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Exploring the Pharmacophore of Novel Synthetic Peptide Activators of type I-alpha cGMP-Dependent Protein Kinase

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Exploring the Pharmacophore of Novel Synthetic Peptide Activators of type Iα cGMP-Dependent Protein Kinase

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ABSTRACT

cGMP-dependent protein kinase (PKG, cGPK) is a serine-threonine kinase of the AGC-kinase family. Although PKG type Iα plays a prominent role in the control of blood-flow and blood pressure, no current hypertension therapies target this enzyme.[1] A PKG-targeted therapy would establish a first-in-class treatment for patients with uncontrolled blood pressure and may provide a clinically relevant alternative to existing antihypertensive therapies.

Dostmann et al. developed novel peptides derived from the crystal structure of PKG Iα, which are helical in solution and found to activate the kinase independent of cGMP and were demonstrated to lower blood pressure *in vivo*.[2,3] Two consecutive phenylalanine residues were identified as a central element of the putative pharmacophore within the full-length peptide. However, a shortened peptide derivative, called S1.5 (here SP), was found to have increased potency and thus has been suggested as a potential lead compound for further development. The aim of this thesis is to reevaluate the pharmacophore and specifically the role of the phenylalanine residues in context of the SP peptide.

In this study, analogs of SP were designed using an alanine-scanning approach to analyze the role of specific amino acids in the activation mechanism. By employing recombinant PKG Iα, kinetic parameters were determined using a specific radiometric assay.[4,5]

We found that substituting either or both phenylalanine residues for alanine did not impair the peptide's potency or efficacy. However, deleting one phenylalanine greatly diminished activity. This phenotype could be rescued by substituting either of two positively charged lysine residues N-terminal to the pharmacophore for alanine. These results indicate that the interactions between the kinase and these novel synthetic peptides are more complex and involve unidentified amino acids. Furthermore, the results presented here will serve to evaluate the role of N-terminal residues in peptide binding and the rescue phenotype discovered in this study.

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I would first like to extend my sincerest gratitude to Dr. Dostmann. He has gone above and beyond both as a professor and as an advisor. He always has a story or anecdote to put things into perspective, jokes to make everyone smile, and invaluable insight that I'll keep with me. I'm thankful to have an advisor that constantly promotes growth and selflessly gives others the skills to succeed.

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To my parents and family, thank you for getting me through college in every sense of the phrase. Your love and support has allowed me to accomplish everything I have envisioned since I was a kid. To my amazing friends, your emotional support was everything I needed to stay sane during my senior years and the three years prior. Brady (and Hawkeye), thank you for listening to me rant and for providing jokes and kind words whenever I needed them. You all keep me going.

Lastly I would like to thank Dr. Helms Cahan and Dr. Ballif for their time and support during these very busy and very strange times. You've been instrumental in my growth as a student and scientist at UVM. I truly love the university and all the different communities I've come to be a part of within it.

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INTRODUCTION

Overview of PKG and cGMP

Cyclic-GMP dependent protein kinase (PKG, cGPK) is a signaling protein which is widely distributed in eukaryotes. PKG isozymes are part of a larger family of basophilic AGC protein kinases which also includes PKA and PKC.[6] These kinases catalyze the transfer of the γ-phosphate from ATP to serine or threonine residues on basic substrate peptides and proteins.[7] PKG is activated allosterically by 3',5'-cyclic guanosine monophosphate (cGMP), unlike some other protein kinases which are activated covalently through modifications such as phosphorylation.[8]

Three variants of PKG are known to exist in mammalian tissue: PKG Iα, PKG Iβ, and PKG II. PKG I isozymes are derived from the gene *prkg 1* while PKG II is derived from a separate gene, *prkg 2*.[7] PKG I isozymes are found in smooth muscle, platelets, and specific neuronal areas. Specifically, PKG Iα is most common in lung tissue, heart tissue, and dorsal root ganglia while PKG Iβ is found in platelets and hippocampal neurons. PKG II is found in small intestine epithelium, the adrenal cortex, juxtaglomerular cells, chondrocytes, and neurons.[7] All PKG isoforms are essential signaling molecules in mammalian tissue, and they serve as the principle receptors for cGMP.[9] Additionally, all isoforms have the same general, highly homologous domain organization. The parallel homo-dimers are composed of N-terminal regulatory domains encompassing two nucleotide binding sites, followed by C-terminal catalytic domains.[10]

As a classic second messenger molecule, cGMP plays a prominent role across a myriad of species. In smooth muscle, production of cGMP can occur by nitric-oxide mediated activation

of soluble guanylyl cyclase (sGC) or by natriuretic peptide activation of the membrane associated particulate guanylyl cyclase (pGC). In vascular smooth muscle, sGC is responsible for synthesis of cGMP,[11] however both sGC and pGC covert guanosine triphosphate (GTP) to GMP in other mammalian tissues. Activated cGMP has downstream targets in other tissues that contribute to processes such as regulating smooth muscle tone, platelet aggregation, bone growth and development, renin release in the kidney, intestinal fluid secretion, and serotonin transport in the brain.[11] Phosphodiesterases (PDEs) hydrolyze cGMP to 5'-GMP. PDE-5 can be phosphorylated by PKG itself which activates the enzyme and decreases intracellular levels of cGMP.[12]

PKG Iα and Smooth Muscle Regulation

PKG Iα plays a role in controlling constriction and relaxation of smooth muscle cells in the central and peripheral vasculature.[13] Smooth muscle contraction is a calcium-dependent process. Ca^{2+} binds calmodulin, which activates the myosin light chain kinase (MLCK). This kinase phosphorylates myosin light chains, which leads to cross-bridge formation between myosin and actin and therefore causes muscle contraction.[14]

PKG relaxes smooth muscle by decreasing calcium levels. First, PKG is activated through a series of biological events referred to as the nitric-oxide (NO) signaling cascade. NO, generated in the endothelium, diffuses into cells and binds to the heme group of soluble guanylyl cyclase (sGC), which converts GTP to cGMP. This cGMP activates PKG Iα. PKG decreases intracellular calcium levels by several mechanisms. The primary mechanism is such that PKG phosphorylates the large conductance Ca^{2+}-activated K^+ channel ($K_{Ca}1.1$, BK_{Ca}), which hyperpolarizes the membrane by increasing efflux of potassium. Hyperpolarization of the membrane inhibits voltage-dependent calcium channels, decreasing intracellular levels of Ca^{2+} responsible for

muscle contraction.[3,15] Other substrates for PKG include phopsholamban (PLN), the inositol triphosphate receptor-associate cGMP-kinase substrate (IRAG), the regulator of G-protein signaling 2 (RGS2), and the Ras homolog gene family member A (RhoA)[2] (**Figure 1**).

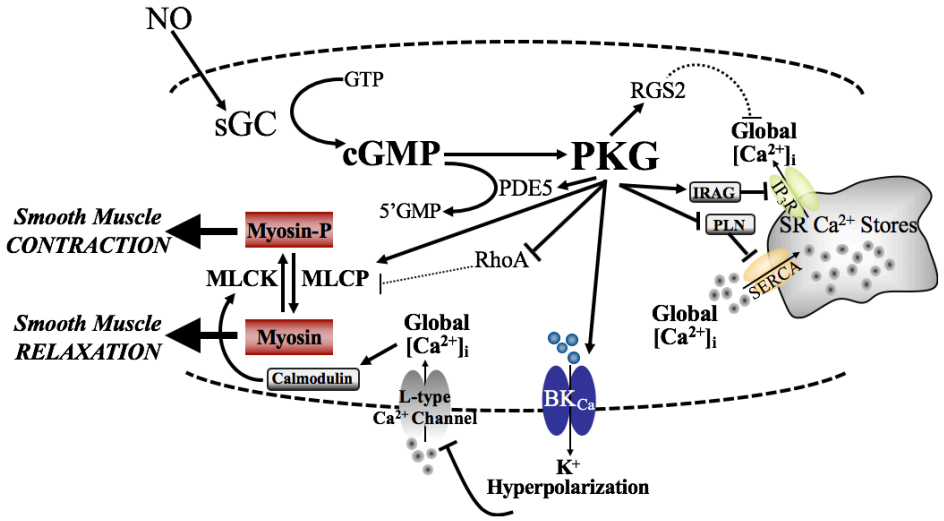


Figure 1. PKG signaling in vascular smooth muscle cells. Image from Osborne, 2011.[2]

PKG Iα Structure

PKG Iα shows significant sequence homology to the other PKG isoforms. PKG Iα, like the other isoforms, is a homodimer in which each monomer has an N-terminal regulatory domain and a C-terminal catalytic domain (**Figure 2**). At the N-terminus of the kinase there is a dimerization domain (DD) where the two protomers interact.[16,17] Facilitation of the homodimer at the DD contributes to cooperative binding of cyclic nucleotides.[18] A linker region connects the DD to an auto-inhibitory (AI) domain and another linker region.[19] Following this, there are two cyclic-GMP binding sites. The N-terminal binding site, or A site, demonstrates higher binding affinity than the C-terminal B site. This contrasts cyclic-nucleotide binding sites of other protein kinases such as PKA, where the order of the high and low affinity sites is reversed.[9,20] In addition, examination of cyclic nucleotide binding revealed that the A site was able to bind both cGMP and cAMP.[9] The difference between these sites may be due to the A-site being more solvent-exposed as well as the presence of an arginine residue in the B site. This could serve to prime the kinase to be more sensitive to changes in cGMP levels under conditions of elevated cAMP while still activate normally by cGMP when cAMP levels are low.[9] At the C-terminus of the B-site there is a region referred to as the switch helix domain (SW) which plays a role in interacting with the opposing protomer.[20] This transitional domain bridges between the regulatory and catalytic domains and is only found in type I isoforms. At the C-terminus of the SW helix lies a motif called the "knob," comprised of the amino acids FFANL. The "knob" interacts with a hydrophobic "nest" located within the B binding site on the opposing protomer (**Figures 3, 4**).[9] The successive phenylalanine residues provide the hydrophobicity for this interaction and the asparagine residue provides hydrogen bonding. Recent analysis of PKG Iα structure revealed this

novel domains is critical to kinase's function as disruption of the knob/nest interaction diminished both cooperativity and activation of the kinase.[9] In the context of the full, dimeric enzyme, creating a quadruple mutant with FF and NL residues in the knob changed to alanine caused a marked shift in activation and cooperativity of the enzyme, demonstrating the importance of the knob-nest interface.[20] Finally, the SW is followed by the catalytic domain which contains binding regions for ATP and substrate.

