyanaraman et al., 2017; Muller et al., 2012). To examine the source of the oxidative phenotype, we expressed constructs of PKG Iα that contained site mutations at cysteine residues previously suggested to form disulfide bonds in the presence of oxidizing agents, with a specific focus on C42 in the dimerization domain and C117 and C195 within the cyclic nucleotide binding A-site (CBD-A) (Landgraf et al., 1991; Osborne et al., 2011; Qin et al., 2015).

### 3.4.1 Characterization of C42S provides insight into rodent models of oxidation-dependent activation of PKG Iα

When cGMP-dependent activation of C42S was examined under reducing conditions, we observed that the presence of the serine mutant significantly altered enzyme activity. This was demonstrated by the shift in its activation constant by 3-fold to 580 nM and a decrease in the measured maximal velocity compared to wild-type (Figure 3A). This overall shift in efficacy and potency culminated in a 90% reduction in activity for C42S under cGMP concentrations near the K_D for PKG Iα wild type. Even under conditions of maximal cGMP concentrations typically observed in smooth muscle cells (1-2 µM), C42S exhibited about 30% of the
maximal activity of PKG Iα wild-type (Figure 3A) (Nausch et al., 2008). Based upon these results, we conclude that, while the C42S mutant retains sensitivity to oxidation — unlike the wild-type enzyme — the reduced form of C42S displays significantly altered kinetic activity toward the synthetic peptide substrate used in the in vitro phosphotransferase assays. In agreement with previous results, C42S did not form an interprotomer disulfide bond in the presence of H₂O₂ (Figure 3E) (Burgoyne et al., 2007; Kalyanaraman et al., 2017). These results reinforce the findings of a recent study observing that oxidation of PKG Iα resulted in interprotomer disulfide formation, but did not increase phosphorylation of the PKG substrates RhoA and VASP (Kalyanaraman et al., 2017). In agreement with our study, the authors also found that the reduced form of C42S exhibited a 5-fold shift in its activation constant for cGMP. Taken together, these results suggest that the interprotomer disulfide bond does not mediate the oxidative phenotype, but does offer an explanation of the detrimental physiological phenotypes associated with this mutation.

To this end, a PKG Iα C42S knock-in mouse model has been used in previous studies to investigate the physiological effects of the interprotomer disulfide bond (Prysyazhna et al., 2012). These mice were found to be hypertensive compared to littermate controls. Based upon our kinetic analyses of PKG Iα C42S, we suggest that this phenotype may result from the compromised kinetic profile of the enzyme rather than an inability to be activated by oxidation. This conclusion is further substantiated by the effects of PKG Iα constructs on KᵥCa1.1 channel activity. Application of PKG Iα activated either with cGMP (under reducing conditions) or by oxidation increased channel activity (Figure 4A). The similarities in channel responses between the two conditions suggests that substrate targeting of PKG through its dimerization domain to KᵥCa1.1 is not compromised by formation of the interprotomer disulfide bond between C42 and C42’ in the dimerization domain.

However, a comparison of KᵥCa1.1 channel characteristics subjected to either C42S or WT, exposed that the mean dwell times were shorter for those patches treated with the mutant kinase. Moreover, we observed an overall increase in the number of channel opening events over an equivalent measurement time (Figure 4Bb, S4, S5). These short channel openings (flickers) may impact the calculated -fold activation values reported in Figure 4A. Moreover, the KᵥCa1.1 channel flickers were observed for all patches exposed to C42S (± cGMP, and oxidizing conditions) (Figure 4Bb, S4, S5). Within this context, a study examining pressure-induced constriction of third order mesentery arteries expressing C42S observed that application of the KᵥCa1.1 channel blocker, paxilline, did not induce vessel constriction (Khavandi et al., 2016). In light of the modified KᵥCa1.1 channel characteristics mediated by C42S, the mutation may possess hitherto unknown
properties, such as unique interactions with the channel pore or altered targeting to other PKG Iα substrates.

As a corollary, the effects of disrupted substrate targeting has been observed previously for a mutant leucine zipper knock-in mouse model in which the leucine residues in the dimerization domain were mutated to alanine residues. This mutant was unable to phosphorylate myosin binding subunit (MBS) and Ras homolog A (RhoA), despite retaining its sensitivity to cGMP and ability to phosphorylate the synthetic substrate BPDEtide (Blanton et al., 2013). Furthermore, these mice, like the C42S knock-in model, were also hypertensive. Since activation and substrate targeting through the dimerization domain are both required for phosphorylation of substrates by PKG Iα, we suggest that either explanation is possible.

3.4.2 C117S drives constitutive activation of PKG Iα by oxidation

To test our hypothesis that the oxidative phenotype is driven by residues that lie outside of the N-terminus, we next tested the PKG Iα truncation construct, Δ53 (Moon et al., 2018). Similar to C42S, Δ53 does not form an interprotomer disulfide bond (Figure 3E), and reduced Δ53 was activated by concentrations of cGMP similar to wild-type (Fig. 3B). Moreover, oxidation had the most pronounced effect on the cGMP-independent activation of the kinase as indicated by the complete insensitivity to cGMP. These results corroborate the C42S data and overwhelmingly demonstrate that the interprotomer disulfide bond is not required for oxidation-induced activation.

Finally, we tested the contribution of the intraprotomer disulfide bond that has been shown to form between C117 and C195 in the cGMP-A domain (Landgraf et al., 1991; Osborne et al., 2011). We observed that, while the cGMP-dependent activation of reduced C117S was similar to reduced PKG Iα WT, oxidized C117S exhibited only a modest increase in basal activity and no reduction in maximal velocity compared to its reduced form (Fig. 3C). We also consistently observed robust activation by cGMP under oxidizing conditions, indicating that the C117S mutation nearly abolishes the oxidative phenotype. These results are corroborated by the inside-out patching experiments, which found that cGMP-activated, reduced C117S stimulated an increase in the fold-activation of K_Ca1.1 that was similar to wild-type PKG Iα. However, oxidized C117S had no effect on channel activity. To determine whether this is controlled by disulfide formation between C117 and C195 or by another oxidation state, we also tested the C195S mutant. Under reducing conditions, we observed an elevated basal activity compared to wild-type; however, in response to oxidation, C195S exhibited a profile similar to
WT. Thus, we conclude that C117 plays a unique role in mediating the oxidative activation of PKG Iα in vitro.

The initial observation that PKG Iα and Iβ exhibit differing responses to H₂O₂ has predominantly focused the field on C42, the cysteine residue specific to the α isoform. However, the results described herein indicate the oxidative phenotype is controlled by C117 in the cyclic nucleotide binding domain-A (CBD-A). PKG Iα and Iβ are 100% identical in this region, and the residue analogous to C117 in PKG Iβ is C132. Examination of reduced and oxidized PKG Iβ showed that oxidation had a significant impact on its maximal velocity that was similar to the effect on PKG Iα. However, only a slight increase in basal activity was observed. These results are consistent with a previous study that focused solely on activation of PKG Iβ with H₂O₂ in the absence of cGMP (Burgoyne et al., 2007) and indicate that examination of the cGMP-dependent activation profile is necessary to observe the full effects of oxidation on PKG Iβ activity. Differences in PKG Iα and Iβ—cGMP-dependent activation profiles and targeting to substrates—are attributed to their distinct N-termini (Schlossmann and Desch, 2009; Dostmann et al., 2000; Ruth et al., 1997). We propose their N-termini also dictate their oxidation-dependent phenotypes and suggest a model (described below) by which this could occur.

3.4.3 A model for oxidation-driven activation in PKG Iα and considerations for pathophysiological conditions

The results obtained using the PKG Iα mutant constructs suggest that C117 is necessary and sufficient to stimulate constitutive activation of PKG Iα. Under mild oxidizing conditions, C117 and C195 would form a sulfenic acid intermediates, which can be reduced to the free thiol or condensed to a disulfide form (Figure 5A). Disulfide bond formation between C117 and C195 likely mitigates oxidative activation as no discernible structural changes have been observed within the CBD-A when comparing the reduced and disulfide-bonded forms (Osborne et al., 2011; Kim et al., 2016, 2017). This suggests that the site maintains an inactive conformation. As determined by this study and Landgraf et al., at higher concentrations of oxidant, the maximum velocity of the kinase begins to decay, indicating a rise in constitutive activity. The reversibility of the oxidized phenotype measured previously was determined at concentrations that did not affect the maximum velocity (Burgoyne et al., 2007; Landgraf et al., 1991). This may suggest that formation of the sulfenic and sulfonic acid species at C117 drives the cGMP insensitivity that is observed at high concentrations of H₂O₂. Based upon this, we propose a model of constitutive cGMP-independent activation of
PKG Iα driven by oxidation to either the sulfinic, sulfonic, or sulfonic acid intermediates at C117.

A sequence alignment of PKG Iα and Iβ paired with a structural alignment of the PKG I CBD-A and the PKA RIα:C holoenzyme shows that C117 is oriented proximal to the linker region that connects the autoinhibitory domain and CBD-A. This region contain very low sequence identity (approximately 6%) between Iα and Iβ (Figure 5B). We propose that the presence of the basic residues (Arg82 and Lys83) within the linker region of PKG Iα may act to form hydrogen bonds or a salt bridge with the sulfur acid forms of C117 (Figure 5C). In comparison, this region in PKG Iβ contains hydrophobic residues (Pro97 and Phe98) at the equivalent positions. In this model, we propose that an interaction between the acid form of C117 and the linker region in PKG Iα could alter the conformation of the linker to release the autoinhibitory segment from the catalytic domain, resulting in constitutive activation. Since previous mass spectrometry experiments using isotope-coded affinity-tag (NOxICAT)-labeled PKG Iα peptides showed that C117 and C195 were equally modified by oxidizing agents, the solvent accessibilities and oxidizing potentials of the two residues are likely similar. Thus, differences between the C117S and C195S constructs could be attributed to their locations proximal to other residues within the domain rather than differing oxidizing propensities (Donzelli et al., 2017). Furthermore, this model would provide an explanation of the differing responses to oxidation between the two isoforms.

Signaling by PKG I isoforms is mediated by both substrate targeting and activation (Aitken et al., 1984; Alverdi et al., 2008; Blanton et al., 2013; Casteel et al., 2005; Francis et al., 1996; Kato et al., 2012; Lee et al., 2007; Surks et al., 1999; Zhou, 2011). More importantly, they can be considered independent since disruption of either facet disrupts PKG I signaling pathways (Blanton et al., 2013; Casteel et al., 2005; Feil et al., 1995; Surks et al., 1999). In this study, we sought to separate these aspects of PKG I signaling to better understand how oxidation may regulate each of these processes. The use of a synthetic peptide substrate allowed for a thorough examination of the effect of oxidation independent of substrate targeting through the dimerization domain. Moreover, observations of PKG Iα activation of the KCa1.1 channel indicate that oxidation does not diminish targeting of PKG Iα to the KCa1.1 channel.

PKG Iα has been reported to be activated by cGMP-independent mechanisms in both physiological and pathophysiological states (Burgoyne et al., 2007; Landgraf et al., 1991; Muller et al., 2012; Nakamura et al., 2015; Rudyk et al., 2012). Oxidizing agents, particularly H2O2, have been suggested to play important roles in a number of conditions associated with vascular remodeling, including angiogenesis, atherosclerosis, and hypertension (Chan and Baumbach, 2013a,b; Gray et al., 2016; Kim et al., 2017; Patel et al., 2017; Rashdan and Lloyd, 2015;
Schiffrin, 2012; Warren et al., 2015). Typically, hydrogen peroxide concentrations in mammalian cells are estimated to average between 1-700 nM (Brewer et al., 2015). However, local levels caused by bursts in H$_2$O$_2$ production are suggested to greatly exceed the average value and depend on the distance from the source and local concentrations of antioxidants, such as catalase, glutathione peroxidases, and thioredoxin reductases. A recently developed H$_2$O$_2$ sensor (HyPer-Tau) coupled with super-resolution microscopy suggested that local bursts of hydrogen peroxide can reach 50-100 µM (Warren et al., 2015). The data presented herein indicate that constitutive PKG Iζ activation via oxidation does not occur until 500 µM and involves long-lived exposure to the oxidant. Thus, this mechanism would be specifically relevant to PKG Iζ anchored near hydrogen peroxide sources or under pathophysiological states associated with high global intracellular concentrations of hydrogen peroxide.

3.5 Experimental Procedures

3.5.1 Site-directed mutagenesis of wild-type PKG Iα

Wild-type PKG Iα and PKG Iβ from Bos taurus (NCBI entries NP_776861.1 and CAA70155.1) were cloned into pFAST Bac HTA as previously described (34, 49). The PKG Iα construct was used as a template for site-directed mutagenesis to produce the following mutants: PKG Iα C42S, PKG Iα C42A, PKG Iα C117S, and PKG Iα C195S. The forward and reverse primers used to generate these constructs were synthesized as follows: C42S- 5-AAG AGG AAA CTC CAT AAA AGC CAG TCA GTG-3' (sense) and 5-GGG CAG CAC TGA CTG GCT TTT ATG GAG-3 (antisense); C42A- 5-AAG AGG AAA CTC CAT AAA GCC CAG TCA GTG-3 (sense) and 5-GGC AGC ACT GAC TGG GCT TTA TGG-3 (antisense); C117S- 5-ATC CAA GAG ATT GTG GAT AGT ATG TAC CCA GTG-3 (sense) and 5-GCC GTA CTC CAC TGG GTA CAT ACA ATC CAC AAT-3 (antisense); C195S- 5-ATC CAA GAG ATT GTG GAT AGT ATG TAC CCA GTG-3 (sense) and 5-GCC GTA CTC CAC TGG GTA CAT ACA ATC CAC AAT-3 (antisense); C195S- 5-GCC GTA CTC CAC TGG GTA CAT ACA ATC CAC AAT-3 (antisense); C195S- 5-GCC GTA CTC CAC TGG GTA CAT ACA ATC CAC AAT-3 (antisense). All site-directed mutagenesis experiments were carried out using the QuickChange (Stratagene) method.

3.5.2 Expression and Purification of PKG I constructs

PKG Iα and Iβ wild-type, PKG Iαmutant constructs, and PKG Iα Δ53 were expressed in Sf9 cells using the Bac-to-Bac Baculovirus Expression System as previously described (Moon et al., 2015, 2018). Constructs were purified from
Sf9 cell pellets by Ni immobilized metal ion affinity chromatography (IMAC) using either prepacked (5 mL, BioRad) or hand-packed Ni-NTA columns. Protein purified for use in inside-out patch clamp experiments were prepared without the N-terminal hexahistidine tag, by incubation with His-tagged tobacco-etch virus (TEV) protease at a 1:20 mass ratio (TEV:PKG) during the first dialysis step (50 mM MES, 300 mM NaCl, 1 mM TCEP, pH 6.9, 4°C, 16 h). TEV and the histidine tag were subtracted from the cleaved protein by flowing over a Ni-IMAC column. The resulting flow-through containing PKG I was concentrated to 1 mg/mL and dialyzed against 1 L of 50 mM MES, 150 mM NaCl, 1 mM TCEP, 10% glycerol, pH 6.9 at 4°C. The resulting protein solution was divided into 20-100 µL aliquots, flash frozen in liquid nitrogen, and stored at -80°C.

