Optical Perturbation of Protein Kinase A Activity via Photoactivatable Inhibitor Peptides

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Optical Perturbation of Protein Kinase A Activity via Photoactivatable Inhibitor Peptides
by
Peter Chen

A thesis presented to
the McKelvey School of Engineering
of Washington University in
partial fulfillment of the
requirements for the degree
of Master of Science

May 2023
St. Louis, Missouri
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May 2023
Protein Kinase A (PKA) plays important roles in diverse biological processes such as sleep, long term memory, and synaptic plasticity. In addition, PKA also acts as an integrator of neuromodulator signaling though G protein-coupled receptor activation. However, despite genetic knockout and pharmacological inhibition experiments that demonstrate the importance of PKA, it is unclear where, when, or how PKA plays these roles in cellular physiology and behavior. In order to better understand the function of PKA in these processes, and how neuromodulator signaling drives complex behavioral changes, there exists a need for a method to selectively activate/inactivate PKA with high spatial and temporal precision. The photoreactive light, oxygen, or voltage 2 (LOV2) domain from Avena sativa phototropin 1 can be used to facilitate the activation/inactivation of an endogenous inhibitory peptide targeting the PKA catalytic subunit. The blue light induced conformational changes in LOV2 results in potent inhibition of PKA activity in the presence of blue light, and effective blocking of kinase-inhibitor interactions in the absence of light. We demonstrate the potency of this photoactivatable inhibitor peptide under dark and lit conditions, as well as the kinetics of caging/uncaging.
Chapter 1: Introduction

Protein phosphorylation is a vital intracellular regulatory process for cells, often catalyzed by protein kinases (Cohen, 2002). Protein kinase A (PKA), also known as cyclic AMP (cAMP) dependent protein kinase, is implicated in numerous biological processes including long-term potentiation (LTP), learning, memory, sleep, and the development of neurodegenerative diseases (Brandon et al. 1997; Isiegas et al. 2006; Abel et al. 1997; Hellman et al. 2010; Bernabeu et al. 1997; Capece & Lydic, 1997; Dagda & Banerjee, 2015). Although much is known about the importance of PKA in these processes, the dynamics of PKA activity and how they influence function remain unclear. Recently developed fluorescent biosensors now allow for real time interrogation of PKA activity in living cells (Chen et al. 2014, Zhang et al. 2021). This technology has aided in our understanding of how PKA dynamically relates to both neuromodulator signaling, and downstream behavior effects (Lee et al., 2021). While these tools are incredibly powerful in visualizing dynamic changes in PKA activity across time, there still exists a need for tools to perturb PKA activity with similarly high spatiotemporal precision. These manipulations will help reveal exactly when and where PKA activity is crucial in these complex behaviors.

Existing methods to perturb PKA activity primarily utilize pharmacological techniques or genetic modification. For example, chemicals such as H89 or KT5720 serve as potent inhibitors of PKA activity (Murray 2008), but delivery methods for these drugs lack spatiotemporal precision. Intraperitoneal (IP) injections require agents to cross the blood brain barrier, application is not spatially confined within the brain, and while inhibition can occur within minutes after delivery, the washout period is long (Al Shoyaib et al., 2019). This set of
limitations makes IP injections an unideal method to study dynamic processes. Furthermore, commonly used inhibitors of PKA are nonspecific and impact other systems as well (Murray, 2008; Lochner & Moolman, 2006).

Genetic alterations of PKA activity are similarly blunt. PKA exists as a tetramer in the inactive state, with two regulatory subunits (PKAr) bound to two catalytic subunits (PKAc). Each PKAr dimer has four binding sites for cAMP. Upon cAMP binding, PKAc will separate from PKAr and become active (Murray, 2008). Genetic disruption of PKA activity generally works via removal of PKAc, overexpression of PKAr, or overexpression of endogenous protein kinase inhibitor proteins (PKI) to decrease overall PKA activity (Kirschner et al., 2009). These methods can perturb PKA with high specificity and potency, but still lack spatiotemporal precision. Spatial precision can be somewhat improved compared to pharmacology through cell type specific genetic modifications. However, temporal precision of genetic methods is even worse as inhibition begins as soon as expression begins, and can impact development (Kirschner et al., 2009).