Current models suggest that under inactive conditions, the catalytic domain interacts with the regulatory domain and the switch helix. When bound to cGMP, the catalytic domains are released and the knobs on the switch helices interact with the two nests. It is believed that the dimer stays intact upon activation but undergoes significant conformational change. This lack of dissociation marks the most distinct difference between it and protein kinase A (PKA).[20]

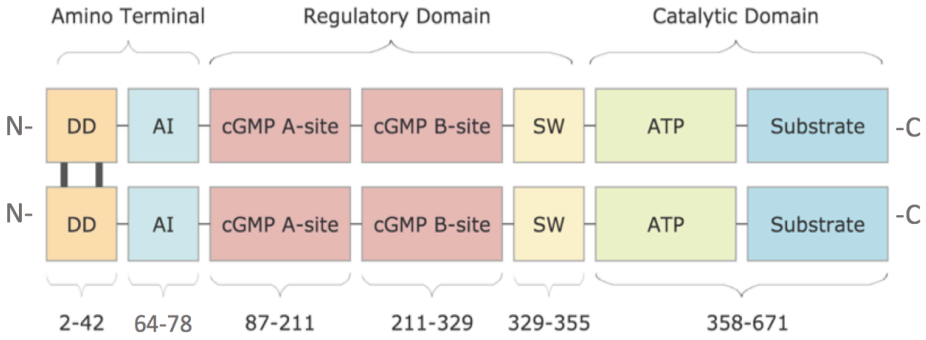


Figure 2. Organization of domains within PKG Iα: Dimerization Domain (DD), Auto-inhibitory Domain (AI), cyclic-nucleotide binding sites A and B, Switch Helix (SW) ATP-binding site (ATP), and substrate binding site.

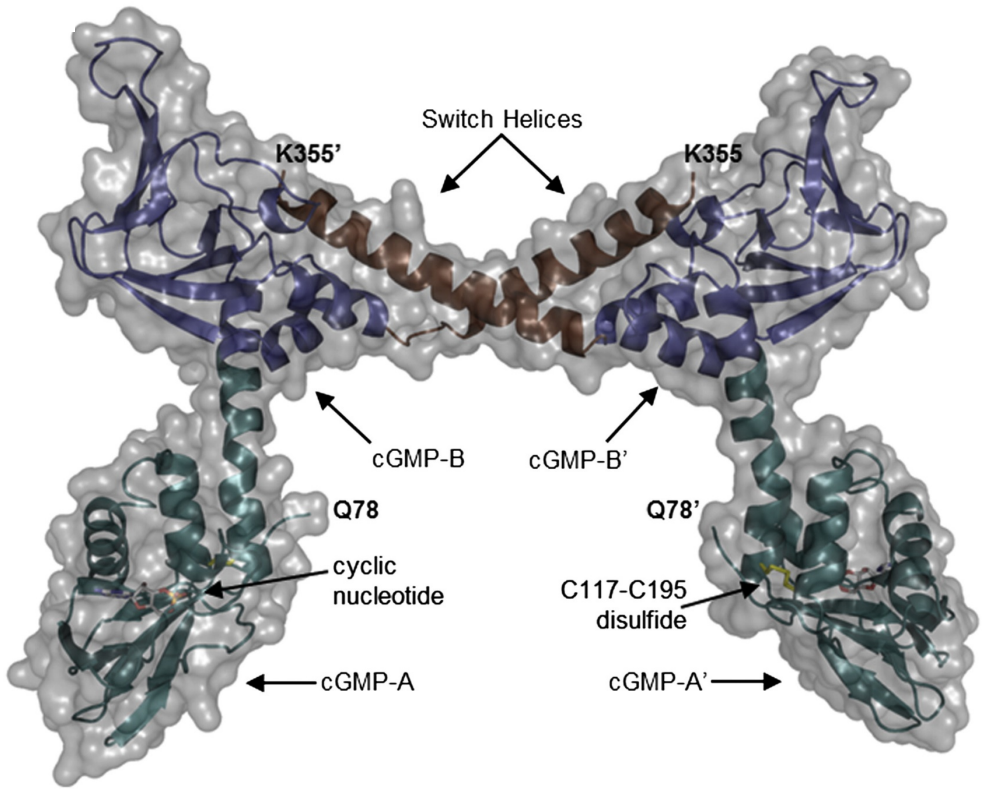


Figure 3. Dimeric view of PKG$^{78\text{-}355}$ crystal structure with cyclic-nucleotide binding sites and switch helices labeled. Image from Osborne, 2011.

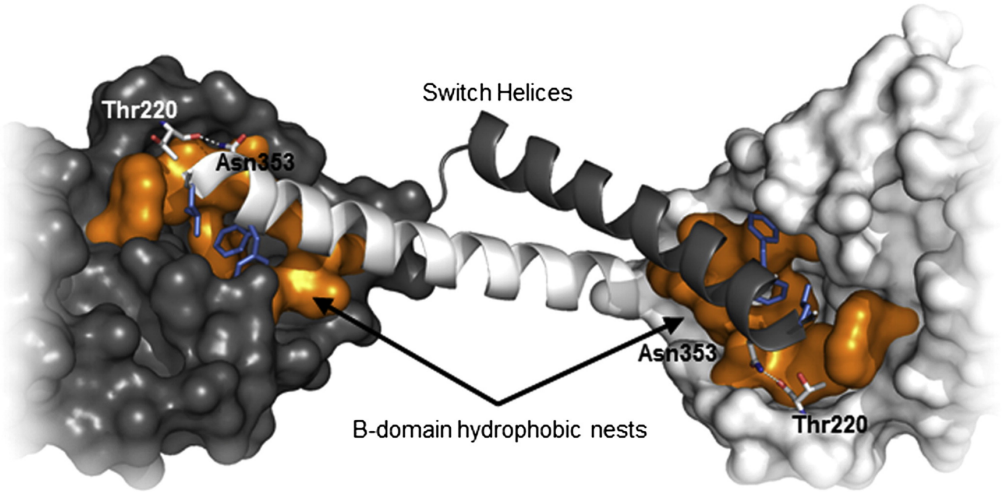


Figure 4. Top view of switch helices with their neighboring protomer. Hydrophobic knobs are located at the end of the SW (blue) and bind within the hydrophobic nest (orange) within the B-domain. Image from Osborne, 2011.

Synthetic Peptide Activators

Recent developments in PKG-targeted therapy have looked to modulate PKG activity independent of cGMP. Upon solving the crystal structure for the fragment PKG^{78-355}, it was determined that the two protomers interact via hydrophobic interactions between the switch helix segment and the B site on the opposing protomer. The isolated switch helix segment (residues 329-358) proved to be helical in solution and was assessed to determine if this segment alone could activate the kinase. Moon et al (2015) introduced a set of synthetic peptide activators (S-tides) derived from the structure of the SW helix (**Table 1**). The full length peptide, named S1.1, was able to activate PKG Iα with 80% efficacy compared to cGMP.[3] To confirm that the primary structure of this peptide is crucial to its function, a scramble peptide (S1.7) served as a negative control.

Derivative S-tides were created that sequentially removed C-terminal amino acid residues until the peptides no longer activated the kinase. This activity dropped off when knob residues (FFANL) were deleted from the synthetic peptides (S1.3). This truncation also partially diminished helicity of the peptide. This truncation removes the proposed hydrophobic interactions provided by the phenylalanine residues as well as hydrogen bonding provided by the asparagine residue. To further probe the hydrophobic interaction provided by the phenylalanine residues, the wild type sequence was next mutated in the knob region. Both phenylalanine residues in the knob region were replaced with alanine, following the logic of alanine-scanning mutagenesis. In brief, mutating residues to alanine removes side chain interactions due to the small, nonpolar character of alanine. This peptide, termed S1.6, was unable to activate the kinase at all, supporting the hypothesis that the two phenylalanine residues are crucial for the peptide's ability to activate the kinase.

Next, sequential truncations were made on the N-terminus of the peptide. These truncations increased the potency of each peptide to activate the kinase up until removal of the tyrosine residue at position #7 in the parent peptide. Truncations beyond this point gradually lowered activity to zero. The peptide that showed the best activation, called S1.5, activated PKG Iα at concentrations as low as 3 μM, but failed to activate PKG Iβ. In physiological context, these peptides were shown to increase open probability of large-conductance calcium-activated potassium channels. In the same study, it was also demonstrated that S1.5 reduced myogenic tone in endothelium-denuded cerebral arteries via this mechanism.[3]

Though the role of the phenylalanine knob residues was assessed in context of the full-length peptide (S1.1), it was never re-evaluated in the context of S1.5. This provided the basis for the aims of this study. In this study, the S1.5 peptide is referred as "switch peptide" or SP.

Name	Sequence	K_a (μM)	Hill
S1.1	Ac-DVSNKAYEDAEAKAKYEAEAAFFANLKLSD-NH_2	35 ± 4	2.2 ± 0.4
S1.7	Ac-ALKSENYADKVEFKDAKYEASALANEFADA-NH_2	-	-
S1.6	Ac-DVSNKAYEDAEAKAKYEAEAA**AA**ANLKLSD-NH_2	-	-
S1.3	Ac-DVSNKAYEDAEAKAKYEAEAA-NH_2	-	-
S1.5 (SP)	Ac -YEDAEAKAKYEAEAAFFANLKLSD-NH_2	3 ±1	2.4 ± 0.6

Table 1. Novel S-tide names and sequences are listed with corresponding activation constants (K_a) and Hill coefficient (n_H).[3] K_a indicates the concentration of activator which produces half maximal activation of the enzyme. A Hill coefficient above one indicates positive cooperativity in binding such that binding of one ligand facilitates binding of another.

Relevance

Hypertension is a highly prevalent condition both in the United States and across the globe and it has very serious outcomes. Despite there being a myriad of mediations to treat hypertension through various mechanisms, there remains significant unmet need for those individuals who do not respond to available treatments. The nitric oxide pathway has been a major target for these drugs for decades, however there is no current treatment which targets PKG. Though PKG may theoretically be a good target for this pathway, it is highly expressed throughout the body and therefore may cause off-target effects. However, novel S-tides are isozyme specific and therefore would target PKG Iα localized to the vascular smooth muscle and minimize off-target effects. Additionally, these S-tides have been demonstrated to lower blood pressure *in vivo* in animal models of hypertension with L-NAME treated rats (B. Osborne and W. Dostmann unpublished data). Therefore, this research provides a pharmaceutical platform for first-in-class agents to treat hypertension.

Experimental Methods for Measuring Kinase Activity

The kinetics of kinase enzymes can be determined by various methods. Enzyme-coupled spectrophotometric assays couple the kinase-mediated phosphorylation of a substrate to colorimetric measurable reactions such as the conversion of phosphoenolpyruvate (PEP) to pyruvate and lactate dehydrogenase (LDH)-mediated conversion of pyruvate and nicotinamide adenine dinucleotide (NADH) to lactate and NAD^+. The reaction is set up such that the kinase-mediated reaction is the rate limiting step.[21] By measuring NADH oxidation with a spectrophotometer the

decrease in absorbance is used to determine the reaction rate. This assay type is particularly useful in that it takes measurements continuously, though other methods may be more efficient for rapidly analyzing large sample numbers.[22]

Fluorescence can also be a useful tool in determining kinase kinetics. Fluorescence polarization (FP) and anisotropy (FA) are two widely used techniques used for binding assays of numerous biological signaling molecules. These assays rely on the principle that the polarization of a fluorophore is inversely related to its rotation.[23] The FP measurement is equal to the difference between the intensity of emission light parallel and perpendicular to the excitation light, and changes in this measurement are used to determine enzymatic velocity. Because Brownian motion decreases as molecular weight increases, the polarization of the fluorescent label is low when attached to a small molecule and high when attached to a larger molecule.[24] For assays examining serine-threonine kinases such as PKG, fluorescently-labeled high-affinity antibodies are used which can discriminate between phosphorylated and non-phosphorylated residues such as tyrosine.[25]

The transfer of radio-labeled phosphate groups from ATP to a kinase's substrate, as it was utilized in this study, can be measured to determine the enzyme's kinetics. In this method, ^{32}P in the γ position of ATP is transferred to a peptide or protein substrate by the kinase. This ^{32}P-labeled substrate can then be caught on a filter and quantified by scintillation counting.[4,5] The ionizing radiation given off by the decay of ^{32}P interacts with aromatic hydrocarbons in the scintillation materials, either liquid or crystal, and results in an energy transfer. Electrons in the scintillation materials are transiently excited and emit photons upon returning to the ground state.[26] These photons are caught by a detector, hereafter referred to as a "counter." Radioactive assays with scintillation counting were used in this experiment and are detailed in the methods section.