3.5.3 Determination of H$_2$O$_2$ concentration

The primary H$_2$O$_2$ stock (stored at 4°C) was measured by absorption spectroscopy at $\lambda = 240$ nm (\(\varepsilon = 43.6 \text{ M}^{-1}\text{cm}^{-1}\)) to determine its concentration (Han et al., 1996; Tan et al.). Fresh dilutions were prepared daily from the stock for use in experiments.

3.5.4 Measurement of interprotoper disulfide bond formation by SDS-PAGE gel electrophoresis

PKG I constructs were buffer exchanged (1-4 times) into 50 mM MES, 150 mM NaCl, 1 mM TCEP, pH 6.9 using 3 kDa MWCO ultracentrifugal filter units (Amicon) to remove glycerol (to below 1%) in the storage buffer. PKG concentration was determined by measuring the absorbance at 280 nm (\(\varepsilon = 81,250 \text{ M}^{-1}\text{cm}^{-1}\)) as calculated by ProtParam [51]). All constructs were adjusted to 1 mg/mL (13.1 µM of monomer) and PKG (3.6 µL) was added to 1.4 µL of H$_2$O$_2$ (freshly prepared 10× stocks that ranged from 0-50 mM) and 9 µL of 50 mM MES, 150 mM NaCl, pH 6.9 (in the absence of reducing agent) (14 µL reactions). Reactions proceeded for 30 min at 24°C and were subsequently quenched by the addition of 2 L of 1M maleimide (10% DMSO stock) and 4 L of 5x SDS-loading buffer without reducing agent. Samples were boiled for 2 min at 95°C and separated by SDS-PAGE using 4-20% TGX gradient gels (BioRad). The resulting gels were stained with InstantBlue Protein Stain (Expedeon) and imaged using an Odyssey Infrared Scanner (LI-COR) at 700 nm. Bands were selected and quantified in the Image Studio Software (LI-COR) using intensity analysis relative to background. The background was subtracted by implementing the mean intensity background subtraction method. Images were exported as 600 DPI grey-scale TIFF files for use as figures. Data analysis was achieved using Prism v7 (GraphPad).
3.5.5 In vitro phosphotransferase assays

Activation of reduced PKG I constructs by cyclic 3,5-guanosine monophosphate (cGMP; BioLog) was assessed by measuring phosphorylation of a synthetic peptide substrate (W15, TQAKRKKSLAMA) with γ-32P-ATP as previously described (Dostmann et al., 2000). To measure the cGMP-dependent activation under oxidized conditions, PKG I constructs were pre-incubated with 500 µM H₂O₂ in 50 mM MES pH 6.9 for 30 min at 24°C. Under these conditions, the 1 mM TCEP in the PKG storage buffer was diluted to a final concentration of 500 nM. Oxidized PKG I was then added to the other reaction components (in the absence of reducing agent) to initiate the reactions. All subsequent steps were performed following the same procedure as the reduced PKG conditions.

3.5.6 Measurement of K_{Ca1.1} activation by inside-out patch clamp of vascular smooth muscle cells

Male C57BL/6 mice (3-6 month old) were euthanized using procedures approved by the Institutional Animal Care and Use Committee at the University of Vermont and performed in accordance with the National Institutes of Health Policy on the care and use of laboratory animals. The anterior and posterior pial and superior cerebellar arteries were isolated and cleaned of connective tissue. The arteries were then incubated at 37°C in dissociation solution (55 mM NaCl, 80 mM Na-glutamate, 6 mM KCl, 2 mM MgCl₂, 10 mM glucose, 10 mM HEPES, pH 7.3) containing 0.5 U/mL papain (Worthington) and 1 mg/mL dithioerythritol for 12 min followed by 10 min incubation in dissociation solution containing 1 mg/mL collagenase (Worthington Type 4). Arteries were then removed and placed back in the isolation solution without enzymes for an additional 10 min before trituration with a polished Pasteur pipette to yield single smooth muscle cells. Cells were left to stick to the glass coverslip in the experiment chamber for 10 min before use.

Membrane patches were pulled from the isolated vascular smooth muscle cells to achieve the inside-out configuration and contained 2-10 K_{Ca1.1} channels each. K_{Ca1.1} single channel currents were recorded under the following conditions: the pipette solution contained 6 mM KCl, 134 mM NaCl, 1 mM MgCl₂, 10 mM HEPES, pH 7.4 (NaOH); the bathing solution contained 135 mM KCl, 2 mM MgCl₂, 1.4787 mM CaCl₂, 2 mM EGTA, 10 mM HEPES, adjusted to pH 7.2 with 14.85 mM KOH. The concentrations of free Ca²⁺ on the intracellular face was calculated to be 500 nM using the online software WEBMAXC Standard (https://web.stanford.edu/~cpatton/webmaxcS.htm; Stanford University, Stanford, CA, USA). Single K_{Ca1.1} channel recordings were obtained at room temperature (25°C) for at least 5 min at 0 mV and using an Axopatch 200 B amplifier coupled to a Digidata 1332A digital-to-analog converter. Currents were filtered at
2 kHz, digitized at 20 kHz using pCLAMP v9.2 software (Axon Instruments), and then analyzed with Clampfit v9.2 using the half-amplitude threshold method. The baseline activity was recorded for at least 20 min to ensure patch stability. The experimental condition was measured after the addition of 100 µM ATP and either reduced, inactive PKG Iα (no cGMP added), PKG Iα constructs activated with 5 µM cGMP, or PKG Iα constructs activated with 500 µM H$_2$O$_2$. For the oxidized condition, PKG Iα constructs were first buffer exchanged into 50 mM MES, 150 mM NaCl, 1 mM TCEP, pH 6.9 using 3 kDa MWCO ultracentrifugal filter units (Amicon) to remove the glycerol (to below 1%) in the storage buffer. Assuming a 1:1 negation of TCEP with H$_2$O$_2$ (Han et al., 1996; Tan et al.), PKG Iα constructs were oxidized with a final concentration of 500 µM H$_2$O$_2$ at 24°C for 30 min before use in patch clamp experiments. Addition of PKG Iα constructs to the bath solution (5 mL) resulted in a final concentration of H$_2$O$_2$ that was less than 500 nM. The fold activation was determined as the open probability of the channels (NPo) during the experimental condition/ NPo at baseline. The fold activations of the experimental conditions were compared to the control condition (reduced PKG Iα, 0 cGMP) using unpaired t-tests with Welch’s correction in Prism 7 (GraphPad). K$_{Ca}$1.1 channel open times within 5 min windows were measured from full-length PKG Iα, C42S, and their respective baseline recordings using Clampfit v9.2. The mean channel open dwell times (τ) and interquartile ranges were calculated using the Mann-Whitney test and the differences between the baseline control and experimental dwell times were compared using a non-parametric Hodges-Lehmann analysis in Prism 7 (GraphPad). Channel recordings examining the effects of C42S on K$_{Ca}$1.1 were run under symmetrical K$^+$, 5 µM Ca$^{2+}$ in the presence of reduced C42S (-cGMP) at +40, 0, and -40 mV.

### 3.5.7 Sequence Alignments

Sequences for PKG Iα and Iβ sequences were downloaded from the National Center for Biotechnology Information (NCBI) database, and a multiple sequence alignment was performed with JalView Desktop using CLUSTAL-O (Waterhouse et al., 2009; Sievers et al., 2011). The following accession numbers were used: *H. sapiens*: Iα-NP_001091982.1 and Iβ-NP_006249.1; *M. musculus*: Iα-NP_001013855.1 and Iβ-NP_035290.1; *F. catus*: Iα-XP_023096056.1 and Iβ-XP_023096055.1; *B. taurus*: Iα-NP_776861.1 and Iβ-CAA70155.1.

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Figure 3.6: PKG Iα oxidation by ambient air. Ambient air oxidation of reduced PKG Iα was achieved through four rounds of buffer exchange into 50 mM MES, 150 mM NaCl, pH 6.9 to remove the TCEP. Oxidation was visualized by denaturing, non-reducing SDS-PAGE in the absence of maleimide.

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3.7 Conflicts of Interest

The authors declare that there are no outstanding conflicts of interest.

3.8 Author Contributions

J.L.S., T.M.M., and W.R.D. conceived of and designed the experiments. J.L.S. and A.B. performed experiments. J.L.S., T.M.M., and W.R.D. wrote the manuscript with contributions from A.B. and M.T.N.

3.9 Supplemental Figures
Figure 3.7: Sequence alignment of mammalian PKG Iα and Iβ isoforms denoting the locations of cysteine residues confirmed to form disulfide bonds. Sequence alignment colored by BLOSUM62 score denoting A) the placement of C42 within the dimerization domain and B) the location of C117 and C195 in relation to nearby structural features within the CNB A-site of PKG Iα, including the A helix, the B/C helix, and the phosphate-binding cassette.

Figure 3.8: Oxidation-dependent activation of PKG Iβ. A) The cGMP-dependent activation of PKG Iα (black) and Iβ (orange) under reducing (solid circles and line) and oxidizing (open circles and dashed line) conditions by an in vitro phosphotranspherase assay. B) Non-reducing, denaturing SDS-PAGE of PKG Iβ exposed to increasing concentrations of H₂O₂ (0-2 mM). The dashed line indicates the point at which the concentration of hydrogen peroxide overcomes the concentration of TCEP in the buffer. H₂O₂ values corrected for the presence of TCEP are shown in red.
Figure 3.9: **Activity of $\text{K}_{\text{Ca}1.1}$ treated with C42S by patch clamp in the inside-out configuration.** $\text{K}_{\text{Ca}1.1}$ channels were exposed to C42S under reducing conditions in the absence of cGMP and ATP. Activity was measured under symmetrical $\text{K}^+$ at +40, 0, and -40 mV.
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Chapter 4

Discussion and Future Directions

The type Iα and Iβ cGMP-dependent protein kinases (PKG Iα and Iβ) are essential regulators of vascular tone and systemic blood pressure. Located in the smooth muscle layer of resistance vessels, these proteins phosphorylate multiple intracellular targets, culminating in smooth muscle relaxation and subsequent vasodilation. Both isoforms are activated by the second messenger, 3’5’-cyclic guanosine monophosphate (cGMP) (Hofmann et al., 2009). In addition, PKG Iα can be activated by oxidation (Landgraf et al., 1991; Burgoyne et al., 2007). The physiological importance of the cGMP-dependent activation of PKG I isoforms is well-established; however, the importance of the oxidation-dependent mechanism is an active area of investigation (Nakamura et al., 2015; Prysyazhna et al., 2012; Prysyazhna and Eaton, 2015; Rudyk et al., 2013; Scotcher et al., 2016). For both mechanisms, there are multiple gaps in our understanding of how the PKG I isoforms respond to cGMP and/or oxidizing conditions and become active. Therefore, this dissertation sought to address some of these gaps and—in regard to the oxidation-dependent mechanism—provide a solid biochemical framework to support future studies investigating its physiological or pathophysiological roles.

Chapters 2 and 3 present our findings on the cGMP- and oxidation-dependent activation mechanisms of PKG Iα, respectively.

In Chapter 2, we hypothesized that a functional, monomeric form of PKG Iα could be expressed that is autoinhibited and activated in a cGMP-dependent manner, and, furthermore, that this construct could be used to determine the influence of the dimeric state on cGMP-dependent activation and binding. As anticipated, the truncation construct, Δ53, was expressed and found to be monomeric, autoinhibited, and activated with cGMP. We also found that Δ53 adopts a similar 3D structure to the PKA holoenzymes, RIIα:C and RIIβ:C. While measuring cyclic nucleotide-dependent activation and binding, we observed approximately 40-fold discrepancies between the $K_a$ and $K_D$ for both cGMP and cAMP, that cyclic nucleotide binding is uncooperative, and that Δ53 lost selectivity for cGMP over cAMP compared to wild-type. These results led to the following conclusions: 1) Autoinhibition occurs in cis; 2) Binding to the A-site is necessary and sufficient for cGMP-dependent activation; 3) Selectivity for cGMP over cAMP within the A-site is driven through contacts outside of the regulatory domain A-site (likely...
located in the N-terminus); 4) Cooperativity is driven in trans through interprotomer contacts that are facilitated by the N-terminus which ensures monomers are within close proximity to form these contacts. These conclusions suggest that cooperative activation must be controlled through an inducible-dimeric interaction and not through cooperative binding of cyclic nucleotide sites within the same protomer (these models were described in Chapter 1.2.5). Furthermore, binding to 1/4 of the sites appears to be sufficient for full activation of PKG Iα (i.e. activation of two catalytic domains within the homodimer). Thus, this model further implies that its structure facilitates rapid activation of two catalytic domains in response to cGMP.

In Chapter 3, we hypothesized that cysteine residues present in the cGMP-binding A-site regulate oxidation-dependent activation of PKG Iα. To test this, we introduced mutations at cysteine residues in PKG Iα that have been previously implicated in mediating this activation mechanism (Landgraf et al., 1991). These constructs, as well as the truncation construct that was characterized in Chapter 2, were used to test oxidation-dependent activation using both a synthetic peptide substrate and the full-length substrate, K Ca 1.1. Using the synthetic peptide substrate, we found that C42S and Δ53 were activated by oxidation with 500 µM H2O2, and furthermore, that C117S was not activated by oxidation whereas C195S was. These results were corroborated by the patch clamp experiments in the inside-out configuration and led to the following conclusions: 1) The interprotomer disulfide bond between C42 and C42 does not mediate oxidative activation of PKG Iα; 2) Oxidation at C117 is necessary and sufficient for activation via this mechanism; 3) Activation requires high concentrations of H2O2 that may only be achieved under pathophysiological conditions. Based on these conclusions, we proposed a model wherein activation via oxidation is achieved through contacts between the oxyacid at C117 and residues in the linker region between the autoinhibitory and regulatory domains rather than through disulfide formation with C195.

4.1 Determining the source of cGMP selectivity in the A-site

The data presented in Chapter 2 indicate that cyclic nucleotide selectivity in the cGMP-binding site A (A-site) must be conferred through residues outside of that domain. Δ53 was shown to have decreased selectivity for cGMP over cAMP based on activity and cyclic nucleotide affinities for the A-site. Furthermore, PKG Iα1-326 also showed decreased selectivity for cGMP based on relative affinities. Thus, both the catalytic domain and the N-terminus contribute to se-
selectivity for cGMP. The SAXS analysis of Δ53 suggested that this construct was similar in overall shape to the PKA holoenzymes (RIα:Cα and RIIβ:Cα). The structure of PKA (PDB 2QCS) aligned with the crystal structure of the PKG regulatory domain (PDB 3SHR) supports this hypothesis and indicate that the guanine moiety of cGMP would be exposed to residues in the catalytic domain as well as residues upstream of the autoinhibitory domain in the inactive state.

