INTRODUCTION

Reviewer	Signif.	Invest.	Innov.	Appr.	Envir.
#1	2	1	2	3	1
#2	2	2	2	3	2
#3	2	2	2	4	2

We are grateful to the reviewers for their thoughtful critique of our application. We appreciate their enthusiasm for the significance and innovation of the proposed work, and confidence that our laboratory has the expertise and resources to conduct the proposed experiments. The table to the right shows the reviewers' scores for our original submission, which indicate that most of their concerns were with our Approach. In retrospect, we agree that our experimental approach was overly ambitious and understand how inclusion of too many sub-aims distracted from our focus. We also failed to provide sufficient preliminary data to allay concerns about the feasibility of some of the proposed work. We have fully addressed these concerns in the revised proposal by: (1) removing Aim 3A-2 (identifying novel PDE6 binding partners) and refocusing the remainder of this aim on hypothesis-driven experiments with known PDE6 interacting proteins; (2) providing new preliminary data demonstrating functional expression of cone PDE6 GAFab that will permit us to carry out the experiments proposed in Aim 1C-2 (rod-cone differences in PDE6 GAF domains) and in Aim 2B-2 (structural determination of PDE6 GAFab with bound Pγ); (3) improving the organization and clarity of Aim 2 by focusing on the most innovative and significant experiments and eliminating sub-aims that stray from the main objectives; (4) increasing the duration of the project from 4 to 5 years in order to realistically accomplish the work.
Our responses to the reviewers' criticisms are organized by the core review criteria. Because we have revised the proposal throughout to improve clarity, we have not marked minor changes. Significant content changes in the proposal are marked with a vertical line and/or an italic remark at the beginning of the revised section.

INNOVATION: No weaknesses noted by reviewers.

ENVIRONMENT: No weaknesses noted by reviewers.

SIGNIFICANCE: Reviewer 2 expressed concern about the relevance of *in vitro* experiments to examine PDE6 interactions to the *in vivo* situation in the rod outer segment (addressed below in Approach responses).

INVESTIGATOR(S): Reviewer 2 mentioned "slowed productivity." Since the original grant submission, we have published a 5th *J. Biol. Chem.* publication (Cahill et al., 2012). We also submitted three abstracts for the Experimental Biology 2013 meeting, all for work completed during the 3-year project period. The latter represent the content for two additional manuscripts to be submitted within the next three months.

APPROACH:

- Aim 1C-2 and Aim 2B: Both Reviewers 1 and 2 expressed concern regarding the expression of biologically active PDE6 GAFab domains, which has proven difficult in the past. This concern is addressed by the inclusion of new preliminary data showing expression of functionally active PDE6 GAFab dimer. Our new results demonstrate that we can express in bacteria dimeric cone PDE6 GAFab domains capable of binding cGMP and Pγ. The revised approach section for Aim 2B also includes information to address Reviewer 2's concern about limitations to the use of a truncated PDE6 GAFab dimer to examine the allosteric/regulatory properties of the full-length, native enzyme. We also more thoroughly discuss potential pitfalls and alternative strategies to increase the likelihood of achieving our objectives.
- Aim 2A: To address the criticism that Aim 2 was too ambitious/unfocused, we have eliminated Aim 2A-2 (characterization of structural changes in Pγ upon transducin and RGS9-1 binding) in order to focus on the mechanism by which Pγ potentiates the GTPase activity of transducin (2A-3).
- Aim 3A: Reviewers 1 and 2 both cited a lack of focus and insufficient justification for Aim 3A-2 (proteomic identification of novel PDE6 binding proteins). We have removed Aim 3A-2 from the current proposal. Reviewer 1 also criticized the absence of feasibility studies for using chemical cross-linking to determine the topological relationships of PDE6 subunits and of PDE6 in a complex with transducin and/or RGS9-1. We have provided additional cross-linking results documenting the feasibility and power of this approach to define the interactions between subunits of the PDE6 heterotetramer ($\alpha\beta\gamma\gamma$), as well as increasing confidence that we can determine the architecture of the PDE6-transducin-RGS9-1 signaling complex.
- New supporting data: We have also included additional preliminary data relevant to the revised proposal: (1) major advances in identifying preferential interactions of Pγ for the catalytic subunits of PDE6 (Aim 1B) and in defining the topology of the PDE6 holoenzyme (Aim 3A) using chemical cross-linking and MS-based sequencing. (2) discovery that GARP2 reduces the efficiency of transducin activation of PDE6 (Aim 3B-1), and; (3) the stoichiometry of transducin and GARP2 binding to PDE6 holoenzyme has been unequivocally determined using sedimentation equilibrium with the fluorescence detection system (Aims 1B and 3B).