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Effect of the *ILE86TER* mutation in the γ subunit of cGMP phosphodiesterase (PDE6) on rod photoreceptor signaling☆

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ABSTRACT

The light-dependent decrease in cyclic guanosine monophosphate (cGMP) in the rod outer segment is produced by a phosphodiesterase (PDE6), consisting of catalytic α and β subunits and two inhibitory γ subunits. The molecular mechanism of PDE6γ regulation of the catalytic subunits is uncertain. To study this mechanism *in vivo*, we introduced a modified *Pde6g* gene for PDE6γ into a line of $Pde6g^{tm1}/Pde6g^{tm1}$ mice that do not express PDE6γ. The resulting *ILE86TER* mice have a PDE6γ that lacks the two final carboxyl-terminal Ile^{86} and Ile^{87} residues, a mutation previously shown *in vitro* to reduce inhibition by PDE6γ. *ILE86TER* rods showed a decreased sensitivity and rate of activation, probably the result of a decreased level of expression of PDE6 in *ILE86TER* rods. More importantly, they showed a decreased rate of decay of the photoresponse, consistent with decreased inhibition of PDE6 α and β by PDE6γ. Furthermore, *ILE86TER* rods had a higher rate of spontaneous activation of PDE6 than WT rods. Circulating current in *ILE86TER* rods that also lacked both guanylyl cyclase activating proteins (GCAPs) could be increased several fold by perfusion with 100 μM of the PDE6 inhibitor 3-isobutyl-1-methylxanthine (IBMX), consistent with a higher rate of dark PDE6 activity in the mutant photoreceptors. In contrast, IBMX had little effect on the circulating current of WT rods, unlike previous results from amphibians. Our results show for the first time that the Ile^{86} and Ile^{87} residues are necessary for normal inhibition of PDE6 catalytic activity *in vivo*, and that increased basal activity of PDE can be partially compensated by GCAP-dependent regulation of guanylyl cyclase.

1. Introduction

The absorption of a photon in the outer segment of a rod photoreceptor [see1] produces an excited form of rhodopsin (Rh*), which binds a heterotrimeric G-protein called transducin and catalyzes the exchange of GTP for GDP on the transducin alpha subunit (Tα). The TαGTP then binds rod phosphodiesterase6 (PDE6), an enzyme complex that consists of catalytic PDE6α and PDE6β subunits and two regulatory PDE6γ subunits. In the dark, PDE6γ is bound to PDE6 α and β and inhibits catalytic activity. Upon light exposure, the newly formed TαGTP binds to PDE6γ, causing the inhibitory subunit to be displaced from the active site of a catalytic subunit. The PDE6 is then free to hydrolyze cGMP, and this hydrolysis decreases the outer segment cGMP concentration and produces a closing of cGMP-gated ion channels, which alters the rod membrane potential.

Because the PDE6γ subunit acts as the control point for regulating cGMP hydrolysis, it plays a key role in the transduction cascade. Little is known, however, about the molecular mechanism by which PDE6γ regulates PDE6 catalytic activity, though some information has been obtained from reconstituted systems. The PDE6γ contains a central

Abbreviations: BSA, bovine serum albumin; cGMP, cyclic guanosine monophosphate; ES, embryonic stem cell; GAP, GTPase accelerating protein; GCAPs, guanylyl cyclase activating proteins; GDP, guanosine diphosphate; GTP, guanosine triphosphate; IBMX, isobutylmethylxanthine; IDV, integral density value; OS, outer segment; PDE, cGMP phosphodiesterase; PDE6, cGMP phosphodiesterase 6; Rh*, active form of bleached rhodopsin (metarhodopsin II); PBS, phosphate-buffered saline; PCR, polymerase chain reaction; T, transducin; Tα, alpha subunit of transducin; WT, wild-type.

☆ Author contributions: Experiments were performed in New York, New York, and in Los Angeles, California. The conception and design of the experiments were done by S. H. Tsang, J. Chen, M. L. Woodruff, and G. L. Fain; the collection, analysis and interpretation of data were done by C.-S. Lin, M. L. Woodruff, B. D. Jacobson, M. C. Naumann, C. W. Hsu, R. J. Davis, Marianne Cilluffo, S. H. Tsang, and G. L. Fain; and the drafting of the article or revising it critically for important intellectual content was done by J. Chen, S. H. Tsang, M. L. Woodruff and G. L. Fain.