Similar analyses of the N- and C-terminally truncated forms of PKG Iβ have not been performed to determine whether this isoforms confers selectivity through contributions from these regions. Since the regulatory domains of both isoforms are identical, selectivity must be conferred through one or both regions. PKG Iα and Iβ are only 29% and 6% identical in the dimerization domain and linker region, respectively (Appendix 2). Assuming the N-terminally truncated isoforms exhibit the same phenotype of cyclic nucleotide selectivity loss, their structural dissimilarity would be advantageous for narrowing down which residues are involved. In the B-site, selectivity for cGMP over cAMP is driven through binding to O6 of the guanine moiety by a conserved Arg residues at position 297 (Huang et al., 2014). In PKA, selectivity for cAMP appears to be driven by the lack of residues that bind to the moiety—since the residues in that region are aliphatic (Gly314 and Ala334)—rather than through specific residues that select for adenine.

Figure 4.1: cGMP Binding Pocket of CNB-B and Its Comparison with the PKA:cAMP Complex. Comparison between the PKG Iβ cGMP binding pocket and PKA RIα cAMP pocket. The cGMP pocket of PKG Iβ in red is shown on the left panel, and the cAMP binding pocket of PKA RIα (PDB code: 1RGS) in gray is shown on the right. Key cyclic nucleotide binding residues are shown as sticks. An ordered water molecule is shown as a blue sphere. (Huang et al., 2014)
4.2 The physiological role of oxidized PKG I

4.2.1 Observations based on the interprotomer disulfide bond that forms between C42 and C42’

The observations described in Chapter 3 support the conclusion that oxidation at C42 does not mediate oxidation-dependent activation of PKG Iα. In addition, we found that C42S displayed dramatically different characteristics from PKG Iα wild-type. The in vitro phosphotransferase assay showed that C42S has a 3-fold higher $K_a$ and a maximal activity that is half that of the wild-type enzyme. Furthermore, application of inactive, reduced PKG Iα C42S, the reduced form activated with cGMP, and the form oxidized with $H_2O_2$ caused an abundance of short $K_{Ca}1.1$ channel openings (channel flickers), which were not observed with PKG Iα wild-type. The phenylalkylamine-type $K_{Ca}1.1$ channel blockers, such as verapamil and gallopamil, similarly induce flickers in dose dependent manners by preventing transition of the channels to other states (Ballesteros et al., 2000). C42S may induce flickers through a similar mechanism. Regardless, these observations collectively indicate the C42S construct does not behave the same as PKG Iα wild-type.

The physiological significance of oxidation-dependent activation of PKG Iα has been supported by experiments using the C42S knock-in mouse model (Prysyazhna et al., 2012; Rudyk et al., 2013). However, the conclusions of those studies rely on the assumption that reduced C42S behaves the same as PKG Iα wild-type. Mutations in the leucine zipper of PKG Iα have been shown to disrupt substrate targeting and result in hypertensive mouse models (Michael et al., 2008; Surks and Mendelsohn, 2003; Surks, 2007). Thus, the hypertensive phenotype observed with the C42S knock-in mouse model may also be due to augmented substrate targeting abilities. This is supported by the complete inability of C42S...
to bind MYPT1 (Burgoyne et al., 2007). The cysteine to serine mutation introduces hydrogen bond potential and can form different rotameric configurations from cysteine, which could cause the C-terminal region of the helices to kink (Ballesteros et al., 2000). This hypothesis could be tested by circular dichroism to compare the helical contents of isolated wild-type and C42S leucine zipper constructs (residues 2-47). The channel flickering caused by application of inactive, reduced C42S suggests that this phenomenon is independent from kinase activity, and, thus, not caused by phosphorylation of a different residue on the channel, and also suggests that K\textsubscript{Ca}1.1 channel activity may be compromised even under stimuli that induce vasoconstriction.

4.2.2 Measuring differences in substrate targeting between reduced and oxidized PKG I\alpha

As discussed in Chapter 3, substrate phosphorylation by PKG I\alpha appears to be driven through two independent facets: activation of PKG (i.e. exposure of the catalytic domain through release of the autoinhibitory segment) and substrate targeting through the dimerization domain. Moreover, loss of either abolishes PKG I\alpha’s ability to phosphorylate substrates. Loss of substrate targeting independent from catalytic activity was shown through mutational disruption of the leucine zipper; this mutant was unable to phosphorylate the full-length substrates RhoA and the MBS of MYPT1, but retained cGMP-dependent activation and ability to phosphorylate the peptide substrate, BPDEtide (Blanton et al., 2013). Catalytic activity was abolished by the T517A mutation which prevented phosphorylation of the activation loop residue required to coordinate the catalytic core (Feil et al., 1995). However, studies have rarely separated effects on targeting from catalytic function, relying heavily on measurements of the endpoint—substrate phosphorylation. In Chapter 3, reduced and oxidized PKG I\alpha wild-type were shown to increase the fold activations of the K\textsubscript{Ca}1.1. This was surprising since there is an approximate 4-fold difference in the maximal velocities of these two mechanisms of activation (Reduced=4.39 vs. Oxidized=1.13 μmol×min\(^{-1}\)×mg\(^{-1}\)). Thus, one could hypothesize that oxidation at C42 in the dimerization domain increases the binding affinity for K\textsubscript{Ca}1.1, which compensates for the poor catalytic activity. Increased substrate affinity of oxidized PKG I\alpha has been suggested previously, but has not been highly scrutinized experimentally (Burgoyne et al., 2007; Kalyanaraman et al., 2017).

To extend this further, one could hypothesize that the dimerization domains of reduced and oxidized PKG I\alpha have different substrate targeting capabilities, and by extension, that the cGMP and oxidation-dependent activation mechanisms preferentially target different substrates. Since cGMP has been shown to
be ubiquitously produced throughout vascular smooth muscle cells (Nausch et al., 2008), this would be pertinent to pools of PKG Iα close to sources of reactive oxygen species (ROS) production. A two-pronged approach would be more rigorous in testing this hypothesis:

1. **A dual-labeling proteomics approach to detect differential phosphorylation patterns of PKG Iα in isolated smooth muscle cells stimulated with either a nitric oxide (NO) donor to stimulate cGMP production or with H₂O₂.** H₂O₂ production could be stimulated by direct micro-injection into the cytoplasm. Initially, a global cytoplasmic approach would be beneficial since there are multiple intracellular ROS sources. Phosphorylation patterns caused by stimulation of specific ROS sources could be used to discern their individual contributions. However, this would require measuring the dose dependencies of the stimuli used to generate ROS since catalytic activity of PKG Iα is compromised by high H₂O₂ concentrations, possibly through formation of irreversible cysteine oxidation states at C516 in the activation loop (the region of the catalytic domain that coordinates the active site components).

2. **Direct binding measurements of reduced and oxidized full-length PKG Iα and the isolated leucine zipper domain to full-length PKG Iα substrates.** The full-length substrates could be limited to those that are firmly established in the PKG field, such as KᵥCa1.1, RhoA, RGS2, and MBS. To further cement the observations of the PKG Iα C42S construct described in Chapter 3, the binding affinities between substrates and the full-length C42S construct or the isolated leucine zipper with the C42S mutation could also be tested.

Another critical experiment is to determine the oxidation potentials of the cysteine residues in PKG Iα through cyclic voltammetry. If C42 is more sensitive to oxidation than C117 (and assuming the hypothesis above regarding substrates targeting is true), this would suggest that PKG Iα could have increased affinity for substrates through its dimerization domain while retaining the ability to be activated with cGMP. Furthermore, lower concentrations required to oxidize C42 would increase the likelihood that this effect is relevant under physiological conditions. H₂O₂ concentrations in mammalian cells are estimated to average between 1-700 nM (Brewer et al., 2015). However, local levels caused by bursts in H₂O₂ production are suggested to greatly exceed the average value and depend on the distance from the source and local concentrations of antioxidants, such as catalase, glutathione peroxidases, and thioredoxin reductases. A recently developed H₂O₂ sensor (HyPer-Tau) coupled with super-resolution microscopy suggests local bursts can reach 50-100 µM (Warren et al., 2015). Our results in Chapter 3 show that 100 µM H₂O₂ slightly increases the basal activity of PKG Iα, though it is
unclear whether this is sufficient for phosphorylation of physiological substrates (particularly if it is compensated by increased substrate affinity).

4.2.3 PKG Iβ as the REDOX resistant isoform

In Chapter 3, we found that PKG Ix exhibited a much greater response to H$_2$O$_2$ compared to PKG Iβ, as shown by the greater increase in minimal velocity. Since both isoforms displayed similar decreases in their maximal velocities, the loss in cGMP sensitivity was much greater for oxidized PKG Ix. Conversely, these data also suggest that PKG Iβ retains more of its cGMP-dependent activation characteristics and may be considered the more "oxidation resistant" isoform.

PKG Iβ in vascular smooth muscle cells is found tethered to the endoplasmic reticulum (ER) membrane where it phosphorylates the IP3- receptor associated cGK substrate (IRAG), resulting in decreased Ca$^{2+}$ efflux from the ER into the cytoplasm (Kato et al., 2015; Schlossmann et al., 2000). ER and mitochondria can be in close proximity within cells, which facilitates crosstalk between the two organelles (Marchi et al., 2014). Thus, some PKG Iβ localized to the ER would be susceptible to ROS (superoxide) generated during ATP production. The presence of PKG Iβ, rather than PKG Ix, would ensure there is functional (cGMP sensitive) PKG I in that cellular microdomain despite fluctuating ROS levels.

PKG Iβ is the only PKG isoform expressed in platelets and controls von Willibrand factor-induced platelet activation and subsequent thrombus formation (Li et al., 2003). These cells appear to have the highest metabolic demands relative to their size (diameter = ~ 2-3 µm; volume = ~ 10 µm$^3$) with over half of their ATP requirements achieved through oxidative phosphorylation (OXPHOS) (Kramer et al., 2014). In contrast, vascular smooth muscle cells range between 20-500 µm in diameter (on the short and long axes, respectively) with an approximate cell volume of 800 µm$^3$ (depending on the type of resistance vessel) and have highly efficient metabolic processes to sustain actin/myosin-dependent contractility (Luo et al., 2016; Yang et al., 2017). Thus, higher ROS generation owing to the intrinsic characteristics of platelets (high OXPHOS rates in a small space) may have exerted selective pressure for PKG Iβ expression over PKG Ix to maintain cGMP-dependent activation capacity.


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Appendices
Appendix A

Synthetic Peptides as cGMP-Independent Activators of cGMP-Dependent Protein Kinase Iα
Supplemental Information

Synthetic Peptides as cGMP-Independent Activators of cGMP-Dependent Protein Kinase Iα

Thomas M. Moon, Nathan R. Tykocki, Jessica L. Sheehe, Brent W. Osborne, Werner Tegge, Joseph E. Brayden, and Wolfgang R. Dostmann
Synthetic peptides as cGMP-independent activators of cGMP-dependent protein kinase Iα

Thomas M. Moona, Nathan R. Tykocki, Jessica L. Sheehe, Brent W. Osborne, Werner Tegge, Joseph E. Brayden, and Wolfgang R. Dostmann

a The University of Vermont, College of Medicine, Department of Pharmacology, 89 Beaumont Ave, Burlington, VT 05405, USA
b Defined Health, 25-B Hanover Road Suite 320, Florham Park, NJ 07932, USA
c Helmholtz Centre for Infection Research (HZI), Department of Chemical Biology, Inhoffenstraße 7, Braunschweig, Germany

Abstract

PKG is a multifaceted signaling molecule and potential pharmaceutical target due to its role in smooth muscle function. A helix identified in the structure of the regulatory domain of PKG Iα suggests a novel architecture of the holoenzyme. In this study, a set of synthetic peptides (S-tides), derived from this helix was found to bind to and activate PKG Iα in a cGMP-independent manner. The most potent S-tide derivative (S1.5) increased the open probability (NPo) of the potassium channel KCa1.1 to levels equivalent to saturating cGMP. Introduction of S1.5 to smooth muscle cells in isolated, endothelium-denuded cerebral arteries through a modified reversible permeabilization (RP) procedure inhibited myogenic constriction. In contrast, in endothelium-intact vessels, S1.5 had no effect on myogenic tone. This suggests that PKG Iα activation by S1.5 in vascular smooth muscle would be sufficient to inhibit augmented arterial contractility that frequently occurs following endothelial damage associated with cardiovascular disease.

Introduction

In arterial smooth muscle, type I cGMP-dependent protein kinase (PKG I) functions as a central signaling node for the nitric oxide (NO) and natriuretic peptide pathways that activate soluble and particulate guanylyl cyclases, respectively (Bian and Murad, 2007; Hofmann et al., 2009; Kemp-Harper and Schmidt, 2009; Kuhn, 2003; Potter et al., 2009). The subsequent production of cyclic guanosine-3′,5′-monophosphate (cGMP) by these enzymes leads to the activation of PKG. This is accomplished through direct binding of the...
PKG I isoforms modulate excitation-contraction coupling in smooth muscle by reducing intracellular Ca\(^{2+}\) levels and promoting dephosphorylation of regulatory myosin light chains (Carrier et al., 1997; Feil et al., 2002; Kawada et al., 1997; Wellman et al., 1996; Wu et al., 1998). This is complemented by PKG I\(\alpha\) phosphorylating the large conductance Ca\(^{2+}\)-activated potassium (K\(_{Ca}\)1.1/BK) channel (Schubert and Nelson, 2001). The PKG-mediated activation of the channel increases the flow of potassium ions across the membrane, hyperpolarizing the cell, inhibiting voltage dependent calcium channels (VDCCs), and thereby promoting vasodilation (Hofmann et al., 2014; Robertson et al., 1993; Sausbier et al., 2000).

All major therapies aimed at modulating blood flow through PKG-dependent mechanisms rely on regulating the rate of cGMP turnover, rather than the activity of PKG itself (Bryan et al., 2009; Evgenov et al., 2006; Kots et al., 2011; Salloum et al., 2012; Schlossmann and Hofmann, 2005). The use of PKG isoforms as pharmaceutical targets has been hampered by a lack of understanding of their mechanisms of activation due to their complex architectures. However, elucidating the regulatory mechanism of the PKG holoenzymes is central to guiding pharmacological discoveries relevant to prevention and treatment of vascular diseases. Recently, we solved the crystal structure of the intact regulatory domain of PKG I (Osborne et al., 2011). This structure exposed a unique helical domain that stabilizes an unexpected dimer interface between monomers in the asymmetric unit. Here we present the development of cGMP-independent peptide activators of PKG I\(\alpha\), derived from this helical segment found bridging the regulatory and catalytic domains, and demonstrate their utility in intact tissue.