A photoactivatable inhibitor of PKA has the potential to modulate PKA activity with high potency, specificity, and spatiotemporal precision. Using this method, subcellular spatial precision can be achieved via focusing of the stimulation light (Wu et al., 2009). Photoactivatable protein-based systems can also be delivered with cell type specificity via Cre/lox recombination. Temporal precision is also greatly improved compared to previous methods. Photoactivatable proteins can quickly and reversibly change conformation, allowing for inhibition and reversal on the seconds scale (Lungu et al., 2012; Nakasone et al., 2006).

The light, oxygen, voltage domain from *Avena sativa* phototropin 1 (LOV2) has shown promise in its ability to regulate peptide activity (Lungu et al., 2012). LOV2 is a member of the
PAS superfamily and contains a flavin mononucleotide (FMN). Upon irradiation with blue light, a covalent adduct is formed between a cysteine residue in the core domain and the FMN (Halavaty & Moffat, 2007; Harper et al., 2003). This initiates structural changes throughout the LOV2 domain, ultimately resulting in the unfolding of a large c-terminal jα helix (Crosson & Moffat, 2001; Harper et al., 2004; Swartz et al., 2002). When light stimulation is stopped this process reverses and LOV2 returns to the original dark state (Figure 1).

Previous work has demonstrated the ability for LOV2 to modulate activity of a fragment of the endogenously expressed PKIα appended to the c-terminus of the LOV2 jα helix (Yi et al., 2014). However, this construct showed limited inhibitory potency and dynamic range, preventing its use in experimental settings. Furthermore, one of the primary benefits of a photoactivatable inhibitor is the temporal precision, so it is important to characterize the temporal dynamics of PKA inhibition that can be achieved using such a system. Therefore, further work is needed to first optimize the dynamic range of these constructs, and second better understand the temporal dynamics with which PKA activity can be modulated using this system.

**Figure 1:** Crystal structure of the LOV2 domain from *Avena sativa* phototropin 1 (PDB ID: 2V0W)
Chapter 2: Methods

2.1 Cell Culture

HEK 293T cells were cultured in Dulbecco’s Modified Eagle Medium with 10% FBS (Millipore Sigma), GlutaMAX (Invitrogen), and penicillin /streptavidin (50 U/ml, Corning) at 37°C in 5% CO2. The cells were plated on 12mm coverslips in 24-well plates and transfected with plasmids one day later using lipofectamine 2000 (Invitrogen). One to two days after transfection the cells were imaged using two-photon fluorescence lifetime microscopy in a HEPES based buffer solution (138mM NaCl, 1.5mM KCl, 10mM HEPES, 1.2mM MgCl2, 2.5mM CaCl2, 10mM glucose, titrated with NaOH to a pH of 7.35).

2.2 DNA Plasmids

Plasmids paPKIv1-mCherry-C1, paPKI9-22-mCherry-C1, paPKI2.1.3-mCherry-C1, paPKI2.1.5-mCherry-C1, EGFP-PKACalphaQR, and pmCherry-C1 were gifts from Dr. Jason Yi’s laboratory. AAV-AKAR6 (Addgene plasmid # 63058) and AAV-FLEX-PKIApha-IRES-nls-mRuby2 (Addgene plasmid # 63059) were made previously by Dr. Yao Chen (Chen et al. 2014). mEGFP was purchased from Addgene (Addgene plasmid #18696).

2.3 Two Photon Fluorescence Lifetime Microscopy

Two photon fluorescence lifetime microscopy (2pFLIM) was performed on a custom-built system. An 80MHz laser (Insight X3, Spectra Physics) was used for excitation. Photon collection was performed with fast photomultiplier tubes (H10770PB-40, Hamamatsu). A 60x NA1.1 objective (Olympus) was used. Imaging of GFP/GFP based sensors was performed using 920nm excitation and a 13.2 μs pixel dwell time. Emission light was separated from excitation
using a dichroic mirror (FF580-FDi01-25X36, Semrock) and then filtered using a 525/50-25
bandpass filter (FF03- 525/50-25, Semrock). Time correlated single photon counting was
performed in 256-time channel mode with a FLIM board (SPC-150, Becker and Hickl GmbH).
The system performs scanning/image acquisition via a custom software (ScanImage) as
described previously (Pologruto et al., 2003). 128x128 pixel frames were acquired at a scan rate
of 4Hz and photons were pooled from 10 frames per acquisition for analysis.