AIMS AND HYPOTHESIS

<u>Aim:</u> To evaluate the pharmacophore of the switch peptide and to probe specific interactions between SP derivatives and PKG Iα.

Substitution of the two phenylalanine residues within the knob region of the full-length synthetic helix (S1.1) were substituted for alanine, the resulting peptide (S1.6) exhibited no activity. This suggested that the phenylalanine residues interact with the nest and in way that is necessary for kinase activation. N-terminal truncations of the full-length peptide led to a shortened peptide which displayed improved potency and now serves as the lead peptide for a new pharmacological platform. However, the pharmacophore of the switch peptide (S1.5) has not been revisited and solidified.

<u>Hypothesis:</u> The phenylalanine residues within the knob of the switch peptide provide a crucial hydrophobic interaction with the nest which allow this peptide to activate the kinase.

Derivative peptides without these phenylalanine residues will be unable to efficiently interact with the kinase. Therefore, they will have a significantly reduced activation constant and maximum velocity compared to the parent peptide, SP. This will be explored by using an alanine scanning approach to create derivative peptides which have altered knob sequences. Peptide kinetics will be determined using a radioactive protein kinase assay.

MATERIALS AND METHODS

Peptide Synthesis and Quality Control

The synthetic peptides were synthesized by Dr. Werner Tegge at the Helmholtz Centre for Infection Research (Braunschweig, Germany). Peptides were synthesized via solid-phase synthesis on Rapp S RAM Resin with a Syro multiple peptides synthesizer. Fmoc chemistry was used with activation by TBTU / diisopropylethyl amine activation in tenfold excess. The reaction time was 1 hour. The side chain protections were as followed: Asp, Glu, Syr, Tyr, and Thr with *t*-Bu; Asn, Gln, and His with trityl group; Arg with Pmc; Lys and Trp with Boc. Peptides were then cleaved from the resin and deprotected with TFA. Crude peptides were purified by high-performance liquid chromatography (HPLC) and characterized by MALDI-MS. Final products were lyophilized from water.

Peptide Solutions

Peptides were put into solution and serially diluted to be used in the kinase assay. Top stocks were made by calculating the milligram mass and volume of ddH_2O required to create 1 mL of 2 mM solution for each peptide. Physical differences between peptides caused variability in the amount of peptide that was able to be dissolved into solution. Once a top stock was made, a serial dilution was performed such that concentrations were halved with each successive dilution. Final stock concentrations ranged from 2.5 μM to 1.25 mM. These concentrations were confirmed using Nanodrop technology at λ=276 nm with a molar coextinction coefficient of 2,840.

Protein Expression, Purification, and Storage.

PKG Iα used in this study was expressed by Joseph Charles (Dostmann laboratory) using the insect cell (Sf9) Bac-to-Bac baculovirus expression system.[18] Nickel affinity chromatography was used for purification and purified kinase was assayed with cGMP to ensure the resulting activity was consistent with previously determined data. The purified protein was stored in a buffer containing 50 mM MES, 150 mM NaCl, 1 mM TCEP and 10% glycerol and stored in 20 μL aliquots at -80 °C.

P81 Phosphotransfer-Assay

Overview:

In order to measure the enzyme kinetics of PKG with various activators, a radioactive assay was used. This assay measures PKG-mediated phosphorylation of the substrate with the γ-phosphate of a ^{32}P tagged ATP.[4,27] The peptide substrate, called W15, has the sequence TQAKRKKSLAMA, and its many positively charged residues allows it to bind to a phosphocellulose filter.[28] The filter paper is then placed in a liquid scintillation counter where CPM can be measured. These counts are analyzed to determine reaction velocity and the concentration at which half-maximal activation is achieved (K_a).

Protocol:

To prep for the cGMP assay, reaction tubes were set up with increasing concentrations of cGMP. Each reaction tube received 10 μL of the respective 10x cGMP stock. For each reaction, a cocktail of reagents, hereafter referred to as "reaction mix," was created. This reaction mix consisted of 20 μL of 5x MES (250 mM MES pH 6.9, 5 mM MgOAc, 50 mM NaCl), 10 μL of

100 mM DTT, 10 μL of 10 mg/mL BSA, 10 μL of 100 μM W15, 10 μL of ddH_2O, and 10 μL of ^{32}P-ATP.

Before the reaction mix was added to each tube, ATP was spiked with the radio-labeled isotope. ^{32}P -ATP was added to 1 mM ATP such the volume of P^{32}-ATP was $1/100^{th}$ the total volume at the calibration date, and $1/50^{th}$ the total volume after one half-life (14 days). To find the specific activity of the spiked ATP, 15 μL of the mixture was blotted onto the P81 filters and counted by liquid scintillation. Once the specific activity of the spiked ATP was determined, 70 μL of the reaction mix was added to each tube.

Each reaction was initiated with the addition of the kinase. PKG Iα was suspended in Enzyme Dilution Buffer (EDB) in 1:1000 dilution resulting in 8 ng of PKG per reaction. This dilution was used to initiate the phosphotransferase reaction. The reaction was allowed to run for 3 minutes and was terminated by blotting 25 μL of the reaction contents onto individually labeled Whatman P81 filter papers. The filters were then washed four times in 1% phosphoric acid for three minutes per wash. The purpose of the wash is to prevent non-specific binding and remove excess ^{32}P -ATP. The filters were then dried and placed into vials containing fluid comprised of toluene, 45 mM 2,5-diphenyloxazole (PPO), and 0.36 mM 1,4-bis(5-phenyl-2- oxazolyl)benzene (POPOP) so that CPM could be measured in a liquid scintillation counter.

Radiation given off by the ^{32}P, now attached to the substrate which was caught on the P81 filters, transiently elevates electrons in the aromatic hydrocarbons of the scintillation fluid. As they return to the ground state, a photon is emitted which is detected by the counter. The output for each filter is reported in counts per minute measurement which was used to calculate the enzyme velocity at each activator concentration.

Variation for Peptide activators:

The study by Moon et al. (2015) illustrated that the binding of the peptide activators to PKG is markedly slower than that of the natural activator, cGMP.[3] Because of this, the experimental designed was modified such that the activators were allowed to incubate with the kinase prior to the reaction being started. The protocol is as follows: 1.5 mL Eppendorf tubes were labeled and aliquoted with 10 μL of the experimental peptide at varying concentrations. The blank and zero received 10 μL of ddH_2O in place of the experimental peptide. PKG Iα was diluted in the same manner as previously described and 20 μL of this PKG/EDB solution was added to the reaction tubes—excepting the blank—and allowed to incubate at 30 °C with the peptide for 30 minutes. The necessity of this incubation time was previously established by the Dostmann lab and it was demonstrated that the incubation had no negative effect on the enzyme itself. This reaction was initiated with 70 μL of the reaction mixed described above, consisting of 5x MES, DTT, BSA, W15, ^{32}P-ATP and ddH_2O. The reaction was allowed to run for 3 minutes and terminated in the same manner as above. The P81 filters were washed and submerged in scintillation fluid for counting as described (**Figure 5**).

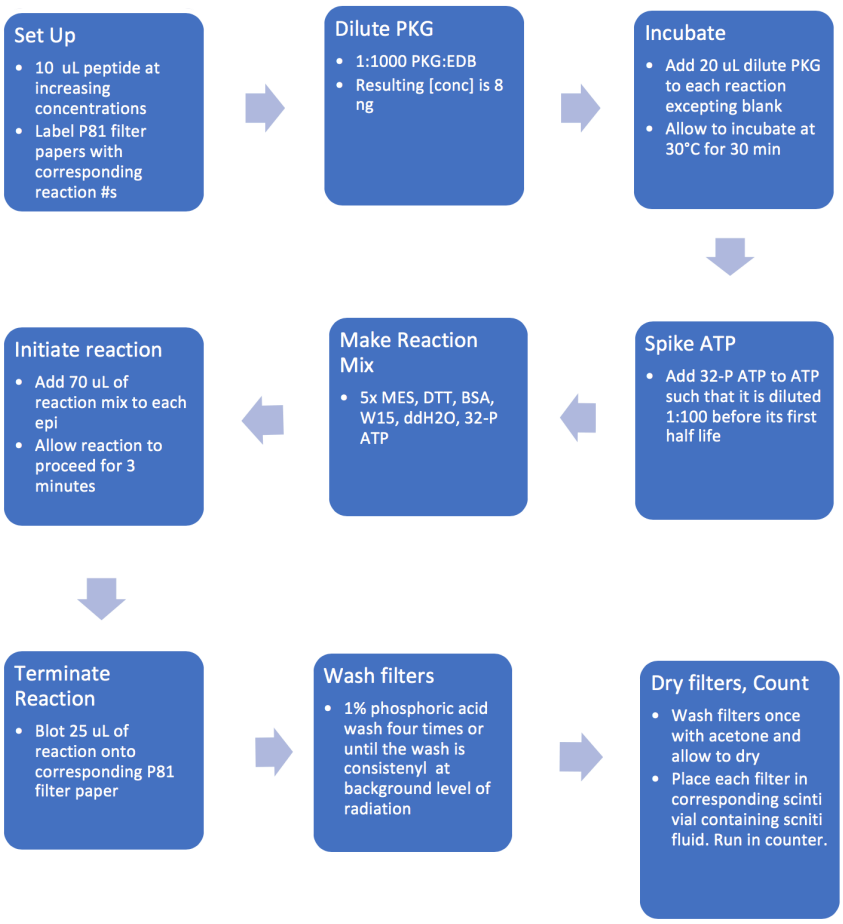


Figure 5. Work flow diagram for the phosphotransferase assay activating PKG with synthetic peptide activators.

Data Analysis

In order to evaluate the kinetics of each activator, raw experimental data was analyzed in Microsoft Excel and Graphpad Prism 8™. The liquid scintillation counter gives an output of counts per minute (CPM) for each filter. After importing this data into Excel, the velocity for each concentration is calculated by multiplying CPM by the blotting correction (0.25) and purity correction (0.98) and divided by reaction time (3 minutes), concentration of protein (8 ng), and specific activity (variable). This gives each velocity measurement with the units of μmol (P-W15) / min*mg (PKG).

The resulting velocity measurements were then plotted against their corresponding activator concentration in Graphpad Prism 8^{TM}. A nonlinear regression curve fit was performed with log concentration versus velocity to determine the activation constant (K_a), Hill coefficient (n_H), V_{max}, and V_{min}. For each parameter, 95% confidence intervals calculated by GraphPad Prism 8^{TM} are provided.