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lysine-rich region, in which 10 of 13 amino acids are basic [2]. These residues apparently contain one site for interaction with Tα [3] and are essential for binding of PDE6γ to the PDE6 α and β catalytic core [4]. The region involved in inhibiting PDE catalytic activity is thought to lie near the carboxyl terminus; deletions and point mutations in the carboxyl terminus have been shown *in vitro* to decrease inhibition of PDE activity [5–7]. Furthermore, the corresponding peptides with a mutated carboxyl terminus of PDE6γ fail to inhibit trypsin-activated PDE6 *in vitro* [8].

In order to test the function of specific amino acids or protein domains of PDE6γ *in vivo*, we constructed mutant PDE6γ cDNA under the control of the opsin promoter and generated transgenic mice by conventional means [9,10]. The transgenes were then transferred by breeding to *Pde6g*^{tm1}/*Pde6g*^{tm1} mice, homozygous for a targeted disruption of the endogenous PDE6 gene [11].

In this study, we examined the *ILE86TER* mutation lacking Ile^{86} and Ile^{87}, the last two amino acids in PDE6γ. These amino acids have been previously shown *in vitro* to play an essential role in PDE6 function [2,12]. We show that the light responses of mutant rods have a dramatically slower time course of decay, and that PDE6 in the mutant photoreceptors has a higher level of spontaneous activity in darkness. We conclude that Ile^{86} and Ile^{87} are essential for controlling PDE6γ inhibition of PDE6αβ *in vivo*.

2. Materials and methods

2.1. Generation of ILE86TER animals

Experiments were performed in accordance with the rules and regulations of the NIH guidelines for research animals, as approved by the institutional animal care and use committees (IACUCs) of Columbia University, University of California, Los Angeles and University of Southern California. Animals were kept in cyclic 12-on/12-off lighting in approved cages and supplied with ample food and water. Animals in all experiments were sacrificed before tissue extraction by an approved procedure, usually decerebration or with an intraperitoneal injection of Nembutal.

The *ILE86TER* DNA construct for expression of *Pde6g* [13], together with the polyadenylation signal of the mouse protamine gene [14], was injected into the male pronucleus of oocytes. The *ILE86TER* point mutation was introduced by a standard PCR-based site-specific mutagenesis strategy [11]. The entire *Pde6g* cDNA coding region in the transgenic construct was sequenced to confirm the introduction of the point mutation and to verify that no other changes had been created inadvertently. KpnI and XbaI were used to excise vector sequences from the constructs. Fertilized oocytes were obtained from superovulated F1 (DBA X C57BL6) females mated with homozygous *Pde6g*^{tm1}/*Pde6g*^{tm1} males. The construct was injected into the male pronuclei of oocytes under a depression slide chamber. These microinjected oocytes were cultured overnight in M16 and transferred into the oviducts of 0.5-day post coitum pseudopregnant F1 females. The resulting transgenic mice were then backcrossed to *Pde6g*^{tm1}/*Pde6g*^{tm1} mice to place the transgene into the knockout background. The mice were also tested for the absence of the *rd1* mutation [15].

2.2. Identification of transgenic mice

DNA was isolated from tail tips or liver samples by homogenizing the tissue, digesting extensively with proteinase K and extracting with phenol. DNAs were analyzed by the polymerase chain reaction (PCR). The DNAs were also digested by SacI and analyzed by Southern blot hybridization with a *Pde6g* cDNA probe. Additional restriction digests were performed to analyze the structure of the integrated sequences, and to ensure that the DNA flanking the transgene was intact.