**Results**

**PKG structure, design and synthesis of S-tides**

The structure of of PKG I\(\alpha\) (78–355; PDB ID: 3SHR) identified an interaction formed by two bridging alpha helices between opposing protomers (Figure 1A) (Osborne et al., 2011). The complementary docking sites observed in either subunit are characterized by hydrophobic pockets located adjacent to the low-affinity cGMP binding sites. Each pocket buries over 1350 Å\(^2\) of surface area, and these interacting elements are conserved in PKG I isoforms (Moon et al., 2013). The C-terminal residues on the helix (F350, F351, N353, and L354) and a constellation of van der Waals and hydrogen bonds that form the aforementioned pocket were termed the “knob” and “nest,” respectively (Figure 1B). The hallmark of this interaction is the direct contact between the knob and residues that compose the nest, which also contribute to the formation of the phosphate binding cassette (PBC) (Figure 1C). It is the PBC — and its closure upon cyclic nucleotide binding — that facilitates the structural reorganization to release the catalytic domain in PKG. In full length PKG I\(\alpha\), cooperativity was abolished through disruption of this unique interaction site (Osborne et al., 2011). Thus, we concluded that targeting the knob-nest site through synthetic, helical peptides may affect kinase activity. To this end, we synthesized the full alpha helical segment in PKG I comprising residues 329–358 (S1.1) and a series of peptides derived from this central sequence, subsequently denoted “S-tides” (Table 1).
Preparation of cyclic nucleotide-free type I PKG isoforms

Previous purification methods of recombinant PKG I isoforms involved the use of cyclic nucleotide affinity chromatography, typically via agarose-linked cAMP (Dostmann et al., 2000; Feil et al., 1993). However, in order to examine the cyclic nucleotide-independent effects of the aforementioned peptides on PKG I activity, enzyme preparations were required that did not encounter elevated cyclic nucleotide concentrations during the purification process. To meet this need, we engineered PKG Iα and Iβ constructs carrying a cleavable N-terminal hexahistidine tag for isolation by Ni-IMAC chromatography (see methods).

For both isoforms, purified kinase preparations reconstituted in in vitro assays demonstrated similar kinetic constants as have been previously reported (Figure S1A, S1B) (Dostmann et al., 1999; Dostmann et al., 2000; Feil et al., 1995; Feil et al., 1993; Landgraf et al., 1991; Ruth et al., 1991; Scholten et al., 2007). We found that cleavage of the hexahistidine tag did not alter kinase activation kinetics (Figure S1C). To further examine the influence of the purification method on the activity of the enzyme, we isolated the His-tagged PKG Iα by 6-AEA-cAMP agarose affinity chromatography and observed no significant differences in its activation constants (Figure S1D). Although residual quantities of cAMP were detected following dialysis of PKG Iα purified by 6-AEA-cAMP agarose, no detectable levels of cGMP or cAMP were found following the Ni-IMAC purification procedures of PKG Iα (Figure S2). In general, for our Ni-IMAC-purified PKG preparations, we observed low basal activities. This resulted in 20-fold and 15-fold increases in activity under saturating quantities of cGMP for PKG Iα and Iβ, respectively (Figure S1A, S1B).

S-tides act as cGMP-independent activators of PKG Iα

Any effect of S-tides on PKG Iα activity must be preceded by a binding event. Using surface plasmon resonance spectroscopy (SPR), we first examined the binding of the kinase to immobilized peptides. Repeated, single injections of PKG Iα on sensor chips covalently bound to a subset of S-tides found that PKG Iα associated with immobilized S1.1 and S1.5 (Figure S3Aa, S3Ab), but not S-tides lacking the knob residues by deletion or mutation (S1.3 and S1.6). The sequence-scrambled derivative (S1.7) also did not bind to the kinase (Figure S3Ac). While the projected maximal association was predicted to be within 10 minutes, this slow association was accompanied by an even slower dissociation of the kinase from the peptide-affixed surface (Figure S3Aa). To assess the reversibility of binding, peptide-affixed sensor chips were cleared of PKG using 1% SDS. Identical binding profiles were obtained for repeated injections of PKG followed by SDS washes. Next, PKG Iα was immobilized on the sensor chip and then probed with S-tides. Using this method, similar results were obtained (Figure S3B). However, these surfaces were unable to be regenerated by SDS, indicative of the sensitivity of PKG to denaturation by the detergent.

To examine the concentration-dependent effect of the S-tides on PKG activity, we needed to account for their apparent slow rate of association as assessed by SPR. Thus, all activity assays were performed under pre111 incubation conditions (15 minutes at 30°C, see methods). We observed robust and cooperative activation of PKG Iα by the parent compound, S1.1. As a control, we verified that S1.1 did not affect the K₅₅ for the substrate.
peptide W15 (Figure S4) (Dostmann et al., 1999). An activation constant of 35 µM in the absence of cGMP reached maximal efficacy of 80% as compared to cGMP controls (Figure 2A, Table 1). Shorter incubation periods resulted in dampening of the peptide’s potency (K_a = 300 µM) (Figure S5). Based on this observation, we concluded that our preincubation strategy provides the time necessary for S-tide association with the kinase, as corroborated by SPR.

Next, a series of S1.1 derivatives was analyzed to probe the roles of the C-terminal amino acids in activating PKG Iα (Table 1). Deletion of the first three C-terminal residues (S1.2) maintained native potency (K_a = 35 µM) but exhibited decreased efficacy (60% of maximum). Further deletion of C-terminal residues that include the knob (S1.3) completely abolished kinase activity (Figure 2A), suggesting that these residues play a vital role in promoting kinase activation. Finally, we found that alanine substitution of two knob residues (F350A and F351A, as found in peptide S1.6) was equivalent to removal of the entire knob. Likewise, a scrambled control of the parent peptide (S1.7) did not activate the kinase (Figure S5A).

In general, we found that this first set of S-tides possessed helical secondary structure as assessed by circular dichroism (CD) spectroscopy (Figure 2C). Peptides S1.1 and S1.2 displayed a similar degree of helicity; however, when the knob resides were deleted (S1.3), helicity was compromised but not completely lost. Mutation of the knob (S1.6) resulted in an increased degree of helicity. In contrast, the sequence-scrambled control (S1.7) was found to be helical (Figure S5B).

Next, we probed N-terminal deletions of S1.1. The S-tides S1.4 and S1.5, which had three and 6 amino acids truncated (Table 1), showed step-wise increases in potency to a minimal activation constant of 3 µM (S1.5), while retaining similar maximal velocities at 80% (Figures 2B and S6A). Further N-terminal truncations through single amino acid removal (S1.9–S.11) reversed this trend (Figures 2B and S6B). While the activation constants for S1.9 and S1.10 returned to values similar to S1.1, removal of one additional amino acid (D337, S1.11) resulted in a complete loss of activity. Moreover, for all N-terminal deletions, we observed a correlation between the loss in helicity and the loss in potency (Figures 2B, 2D, and S7). Given that S1.5 was found to be the most potent activator for PKG Iα, we investigated its efficacy and potency for activating PKG Iβ. Interestingly, we observed no detectable activation of PKG Iβ using the S1.5 peptide (Figure S8).

**S1.1 and S1.5 but not scrambled peptide increases K_{Ca}1.1 channel open probability in inside-out patches from VSM**

To assess the functional relevance of the S-tides, we investigated their effects on the large conductance calcium-activated potassium channel (K_{Ca}1.1), as it is one of the few well-established molecular targets of PKG Iα in VSM (Robertson et al., 1993; Sausbier et al., 2000). Inside-out membrane patches derived from freshly isolated cerebral artery myocytes demonstrated consistent K_{Ca}1.1 channel openings in the presence of 500 nM Ca^{2+} for every experimental condition tested (Figure 3A, *left panel*). Upon addition of recombinant PKG Iα to the bath solution (cytosolic face) and in the presence of 50 nM cGMP to mimic basal levels of cGMP typically found in VSM (Francis et al., 1988; Jiang et al., 1992), we...
observed no significant change in $\text{K}_{\text{Ca}}1.1$ open probability (NPo) (Figure 3A, right panel, 3B). The addition of 5 µM cGMP to evoke saturating conditions was sufficient to raise the NPo eight-fold above the control ($p<0.002$) (Figure 3B). Similar to the activation by saturating cGMP, we observed that 100 µM S1.1 pre-incubated with PKG Iα substantially increased (6-fold, $p<0.05$) $\text{K}_{\text{Ca}}1.1$ channel activity. Likewise, 10 µM S1.5 gave near-identical results (5-fold, $p<0.002$). This increase in NPo is consistent with PKG-mediated stimulation of the channel as previously described (Alioua et al., 1995; Alioua et al., 1998). In contrast, introduction of the scrambled control peptide S1.7 did not stimulate $\text{K}_{\text{Ca}}1.1$ (Figure 3A, lower panel).

**Paxilline-induced constriction is augmented in S1.5-treated arteries**

Smooth muscle $\text{K}_{\text{Ca}}1.1$ activation represents a predominant vasodilatory, negative feedback mechanism in myogenically-active blood vessels (Nelson et al., 1995). Thus, we investigated the physiological effects of S1.5 on vessel diameter in the presence or absence of the $\text{K}_{\text{Ca}}1.1$ blocker paxilline. We selected S1.5 for its potency and selectivity toward recombinant PKG Iα. Because S1.5 was unable to cross the plasma membrane unaided, we introduced the molecule to intact arteries using a reversible permeabilization (RP) procedure as previously described (Earley et al., 2004) (see methods). RP utilizes the activation of P2X7 receptors that are specifically expressed in endothelium and VSM cells (Lesh et al., 1995).

To test whether S1.5 was able to enter VSM cells through RP, we synthesized an S1.5 analog containing an N-terminal fluorescein tag (FITC-S1.5). Posterior cerebral arteries (PCA) were exposed to either S1.5 or FITC-S1.5 using the RP protocol. Individual VSM cells were dissociated using papain and collagenase, and analyzed by confocal fluorescence microscopy. Smooth muscle cells from arteries treated with FITC-S1.5 showed clear internalization of the peptide with diffuse staining throughout the cytosol. VSM cells from arteries treated with non-labeled S1.5 did not fluoresce (Figure 4).

Next, we studied the functional effects of S1.5 in isolated arteries. S1.5 significantly reduced myogenic tone development in endothelium-denuded PCAs, as compared to controls (39% vs. 66%; $p<0.05$) (Figure 5Aa, 5Ab, 5Ac). Subsequent addition of the $\text{K}_{\text{Ca}}1.1$ inhibitor paxilline (1 µM) resulted in an additional constriction that was significantly greater in S1.5-treated arteries than controls (26% vs. 5%; $p<0.001$) (Figure 5Ad). Interestingly, the maximal constriction (% constriction in the presence of paxilline) was not different between control and S1.5 treated arteries (Figure 5Ae). This indicates that the reduction in myogenic tone in the S1.5-treated arteries was due primarily to augmented $\text{K}_{\text{Ca}}1.1$ activity.

In PCAs with intact endothelium, we observed no significant difference in myogenic tone between control and S1.5-treated arteries, but found that paxilline caused a larger constriction in S1.5 treated arteries as compared to controls (12% vs. 4%; $p<0.02$) (Figure 5Ba–d). However, as with endothelium-denuded vessels, maximal constriction in the presence of paxilline was not different between control and S1.5 treated arteries (Figure 5Be). These data indicate that activation of endogenous PKG Iα by S1.5 leads to a net, $\text{K}_{\text{Ca}}1.1$ channel-driven relaxation of smooth muscle cells in intact posterior cerebral arteries.
Discussion

PKG I activation is mediated through the modulation of cellular levels of cGMP and the activity of this kinase is a critical component of vascular function (Francis et al., 2010; Hofmann et al., 2009). The cGMP-independent modulation of PKG I activity in relation to KCa1.1 activity represents an attractive avenue toward novel therapeutic treatments of age-related cardiovascular disease. Traditionally, exogenous stimulation of PKG has been accomplished by the use of cGMP analogs (Butt, 2009; Schwede et al., 2000). Although these compounds have served as valuable tools for studying PKG signaling, their clinical utility has remained elusive due to off-target effects, such as cross-activation of other cyclic nucleotide-binding proteins and modulation of phosphodiesterase activity. Here, we introduce a set of synthetic peptide activators (S-tides) derived from a novel structural element within the PKG I protein kinase. S-tides represent a potential new class of compounds, capable of eliciting vasodilation through a mechanism similar to that of nitrates and phosphodiesterase inhibitors (i.e., activation of PKG), but without the need for cGMP (Bryan et al., 2009; Kots et al., 2011; Schlossmann and Hofmann, 2005).

Biochemistry

We observed that isoform-specific PKG Iα activity can be promoted by S-tides in a cGMP-independent manner. Truncation of the C-terminus of the S1.1 parent peptide in a form that removes or modifies the knob residues (F350, F351) produces peptides that do not stimulate kinase activity. The N-terminal truncations of the parent molecule provided an increase in potency followed by a dramatic reduction and, ultimately, a complete loss of activity that was dependent on how many amino acids were removed. Concomitant with these shifts in the activation constant, we observed a positive correlation between activation of the kinase and helicity (Figure S7). We propose that stabilization of this helix by chemical means, as has been demonstrated with AKAP disruptors, may pose an additional avenue for further development of these activators (Kennedy and Scott, 2015; Wang et al., 2015).

The most potent peptide activator for PKG Iα (S.15) was unable to activate PKG Iβ, even though the type I isoforms of PKG retain 99% identity outside of their dimerization and autoinhibitory domains (Hofmann, 1995; Orstavik et al., 1997; Pfeifer et al., 1999; Sandberg et al., 1989; Wernet et al., 1989). The 10-fold difference in activation constants observed for PKG I isoforms by cGMP (Figure S1A, S1B) has been linked directly to these regions in the N-terminus (Ruth et al., 1997). The selectivity of S-tides for PKG Iα over Iβ may be governed by these differences.

The cGMP B-site can be divided into two parts; one containing the nest, and the other containing the cGMP-binding site (Figure 1C). Of note, part of the nest is formed by residues that construct the cGMP binding site. Residue W288 in addition to E291 and L294 from the phosphate binding cassette (PBC) help form the ridge of the nest (Figure 1B). In the reported structure, L294 comes into proximity with F351 upon docking of the knob. It is feasible that the interaction of the knob with the nest may affect the geometry of the PBC to mimic a closed, cGMP-bound state, not captured in the reported structure. Even in the absence of supporting structural data, we found that deletion or mutation of the knob residues in S-tides resulted in retarded activation of the kinase. This suggests a mechanism...
indicative of the knob/nest interaction observed in the crystal structure of the intact regulatory domain. In any case, these molecules are efficacious and provide an unexplored avenue for the development of potent and selective cGMP-independent activators of PKG Iα.