To correct for variability in the V_{max} of the cGMP control for each assay, results from each peptide-activated assay were normalized such that the V_{max} of the cGMP control represented 100%. This is referred to hereafter as "normalized velocity."

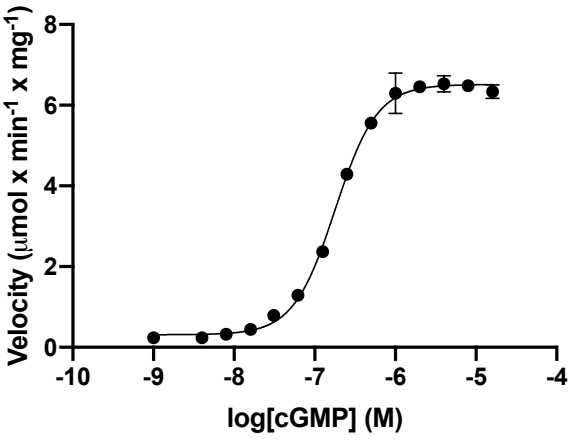
3D Modeling

In order to further probe the interactions between the switch peptide and the kinase, 3D modeling was implemented. The PyMol visualization program was used and PKG crystal structures were obtained from the protein data bank (PDB). The PDB entry used in this study, 3SHR, was crystalized by Osborne et al (2011) and provides an interactive representation of PKG Iα as shown in Figure 3.

RESULTS

Controls: cGMP and SP

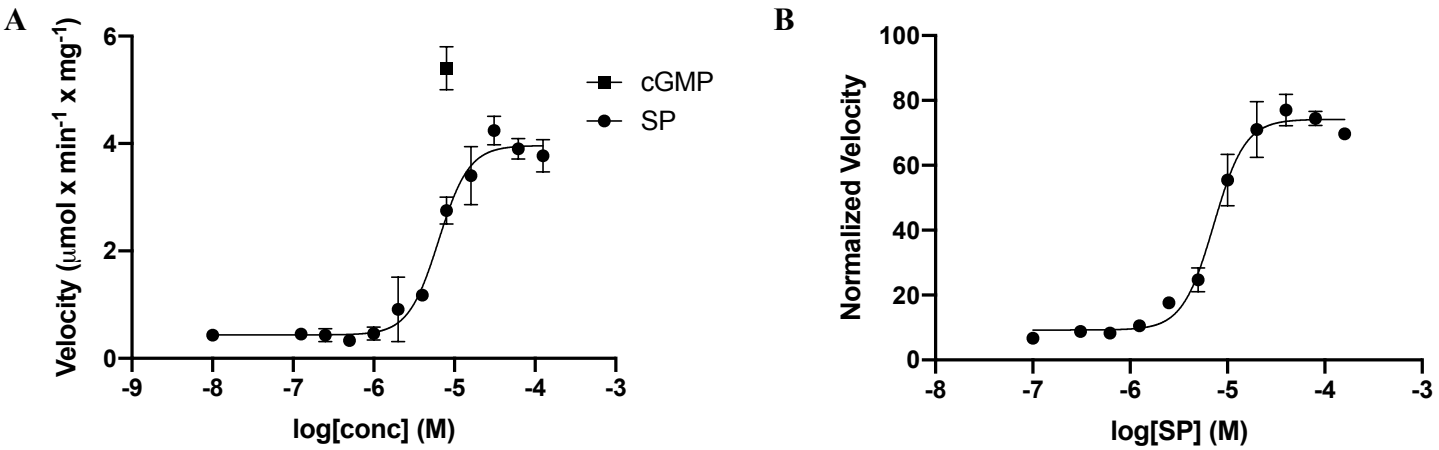
Control trials were run for both cGMP and the switch peptide SP. All results for the control were found to be within a similar range relative to previously published data. Assays performed using cyclic nucleotides, required a protocol as outlined in the methods, whereby the reactions were initiated by adding kinase to the individual reaction mixes. cGMP had an activation constant (K_a) of 180 nM and it reached a maximum velocity of 6.5 μmol per min*mg while V_{min} was comparatively low with 0.3 μmol per min*mg as is typical for enzyme preparations performed in the presence of strong reducing agents such as TCEP, see Methods (**Figure 6**).[13,18] Concentrations that resulted in maximal kinase activation level (8 μM cGMP) were used as a control for each subsequent assay involving peptides. This allowed us to normalize each peptide's maximum velocity to the velocity of cGMP.



Activator	V_{min}	V_{max}	Hill (n_H)	K_a (nM)	n
cGMP	0.31 ± 0.12	6.51± 0.12	1.68 ± 0.2	179 ± 14	4

Figure 6. Activation of PKG Iα with cGMP. The enzyme (1 nM, 8 ng per assay) was activated with increasing concentrations (4 nM – 16 μM) of cGMP under non-preincubation conditions as described in the methods. The adjacent table summarizes the kinetics constants derived from the reaction. Reaction velocities are given in μmols of substrate per minute and mg of PKG.

The parent peptide, SP, was also used as a control. However, the assay had been changed to accommodate the need for preincubation of the peptide with kinase, due to the peptide's relatively slow activation of the kinase relative to cGMP, demonstrated by Moon et al (2015). Following a 30-minute preincubation period of kinase and various concentrations of peptide, reactions were initiated by addition of reaction mix. The activation constant for SP in this study was found to be 7.2 μM and its maximal activation was 74% of cGMP (**Figure 7**). Derivative peptide results are compared to these values. This K_a value is approximately 2-fold higher than previously reported. However, it was highly reproducible and is likely a reflection of the differences in enzyme preparation protocols.[29]

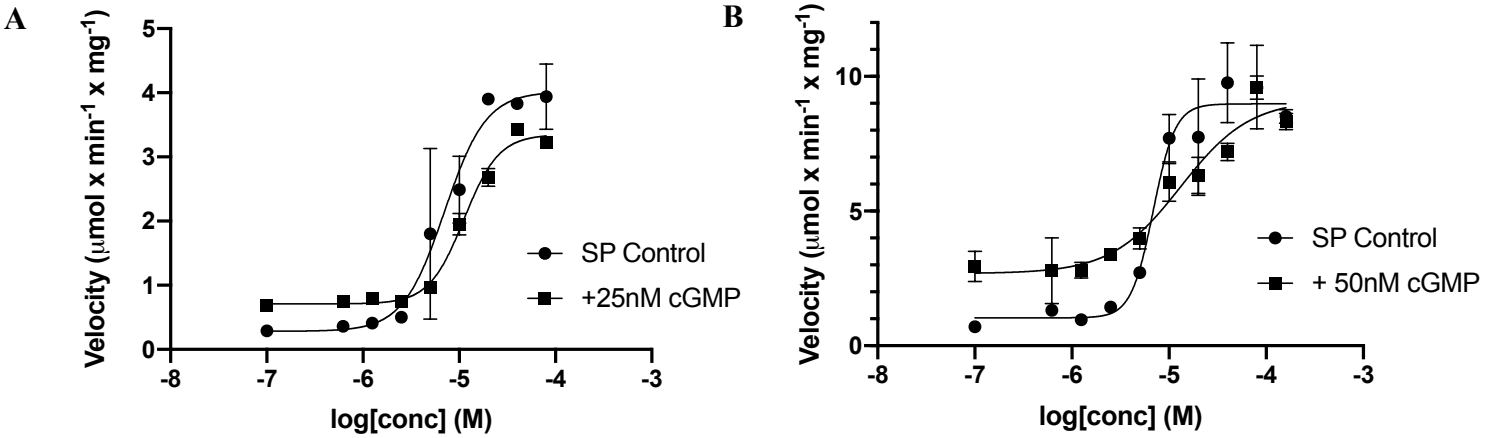


Name	Sequence	N. V_{min}	N. V_{max}	Hill (n_H)	K_a (μM)	n
SP	YEDAEAKAKYEAEAAFFANLKLSD	9.20 ± 3.88	74.18 ± 4.29	2.70 ± 1.19	7.23 ± 1.25	8

Figure 7. Activation of PKG Iα with the with peptide (SP). The kinase was preincubated with increasing concentrations of the peptide as described in the variation protocol. 8 μM cGMP was used as a control. A: representative trace. B: trials had been combined and normalized to each trial's respective cGMP control. The accompanying table gives the kinetic constants for SP with normalized velocities.

Simultaneous Activation with cGMP and Switch Peptide

Due to the close physical proximity of the nest and the cGMP binding site B, it had been hypothesized that the presence of basal levels of cGMP would significantly affect the activation kinetics of switch peptides. This was assessed by activating the kinase with the switch peptide in the presence of low levels of cGMP (25 nM and 50 nM) (**Figure 8**). These concentrations are well below those required for half-maximal PKG activation. In addition, previous reports estimated that the basal intracellular levels of cGMP are likely ranging from 20-50 nM.[30,31] Though the presence of cGMP slightly shifted the activation constant for the peptide, this change was found to be insignificant in both cases. As predicted, the V_{min} was increased with the addition of cGMP, however the V_{max} was slightly reduced in the 25 nM cGMP experiment. Furthermore, in the 50 nM cGMP experimental set-up the cooperativity of peptide activation was markedly reduced, while the K_a values were unchanged.



Activator	V_{min}	V_{max}	Hill	K_a (μM)
SP Control	0.3 ± 0.3	4.0 ± 0.5	2.0 ± 1.1	7.0 ± 2.5
SP + 25 nM cGMP	0.7 ± 0.2	3.4 ± 0.3	2.4 ± 1.3	11 ± 2.9

Activator	V_{min}	V_{max}	Hill	K_a (μM)
SP Control	1.0 ± 0.9	9.0 ± 0.9	3.8 ± 2.2	6.9 ± 1.9
SP + 50 nM cGMP	2.7 ± 0.5	9.1 ± 1.5	1.2 ± 1.5	13 ± 7.6

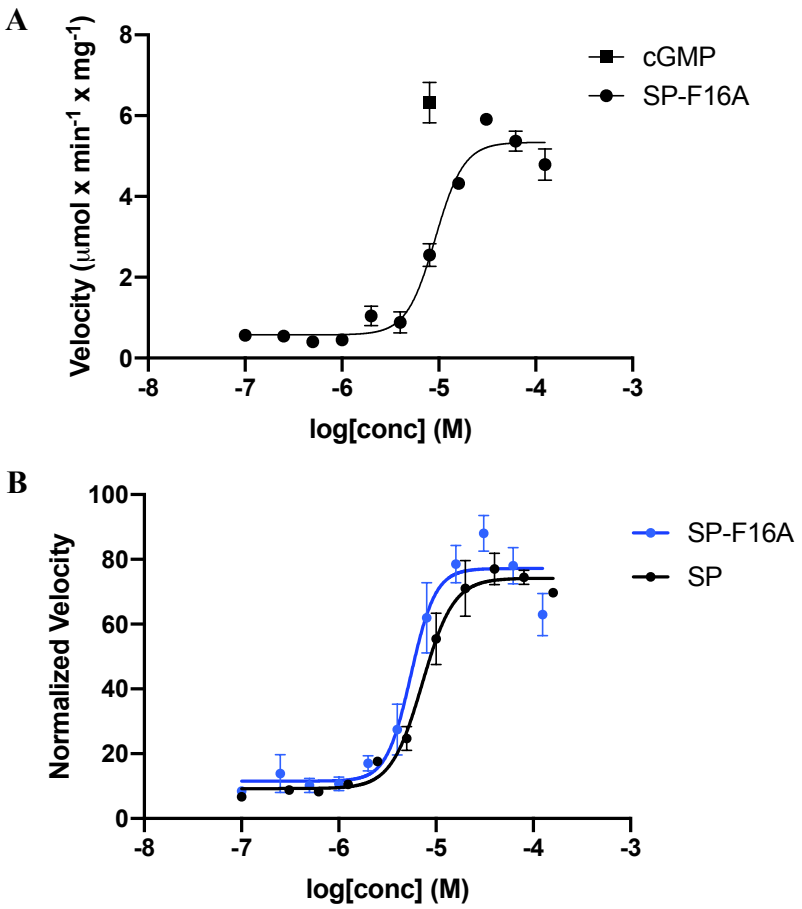
Figure 8. PKG activation with the switch peptide compared to activation with the switch peptide and basal levels of cGMP. Co-activation with both 25 nM cGMP (A) and 50nM cGMP (B) was analyzed. In the accompanying tables, kinetic data is given comparing the coactivation trials with their respective SP control. Velocity is given in μmol of W15 per minute*mg of PKG and Ka is given in μmol.