2.4 Fluorescence Lifetime Image Analysis

Fluorescence lifetime was calculated using the empirical lifetime corrected via a figure
offset term. The empirical lifetime is defined as the average photon arrival time as described in
equation 2.1.

\[
\text{Empirical Lifetime} = \frac{\sum F(t)\cdot (t-t_0)}{\sum F(t)} \quad (\text{eq. 2.1})
\]

Where \(F(t)\) represents the fluorescence intensity in each time channel, \(t\) represents the time
correlating to each time channel, and \(t_0\) represents the figure offset term, calculated through
fitting. The figure offset is defined as the difference between the lifetime calculated through
fitting and the average photon arrival time. The histogram of photon arrival times for each image
was fit to the following double exponential decay equation convolved with the empirically
determined IRF as show in equation 2.2.

\[
F(t) = [F_0(P_D e^{-\frac{t}{\tau_D}} + P_{AD} e^{-\frac{t}{\tau_{AD}}})] \ast IRF \quad (\text{eq. 2.2})
\]

Where \(F(t)\) is the fluorescence at time \(t\), and \(F_0\) is the peak fluorescence. \(P_D\) and \(P_{AD}\) represent
the populations of donor fluorophores that are either free (\(P_D\)) or undergoing Förster resonance
energy transfer (FRET). \(\tau_D\) and \(\tau_{AD}\) are the fluorescence lifetimes of the free donors and donors
undergoing FRET respectively. The 2pFLIM instrument response function (IRF) was empirically

determined by 2nd harmonic generation with collagen fibers from a mouse tail and 1050nm excitation. Cytoplasmic sensor fluorescence lifetime changes were calculated by performing image registration and region of interest (ROI) analysis to exclude signal from the nucleus and background. The nucleus was excluded because PKA activity changes occur on a different timescale compared to cytoplasmic activity.

The amplitude of change for dark/lit state activity was calculated using the difference between the average of the final 3 acquisitions in the baseline period and the minimum of the following time period. Response time was limited to 6 minutes when examining the magnitude of the forskolin driven PKA activity increase.

For quantification of intermolecular FRET experiments, the percent binding fraction $(P_{AD})$ was calculated from the empirical lifetime via equation 2.3 (Yasuda et al., 2006).

$$P_{AD} = \frac{\tau_d(\tau_d-\tau)}{(\tau_d-\tau_{AD})(\tau_d+\tau_{AD}-\tau)} \quad (eq. \ 2.3)$$

Where $\tau$ is the figure offset corrected empirical lifetime from equation 2.1. $\tau_d$ was fixed to 2.59ns as empirically determined from cells expressing EGFP without a FRET acceptor. $\tau_{AD}$ was determined through fitting and fixed at 1.45ns.

2.4 Statistical Analysis
GraphPad Prism 9 was used to perform non-parametric, unpaired population comparison (Mann Whitney test). Intermolecular FRET experiments for binding kinetics were fit to a single-phase exponential decay equation using GraphPad Prism 9 to extract the time constant.

2.5 Optical Stimulation
Optical stimulation was performed using a 470nm LED (Thorlabs M470F3) coupled to a 400-micron core diameter 0.39NA fiber (Thorlabs FT400EMT Custom Fiber). The fiber tip was
placed within 1mm of the sample. 9mW stimulation was delivered via a single 5s pulse between each image acquisition, with a resulting interstimulus interval (ISI) of 2.5s.

2.6 Pharmacology
Pharmacological agents were delivered via bath application. Forskolin (Cayman Chemical Company) was dissolved into DMSO at 100mM, and was spiked into the HEPES based buffer perfusion bath for a final concentration of 50uM.
Chapter 3: Results

3.1 Construct Description

Numerous constructs containing different combinations of mutations to the LOV2 domain and different lengths of the PKIα peptide were created by Dr. Jason Yi’s lab and screened in our two labs with independent assays (data not shown). Photoactivatable PKI (paPKI) constructs were N-terminally tagged with the red fluorescent protein mCherry to verify expression (Figure 2). Of the constructs screened, three showed potential in maintaining a high dynamic range (constructs described in Table 1). These three constructs were fully characterized and compared against the previously published construct (paPKIv1) in this work.

