Phosphorylation of the Ca^{2+} -Binding Protein CaBP4 by Protein Kinase C ζ in Photoreceptors

Amy Lee,¹ Amber Jimenez,² Guiying Cui,¹ and Françoise Haeseleer²

¹Department of Pharmacology and Center for Neurodegenerative Disease, Emory University School of Medicine, Atlanta, Georgia 30322, and ²Department of Ophthalmology, University of Washington, Seattle, Washington 98195

CaBP4 is a calmodulin-like neuronal calcium-binding protein that is crucial for the development and/or maintenance of the cone and rod photoreceptor synapse. Previously, we showed that CaBP4 directly regulates Ca_v1 L-type Ca^{2+} channels, which are essential for normal photoreceptor synaptic transmission. Here, we show that the function of CaBP4 is regulated by phosphorylation. CaBP4 is phosphorylated by protein kinase C ζ (PKC ζ) at serine 37 both *in vitro* and in the retina and colocalizes with PKC ζ in photoreceptors. CaBP4 phosphorylation is greater in light-adapted than dark-adapted mouse retinas. In electrophysiological recordings of cells transfected with $\text{Ca}_v1.3$ and CaBP4, mutation of the serine 37 to alanine abolished the effect of CaBP4 in prolonging the Ca^{2+} current through $\text{Ca}_v1.3$ channel, whereas inactivating mutations in the CaBP4 Ca^{2+} -binding sites strengthened $\text{Ca}_v1.3$ modulation. These findings demonstrate how light-stimulated changes in CaBP4 phosphorylation and Ca^{2+} binding may regulate presynaptic Ca^{2+} signals in photoreceptors.

Key words: CaBP4; Ca^{2+} -binding proteins; phosphorylation; protein kinase C ζ ; Ca^{2+} channel; photoreceptors

Introduction

CaBP4 belongs to a subfamily of neuronal Ca^{2+} -binding proteins (CaBPs) with high similarity to calmodulin (CaM) (Haeseleer et al., 2000, 2002, 2004; Haeseleer and Palczewski, 2002; Maeda et al., 2005). CaBPs bind to and yet differentially modulate a number of CaM targets, including voltage-gated Ca^{2+} channels (Lee et al., 2002; Zhou et al., 2004; Yang et al., 2006), transient receptor potential TRP channels (Kinoshita-Kawada et al., 2005), and inositol 1,4,5-trisphosphate (IP_3) receptors (Yang et al., 2002; Haynes et al., 2004; Kasri et al., 2004). Through interactions with these and potentially other effectors, CaBPs may enhance the Ca^{2+} signaling potential in neurons and other excitable cells.

In the retina, CaBP4 is localized in the photoreceptor synaptic terminal and is required for normal neurotransmission to bipolar cells (Haeseleer et al., 2004; Maeda et al., 2005). Mice lacking CaBP4 (*Cabp4*^{−/−}) exhibit synaptic defects and electroretinograms, indicating impaired cone and rod synaptic function. Multiple lines of evidence suggest that visual deficits in *Cabp4*^{−/−} mice result from dysregulation of presynaptic Ca_v1 L-type Ca^{2+} channels that mediate transmitter release. First, CaBP4 interacts with the pore-forming α_1 subunit of Ca_v1 L-type Ca^{2+} channels and modulates the activation properties of these channels in transfected human embryonic kidney HEK293T cells (Haeseleer et al., 2004; Yang et al., 2006). Second, defects in visual transmission and retina morphology in *Cabp4*^{−/−} mice are similar to

those in mice lacking $\alpha_1 1.4$ or the auxiliary $\text{Ca}_v \beta_2$ subunit (Ball et al., 2002; Mansergh et al., 2005; Chang et al., 2006). Third, human mutations in genes encoding CaBP4 (Zeitz et al., 2006) and $\alpha_1 1.4$ (Bech-Hansen et al., 1998; Strom et al., 1998) are linked to incomplete congenital stationary night blindness (CSNB2). Thus, elucidating the factors controlling CaBP4 activity may yield insights into the underlying pathology of CSNB2.