C-Terminal Substitutions: Alanine Substitutions of Phenylalanine Residues

In order to probe the function of the phenylalanine residues within the knob, derivative peptides were made. These derivatives were designed with an alanine-scanning approach. Alanine mutagenesis is a powerful technique to identify the functional role of amino acid side-chains as each substitution removes side-chain atoms beyond the β-carbon.[32] Alanine residues are used because the methyl side chain is small in size and chemically inert. Because it retains a beta-carbon, it has the propensity to form alpha-helices. Glycine, which does not have a beta-carbon, is more flexible and can cause conformational changes.[32] In this study, alanine-scanning mutagenesis was used to determine the relationship between primary amino acid sequence and peptide activity. Amino acid side chains provide crucial noncovalent interactions between the peptide and their respective binding site on the kinase.

In Moon et al (2015), it was posited that the dual phenylalanine residues constituting the "knob" of the peptide, provide hydrophobic interactions on the B-site of the opposing protomer. Each phenylalanine residue was mutated to alanine sequentially to assess the validity of this claim in the context of the switch peptide. In a third peptide, both were substituted to alanine.

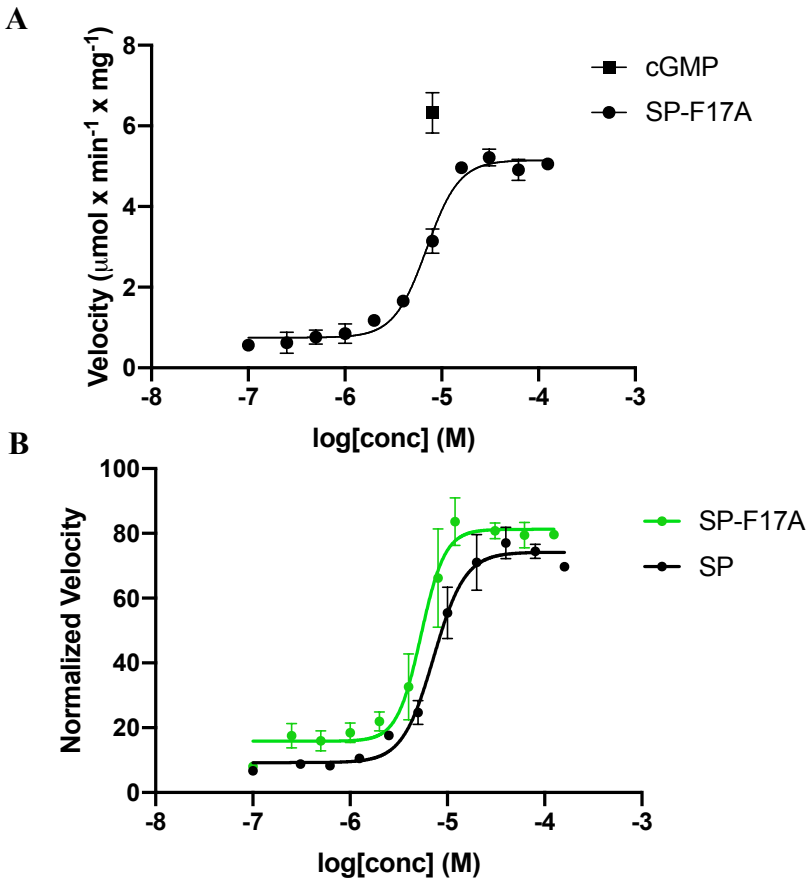
SP-F16A had the sequence YEDAEAKAKYEAEAA**A**FANLKLSD such that compared to SP, the first phenylalanine residue has been substituted for an alanine residue. This peptide showed similar efficacy and potency to the parent peptide with an activation constant of 5.53 μM and a normalized velocity of 77%. The increase in cooperativity with a Hill coefficient of 3.44 was not significantly different from that of SP (**Figure 9**).



Name	Sequence	N. V_{min}	N. V_{max}	Hill	K_a (μM)	n
SP	YEDAEAKAKYEAEAAFFANLKLSD	9.20 ± 3.9	74.18 ± 4.3	2.70 ± 1.2	7.23 ± 1.3	8
SP-F16A	YEDAEAKAKYEAEAA**A**FANLKLSD	11.55 ± 8.3	77.19 ± 9.0	3.44 ± 1.8	5.53 ± 1.9	6

Figure 9. Kinase activation with SP-F16A showed results similar to parent peptide, SP. A: representative trace for one trial is given, B: all trials (n=6) are represented. This curve reached 77% of the cGMP control and had an activation constant of 5.5 μM. On the right, SP-F16A is compared to SP which had activation at 74% of cGMP and a K_a of 7.2 μM. The accompanying table provides kinetic constants for SP and SP-F16A with normalized velocities as a percent of the cGMP control.

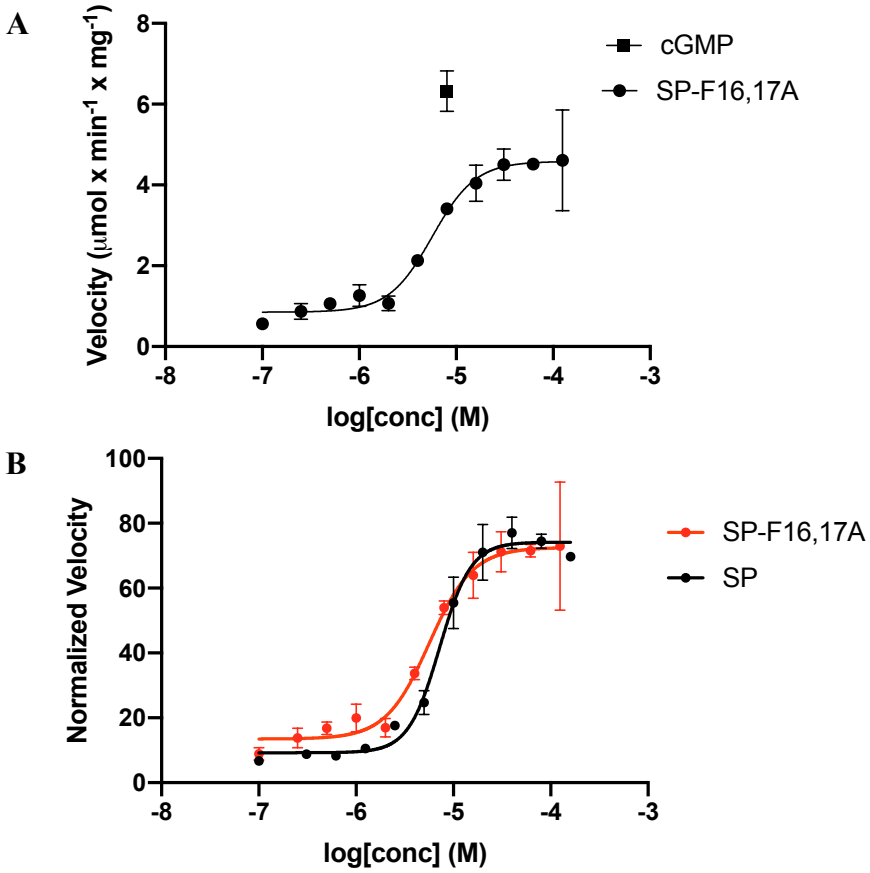
Similar results were observed when the second phenylalanine residue was substituted for alanine. This peptide was termed SP-F17A and had the sequence YEDAEAKAKYE-AEAAF**A**ANLKLSD. The activation constant for this peptide was 5.36 μM and the normalized velocity reached 81% of cGMP (**Figure 10**). With a Hill coefficient of 3.53, all three major kinetic constant values were nearly identical between SP-F16A and SP-F17A.



Name	Sequence	N. V_{min}	N. V_{max}	Hill	K_a (μM)	n
SP	YEDAEAKAKYEAEAAFFANLKLSD	9.20 ± 3.90	74.18 ± 4.3	2.70 ± 1.2	7.23 ± 1.3	8
SP-F17A	YEDAEAKAKYEAEAAF**A**ANLKLSD	15.84 ± 5.0	81.26 ± 5.7	3.53 ± 2.1	5.36 ± 1.1	8

Figure 10. Activation of PKG Iα with SP-F17A. A: a representative trace for one trial is given, B: all trials are represented (n=8). The accompanying table gives the kinetic constants for this peptide with normalized velocity as a percent of the cGMP control.

Results from SP-F16A and -17A show that with only one phenylalanine residue in either position, the peptides will still activate PKG. This led to the development of a third analog with both phenylalanine residues substituted for alanine, creating a chain of 5 sequential alanine residues. This peptide was referred to as SP-F16,17A and it had the amino acid sequence YEDAEAKAKYEAEAA**AA**ANLKLSD. The activation constant of this peptide was 5.65 μM and the normalized velocity reached 73% (**Figure 11**).

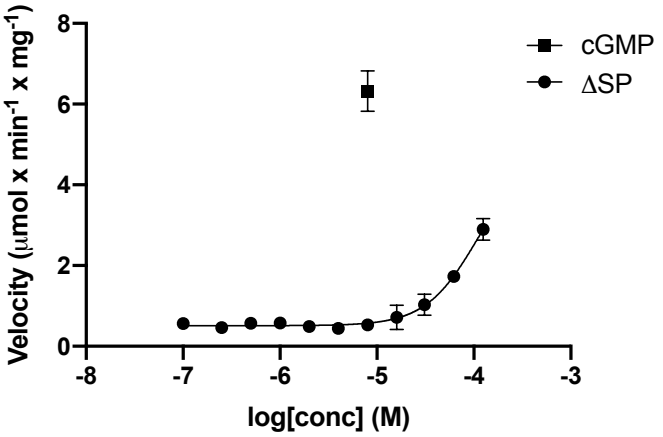


Name	Sequence	N. V_{min}	N. V_{max}	Hill	K_a (μM)	n
SP	YEDAEAKAKYEAEAAFFANLKLSD	9.20 ± 3.90	74.18 ± 4.3	2.70 ± 1.2	7.23 ± 1.3	8
SP-F16,17A	YEDAEAKAKYEAEAA**AA**ANLKLSD	13.47 ± 4.3	72.53 ± 5.4	1.95 ± 1.0	5.65 ± 1.4	2

Figure 11. Activation of PKG Iα with SP-F16,17A. A: a representative trace is given, B: trace represents n=2. It is overlaid with the trace for SP to show that this peptide's activation kinetics are not significantly shifted from SP. The accompanying table gives the kinetic constants with normalized velocity given as a percent of the cGMP control.