Figure 2: Schematic of paPKI. Endogenous PKIα fragments were appended to the C-terminal jα helix of LOV2 from Avena sativa phototropin 1. Upon 470nm optical stimulation PKI is uncaged, allowing for binding to PKA and inhibition of PKA activity.

The first construct identified utilized the LOV2 domain (amino acids 404-541) with PKIα peptides 14-22 appended on the C-terminus of the jα helix. The LOV2 domain contained two sets of mutations which were previously found to improve the speed and dynamic range of light activation. First the T406, 407A mutations were introduced to increase speed of photoactivation (Zayner et al., 2012). Second the I532A mutation was introduced into the LOV2
domain to increase caging efficiency (Strickland et al., 2010). This construct was named paPKI2.1.3.

The second construct identified was the same as paPKI 2.1.3 but with two additional modifications. First, amino acids 404-539 of the LOV2 domain were used. Second, PKIα peptides 10-22 were used. This construct was named paPKI2.1.5.

The third construct identified in the preliminary screening again utilized amino acids 404-539 of the LOV2 domain in combination with PKIα peptides 9-22 appended to the C-terminus. This construct contained the same T406, 407A and the I532A mutations to the LOV2 domain. This construct was named paPKI9-22.

Table 1: Descriptions of mutations in the paPKI constructs tested.

<table>
<thead>
<tr>
<th>Construct</th>
<th>LOV2 Peptides</th>
<th>LOV2 Mutations</th>
<th>PKIα Peptides</th>
</tr>
</thead>
<tbody>
<tr>
<td>paPKI v1</td>
<td>404-541</td>
<td>NA</td>
<td>14-22</td>
</tr>
<tr>
<td>paPKI2.1.3</td>
<td>404-541</td>
<td>T406A, T407A, I532A</td>
<td>14-22</td>
</tr>
<tr>
<td>paPKI2.1.5</td>
<td>404-539</td>
<td>T406A, T407A, I532A</td>
<td>10-22</td>
</tr>
</tbody>
</table>

3.2 Dynamic Range

Each construct was co-transfected with a FRET based fluorescent biosensor for PKA activity (FLIM-AKAR), which shows a fluorescence lifetime decrease in response to increases in PKA activity (Chen et al. 2014). Sensor fluorescence was imaged using 2pFLIM. The adenylate cyclase activator forskolin (FSK) was added to increase PKA activity, and the resulting FLIM-AKAR response magnitude in the presence/absence of blue light stimulation is representative of the construct inhibitory potency in the lit and dark state respectively (Figure 3A-3C). Constructs with high inhibition under blue light stimulation and low inhibition in the absence of blue light are desirable. The mCherry-paPKI constructs were replaced with mCherry alone or PKI to serve
as a control for the dark and lit conditions respectively. Light stimulation had no effect on the FSK driven PKA activity increase in cells expressing either mCherry alone or PKI.

Figure 3: (A) Example 2pFLIM images of HEK293T cells expressing FLIM-AKAR and paPKI9-22. Cells in the top row were imaged in the dark, and cells in the bottom row were given 5s pulses of 9mW 470nm optical stimulation between each acquisition (ISI = 2.5s). (B-C) Example traces of the cytoplasmic FLIM-AKAR response to FSK (applied at time = 0 min) in either the dark or lit conditions with different paPKI constructs. mCherry was included as a control for dark state activity and PKI was included as a control for lit state activity. Optical stimulation was applied in 5s pulses (ISI = 2.5s) of 9mW, 470nm light.
**Figure 4:** Summary data representing the magnitude of the cytoplasmic FLIM-AKAR response following addition of FSK when cells express different paPKI constructs, and are either under blue light stimulation (9mW, 470nm, 5s pulses, ISI = 2.5s) or in the dark. mCherry was included as a control for responses with no inhibition and PKI was included as a control for maximum inhibition. *p<0.0001, Mann Whitney test.