Light-dependent phosphorylation regulates the physiological activity of several key players in the phototransduction cascade, including rhodopsin, opsin, phosducin, RGS-9 (regulator of G-protein signaling 9), centrins, β -subunit of the insulin receptor, abLIM (actin binding LIM protein family), peripherin, and phosphodiesterase (Kuhn and Dreyer, 1972; Lee et al., 1984; Boesze-Battaglia et al., 1997; Roof et al., 1997; Hayashi et al., 2000; Hu et al., 2001; Rajala et al., 2002; Trojan et al., 2003). Phosphorylation of Ca^{2+} -binding proteins, including CaM, can affect their Ca^{2+} -binding capability and interaction with target proteins (Benaïm and Villalobo, 2002). Casein kinase 2 (CK2) phosphorylates several amino acid residues in CaM, including serine 101, which decreases the affinity of CaM for its substrates (Quadroni et al., 1998; Bildl et al., 2004; Kasri et al., 2004; Allen et al., 2007). Similar to CaM, an analogous residue (Serine 120) in CaBP1 is phosphorylated by casein kinase 2, which weakens the effect of CaBP1 in inhibiting Ca^{2+} release by the IP_3 receptor (Kasri et al., 2004).

In this study, we investigated whether phosphorylation of CaBP4 might similarly regulate the activity of CaBP4. We provide evidence that CaBP4 is phosphorylated by protein kinase C ζ (PKC ζ) in the retina and that phosphorylation and Ca^{2+} binding have opposite effects on CaBP4 modulation of Ca_v1 L-type Ca^{2+} channels. We also show that CaBP4 phosphorylation levels change with different light conditions, which may underlie light-dependent regulation of CaBP4 targets.

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Correspondence should be addressed to Françoise Haeseleer, University of Washington, Department of Ophthalmology, 1959 North East Pacific Street, Box 356485, Seattle, WA 98195. E-mail: fanfan@u.washington.edu.

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Materials and Methods

Animals. C57BL/6J mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME). C57BL/6J, *CaBP4*^{−/−} (Haeseleer et al., 2004), *Gnat1*^{−/−} (rod transducin α -subunit null; provided by Dr. Janis Lem, Tufts-New England Medical Center, Boston, MA) (Calvert et al., 2000) and *Gnat1*^{−/−} *CaBP4*^{−/−} (Maeda et al., 2005) mice were bred in the animal facility at the University of Washington. Their eyeballs were used as a source of retinal proteins and for immunohistochemistry. All the procedures for the maintenance and use of animals were approved by the Institutional Animal Care and Use Committee of the University of Washington.

Reagents. Casein kinase II inhibitor I [4,5,6,7-tetrabromobenzotriazole (TBB)], H-89 (*N*-[2-(*p*-bromo-cinnamylamino)-ethyl]-5-isoquinoline-sulfonamide 2HCl), Bisindolymaleimide I (Bis), 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo [3,4-*d*] pyrimidine (PP2), Rottlerin, G66976 [12-(2-cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5*H*-indolo[2,3-*a*]pyrrolo[3,4-*c*] carbazole], G66983(2-[1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl]-3-(1*H*-indol-3-yl)maleimide), protein kinase C ζ pseudosubstrate inhibitor, phorbol-12-myristate-13-acetate (PMA), 1,2-dioleoyl-*sn*-glycerol (DAG), and recombinant PKC α were purchased from Calbiochem (La Jolla, CA). L- α -Phosphatidyl-L-serine (PS) was purchased from Sigma (St. Louis, MO). Recombinant PKC α and lipid activator (mix of PS and DAG) were obtained from Upstate Biotechnology (Lake Placid, NY). Doxycycline and hygromycinB were purchased from BD Biosciences (Palo Alto, CA). The 1 kb DNA ladder was purchased from Invitrogen (Carlsbad, CA).

Antibodies. Commercially available antibodies were as follows: alkaline phosphatase-conjugated anti-mouse and anti-rabbit (Promega, Madison, WI); mouse anti-polyhistidine (6His) tag (EMD Biosciences Novagen, Madison, WI); mouse anti-glutathione *S*-transferase (GST) and anti-PKC α (Santa Cruz Biotechnology, Santa Cruz, CA); rabbit anti-PKC ζ (Oxford Biomedical Research, Oxford, MI); cyanine 3 (Cy3) goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA); and Alexa Fluor 488 goat anti-mouse (Invitrogen).