Deletion Peptide

Next, a peptide was synthesized which had one phenylalanine residue removed. This peptide is referred to as ΔSP and has the sequence: YEDAEAKAKYEAEAAF_ANLKLSD. This peptide exhibited significantly reduced potency and efficacy. In fact, an activation constant and maximal velocity could not be determined (higher concentrations could not be achieved due to solubility issues of the peptides (**Figure 12**). Though this result was expected, it was hypothesized that further modifications to this shortened peptide may restore its functionality. Recalling that N-terminal residues constitute the difference between S1.6, which failed to activate the kinase, and SP-F16,17A which activated just as well as SP, N-terminal residues of ΔSP were modified in assessing rescued the phenotypes. In addition, the C-terminus of the switch peptide has been heavily investigated in previous studies, where C-terminal truncations resulted in inactive peptides.



Name	Sequence	V_{min}	V_{max}	Hill	K_a (μM)	n
SP	YEDAEAKAKYEAEAAFFANLKLSD	9.2 ± 3.9	74.18 ± 4.3	2.70 ± 1.2	7.23 ± 1.3	8
ΔSP	YEDAEAKAKYEAEAAF_ANLKLSD	8 ± 1	N/A	1.6 ± 1	>100	2

Figure 12. Activation of PKG Iα with ΔSP. Kinetic constants for this peptide are reported in the accompanying table. Velocities have been normalized to the cGMP control. All trials are represented.

N-Terminal Substitutions: Negatively Charged Residues

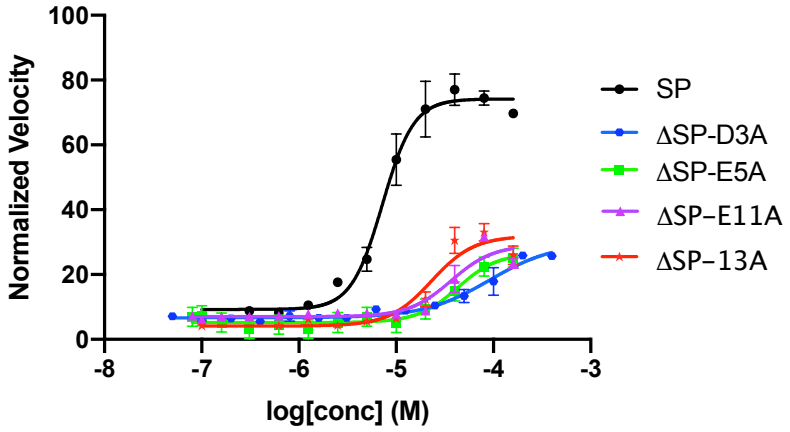
To determine if any N-terminal modifications could rescue the activity of the frame-shifted peptide, a series of alanine scanning peptides were made. Charged residues were chosen to be sequentially substituted because charged amino acids are critical to binding specificity.

The first of these peptides examined was named ΔSP-D3A and had the sequence YE**A**AEAKAKYEAEAAFANLKLSD, such that compared to ΔSP, the third amino acid was changed from a glutamic acid residue to alanine. ΔSP-D3A showed minimal efficacy and potency. The activation constant was 90.54 μM, demonstrating over a ten-fold shift in activity compared to SP (**Figure 13**). Additionally, the normalized velocity was only 29.77% of cGMP while SP consistently shows ~70% activation. This suggests that the aspartic acid residue at position # is not the sole caused of the poor activation seen in ΔSP.

ΔSP-E5A followed the alanine scanning rationale described above and consequently had the next non-alanine residue substituted for alanine, giving it the sequence YEDA**A**AKAKYEAEAAFANLKLSD. This peptide gave similar results to ΔSP-D3A, with an activation constant of 44.20 μM and a normalized velocity of 27.02% (**Figure 13**). This supports the results of ΔSP-D3A illustrating that removal of negatively charged amino acids will not rescue the deleterious phenotype caused by the deletion.

Results from the next alanine scanning analog, ΔSP-E11A, followed this pattern as well. This peptide had the glutamic acid residue at position 11 in the parent peptide substituted for alanine (YEDAEAKAKY**A**AEAAFANLKLSD) and demonstrated similar potency and efficacy to ΔSP-D3A and ΔSP-E5A. Specifically, the activation constant for ΔSP-E11A was 39.71 μM and the normalized velocity was 27.62% of the cGMP control (**Figure 13)**.

The next alanine substitution was to the glutamic acid residue at position 13 in the parent peptide giving it the sequence YEDAEAKAKYEA**A**AAFANLKLSD which was termed ΔSP-E13A. This peptide continued the previously described pattern of significantly diminished potency and efficacy compared to SP. This peptide had an activation constant of 20.90 μM and a maximum normal velocity of 29.83% (**Figure 13**).



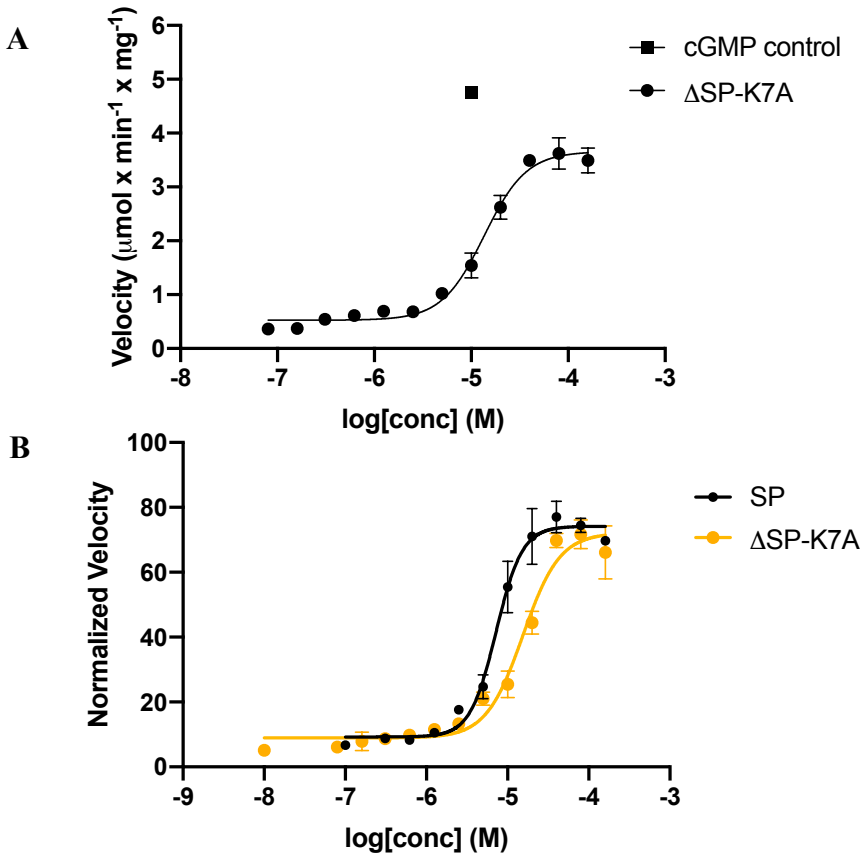
Name	Sequence	N. V_{min}	N. V_{max}	Hill	K_a (μM)	n
SP	YEDAEAKAKYEAEAAFFANLKLSD	9.2 ± 3.9	74.18 ± 4.3	2.70 ± 1.2	7.23 ± 1.3	8
ΔSP	YEDAEAKAKYEAEAAF_ANLKLSD	8 ± 1	N/A	1.6 ± 1	>100	2
ΔSP-D3A	YEAAEAKAKYEAEAAF_ANLKLSD	7 ± 1	30 ± 13	1.3 ± 1	91 ± 33	4
ΔSP-E5A	YEDAAAKAKYEAEAAF_ANLKLSD	5 ± 1	27 ± 13	2 ± 2	44 ± 13	4
ΔSP-E11A	YEDAEAKAKYAAEAAF_ANLKLSD	7 ± 4	29 ± 6	2.00*	37 ± 18	4
ΔSP-E13A	YEDAEAKAKYEAAAAF_ANLKLSD	5 ± 4	32 ± 7	2.00*	23 ± 12	4

Figure 13. Activation of PKG Iα with analog peptides. Traces have been overlaid to compare their kinetics to each other and to the parent peptide, SP. The accompanying table provides kinetic constants for these analogs as well as the parent peptides, SP and ΔSP. Velocities have been normalized as a percent of cGMP controls.

* Hill values for ΔSP-E11A and ΔSP-E13A were fixed at 2.0. Prism8's automatic curve fit function caused an artificially high Hill and ambiguous kinetic constants caused by a steep increase between two points.

N-terminal Substitutions: Positively Charged Residues

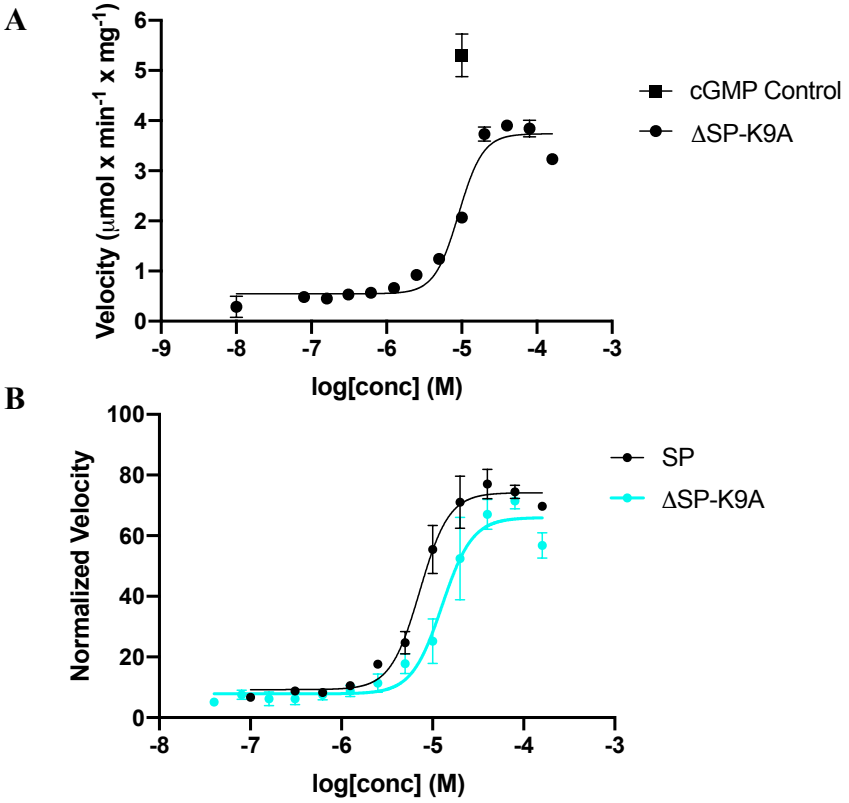
After examining negatively charged residues, positively charged residues were scanned. ΔSP-K7A, had the sequence YEDAEA**A**AKYEAEAAFANLKLSD, which contrasts previously discussed ΔSP derivatives as the single residue substituted in this peptide was positively charged lysine, instead of a negatively charged glutamic acid or aspartic acid. This peptide gave a moderately stored K_a compared at 15.46 μM, and efficacy was regained with a normalized velocity of 72% (**Figure 14**).