**Vascular Biology**

In inside-out patches from posterior cerebral artery myocytes, S-tide incubation with PKG Iα stimulated KCa1.1 activity to a similar magnitude as saturating levels of cGMP. Basal level activity of PKG Iα in the presence of low nanomolar quantities of cGMP did not affect KCa1.1 open probability. Based upon these findings, we advanced the hypothesis that S-tide mediated stimulation of PKG Iα provides a basis for future attempts to modify PKG activity and effect physiological responses. This hypothesis was further supported by our observation that cerebral vascular myogenic tone was reduced in endothelium-demaded arteries exposed to S1.5. Of particular interest was our observation that paxilline-induced constriction was significantly augmented, suggesting that S1.5 stimulates KCa1.1 activity in vascular myocytes *in situ*. The minimal augmentation of tone by paxilline in endothelium-intact vessels treated with S1.5 suggests that simultaneous activation of endothelial PKG and smooth muscle PKG has opposing effects. There is precedent for this interpretation, as activation of endothelial PKG acts as a negative regulator of NO release (Borysova and Burdyga, 2015; Dora et al., 2001). Our finding that endothelium-denuded vessels have decreased myogenic tone in the presence of S1.5 further reinforces this concept, and shows that activation of smooth muscle PKG could be sufficient to oppose the exaggerated myogenic tone that develops in response to endothelial damage associated with cardiovascular diseases such as hypertension and diabetes (Sun et al., 1994; Huang et al., 1993; Zimmermann et al., 1997). An important advantage of this type of potential therapeutic intervention is that S1.5 does not appear to negatively affect the development of tone in vessels where the endothelium is functioning normally.

**Significance Statement**

The control of vascular smooth muscle dilation and the regulation of blood flow are tightly linked to the rise and fall of cGMP, a small molecule that is made by vascular cells and is integral to proper circulatory function. This molecule controls the activation of a key enzyme, the cGMP-dependent protein kinase (PKG Iα), which is responsible for controlling a host of processes that regulate dilation of blood vessels independent of input from other body systems. All existing therapeutics that target this pathway either increase cGMP production or inhibit its breakdown. Here, we describe a novel class of small molecules, called S-tides, capable of activating PKG Iα, directly and selectively. S-tides also blunted the excessive constriction of blood vessels typical of arteries whose innermost layer is destroyed, which is often seen in hypertension and diabetes.

**Materials and Methods**

**Recombinant Expression and Purification of PKG I isoforms**

Expression in Sf9 cells using the Bac-to-Bac Baculovirus system (Life Technologies) was as previously reported (Dostmann et al., 2000). His-tagged proteins were isolated from Sf9...
cells by homogenization followed by trapping on a Mini Profinity IMAC column (BioRad).
Proteins were eluted using 250 mM imidazole and dialyzed into a final buffer of 50 mM MES, pH=6.9, 100 mM NaCl, 1 mM TCEP, and 10% glycerol. Full methods can be found in the SI Materials and Methods.

**Peptide Synthesis**
The solid-phase synthesis of the peptides was carried out as previously described (Nickl et al., 2010; Tegge et al., 2010). A full description of the methods can be found in the SI Materials and Methods.

**Surface Plasmon Resonance**
SPR measurements were conducted on a SR7500 dual-channel surface plasmon resonance spectrometer connected to an SR7100 autosampler using a standard, dual channel flow cell (Reichert Technologies). Gold sensorchips coated with 500 kDa carboxymethyl dextran hydrogel (Reichert Technologies) were used to immobilize PKG Iα or S-tides to the surface using EDC/NHS coupling chemistry at 25°C (Schasfoort and Tudos, 2008). Typically, 250nM PKG Iα was injected onto the surface for 7 minutes. Dissociation times of 15 minutes were recorded. A full description can be found in the SI Materials and Methods.

**Kinetic Analysis**
Activity of recombinant PKG Iα toward the synthetic substrate peptide (W15, TQAKRKKSALMA) was measured by γ32P-ATP incorporation assay as described with some modifications (Dostmann et al., 2000). Briefly, reactions were initiated when 0.1 mM γ32P-ATP (200–300 cpm/pmol) was incubated with vials preincubated with 50 mM MES, pH=6.9, 1 mM MgAcetate, 10 mM NaCl, 10 mM DTT, 1 mg/mL BSA, 10 µM W15 substrate, 1 nM PKG Iα, and 10 µL of 10x S-tide stocks, in 100 µL reaction volume at 30°C. Prior to the initiation of the reaction, all components excluding γ32P-ATP were allowed to pre-incubate for 15 minutes. Each reaction was run for 90 seconds and terminated by blotting on 25 mm phosphocellulose circles (Whatman P81 filter paper, GE Life Sciences). Filters were washed 3 times in 0.8% phosphoric acid and measured by liquid scintillation counting. Data was analyzed using Excel (Microsoft), Prism 6 (GraphPad), and plotted using DataGraph (Visual Data Tools).

**Circular Dichroism Spectroscopy**
CD spectra were collected on a JASCO J-815 circular dichroism spectrometer at 22°C using a 2mm cuvette (Starna Cells). Data were collected on samples containing 10 µM peptide in 10 mM PBS pH=7.4. A sampling range from 260 to 190 nm with a 0.5 nm sampling interval and 2 s integration time. A total of 10 accumulations were collected at 100 nm/min and averaged to produce the final spectra for each peptide.

**Electrophysiology**
Potassium channel activity was recorded as previously described (Brayden and Nelson, 1992). Briefly, inside284 out membrane patches were obtained from enzymatically dispersed cerebral artery smooth muscle cells. The bathing solution (intracellular face
contained (in mmol/L) 140 KCl, 2 MgCl$_2$, 0.1 Mg-ATP, 10 HEPES (pH=7.3), and 3 EGTA. CaCl$_2$ was added to the bath solution to achieve a concentration of 500 nM. The pipette solution contained (in mmol/L) 120 NaCl, 20 KCl, 2 MgCl$_2$, 1 CaCl$_2$, and 10 HEPES (pH=7.4). Single BK channel recordings were obtained over at least 5 minutes at a holding potential of +10mV in the absence and presence of purified PKG and various derivatives of the SW peptide. See SI Materials and Methods for a complete description.

**Reversible Permeabilization and Myography**

To introduce S1.5 peptide into the smooth muscle cytosol, isolated rat posterior cerebral arteries were reversibly permeabilized as described previously, see SI Materials and Methods (Earley et al., 2004; Lesh et al., 1995). Vessels were then placed in physiological saline solution (PSS) containing (in mM): 118.5 NaCl, 4.7 KCl, 1.2 KH$_2$PO$_4$, 24 NaHCO$_3$, 1.2 MgCl$_2$, 2.0 CaCl$_2$, 11 glucose, 0.026 EDTA (pH 7.4 at 37°C), and cannulated to resistance-matched micropipettes filled with PSS. Endothelial denudation was achieved by passing air bubbles through the vessel lumen. Intraluminal pressure was increased to 40 or 80 mmHg using an electronic servo-pressure transducer (Living Systems, St. Albans, VT USA), and myogenic tone was allowed to develop (~45 minutes) prior to the addition of drugs. In endothelium-denuded vessels, denudation was confirmed by the absence of vasodilation to the SK/IK activator NS-309 (1 µM). During the experiment, vessel diameter was monitored continuously using a CCD camera and edge-detection software (Living Systems). At the end of each experiment, vessels were exposed to Ca$^{2+}$-free PSS containing 5 mM EGTA to determine the maximal passive diameter. All data were then normalized to the maximal passive diameter.

**Smooth Muscle Cell Isolation and Confocal Fluorescence**

Microscopy S1.5 peptide or FITC-S1.5 peptide were introduced into rat posterior cerebral arteries as described above. Vessels were then digested, using papain (0.5 mg/ml) with dithioerythritol (1 mg/ml) followed by Type F collagenase (1 mg/ml; Sigma Aldrich, USA). Smooth muscle cells were isolated by gentle trituration, and allowed to adhere to glass coverslips at RT for 30 minutes. Cells were then imaged using a Zeiss LSM 510 Meta confocal laser scanning microscope with a 63x oil-immersion objective (NA=1.4). Identical imaging conditions were used for smooth muscle cells containing control (S1.5) and fluorescent (FITC-S1.5) peptide.

**Statistical Analysis**

Arterial diameter data were analyzed using LabChart 7 Pro software (ADInstruments, Colorado Springs, CO, USA). Electrophysiological data were analyzed using pCLAMP 9 software (Molecular Devices). For comparisons of two samples of equal variance, statistical significance between groups was determined using two-tailed, unpaired Student’s t-tests ($\alpha = 0.05$). For multiple sample comparisons, one- and two-way ANOVA were used followed by Bonferroni’s post hoc analysis to compare individual means. P-values ≤ 0.05 were considered significant. These and all other relevant calculations were performed using Microsoft Excel (Microsoft Corporation, USA) and GraphPad Prism (GraphPad Software Inc., USA).
Acknowledgements

We would like to thank Drs. Frank Schwede at BioLog, Bremen, Germany for HPLC analyses of cyclic nucleotide samples and Matthew Liptak at the University of Vermont Department of Chemistry for use of the CD spectrometer. This research was supported by grants from the National Institutes of Health (NIH) with direct support to TMM (5T32 HL007647) and NRT (1K01 DK103840), JEB (R01 HL095488), and WRD (R01 HL08981) as well as support from the Tottman Trust for Biomedical Research. A U.S. Non-provisional patent application 13/801,235, was filed March 13, 2013, entitled "NOVEL PEPTIDIC ACTIVATORS OF TYPE I cGMP DEPENDENT PROTEIN KINASES AND USES THEREOF" by Wolfgang Dostmann, Brent W. Osborne, and Thomas M. Moon. We would like to dedicate this manuscript to the memory of Matthew J. Tavares.

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Highlights

- S-tides are a new class of molecule capable of selectively activating PKG Iα.
- S-tides target PKG Iα and elevate the open probability of K⁺ channels (BK, KCa1.1).
- S-tides modulate vascular contractility via PKG Iα effects on BK channels.
- S-tides dilate damaged arteries that have exaggerated myogenic tone.
Figure 1.
Structure of the cGMP B-sites (B and B‘) from the regulatory domain of PKG Iα (A) emphasizing the asymmetric interaction of helical segments between protomers (PDBID: 3SHR) (Osborne et al., 2011). The arrow indicates the directional view depicted in (B) detailing the interaction of the knob residues (F350’, F351’, L354’) with the nest (N353’ not depicted). C) Electrostatic surface representation of the cGMP B-site demonstrating its amino acid composition, the boundaries of the nest, and its location relative to the cGMP342 binding site.
Figure 2. Activation of PKG Iα by S-tides. Activity of PKG Iα was measured using peptides with (A) C-terminal truncations, modifications and (B) N-terminal truncation mutants derived from the parent compound S1.1. Activity is expressed as a percentage relative to 5µM cGMP for individual experiments. Values indicated are the mean ± SD. Secondary structure determination of (C) C-terminal and (D) N-terminal truncation mutants as measured by CD spectroscopy. Mean residue ellipticity (θ) is expressed as mdeg x cm²/dmol x residue.
Figure 3. 
Effects of peptide- or cGMP-treated PKG Iα added to the cytosolic face of excised, inside-out membrane patches containing KCa1.1 channels. A) Representative control (left) and experimental traces (right) of single KCa1.1 channel openings in excised membrane patches from cerebral artery myocytes. The combined results of single KCa1.1 recordings (B) demonstrate a substantially increased NPo versus control for saturating cGMP levels and S-tides S1.1 and S1.5 († P<0.002, * P<0.05). Mean values with SEM are represented. Dashed line indicates normalized channel activity from control patches.
Figure 4.
Posterior cerebral arteries were subjected to reverse permeabilization with FITC-βAla$_2$-S1.5 (experimental, left panel) and S1.5 (control, right panel) peptides. Following digestion using papain and collagenase, single smooth muscle cells were imaged using differential interference contrast microscopy (DIC) and confocal fluorescence microscopy (CFM).
Figure 5.
A) Diameter measurements for endothelium-denuded, reversibly permeabilized arteries demonstrating myogenic tone response in (Aa) control and (Ab) S1.5-treated arteries. (Ac-Ae) Summarized results of diameter measurements for endothelium-denuded arteries in control and permeabilized treatments are shown with values represented as the mean +/- SEM. Differences in myogenic tone in response to pressure (Ac) as well as paxilline-induced constriction (Ad) are shown (P<0.05). Diameter measurements from endothelium-intact arteries for controls (Ba) and S1.5-treated arteries (Bb) show unaltered myogenic tone response (Bc) but small differences in paxilline-induced constriction of control and S1.5 treated arteries (Bd, P<0.02). For both sets of experiments, total constriction (Ae, Be) was unchanged between controls and S1.5-treated arteries.

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Supplemental Information

Synthetic Peptides as cGMP-Independent Activators of cGMP-Dependent Protein Kinase 1α

Thomas M. Moon, Nathan R. Tykocki, Jessica L. Sheehe, Brent W. Osborne, Werner Tegge, Joseph E. Brayden, and Wolfgang R. Dostmann
SI Methods: Recombinant Expression and Purification of PKG Iα.

Sf9 Expression: PKG Iα and PKG Iβ from Bos taurus was sub-cloned from vectors containing PKG Iα (pCDNA3.1) and PKG Iβ (pAS2) into the pFAST-Bac HTA plasmid (Invitrogen). Sf9 cells (Invitrogen) were grown on plates in Graces media (Invitrogen) in the presence of 5% fetal bovine serum and 10µg/mL gentamicin. Upon observation of confluency, Cells were transferred into suspension in serum-free SF900-III media (Invitrogen) supplemented with 10 µg/mL gentamicin, lipids, and 0.1% Pluronic F-68 (Sigma). Maximum yield of protein expression was achieved between passages 9 and 14. Typically, 1 L of cell culture at 1.2x10⁶ cells/mL in 2.8 L Fernbach flasks with baffling (Chemglass) were infected in a 1:300 or 1:100 v/v ratio using a third amplification stock of baculovirus containing recombinant PKG Iα. Cell suspensions were incubated by shaking (80 rpm) at 27°C for 72 hours. Cells were harvested via centrifugation at 300 x g for 15 minutes at 4°C. The supernatant was decanted and the pellet was resuspended in 15 mL of lysis buffer (5 mM TES pH=7.4, and 300 mM NaCl) for every 1L of cell culture. The pellets were flash frozen in liquid nitrogen and stored at -80°C.

Purification of PKG I isoforms by Ni-NTA: Pellets were thawed in a 37°C H2O bath for 15 minutes. 1 mL of protease inhibitor cocktail (2 mM AEBSF, 0.3 µM Aprotinin, 130 µM Bestatin, 1 mM EDTA, 14 µM E-64, 1 µM Leupeptin (Sigma)) was added to every liter of cell culture thawed and gently mixed by inversion. For every pellet, an equal volume of fresh lysis buffer was added and gently mixed. The resuspended cells were twice passed through a french pressure cell (SLM-AMINCO) at 25,000 psi. The lysed cells were then centrifuged at 30,000 x g for 30 minutes to separate insoluble cell debris from soluble protein. The pellets were either diverted to Ni-NTA or 6-AE-cAMP agarose purification.