Development and characterization of rabbit anti-CaBP4 (UW145) was described by Haeseleer et al. (2004). Bacterially expressed CaBP4 was used to immunize BALB/c mice to obtain mouse anti-CaBP4 antisera.

The phospho-Ser³⁷-specific antibody was targeted against a sequence in mouse CaBP4 (NH₂-CPALTRRRpSKKESWHP-COOH). The corresponding peptide was obtained from Mimotopes (Clayton, Victoria, Australia) and contained an N-terminal cysteine residue for covalent linkage to keyhole limpet hemocyanin as a carrier protein for generating antibodies. The peptide was injected into a New Zealand white rabbit (Cocalico Biologicals, Reamstown, PA), and polyclonal antibodies were purified from the serum by affinity chromatography on a phospho-peptide–Sepharose column. After elution, the affinity-purified antibodies were depleted of the antibodies that react with the nonphosphorylated peptide by passing through a CaBP4–Sepharose column prepared with nonphosphorylated, bacterially expressed CaBP4.

Kinase assay. Dark-adapted mouse retinas were dissected and sonicated in dim red light in 100 μ l of 10 mM bis-Tris-propane (BTP), pH 8.0, containing a mixture of proteases inhibitor (Sigma). Phosphorylation experiments were performed in the dark using 5 μ l of retinal extract and 5 μ Ci of [γ -³²P]ATP in kinase buffer (10 mM BTP, pH 8.0, 1 mM MgCl₂, 1 mM Na₃VO₄, 1 mM NaF, 1 mM dithiothreitol, and 500 μ M ATP) for 10 min at 30°C. CaCl₂ or EGTA (1 mM each) was added to the kinase assay when indicated. His-tagged or GST-tagged CaBP4 purified from bacteria (1 or 0.2 μ g) was included in the reaction as indicated. The reaction was terminated with the addition of SDS sample buffer. The reaction product was subjected to SDS-PAGE followed by transfer to Immobilon membrane and Western blotting with the anti-CaBP4 antibody (UW145) or Ponceau staining. Alternatively, the gels were treated with Coomassie blue and dried. Incorporation of ³²P in CaBP4 was detected by autoradiography. For characterization of the endogenous kinase, soluble proteins were isolated by centrifugation of the retinal extract for 15 min at 13,000 $\times$ *g* at 4°C. The pellet was washed with 10 mM BTP (100 μ l) and centrifuged for 15 min at 13,000 $\times$ *g* at 4°C. The supernatants represented the soluble fraction. The pellet was resuspended in 10 mM BTP

(100 μ l) and constituted the membrane protein fraction. The following changes were made to the assay buffer when indicated: EDTA (10 mM) was added; MgCl₂ (1 mM) was substituted with MnCl₂ (1 mM); PMA (5 μ M), PS (0.1 mg/ml), and/or DAG (0.01 mg/ml) were added. Protein kinase inhibitor concentrations used in the assay are described in the figures and were added to the reaction mix described above. Analysis of CaBP4 phosphorylation in HEK293 cells was performed as described above except that the retina extract was substituted with a lysate from HEK293 cells.

Kinase assay with recombinant PKC. GST-tagged CaBP4 (10 μ g) and GST-S37A (10 μ g) mutant were phosphorylated *in vitro* with recombinant PKC α (rPKC α) (0.1 μ g) in a 200 μ l reaction containing 20 mM HEPES, pH 8.0, 0.03% Triton X-100, 0.1 mM ATP, 1 mM MgCl₂, 1 mM CaCl₂, 20 μ l of lipid activator (0.1 mg/ml PS, 0.01 mg/ml DAG, final concentration) and 25 μ Ci of [γ -³²P]ATP for 1 h at 30°C. The reactions were terminated by adding an equal volume of 2 $\times$ sample buffer. For phosphorylation of the GST-tagged proteins (10 μ g) by rPKC ζ (0.2 μ g), the reactions were performed in 200 μ l containing 20 mM HEPES, pH 8.0, 0.03% Triton X-100, 0.1 mM ATP, 1 mM MgCl₂, 1 mM EGTA, and 25 μ Ci of [γ -³²P]ATP for 1 h at 30°C. The reactions were terminated by adding an equal volume of 2 $\times$ sample buffer or on ice. Protein kinase inhibitor concentrations used in the assay are described in the figure legends and were added to the reaction mix. For affinity chromatography, purified His-tagged CaBP4 (300 μ g) was coupled to cyanogen bromide (CNBr)-activated Sepharose, which was incubated with rPKC ζ (3 μ g) in a 1.5 ml kinase reaction as described above. The reactions were terminated by washing the CaBP4–Sepharose three times with PBS and 0.5% Triton X100.