We found that paPKI9-22 showed the largest difference between the lit state and the dark state compared to the rest of the constructs (Figure 4). In the absence of 470nm light there was no significant difference in the FSK driven PKA response magnitude when cells expressed mCherry-paPKI9-22 compared to mCherry alone (p = 0.736, Mann Whitney test). The median response magnitude with paPKI9-22 in the dark state was 0.188 ns (IQR = 0.077 ns), similar to the median response amplitude of 0.213ns (IQR = 0.097 ns) in the mCherry control group. When cells expressing paPKI9-22 were placed under 470nm optical stimulation, the median PKA response magnitude was reduced to 0.013ns (IQR = 0.030 ns). The PKI control group had a median response magnitude of 0.002ns (IQR = 0.009). Although the lit state of paPKI9-22 did not show as much inhibition as the PKI control group (p = 0.0069, Mann Whitney test), there
was still a significant difference between the lit and dark states of paPKI9-22 (p<0.0001, Mann Whitney test) demonstrating effective photoactivation. The entire dynamic range of a given construct can be estimated by calculating the fold-change in PKA response magnitude induced by light stimulation as shown in equation 3.1.

\[
Fold \ Change = \frac{\Delta \text{Lifetime}_{\text{Median,Dark}}}{\Delta \text{Lifetime}_{\text{Median,Lit}}} \quad (\text{eq. 3.1})
\]

Where \( \Delta \text{Lifetime}_{\text{Median,Dark}} \) is the median PKA response magnitude in the dark state and \( \Delta \text{Lifetime}_{\text{Median,Lit}} \) is the median PKA response magnitude under 470nm stimulation. With this metric paPKI9-22 has a fold-change of 14.25 that is inducible via light stimulation. This is a 3.6x improvement over paPKIv1 which has a fold-change of 3.91 (Table 2).

**Table 2:** Dynamic range of each construct. Fold change is calculated from the median FSK driven PKA activity response in the dark condition divided by the median response in the lit condition.

<table>
<thead>
<tr>
<th>Construct</th>
<th>Median Fold-Change</th>
</tr>
</thead>
<tbody>
<tr>
<td>paPKIv1</td>
<td>3.91</td>
</tr>
<tr>
<td>paPKI2.1.3</td>
<td>1.25</td>
</tr>
<tr>
<td>paPKI2.1.5</td>
<td>7.32</td>
</tr>
<tr>
<td>paPKI9-22</td>
<td>14.25</td>
</tr>
</tbody>
</table>

### 3.3 Kinetics of Inhibition

After identifying paPKI9-22 as the construct with the largest dynamic range, we aimed to characterize the binding kinetics of this construct by using 2pFLIM to measure changes in intermolecular FRET between the mCherry-paPKI9-22 and an EGFP tagged constitutively active PKAc (EGFP-PKACalphaQR). Blue light stimulation was used to activate paPKI, which should cause paPKI to bind to PKAc. This results in increased FRET between EGFP and mCherry, and a fluorescence lifetime decrease in the EGFP emission. Upon cessation of blue light stimulation, the two species should dissociate, resulting in a fluorescence lifetime increase of the EGFP.
emission (Figure 5). Given EGFP is known to form dimers at high concentrations, only cells showing dim expression of EGFP were imaged (Ilagan et al., 2010; Ward, 2005).

**Figure 5:** Diagram of blue light dependent FRET between mCherry and EGFP. In the dark state the two fluorophores are too far apart for FRET to occur, but upon blue light stimulation, PKI will bind to and inhibit PKA, resulting in FRET between EGFP and mCherry. This process reverses when PKI dissociates from PKA after blue light stimulation is removed.