2.3. Immunoblot analyses

Each retina was homogenized in 100 μl buffer (50 mM Tris, pH 7.4, 1 mM EDTA with protease inhibitor mixture from Roche Diagnostics, Indianapolis, IN), to which another 100 μl of sample loading buffer was added; the samples were boiled for 5 min. From this sample extract, different amounts were loaded onto a 4%–12% gradient gel (1, 2 and 3 μl for WT and 8, 12 and 16 μl for ILE86ter). For the detection of PDE6γ, blots were incubated with a 1:2,000 dilution of a polyclonal antibody directed against amino acid residues 2–16. Other antibodies were: PDE6α (PA1-720, 1:2,000, Thermo Scientific), RGS9 (from M.I. Simon, 1:5,000), Gαt (K60006R, 1:5,000, Meridian Life Science), and Gβ1 (sc-379, 1:2,000, Santa Cruz Biotechnology). The secondary antibody was IRDye-labeled (1:10,000, LI-COR Biosciences), and the bands were detected and the fluorescence intensities were quantified with the Odyssey infrared imaging system (LI-COR Biosciences). In additional control experiments not shown in Fig. 1, we used the following primary antibodies to other phototransduction enzymes: GUCY2E, a polyclonal antibody to guanylyl cyclase 2E (gift of Prof Alexander M. Dizhoor, Pennsylvania College of Optometry, USA); GRK1 (rhodopsin kinase), polyclonal antibody sc-13078 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) to GRK1; rhodopsin, 1D4 monoclonal antibody to opsin (gift R. S. Molday of the University of British Columbia, Vancouver, Canada); and the saryl hydrocarbon receptor-interacting protein-like 1, a polyclonal antibody (gift of Visvanathan Ramamurthy, Morgantown, WV, USA). In some experiments Western blots were visualized with the Duo-Lux Chemiluminescence substrate kit (Vector Laboratories, Inc., Burlingame, CA, USA) with a goat-anti-rabbit IgG-alkaline phosphatase conjugate. Blots were exposed to Hyperfilm-MP (Amersham Pharmacia Biotech, Piscataway, NJ, USA) and were preflashed to increase sensitivity and linearity according to the Sensitize™ protocol (Amersham Pharmacia Biotech).

2.4. Histology

Mice were euthanized with an intraperitoneal injection of Nembutal. Each eye was rapidly removed, punctured at 12:00 along the limbus, and placed in a separate solution of 3% glutaraldehyde in phosphate buffered saline. After fixation for 1–2 days, the eyes were washed with saline and the 12:00 limbal puncture was used to orient the right and left eyes, which were kept in separate buffer so that the posterior segment containing the retina could be sectioned along the

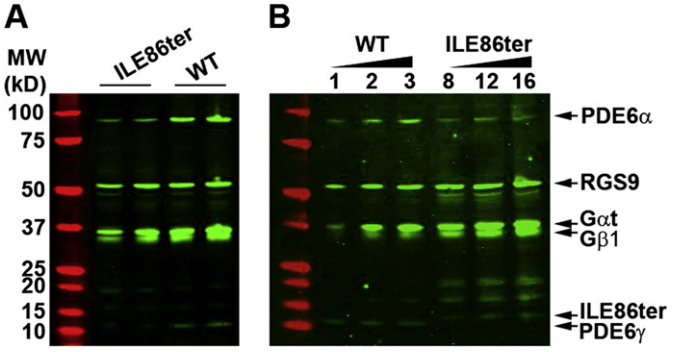

Fig. 1. Immunoblot analysis of the expression of PDE and other rod transduction proteins. (A). The levels of RGS9, Gαt and Gβ1 were comparable between WT and ILE86ter retinas. However, PDE6 α and γ subunits were noticeably lowered in the ILE86ter retinas. Equal fraction of a retina (1/50) from an individual mouse was loaded onto each lane. (B). Quantification of PDE expression levels in ILE86ter and WT retinas. Representative blot of retinal extract prepared from WT and *ILE86TER/GCAPs*$^{-/-}$ mice. Each lane represents the amount loaded (μl) per retina (200 μl total sample volume). Based on the fluorescence signal quantified from each sample, the amount of PDE6α and PDE6γ in ILE86ter was 10 ± 3% of WT (N = 3). Control experiments revealed no difference in PDE6 subunit expression levels between *ILE86TER* and *ILE86TER/GCAPs*$^{-/-}$ mice. Levels of other transduction proteins (RGS9, Gαt and Gβ1) were similar between WT and ILE86ter.