For Ni-NTA purification, the supernatant was filtered (0.22 µm PES, Nalgene) and loaded onto a 5 mL Mini Profinity IMAC column (Bio-Rad) at room temperature. The column was washed with 20 mL of lysis buffer and loaded onto a Profinia purification system (Bio-Rad). The column was washed with 30 mL of 5 mM TES, pH=7.4, 500 mM NaCl, and 20 mM imidazole and eluted by switching to buffer containing 250 mM imidazole. The peak fraction dialyzed twice against 2 L of 50 mM MES, pH=6.9, 100 mM NaCl, 1 mM TCEP and once against 1 L of 50 mM MES, pH=6.9, 100 mM NaCl, 1 mM TCEP, and 10% glycerol using SpectraPore 12-14 kDa MWCO dialysis tubing (SpectrumLabs). Typical concentrations of protein following dialysis were 1 mg/mL as determined by Bradford assay. Proteins samples were aliquoted, flash frozen in liquid nitrogen, and stored at -80°C.

Purification of PKG Iα by 6-AEA-cAMP: 0.6 mL of 6-AEA-cAMP agarose resin was equilibrated with approximately 85 column volumes (CV) of lysis buffer (5mM TES, 300mM NaCl, pH 7.4). Clarified supernatant was passed over the column at a rate of approximately 1 mL/min and flow-through was collected. The resin was washed once with 20 CV of cold lysis buffer. PKG Iα was eluted with lysis buffer supplemented with 3 mM cAMP in 1CV or 2CV fractions. Eluates were collected with 15-minute incubations between collections. Fractions with protein were pooled and dialyzed overnight at 4°C into 50 mM MES, 100 mM NaCl, 2 mM DTT, pH 6.9. The following day, protein was dialyzed three additional times with 3h incubations each at 4°C and then dialyzed overnight into 50 mM MES pH=6.9, 100 mM NaCl, 1 mM TCEP, and 10% glycerol. PKG Iα was filtered post-dialysis and the final concentration was determined using the Bradford method. PKG Iα was aliquoted, flash frozen in liquid nitrogen, and stored at -80°C.
His-tag cleavage: The 3kDa N-terminal hexahistidine tag was cleaved from the purified protein by a 1:20 mass ratio incubation with TEV protease (Sigma) for 14 hours at 4°C in 50 mM MES, pH=6.9, 100 mM NaCl, 1 mM TCEP, and 10% glycerol. The hexahistidine tag and the TEV protease were removed by incubation of the reaction mixture with 0.5mL of PureProteome Ni-magnetic beads (Millipore) for 15 minutes at 4°C. PKG Iα (beginning sequence “GAMDPSELEED...”) was recovered using a magnetic stand (Fisher Scientific) to isolate the beads from the supernatant containing the cleaved, recombinant enzyme. Final protein concentrations were assayed using the Bradford method. Typical protein concentrations were 0.5 mg/mL following TEV cleavage. Protein samples were aliquoted and flash frozen in liquid nitrogen and stored at -80°C.

Peptide Synthesis.
Peptides were synthesized on a scale of 50 µmole with a Syro multiple peptide synthesizer (MultiSynTech, Witten, Germany) on Rapp S RAM resin (Rapp Polymere, Tübingen, Germany) for peptide amide formation. Fmoc chemistry with TBTU / diisopropylethyl amine activation with tenfold excess was employed. Coupling time was 1 h. Side chain protections of the amino acids were as follows: Asp, Glu, Ser, Thr and Tyr: t-Bu; Asn, Cys, Gln and His: Trt; Arg: Pbf; Lys and Trp: Boc. Peptides were cleaved from the resin and deprotected by a 3 hour treatment with TFA containing 3% triisopropylsilane and 2% water (10 ml/g resin). After precipitation with t-butylmethyl ether, the resulting crude peptides were purified by preparative HPLC (RP-18) with water/acetonitrile gradients containing 0.1% TFA and characterized by MALDI-MS. Final products were lyophilized from water. For the calculation of the concentration of stock solutions of the peptides for each basic amino acid (Arg, Lys and His) and for the free N-terminus one counter ion of trifluoroacetic acid (mol. weight 114.02) was taken into account. Fluorescein peptide labeling was carried out as previously described [1].

Surface Plasmon Resonance.
SPR measurements were conducted on a SR7500 dual-channel surface plasmon resonance spectrometer connected to an SR7100 autosampler using a standard, dual channel flow cell (Reichert Technologies). Gold sensors chips coated with 500 kDa carboxymethyl dextran hydrogel (Reichert Technologies) were used to immobilize PKG Iα or S1.X peptides to the surface using EDC/NHS coupling chemistry at 25°C. The standard buffer used for all loading and experimental data collection was composed of 50 mM MES, pH=6.9, 150 mM NaCl, 1 mM EDTA, 1mM TCEP, and 0.05% v/v Tween-20. Surfaces were cleared of electrostatically bound contaminants using a solution of 2 M NaCl and 10 mM NaOH injected for 5 min at 20 µL/min. A mixture of 90 mM N-hydroxysuccinimide (NHS) and 250 mM 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) in 1 ml of 0.5 M MES, pH=5.5 was injected onto the surface for 5 minutes, followed by a buffer wash for 6 minutes. Typically, 150 pmol of PKG Iα or 4 nmol of peptide was bound to the left channel in a buffer of 20 mM Sodium Acetate, pH=5.0 over 10 min. Surfaces for both channels were protected using a 1 M solution of ethanolamine HCl, pH=8.0. To measure peptide binding to the PKG Iα-bound surface, 100 µM peptide in diluted into SPR running buffer was injected onto the surface for 7 minutes. For measurements of PKG binding to S1.X-coupled surfaces, 250nM PKG Iα was injected onto the surface for an equal amount of time. Dissociation time of 15 minutes were typically recorded. Data were analyzed using Integrated SPRAutolink (Reichert Technologies), Scrubber (BioLogic), and plotted using DataGraph (Visual Data Tools).

Cyclic Nucleotide Analysis
Preparations of PKG Iα were precipitated to release bound cyclic nucleotides. Acetonitrile was added at a 1:1 v/v ratio by volume to 1.2 mmol (92.9 μg) of PKG Iα, buffer alone, or a 20 μM cAMP control. Samples were vortexed and incubated on ice for 1 h with additional vortexing at 15 min intervals. Samples were centrifuged at 14,000 x g for 10 min. Analytical gradient HPLC was performed with a LaChrom Elite instrument consisting of a L-2130 pump (set at 1.0 mL/min), a L-2400 UV detector (255 nm), a L-2350 column oven (30 °C), and EZChrom software version 3.3.1 SP1 (all VWR–Hitachi, Hannover, Germany). YMC ODS-A 12 nm, S-11 μm (YMC, Dinslaken, Germany) was used as stationary phase in a 250 x 4.6 mm stainless steel column with Gemini C18, 4 x 3 mm security guard™ column (Phenomenex, Aschaffenburg, Germany). The mobile phase was 2.5 % 2-propanol, 25 mM triethylammonium formate (TEAF), pH 6.9.

**Electrophysiology.**

Middle cerebral, posterior cerebral, and cerebellar arteries were removed from 12- to 16-wk-old Sprague-Dawley rats (Charles River Laboratories; St. Constant, Quebec, Canada) after euthanasia by a lethal dose of pentobarbital sodium and exsanguination. Arteries were then cleaned of connective tissue, cut into 2-mm segments, and placed in the following cell isolation solution (in mM): 60 NaCl, 80 Na glutamate, 5 KCl, 2 MgCl₂, 10 glucose, and 10 HEPES; pH 7.2. The segments were incubated at 37°C in 0.5 mg/ml papain and 1 mg/ml dithioerythritol for 11 min, followed by 13 min incubation in 1.0 mg/ml type F collagenase. The digested segments were then washed three times in ice-cold bath solution and triturated to release myocytes. Cells were stored on ice in isolation solution for use by the same day.

Single-channel currents were recorded from inside-out membrane patches obtained from freshly isolated arterial myocytes using an Axopatch 200B amplifier equipped with a CV203BU headstage (Axon Instruments). Recording electrodes (resistance, 4–5 MΩ) were pulled from borosilicate glass (1.5 mm OD, 1.17 mm ID; Sutter Instrument, Novato, CA). Currents were filtered at 0.5 kHz, digitized at 10 kHz, and recorded using pCLAMP software (Axon Instruments), and were analyzed offline using Clampfit version 9.2. Patches were initially held at a membrane potential of 0 mV and single channel currents were recorded at a holding potential of +10 mV. All recordings were performed at room temperature (22°C). The bathing solution contained (in mmol/L) 140 KCl, 2 MgCl₂, 3 EGTA, 2.415 CaCl₂, and 10 HEPES (pH 7.3). Free calcium in this solution was calculated as 500 nM using the Ca-Mg-ATP-EGTA MaxChelator program (v1.0) (http://www.stanford.edu/~cpatton/CaMgATPEGTA-NIST.htm). The pipette solution contained (in mmol/L) 120 NaCl, 20 KCl, 2 MgCl₂, 1 CaCl₂, 10 HEPES (pH=7.4). Channel open probability (NPo) was calculated using the following equation:

\[ N \cdot P_o = \left( \sum_{j=1}^{N} t_j \cdot j \right) / T \]

Where Po is the open state probability, \( t_j \) = time spent in seconds with \( j = 1, 2, ..., N \) channels open, \( N \) = max number of channels observed, and \( T \) = duration of measurement.

**Reversible Permeabilization**

Segments were first incubated for 20 minutes at 4°C in the following solution (in mM): 120 KCl; 2 MgCl₂; 10 EGTA; 5 Na₂ATP; 20 TES; pH 6.8. Arteries were then placed in a similar solution containing S1.5 peptide (10 μM) for 120 minutes at 4°C and then in a similar S1.5–containing solution with elevated MgCl₂ (10 mM).
Permeabilization was reversed by placing the arteries for 30 minutes in a MOPS buffered physiological solution containing (in mM): 140 NaCl; 5 KCl; 10 MgCl$_2$; 5 glucose; 2 MOPS; pH 7.1, 22°C. Ca$^{2+}$ was gradually increased in the latter solution from nominally calcium free to 0.01, 0.1, and 1.8 mM over a 45 minute period.

**Supplemental Figure Legends**

**Figure S1**
Kinetic characterization of recombinant PKG I$\alpha$ and I$\beta$ expressed in Sf9 cells. A) Ni-NTA purified PKG I$\alpha$ (see inset). An overall robust stimulation (>20-fold) results from relative low basal activity. B) Ni-NTA purified PKG I$\beta$ (see inset) is activated by cGMP and demonstrates >20-fold stimulation. Values for all traces are represented as the mean +/- SD. Velocity units are given in $\mu$mol PKG x min$^{-1}$ x mg$^{-1}$ substrate. C) Activation of PKG I$\alpha$ in the presence (black) and absence (red) of a hexahistidine purification tag showed invariant activation parameters. D) Activation of PKG I$\alpha$ as purified by Ni-NTA (black) or cAMP-agarose (blue) chromatography. Although the $V_{max}$ of the cAMP-agarose purified kinase is somewhat dampened, we observed low basal activity and similar $K_m$ values for both preparations.

**Figure S2**
HPLC-UV analysis of cyclic nucleotide content of purified, recombinant PKG I$\alpha$ from Sf9 cells. HPLC traces for control quantities of buffer blank, 25 $\mu$M cGMP, and 10$\mu$M cAMP are shown (top panel). The elution of cNMPs are plotted and listed at discrete times as standards. Resulting cNMP analysis from the extraction of Ni-IMAC and cAMP-agarose purified PKG I$\alpha$ (bottom panel). The Ni-IMAC purified PKG I$\alpha$ did not show evidence of the presence of any cNMPs whereas the cAMP-agarose purified PKG I$\alpha$ sample contained trace amounts of cAMP.

**Figure S3**
Surface plasmon resonance (SPR) recordings for peptide-affixed (Aa-Ac) and PKG I$\alpha$-affixed (B) sensor chips. (Aa) S1.1, (Ab) S1.5, and (Ac) control peptides were affixed to individual sensor chips and exposed to single injections of PKG I$\alpha$. To confirm these observations, PKG I$\alpha$ was affixed to the surface (B) and exposed to single injections of the indicated peptides. The kinase/peptide complexes did not readily dissociate during either of these experiments.

**Figure S4**
A) Michealis-Menten analysis of substrate (W15) dependence on PKG I$\alpha$ activation by cGMP and S1.1. Normalized activity of PKG I$\alpha$ from activation with cGMP (black) or S1.1 (red) under varying concentrations of peptide substrate. The $K_m$ values (2$\mu$M) for substrate in S1.1 activation is invariant from cGMP. This result indicates that the binding of the parent peptide does not interfere with substrate binding in the catalytic domain.

B) S1.1 activation of recombinant PKG I$\alpha$ normalized to 5$\mu$M cGMP activity under preincubation (black) and direct exposure (red) conditions. For direct exposure conditions, S1.1 was added into the reaction mixture in the absence of PKG. To initiate these reactions, PKG was added to the mixture. The reactions were terminated after 90 sec by blotting on P81 filter paper (see methods). Under direct exposure, the activation of PKG I$\alpha$ by S1.1 is
shifted compared to that of preincubation conditions. The observation corroborates the SPR data collected indicating that the peptide requires minutes to fully associate with the kinase.

**Figure S5**
A) An assay of S1.1 with PKG Iα compared to S1.7 scrambled control shows that the sequence scrambled control does not activate the kinase. Further, the scrambled control is disordered (B) as determined by CD spectroscopy.

**Figure S6**
The complete set of N-terminal truncation peptides and their activation of PKG Iα. Kinase activities were measured using modified peptides with (A) 3 and 6-residue truncations compared to S1.1 activity and (B) single amino acid truncations thereafter. We observed that as single amino acid changes were introduced starting at E336, potency was lost. This loss in potency seems to correlate with the secondary structure of the peptide (C,D). Even though the knob residues were present, the overall secondary structure of the peptide contributes to the ability of the peptide to activate the kinase. Activity is expressed as a percentage of activation relative to that of 5µM cGMP velocities for individual experiments. Values indicated are the mean +/- SD. Mean residue ellipticity (θ) is expressed as mdeg cm²/dmol x residue.

**Figure S7**
Plot of mean residue ellipticity (θ at 222nm) and activation constants observed for PKG Iα for the indicated peptides shows a positive correlation between helicity and activity. In general, we observed that as helicity increased, as seen from S1.1 to S1.5 and S1.5, potency also increased. Moreover, the loss of activation of PKG Iα associated with S1.9-S1.11 was also correlated with a loss of helicity, provided that the knob residues were unmodified.