Application of blue light stimulation resulted in an immediate fluorescence lifetime decrease (increased FRET/binding). When blue light stimulation was turned off, fluorescence lifetime recovered back to the baseline value (Figure 6A). Replacing EGFP-PKACalphaQR with mEGFP alone abolished the blue light dependent fluorescence lifetime change (Figure 6A), indicating that PKAc is required for binding/FRET to occur. The binding fraction was calculated based on the fluorescence lifetime traces as described in the methods (Figure 6B). The decrease in binding as the two species disassociated was fit to a single exponential decay equation, giving an average $K_{off}$ rate of 0.443 min$^{-1}$ (SD = 0.21 min$^{-1}$, n = 19). The initial increase in binding occurs too rapidly to produce a good fit given the imaging speed. The photoactivatable binding process is reversible and repeatable using a series of blue light pulses (Figure 6C).
**Figure 6:** (A) Example fluorescence lifetime trace from cells co-expressing mCherry-paPKI9-22 and either EGFP-PKACalphaQR or mEGFP alone in HEK293T cells. Blue light stimulation was applied via a single 5s pulse of 9mW, 470nm light at time = 0. (B) The same example traces as in panel A, but displaying the calculated binding fraction. The decay to baseline was fit using a single-phase exponential equation, with $K_{off} = 0.252\text{min}^{-1}$. C) Light activated binding and dissociation between mCherry-paPKI9-22 and EGFP-PKACalphaQR is reversible and repeatable using a series of blue light pulses (9mW, 470nm, 5s pulse width).

After characterization of binding kinetics, we went on to test how rapidly paPKI can inhibit PKA activity after light stimulation. Cells were co-transfected with FLIM-AKAR and paPKI9-22, and FLIM-AKAR was imaged using 2pFLIM. FSK was used to increase PKA activity, during which blue light stimulation was applied. FSK driven PKA activity increase was halted after just one 5s pulse of blue light, resulting in a fluorescence lifetime increase as blue
light stimulation continued. After blue light stimulation was turned off, PKA activity began to increase within 30 seconds (Figure 7). These results indicate that paPKI can inhibit PKA activity within seconds of activation via blue light stimulation, and that reversal of inhibition occurs on the seconds-minutes scale following cessation of blue light stimulation.

**Figure 7:** Pulsed light stimulation (9mW, 470nm, 5s pulse width, ISI = 2.5s) of paPKI9-22 can interrupt the FSK driven PKA activity increase in HEK293T cells. Pulsed light stimulation was performed for a total of 2.35 minutes. Fluorescence lifetime began to increase after just one 5s pulse of blue light and began to decrease within 30 seconds of removing the light stimulation.
Chapter 4: Discussion

By leveraging the light induced conformational changes associated with the C-terminal $\alpha$ helix in LOV2, we demonstrate control of PKA activity via light-activated PKI. paPKI9-22 shows no significant inhibition of PKA activity in the dark state, and the ability to quickly change conformation in response to blue light, resulting in a 14.25-fold change in inhibitory potency. This is a 3.6-fold improvement over the previously developed construct, which has only a 3.91-fold change in inhibitory power in response to light stimulation. This large change in inhibitory potential is fully reverseable and repeatable. The increase in dynamic range of paPKI9-22 was achieved via two sets of mutations. First, paPKI9-22 utilized PKI peptides 9-22, which improved the inhibitory potency of this construct in the lit state, while showing minimal inhibition in the dark state. Second, the I532A mutation was introduced into the LOV2 domain which improved the dark state caging efficiency of PKI activity.

Furthermore, we characterized the kinetics of PKA inhibition that are achievable using this system, with an average $K_{off}$ rate of 0.443 min$^{-1}$ (SD = 0.21 min$^{-1}$). However, further work should be done to simplify the interpretation of findings regarding binding kinetics due to the tendency of EGFP to form dimers at high concentrations. Introducing the A206K mutation in EGFP results in a monomeric mutant, which would be ideal for experiments regarding binding kinetics (Zacharias et al., 2002). In addition, surface plasmon resonance can be used to characterize binding affinities in the lit and dark states (Lungu et al., 2012). This could also be used to capture the on rate, which is difficult using 2pFLIM.

We can use paPKI9-22 to inhibit PKA activity with high potency within seconds of blue light stimulation, and inhibition is spatially limited to the region of stimulation. Using pharmacological or genetic methods to study how the dynamics of endogenous PKA activity
relate to function can be difficult due to the lack of spatiotemporal precision. In contrast, paPKI9-22 offers a new, minimally invasive method to perturb PKA activity with high potency and greatly improved spatiotemporal precision. Future work in this area should characterize the function of paPKI9-22 in vivo. After in vivo characterization, this system can be used to perform targeted experiments to discover when and where PKA is crucial during different behaviors.