Name	Sequence	N. V_{min}	N. V_{max}	Hill	K_a (μM)	n
SP	YEDAEAKAKYEAEAAFFANLKLSD	9.2 ± 3.9	74.18 ± 4.3	2.70 ± 1.2	7.23 ± 1.3	8
ΔSP-K7A	YEDAEAAAKYEAEAAF_ANLKLSD	8.93 ± 4.2	72.14 ± 8.8	1.91 ± 1.2	15.46 ± 5.0	4

Figure 14. Activation of PKG Iα with ΔSP-K7A. A: representative trace for a single trial with its respective cGMP control. B: all trials are represented and overlaid with the SP to compare activation of the derivative peptide to SP. The accompanying table gives the kinetic constants for ΔSP-K7A and SP with normalized velocities.

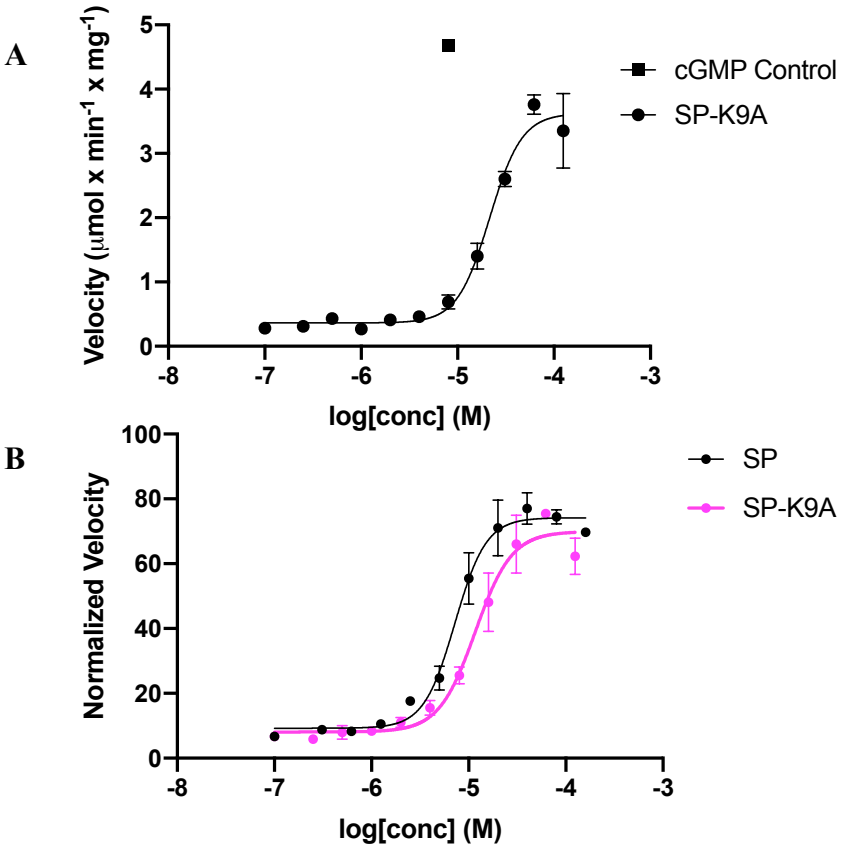
The final ΔSP derivative was made such that lysine residue at position 9 in the parent peptide was substituted for alanine. This peptide, with the sequence YEDAEAKA**A**YE-AEAAFANLKLSD, was termed ΔSP-K9A. Results from this peptide mimicked that of ΔSP-K7A with significantly restored potency, represented by K_a, and efficacy, represented by maximum velocity. The activation constant for ΔSP-K9A was 12.70 μM, which is nearly two-fold shifted compared to SP but significantly improved from ΔSP-D3A, ΔSP-E5A, and ΔSP-E11A. The normalized velocity reached 65.97%, which is relatively comparable to SP (**Figure 15**).



Name	Sequence	N. V_{min}	N. V_{max}	Hill	K_a (μM)	n
SP	YEDAEAKAKYEAEAAFFANLKLSD	9.2 ± 3.9	74.18 ± 4.3	2.70 ± 1.2	7.23 ± 1.3	8
ΔSP-K9A	YEDAEAKAAYEAEAAF_ANLKLSD	7.84 ± 4.2	65.97 ± 7.0	2.61 ± 2.1	12.70 ± 3.5	6

Figure 15. Activation of PKG Iα with ΔSP-K9A. A: representative trace for a single trial with its respective cGMP control. B: all trials are represented and overlaid with the SP curve compare the kinetics of the derivative peptide with SP. The accompanying table gives the kinetic constants for ΔSP-K9A and SP with normalized velocities.

To expand on results from ΔSP-K7A and -K9A, the lysine to alanine substitution was examined in context of the full-length peptide. SP-K9A and had the sequence YEDAEAKA**A**YEAEAAFFANLKLSD such that both phenylalanine residues of the parent pharmacophore are present and the lysine residue at position 9 has been changed to an alanine. The K_a for this peptide was 11.70 μM and it reached a maximal normalized velocity of ~70% (**Figure 16**). The observation that SP-K7A and -K9A yielded similar results to SP-K9A raises questions about the role of the lysine residues in the peptide-kinase interaction.



Name	Sequence	N. V_{min}	N. V_{max}	Hill	K_a (μM)	n
SP	YEDAEAKAKYEAEAAFFANLKLSD	9.2 ± 3.9	74.18 ± 4.3	2.70 ± 1.2	7.23 ± 1.3	8
SP-K9A	YEDAEAKAAYEAEAAFFANLKLSD	8.08 ± 4.8	69.93 ± 7.7	2.32 ± 1.4	11.70 ±0.3	10

Figure 16. Activation of PKG Iα with SP-K9A to assess the effect of the lysine substitution at position 9 in the context of the full-length peptide. A: representative trace for one trial with its respective cGMP control. B: all trials are represented, and the trace is overlaid with that of SP to compare the kinetics of the derivative peptide and SP. The accompanying table provides kinetic constants for SP-K9A and SP with normalized velocities.

Table 2 provides a summary of all peptides examined in this study. Listed is each peptide's name, sequence, normalized minimum and maximum velocity, Hill coefficient, activation constant (K_a) and number of trials.

Name	Sequence	N. V_{min}	N. V_{max}	Hill	K_a (μM)	n
SP	YEDAEAKAKYEAEAAFFANLKLSD	9.20 ± 3.9	74.18 ± 4.3	2.70 ± 1.2	7.23 ± 1.3	8
SP-F16A	YEDAEAKAKYEAEAAAFANLKLSD	11.55 ± 8.3	77.19 ± 9.0	3.44 ± 1.8	5.53 ± 1.9	6
SP-F17A	YEDAEAKAKYEAEAAFAANLKLSD	15.84 ± 5.0	81.26 ± 5.7	3.53 ± 2.1	5.36 ± 1.1	8
SP-F16,17A	YEDAEAKAKYEAEAAAAANLKLSD	13.47 ± 4.3	72.53 ± 5.4	1.95 ± 1.0	5.65 ± 1.4	2
ΔSP	YEDAEAKAKYEAEAAF_ANLKLSD	8 ± 1	N/A	1.6 ± 1	>100	2
ΔSP-D3A	YEAAEAKAKYEAEAAF_ANLKLSD	7 ± 1	30 ± 13	1.3 ± 1	91 ± 33	4
ΔSP-E5A	YEDAAAKAKYEAEAAF_ANLKLSD	5 ± 1	27 ± 13	2 ± 2	44 ± 13	4
ΔSP-E11A	YEDAEAKAKYAAEAAF_ANLKLSD	7 ± 4	29 ± 6	2.00*	37 ± 18	4
ΔSP-E13A	YEDAEAKAKYEAAAAF_ANLKLSD	5 ± 4	32 ± 7	2.00*	23 ± 12	4
ΔSP-K7A	YEDAEAAAKYEAEAAF_ANLKLSD	8.93 ± 4.2	72.14 ± 8.8	1.91 ± 1.2	15.46 ± 5.0	4
ΔSP-K9A	YEDAEAKAAYEAEAAF_ANLKLSD	7.84 ± 4.2	65.97 ± 7.0	2.61 ± 2.1	12.70 ± 3.5	6
SP-K9A	YEDAEAKAAYEAEAAFFANLKLSD	8.08 ± 4.8	69.93 ± 7.7	2.32 ± 1.4	11.70 ±0.3	10

Table 2. Results from all peptide assays with Vmin, Vmax, Hill coefficient, activation constant and number of trials. Velocities have been normalized to each trial's respective cGMP control values.

* Hill values for ΔSP-E11A and ΔSP-E13A were fixed at 2.0. Prism8's automatic curve fit function caused an artificially high Hill and ambiguous kinetic constants caused by a steep increase between two points.

DISCUSSION AND FUTURE DIRECTIONS

Pharmacophore Revisions

This study probed the function of residues within the "knob" of the switch peptide, previously created by Dostmann et al.[3] Two successive phenylalanine residues were thought to provide a hydrophobic interaction with the "nest" located in the cGMP B-binding site of PKG Iα and therefore allow these novel S-tides to bind to the kinase and cause activation.

Results from the SP-F16,17A peptide as well as SP-F16A and SP-F17A indicate that the phenylalanine residues themselves are not necessary for peptide activity, which directly contrasts results from the S1.1 peptide examined by Moon et al (2015). The difference between S1.1 and SP-F16,17A is the length of the peptide, with S1.1 having additional peptides at the N-terminus. These additional N-terminal residues were previously thought to only provide structure however with SP-F16,17A as a direct parallel we can conclude that those additional residues on S1.1 prevented optimal activation of the kinase.

When one phenylalanine was deleted (ΔSP) from the knob, the ΔSP peptide showed nearly no activation of the kinase. We hypothesize that this shift disrupts essential binding interactions either N-terminally or C-terminally from the deletion location. Removing charged residues that may be responsible for this effect could potentially save the phenotype. Both negative and positively charged N-terminal residues were probed. Results from ΔSP analogs clearly demonstrate that positively charged residues play a different role in binding than negatively charged residues. Though substitution of negatively charged residues did not improve activity, substitution of either lysine residue (ΔSP-K7A, -K9A) did improve both efficacy and potency to

approximately the same level as SP. This suggests that positively charged residues may be responsible for the diminished activation by the ΔSP peptide. This hypothesis was further explored with 3D modeling in the PyMol program. After modeling the interaction between the switch peptide and the nest, the location of both lysine residues was examined in an attempt to identify charged or steric interactions that may have caused the phenotype exhibited by ΔSP-K7A, -K9A (**Figure 17**). However, no obvious interactions were noted in the model and thus further studies are required to determine the cause of the rescue effect and the exact role of the lysine residues. To expand upon this work it is suggested that both lysine residues be substituted within the same peptide. If our hypothesis is correct, this derivative should show activation similar to SP.