**Figure S8**
Activation assay of PKG Iα (blue) and Iβ (red) by S1.5 demonstrating that S-tides selectively activate PKG Iα. Activity is represented as a percentage relative to 5µM cGMP. Values indicated are the mean +/- SD.
Bibliography

Fig. 1. Structure of the cGMP B-sites from the regulatory domain of PKG I (A) emphasizing the asymmetric interaction of helical segments between protomers (PDBID: 3SHR) [23]. An arrow indicates the directional view depicted in (B) detailing the interaction of the knob residues (F350, F351, L354) with the nest (N353 not depicted). C) Structure of the cGMP B-site demonstrating the boundaries of the nest relative to the cGMP-binding site.

Fig. 2. Activation of PKG I by S-tides. Activity of PKG I was measured using peptides with (A) C-terminal truncations, modifications and (B) N-terminal truncation mutants derived from the parent compound S1.1. Activity is expressed as a percentage relative to 5M cGMP for individual experiments. Values indicated are the Mean ± SD. Secondary structure determination of (C) C-terminal and (D) N-terminal truncation mutants as measured by CD spectroscopy. Mean residue ellipticity (θ) is expressed as mdeg x cm²/dmol x residue.

PKG Iα

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Fig. 3. Effects of peptide- or cGMP-treated PKG I added to the cytosolic face of excised, inside-out membrane patches containing $Ca_{1.1}$ channels A) Representative control (left) and experimental traces (right) of single $Ca_{1.1}$ channel openings in excised membrane patches from cerebral artery myocytes. The combined results of single $Ca_{1.1}$ recordings (B) demonstrate a substantially increased NP versus control for saturating cGMP levels and S-tides S1.1 and S1.5 ($^\dagger$ P < 0.002, * P < 0.05). Mean values with SEM are represented.
Appendix B

Sequence alignment of PKG Iα and Iβ isoforms expressed in placental mammals.

PKG Iα and Iβ sequences were obtained from the National Center for Biotechnology Information (NCBI) database on Feb. 19, 2018. The initial search for "cGMP-dependent protein kinase" and "placentals" resulted in 4586 hits. The list was filtered by sequence length (600-700) and excluding of predicted and redundant sequences. The final list included 45 sequences (Iα=24; Iβ=21) and were organized by order. Sequences were aligned and analyzed in Jalview V2 using Clustal OWS and colored using BLOSUM62 scoring (Sievers et al., 2011; Waterhouse et al., 2009).
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Appendix C

Protocol Development

C.1 Sf9 cell culture

C.1.1 Growth and maintenance of Sf9 cells

C.1.1.1 Starting Sf9 cell culture from frozen stocks

Reagents: Grace’s Insect Cell Culture Medium (Life Technologies, 11300-043); heat inactivated fetal bovine serum (FBS; Life Technologies, 10082-139); 1000× lipid mix (Sigma, L5146); Sf900 III medium (Life Technologies, 12658-027); 10% Poloxamer 188 Solution (Sigma, P5556).

1. Prepare Grace’s Insect Cell Medium
   (a) Dissolve Grace’s Insect Cell Medium in approximately 500 mL of sterile ddH²O.
   (b) Add 0.35 g NaHCO₃.
   (c) Add 50 mL of FBS (0.5% final).
   (d) Adjust the pH to 6.0.
   (e) Add 1 mL of 10 mg/mL gentamicin (10 µg/mL final).
   (f) Sterile filter the medium into an autoclaved bottle for light sensitive solutions or wrap in aluminum foil.
   (g) Store medium at 4°C.

2. Prepare Suspension Medium
   (a) To 1 L of Sf900 III medium, add 1 mL of 10 mg/mL gentamicin, 1 mL of 1000× lipids, and 10 mL of poloxamer 188 solution (0.01% final).

3. For culturing cells in Grace’s Insect Cell Culture Medium:
   (a) Thaw 10×10⁶ Sf9 cells by warming the vial(s) in your hands.
   (b) Transfer cells to 25 mL of Grace’s medium in a 25 mL cm² flask.
   (c) Let the cells adhere 1 h and then replace the old medium with fresh 25 mL of Grace’s medium.
(d) Incubate cells at 27 °C.
(e) Replace medium the following day and again thereafter every 2 days.
(f) When cells are 85-90% confluent, mechanically detach cells by hitting the side of the flask a few times.
(g) Centrifuge the cells at 500 × g.
(h) Decant the supernatant and suspend the cells in 25 mL of suspension medium; transfer the cells to a sterile, baffled flask. The medium should cover the entire bottom surface of the flask, but the volume should not exceed 30% of the total flask volume (for sufficient aeration).
(i) Incubate cells at 27°C, 80 rpm.
(j) Cells should be passaged every 3.5 days to 1.2×10^6 cells/mL. Check the cells viability and density as described in Section C.1.2. My observations of cell growth patterns have suggested that healthy cells secrete growth factors that promote continued rapid proliferation. Therefore, medium should be added to the flask rather than performing a complete medium change.

4. Frozen cell stocks that are very healthy can be directly grown in Suspension Medium rather than Grace’s Insect Cell Medium. For culturing in Suspension Medium:

(a) Thaw cells as described above. Suspend 10×10^6 Sf9 cells in 25 mL of Suspension Medium.
(b) Transfer the cells to a sterile, baffled flask (see above for more details regarding flask volume).
(c) Incubate cells at 27°C, 80 rpm.
(d) Allow cells to proliferate for 5 days or until cells reach between 7-10 ×10^6 cells/mL (whichever occurs first).
(e) Passage cells every 3.5 days as described above.

C.1.2 Determination of cell density and viability

Goal: Determine Sf9 cell viability and density.

Reagents: 1x Phosphate buffered saline pH 7.4; trypan blue (stock is 20×).

1. Make a 10× stock of PBS: 87.6 g NaCl, 5.52 g NaH_2PO_4 (sodium phosphate monobasic, monohydrate), 16.1 g Na_2HPO_4 (sodium phosphate, dibasic, heptahydrate), 20 mL tween 20. Adjust pH to 7.4 and QS to 1 L.
2. Add 200 µL of Sf9 cells to 800 µL of 1× PBS in a 10 mm tissue culture dish.

3. Let cells adhere for 10 min and then visualize by light microscopy at 10× magnification.

4. Most of the cells should be adherent to the surface of the dish. Move the solution in the dish gently to confirm. Cells should be golden yellow in color with nuclei that encompass approximately 50% of the total cell volume. Cells that are dead/dying will look gray.

5. Dilute cells in 1× PBS with 1×trypan blue using a dilution factor between 20 and 50. The optimal dilution factor (DF) will depend on the density of the cells.

   (a) 20 = 20 µL cells + 380 µL trypan/PBS;
   (b) 30 = 20 µL cells + 580 µL trypan/PBS;
   (c) 40 = 20 µL cells + 780 µL trypan/PBS;
   (d) 50 = 20 µL cells + 980 µL trypan/PBS.

6. Load 10 µL of diluted cells into each chamber of the hemacytometer.

7. Count ten squares (5 from each side) as shown in Figure C.1. There should be between 100-200 cells total in the ten squares. If not, make a new dilution with a different dilution factor and recount.

8. The following formula is used to calculate the approximate cell density:

\[
\frac{\text{Cells}}{\text{Squares}} \times DF \times 10^4
\]

C.2 Expression of PKG I constructs using the Sf9/Baculovirus system

C.2.1 Blue-White Screening

Goal: Transpose the gene of interest from pFAST Bac HTA into bacmid.

Reagents: construct (pFAST Bac HTA containing your protein of interest); LB medium; KGTI plates (LB agar containing 50 µg/mL kanamicin; 7 µg/mL gentamicin; 10 µg/mL tetracycline, and 40 µg/mL isopropyl β-D-1-thiogalactopyranoside (IPTG)).
1. Thaw vial of chemically competent DH10 Bac E. coli on ice for 10 min.

2. Add approximately 50 ng of construct to E. coli.

3. Incubate E. coli tube on ice for 30 min.

4. Incubate E. coli tube at 42°C for 30 sec and then on ice for 2 min.

5. Under semi-sterile conditions, add 100 μL of LB medium to the tube.

6. Place the tube in a 125 mL flask and shake E. coli for 5 h, 220 rpm, 37°C.

7. Prepare a 20 mg/mL stock of X-gal in 100% DMSO; spread 120 μL onto a KGTI plate and let dry. The X-gal and spreading on the plate should always be performed the same day as use.

8. Plate E. coli on KGTIX plate.

9. Grow E. coli at 37°C for approximately 24 h. The cells should be approximately 1-2 mm in diameter without growing into each other.

10. Incubate the plate at 4°C until blue and white colonies are easily distinguishable.

11. Choose two single colonies that are clearly and completely white.
12. For each colony, use a 10 µpipette tip to pick up the colony and deposit it on a new KGTIX plate. Use a sterile loop to streak the colony 3× as shown in Figure XX.

C.2.2 Bacmid Isolation

Goal: Isolate bacmid from single, white DH10 Bac E. coli colonies

Reagents: 1x LB broth, 50 mg/mL kanamicin; 7 mg/mL gentamicin; Qiagen buffers P1, P2, and N3; 100% isopropanol; 70% ethanol; sterile ddH₂O; 3 M KOH (optional).

1. Suspend white colonie(s) in 6 mL of LB with 50 µg/mL kanamicin and 7 µg/mL gentamicin. Grow suspensions overnight (approximately 16 h) at 37°C, 220 rpm.

2. Centrifuge suspensions at approximately 3000 × g for 5 min and discard supernatant.

3. Suspend pellet in 300 µL of 50 mM Tris-HCl, 10 mM EDTA, pH 8.0, 50-100 µg/mL RNase A (Qiagen Buffer P1). Transfer suspension to a 1.5 mL tube.

4. Add 250 µL of 200 mM NaOH, 1% SDS (Qiagen Buffer P2); solution turns blue if the LyseBlue indicator is present.

5. Add 350 µL of 4.2 M guanidine hydrochloride (GuHCl), 0.9 M potassium acetate, pH 4.8 (Qiagen Buffer N3). Invert tubes gently to mix.

6. Centrifuge samples at approximately 15,000 × g for 20 min.

7. Transfer supernatant to a 2 mL tube, add 800 µL of 100% isopropanol, and mix gently by inverting the tube.

8. Store samples at -20°C overnight (the longer the better). Samples can be stored for at least one month to preserve bacmid potency.

9. Centrifuge sample at approximately 15,000 × g for 30 min at 4°C to pellet the DNA. The pellet should be easily visible.

10. Discard supernatant and add 1 mL of ice cold 70% ethanol. Incubate sample at 4°C for 10 min.

11. Centrifuge sample for 10 min. at 4°C

12. Repeat steps 10-11 at least 6 times to ensure sufficient removal of salts.
13. **Discard supernatant.** With the tube cap open, invert the tube over a kim wipe and allow pellet to dry. The pellet will change from white to translucent.

14. Add 200 µL of **sterile** ddH₂O, and store bacmid at 4°C overnight. Do not mix the sample, because pipetting can shear the DNA.

15. The bacmid should be in solution. However, if pellet is still visible, pipette gently a few times to bring into solution. Do not pipette too many times; the bacmid will not go into solution if the concentration is very high. Check the concentration of DNA using a Nanodrop. Add more sterile ddH₂O to achieve a final concentration of 500 ng/µL. Pipette the bacmid up and down a few times to ensure it is fully in solution.

16. If the salt concentration is too high (260/230 < 1.7), add KOH to a final concentration of 0.3 M. Add 2 volumes of cold 100% ethanol (1 V = sample + KOH). Invert to mix and incubate at -20°C for at least 30 min. Return to step 9 and complete procedure to step 15 with more wash steps than were performed previously.

### C.2.3 Bacmid Analysis by M13 PCR

**Goal:** Analysis of bacmid to confirm successful transposition of the gene of interest into pFAST BAC HTA. This protocol uses DreamTaq (Invitrogen); however, it can be adapted to use other polymerase/buffer sets.

**Reagents:** M13 forward primer- CCCAGTCACGCACGTTGTAAAACG; M13 reverse primer-AGCGGATAACAATTTCACACAGG; DreamTaq polymerase; agarose; SYBR Safe.

1. Set up PCR reaction(s) as shown in Table C.1.

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</tbody>
</table>
2. Spin down the samples and run the PCR reaction(s) in the Thermocycler as shown in Table C.2. Note that the annealing temperature is lower than the value recommended in the Bac-to-Bac Baculovirus Expression System protocol (Invitrogen).

Table C.2: M13 PCR Thermocycler Procedure

<table>
<thead>
<tr>
<th>Step</th>
<th>Temperature (°C)</th>
<th>Time (min)</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>95</td>
<td>1:30</td>
<td>Hot start</td>
</tr>
<tr>
<td>2</td>
<td>95</td>
<td>0:45</td>
<td>Denaturation</td>
</tr>
<tr>
<td>3</td>
<td>50</td>
<td>0:45</td>
<td>Annealing</td>
</tr>
<tr>
<td>4</td>
<td>68</td>
<td>5:00</td>
<td>Extension</td>
</tr>
<tr>
<td>5</td>
<td>Go to 2, 30x</td>
<td></td>
<td>Cycle</td>
</tr>
<tr>
<td>6</td>
<td>68</td>
<td>7:00</td>
<td>Final extension</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>Forever</td>
<td>Storage</td>
</tr>
</tbody>
</table>

3. Load the sample(s) and a DNA ladder on a 1% agarose gel. Separate the bands (approximately 100 V for 30 min). Image bands at 230 nm.

4. Successful transposition of the gene of interest into pFAST Bac HTA is determined based on the size of the bands. Bands should be approximately 2430 bp + insert size bp.

C.2.4 First Amplification Baculovirus

Goal: Transfect bacmid into Sf9 cells to produce first amplification baculovirus.

Reagents: Sf9 cells in mid-log phase; Cellfectin (Invitrogen); unsupplemented Sf900 III (Invitrogen) (no lipids, gentamicin, or poloxamer-81), Sf900 III supplemented with 1× lipids (Sigma), 10µg/mL gentamicin (Sigma), and 0.01% poloxamer-81 (Sigma).

Notes: This protocol is for a single, white bacmid isolate that has been confirmed for successful transposition of the gene of interest by M13 PCR. All steps should be performed in the hood under sterile conditions. Sf9 cells should be in mid-log phase of growth and appear healthy as described in Appendix 3.2.

1. Passage Sf9 cells at 0.5 × 10^6 cells/mL in 15 mL of unsupplemented Sf900 III medium.

2. Add 2 mL of cells per well to a 6 well tissue culture plate (tissue culture treated, non-pyrogenic, polystyrene).
3. Incubate cells in the hood for 30 minutes to allow them to adhere to the bottom of the wells. Replace medium with fresh 2 mL of unsupplemented Sf9000 III medium. Label plate as shown in Figure C.2.

![Figure C.2: Plate setup for transfection of Sf9 cells with bacmid](image)

4. Prepare bacmid in 1.5 mL tubes as shown in Table C.3. Bacmid stocks should be 500 ng/µL. There will be two wells per bacmid condition. However, this preparation step assumes there are 3 wells per condition to ensure there is enough material for subsequent steps. Once the bacmid have been added to the tubes, add 300 µL of unsupplemented Sf900 III medium to each tube (100 µL per well).

<table>
<thead>
<tr>
<th>Well Condition (µg Bacmid)</th>
<th>Bacmid (µL)</th>
<th>Wells</th>
<th>Total (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 µg</td>
<td>0</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>1 µg</td>
<td>2</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>2 µg</td>
<td>4</td>
<td>3</td>
<td>12</td>
</tr>
</tbody>
</table>

5. Prepare Cellfectin in a 10 mL conical. Each well will contain 6 µL of Cellfectin during the transfection:
   - 6 µL Cellfectin × 9 wells (3 per condition) = 54 µL Cellfectin
   - + 900 µL of unsupplemented Sf900 III medium to the conical (100 µL per well).

6. Add 300 µL of Cellfectin to each bacmid tube condition. Incubate tubes in the hood at room temperature for 30 min.
7. Add 200 µL dropwise of either Cellfectin alone (control), the 1 µg bacmid/Cellfectin condition, or the 2 µg bacmid/Cellfectin condition to the corresponding wells in the tissue culture plate containing the Sf9 cells. Tilt the plate back and forth a few times to ensure the cells are evenly coated in the added solutions.

8. Incubate cells in the hood at room temperature for 5 h.

9. Remove the medium and replace with Sf900 III medium supplemented with 1× lipids (Sigma), 10µg/mL gentamicin (Sigma), and 0.01% poloxamer-81 (Sigma).

10. Incubate cells at 27°C and monitor daily for signs of infection as indicated in Table C.4 and Figure C.3. The infection should be easily apparent by day 4 post transfection.

<table>
<thead>
<tr>
<th>Stage of Infection</th>
<th>Day</th>
<th>Control Condition</th>
<th>Bacmid Condition</th>
</tr>
</thead>
</table>
| Early              | 1-2 | Some fibroblastic           | Some fibroblastic
Golden yellow cytoplasms
Cessation of cell growth |
| Mid                | 3-5 | 90-100% confluent           | Increased cell diameter
Large, dense, granular nuclei
Golden yellow cytoplasms
Cells detach from the plate
Some cellular debris |
| Late               | 6-7 | Growing on top of each other | Many cells turn dark
Many lysed cells
Much cellular debris |

11. When cells reach late stage infection, scrape cells from the bottom of the wells using a cell scraper. Pool liquid from both wells of the same condition.

12. Centrifuge samples at 500 × g for 5 min.

13. Transfer supernatant to a light sensitive 15 mL conical. Label the conical with the construct information, viral amplification number, colony isolate number, and the date. E.g. PKG Iα P1.1.1 022718 (first amplification; first colony isolate, first batch). The labeling is important if these stocks will be used for subsequent amplification batches after the initial third amplification stock has lost its effectiveness.
Figure C.3: Stages of *Sf9* cell infection. A. Healthy *Sf9* cells. The white box indicates a representative healthy cell characterized by a golden-colored cytoplasm and nucleus that is approximately 50% of the total cell body; B. Overgrown *Sf9* cells. Cells are very dense and starting to grow on top of each other. C. *Sf9* cells that have reached mid stage infection. The white box indicates a representative infected cell characterized by a large, dense nucleus that occupies greater than 50% of the total cell body. D. *Sf9* cells that have reached late stage infection. Most cells have lost their golden color, which is indicative of cell death. There is much cellular debris due to cell lysis. The white box indicates a large, representative dead cell characterized by a dark cytoplasm. All images were captured using an Apple iPhone SE and a light microscope at 10× magnification.
14. Store virus at 4°C.

**C.2.5 Second Amplification of Baculovirus**

**Goal:** Perform second amplification of baculovirus.

**Reagents:** Sf9 cells; Suspension Medium (Sf900 III supplemented with 1× lipids, 10µg/mL gentamicin, and 0.01% poloxamer 188 solution.

**Notes:** Sf9 cells should be in mid-log phase of growth and appear healthy as described in Appendix 3.2.

1. Seed cells at $1 \times 10^6$ cells/mL (25 mL) in a 75 cm$^2$.
2. Allow cells to adhere for 1 h.
3. Add 200 µL of first amplification virus to the cells; tilt the flask back and forth to ensure even distribution of the virus.
4. Monitor cells daily for signs of infection based on Table C.4.
5. At late stage infection, the virus should be harvested (approximately 7 days post infection).
6. Dislodge cells from the bottom of the flask using a cell scraper.
7. Collect media and transfer to a sterile 15 mL conical.
8. Centrifuge the conical for 5 min at 500 × g, 4°C.
9. Transfer the supernatant to a sterile light sensitive 15 mL conical. Label using the same scheme as described for the first amplification virus (e.g. Construct; P2.1.1; Date of harvest; Initials).
10. Store virus at 4°C.

**C.2.6 Third Amplification of Baculovirus**

**Goal:** Perform third amplification of baculovirus.

**Reagents:** Sf9 cells; Sf900 III supplemented with 1× lipids (Sigma), 10µg/mL gentamicin (Sigma), and 0.01% poloxamer 188 (Sigma).

**Notes:** Sf9 cells should be in mid-log phase of growth and appear healthy as described in Appendix 3.2.
1. Seed cells at $1.0 \times 10^6$ cells/mL (40 mL) in a 125 mL baffled flask.

2. Add 2.5 mL of second amplification virus to the flask.

3. Incubate cells at 27°C at 80 rpm.

4. Monitor cells for signs of infection (Table C.4) every other day by viewing a sample by light microscopy.

5. At late stage infection, harvest the virus by centrifuging at $500 \times g$ for 5 min and transferring the supernatant to a 50 mL light sensitive conical.

6. Label conical with virus information as described for the first amplification virus (e.g. Construct; P3.1.1; Date of harvest; Initials).

7. Store virus at 4°C.

C.2.7 Test Expressions of PKG I

Goal: Test the expression potency of third amplification baculovirus.

Reagents: Sf9 cells; Sf900 III supplemented with 1× lipids (Sigma), 10 µg/mL gentamicin (Sigma), and 0.01% poloxamer 188 (Sigma).

Notes: Sf9 cells should be in mid-log phase of growth and appear healthy as described in Appendix 3.2.

1. Prepare 4 flasks of Sf9 cells seeded at $1.5 \times 10^6$ cells/mL (40 mL) in 125 mL baffled flasks.

2. Let cells recover for at least 1 h.

3. Add third amplification baculovirus at 1:100 (400 µL), 1:500 (80 µL), 1:1000 (40 µL), and 1:10,000 (4 µL).

4. Incubate cells at 27°C at 80 rpm.

5. Harvest 1 mL samples daily for 96 h

   (a) Transfer 1 mL of cells from each flask into 1.5 mL epis.

   (b) Centrifuge samples at $500 \times g$ for 5 min.

   (c) Discard supernatant and store pellets at 20°C.

6. Harvest text expressions at 72 h
(a) At 72 h post infection, transfer 25 mL of cells from each test expression to 50 mL conicals.

(b) Centrifuge samples at 500×g for 30 min.

(c) Discard supernatant

(d) Flash freeze pellets in liquid nitrogen and store them at 80°C.

7. Discard remaining cells after taking the 96 h sample.

**C.2.8 Western blot analysis of PKG I test expression samples**

**Goal**: Perform a Western blot to determine the potency of the third amplification baculovirus using the 1 mL samples from the previous step.

**Reagents**: 10× Electrophoresis Buffer (250 mM Tris, 1.92 M glycine); Transfer Buffer stored at 4°C (1 L = 100 mL of electrophoresis buffer, 200 mL methanol, 700 mL dH₂O); 5×Laemmli Buffer with reducing agent.

**Notes**:

**C.2.8.1 Sample Preparation**

1. Prepare 10 mL of lysis buffer: 5 mM TES pH 7, 300 mM NaCl, 1% igepal, 1× protease inhibitor cocktail.

2. Add 500 µL of lysis buffer to each text expression sample.

3. Vortex samples to suspend pellets and incubate on ice for 30 min.

4. Centrifuge samples at approximately 15,000×g for 25 min at 4°C (highest speed on the tabletop centrifuge).

5. Transfer supernatant to fresh tubes.

6. Prepare samples for gel electrophoresis: 80 µL of sample + 20 µL of 5×Laemmli buffer with reducing agent. Boil samples for 2 min at 95°C.

7. Prepare a sample of PKG to use as a control (if available).

**C.2.8.2 Separate Proteins by SDS-PAGE**

1. Using either a hand-made or precast gel, remove the comb and wash wells with electrophoresis buffer. Ensure the sides of the wells are upright. If not, straighten them using a 22.5 gauge needle.
2. Load 15 µL of sample per lane (for a 10 well gel). Remember to include a molecular weight ladder.

3. Separate proteins at constant voltage (between 130-150 V). The starting amperage should be between 0.3-0.8 A. This step will take 0.5-1 h depending on the voltage used.

**C.2.8.3 Transfer proteins to PVDF (wet transfer)**

1. Remove the stacking gels and place the remaining gels in transfer buffer.

2. Cut out a piece of polyvinylidene difluoride (PVDF) membrane. It should be slightly larger than the dimensions of the gel. For two gels, proteins from both can be transferred to the same PVDF membrane.

3. Soak the membrane in 100% methanol for 1 min.

4. Soak the membrane in transfer buffer for 10 min.

5. Soak the other components of the transfer sandwich (Figure C.4).

6. Under completely wet conditions (in transfer buffer), prepare transfer sandwich as shown below. After adding each of the filter papers, use a roller to remove air between the layers.

7. Close sandwich and place in the transfer apparatus.

8. Transfer proteins at constant voltage (100 V) for 1 h.

---

**Figure C.4**
C.2.8.4 Probe PVDF and image bands

1. Remove membrane (molecular weight standard bands should be visible) and transfer to Odyssey blocking buffer (enough to completely cover the membrane).
2. Block membrane overnight at 4°C with rocking.
3. Incubate membrane with RzHis6 (1:500 in Odyssey blocking buffer) for 1 h at 4°C with rocking.
4. Wash membrane 3× in PBS for 20 min per wash (4°C with rocking).
5. Incubate membrane with GzR Alexa 680 (LiCor) for 1 h at 4°C with rocking.
6. Wash 3× with PBS as before.
7. Image membrane using the LiCor system on the 700 channel. Quantify bands to determine which condition resulted in the most protein production.

C.2.9 Full Expression of PKG I

Goal: Prepare a large expression of PKG I based on the optimum conditions determined by the Western blot

Reagents: Sf9 cells; Sf900 III supplemented with 1× lipids (Sigma), 10 µg/mL gentamicin (Sigma), and 0.01% poloxamer 188 (Sigma).

Notes: If the optimum conditions have not been determined, 1:500 of baculovirus for 72 h is usually a good place to start.

1. Prepare 1 L of Sf9 cells at 1.5 × 10^6 cells/mL in a 2.8 L baffled flask.
2. Based on the Western blot analysis, add baculovirus at the dilution which produced the highest PKG expression.
3. Incubate cells at 27°C, 80 rpm.
4. Harvest the cells at the time which resulted in the most PKG. Centrifuge cells at 500×g for 30 min. Discard supernatant and flash freeze the pellet in liquid nitrogen. Store pellet at -80°C.
5. The pellet should be labeled as shown in the example below (description in red):
   FL NHis6 PKG I..................Full-length, N-terminal His6 tag, Construct
   Sf9-SF P3.1.1 1:500 021315.....Cells, Serum free, Virus info
   72 h 1L 042318....................Expression time, Volume, Date of harvest
C.3 Ni-IMAC column Regeneration

Reagents: Deionized or ddH$_2$O 0.5 M EDTA 1 M NaCl 1 M NaOH 1 M NiSO$_4$

Equilibration Buffer: 50 mM NaOAc pH 5.2, 300 mM NaCl Lysis Buffer: 50 mM MES pH 6.9, 300 mM NaCl, 10 mM imidazole

The Lysis Buffer conditions will depend on the protein being purified (close to the protein's PI). Regardless, 10 mM imidazole is always included to prevent nonspecific binding of proteins to the column.

Ensure the column is uniformly packed and has never been introduced to air (without bubbles). If bubbles/nonuniform packing are observed, repack the column before following the regeneration protocol.

$CV =$ column volume

Procedure:

- 10 CV EDTA
- 2 CV H$_2$O
- 1 CV NaCl
- 2 CV H$_2$O
- 1 CV NaOH
- 10 CV H$_2$O
- 5 CV Equilibration Buffer
- 4 CV NiSO$_4$

After this step, it is preferred to incubate overnight NiSO$_4$. This provides time for the NiSO$_4$ to interact with the resin. The column can also be stored longterm in NiSO$_4$ to prevent microbial growth.

- 5 CV Equilibration Buffer
- 10 CV H$_2$O
- 5 CV Lysis Buffer

At this point, the column is ready to load with clarified, filtered cell lysate for purification of His-tagged proteins.
C.3.1 Worksheets

Figure C.5: Checklist for performing the \textit{in vitro} $^{32}$P-γ-ATP phosphotransferase assay.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{checklist.png}
\caption{Checklist for Kinase Assay}
\end{figure}

Specific Activity
ATP 1-________ cpm/pmol
ATP 2-________ cpm/pmol
Figure C.6: Layout sheet for gel electrophoresis- 10-well. Courtesy of Dr. Jay Silvera in the Tracy Laboratory, University of Vermont.
Vitae

Jessica Lynne Sheehe was born in Rutland, Vermont on November 14, 1986, the daughter of Beth Aldene Blaisdell and Michael James Sheehe II. She graduated with honors from Rice Memorial High School in South Burlington, VT, and then attended Saint Michael’s College in Colchester, VT where she earned a Bachelor of Science degree in Biology in 2009. Her daughter, Emily, was born in September, 2010. After working in diverse positions as a cashier, brief tenure as a substitute teacher, wire manufacturer, and geriatric care worker—none of which being in the life sciences —Jessica decided to pursue research. She volunteered in the Laboratory of Dr. Beth Bouchard at the University of Vermont (UVM), which ultimately led to a technician position in Dr. Wolfgang Dostmann’s Laboratory at UVM. With support and encouragement from family, friends, and mentors, Jessica applied and was accepted to UVM’s Cellular, Molecular, and Biomedical Sciences Ph.D. program in the Fall of 2013. She joined Dr. Dostmann’s Laboratory, this time as a graduate student. During that 4 year and 10 month period, she married her best friend, Dr. Thomas Matthew Moon, and received her Ph.D. in May of 2018.

Permanent address: 70 Davis Parkway, South Burlington, VT 05403

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