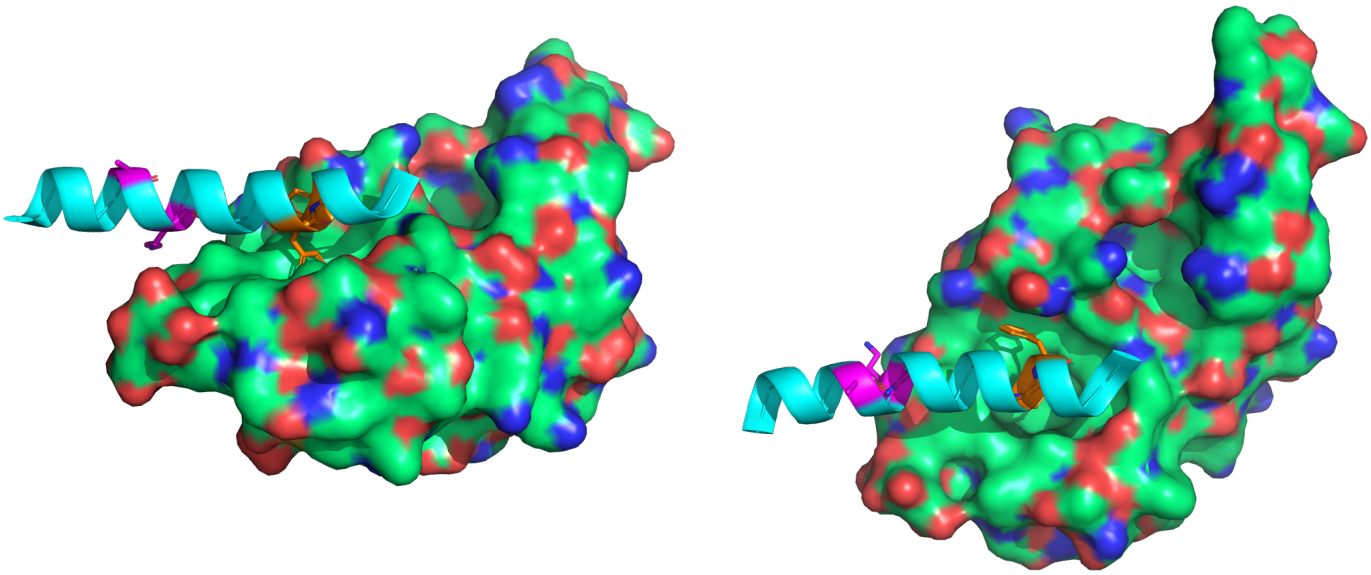


Figure 17. PyMol models of the switch peptide and PKG Iα B-site. Left: side view, right: top-view. Phe residues are represented in orange and reside within the nest. Lys residues are indicated in magenta are and are distant from both charged areas on the B-site or significant structures which would pose steric interaction.

It is also possible that the alanine substitutions made in these peptides, which created a string of multiple alanine residues, impaired the binding specificity of derivative peptides. In this way, the peptide is able to bind to PKG in both the N- to C-terminus direction and the C- to N-terminus direction. This may suggest that the activating interaction between the peptide and PKG is not caused by specific residues at a precise location in the sequence but rather any string of capable residues within the sequence. This theory could be further evaluated with retro-inverted peptides. These peptides would maintain the same sequence as the parent peptide in question, but with the N- and C-termini reversed.

Co-Activation of SP with cGMP

The nest site on PKG Iα and the cGMP-binding site are in close physical proximity. The crystal structure of this protein illustrates a single ridge separating the two pockets (**Figure 18**). It was hypothesized that because of the proximity of these two binding sites, the presence of cGMP may affect the binding kinetics of the switch peptide. This was explored by performing the kinase assay with the switch peptide as the activator with the addition of 25 nM or 50 nM cGMP (**Figure 8**). In both experiments, with the addition of cGMP a predicted increase in V_{min} was observed and V_{max} remained unchanged. Additionally, neither concentration of cGMP caused a significant shift in the activation constant for the peptide. However, in the 50 nM experiment, there was a significant decrease in the Hill coefficient (n_H). The Hill coefficient for the SP control was 3.8, and after the addition of cGMP, n_H was markedly reduced to 1.2. This suggests that cGMP, at elevated concentrations, prevents cooperative activation by the peptides. The phenomenon of cooperativity inherently relies on multiple ligand binding sites, however peptide

studies up to this point suggest the nest is the only location of peptide binding on PKG Iα. Therefore, the role of cGMP on the cooperativity of SP remains unclear.

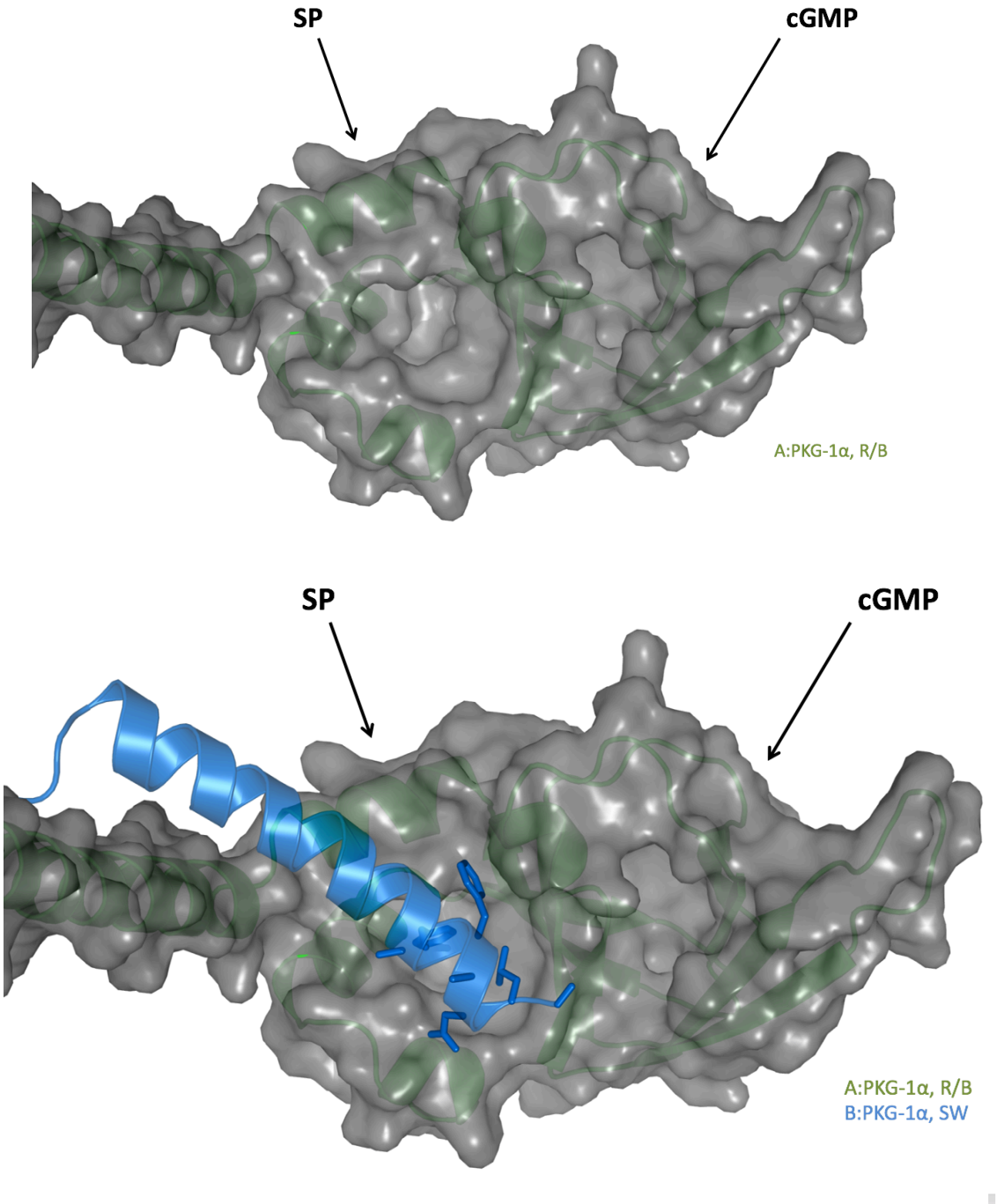


Figure 18. Binding site proximity of the switch peptide and cGMP. The top image shows the crystal structure of the unbound B-site. The bottom image has SP modeled (blue) such that it is interacting via the proposed mechanism with Phe residues providing a hydrophobic interaction with the nest. The two sites are separated by a pronounced ridge, which is located at the center and runs vertically in both images.

Hill Coefficient

The Hill coefficient is used to describe cooperativity in ligand binding. A Hill coefficient above 1 indicates that the binding of one ligand to an enzyme facilitates the binding of a second. All peptides examined in this study had a Hill coefficient above 1, which suggests that the peptides exhibited positive cooperativity. This may be observed because the dimeric structure of the kinase provides two nests which do not interact with the peptide independently. This hypothesis could be explored by using a monomeric mutant PKG Iα in the peptide assays.[18] If the dimerization of the kinase is responsible for the observed cooperativity, using a monomeric version of the kinase should reduce the Hill coefficient below 1.

Concentration-Dependent Effects on PKG Iα activity

At the highest peptide concentrations tested, it was noted that the activity of the kinase began to decrease instead of the predicted plateau (see Figures 9,14, 15, 16). This highly repeatable effect was observed for multiple peptides in this study as well previous studies. This may suggest that at high concentrations, the peptide activators begin to interact with the substrate itself. This hypothesis could be tested by using a different peptide substrate and seeing if the drop in activity occurs. Future studies could also examine this effect by incrementally increasing peptide concentrations beyond concentrations used in this study to determine how far activity could be reduced in this way.

Conclusion

An estimated 73M Americans have hypertension; however most respond poorly to current treatments. Uncontrolled hypertension leads to increased risk of stroke, heart attack, congestive heart failure, and kidney failure. There remains a significant unmet need for novel antihypertensive agents with improved efficacy and fewer side effects. None of the existing hypertension drugs target PKG, an enzyme critically responsible for mediating arterial dilation.

A peptide library directly targeting cGMP-dependent protein kinase (PKG) has been developed, however the pharmacophore of the lead peptide (SP) had not yet been solidified. It was hypothesized that two phenylalanine residues toward the C-terminus of the peptide provide crucial hydrophobic interactions which allow the peptide to activate the kinase. In this study it was observed that in the context of SP, neither phenylalanine residue is necessary for peptide-mediated activation of PKG. Though deleting one phenylalanine residue caused a marked reduction in potency and efficacy, substituting either lysine residue N-terminal from the deletion to alanine rescued the peptide's ability to activate the kinase.

Our peptides show dose-dependent activation specifically for the vascular PKG isoform, resulting in a rapid and reversible onset of action. These peptides have been demonstrated to promote 20-50% blood pressure reductions in rodent models of hypertension. This approach may provide immediate clinical benefit as an IV-administered drug for acute hypertensive crisis or may serve as a platform for oral/subcutaneous therapeutics for resistant hypertension.

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