Immunoprecipitation of radiolabeled native CaBP4. CaBP4 was immunoprecipitated from the kinase assay reactions using anti-CaBP4 polyclonal antibodies. The phosphorylation experiments were performed as described above. The reactions were stopped by incubation on ice. For the immunoprecipitation experiments, the reactions were performed in light conditions unless specified as performed in the dark (dim red light). The samples were diluted with 2 vol of PBS containing 0.5% Triton-X100 and cleared by incubation with 40 μ l of protein-G agarose for 4 h at 4°C. After centrifugation, 10 μ g of affinity-purified anti-CaBP4 antibodies were added to the supernatant and incubated for 1 h at 4°C. Protein G agarose (40 μ l) was added and incubated overnight at 4°C. The pellets were washed five times for 10 min with 1 ml of PBS and 0.5% Triton X-100. The immunoprecipitated proteins were eluted after boiling for 5 min in SDS-PAGE sample buffer and subject to SDS-PAGE followed by transfer on Immobilon membrane and Western blotting with anti-CaBP4 antibodies. The blot was then exposed to an x-ray film.

Generation of CaBP4 mutants. The cloning of all deletion mutants was performed using PCR/subcloning. The deletion mutants of CaBP4 were generated by PCR with primers confined at the 5' and 3' end of the truncated segments and the resulting sequence subcloned into the pENTR-topo vector (Invitrogen). CaBP4–S37A was generated using appropriate mutagenic primers with the QuickChange Site-Directed mutagenesis kit (Stratagene, Cedar Creek, TX) according to the protocol of the manufacturer. To generate the mutant of CaBP4 that does not bind Ca²⁺ [CaBP4-EF-hand Ca²⁺-binding motif (EF)], the glutamic acid in position 12 of the EF1, EF3, and EF4 loops was mutated to a glutamine (Glu149Gln, Glu226Gln, Glu263Gln) using appropriate primers with the QuickChange Site-Directed mutagenesis kit (Stratagene). The sequence of all constructs was confirmed by DNA sequencing. The cDNA sequences encoding wild-type (WT) or mutated CaBP4 were then subcloned, using the Gateway Technology System (Invitrogen), into the Gateway expression vector pDest17 in fusion to a 6His tag and purified using Ni-NTA agarose (Qiagen, Valencia, CA) or into the pDest15 vector for fusion to a GST tag and purified on Glutathione resin (Promega) as recommended by the manufacturer.

The cDNA sequence encoding 6His-tagged wild-type or CaBP4–S37A was also subcloned into the pTRE-hyg vector (BD Biosciences) to generate an inducible stable cell line. T-Rex-293 cells (HEK293 cells stably expressing the tetracycline repressor) (Invitrogen) were transfected with the recombinant plasmids using Lipofectamine 2000 (Invitrogen). After doxycycline-induced CaBP4 expression, stable cell lines were selected for

the highest protein levels from colonies isolated in the presence of hygromycin following the protocol of the manufacturer. The 6His-tagged proteins were purified from HEK293 cells using Ni-NTA agarose (Qiagen) following the protocol of the manufacturer.

Cloning of $\alpha_1.4$ and $\alpha_1.3$ cytoplasmic domains. The mouse $\alpha_1.4$ C-terminal segment (CT1, amino acids 1445–1605) was amplified with primers FH586 (5'-CACCGATAACTTTGATTACCTAACAGAG-3') and FH585 (5'-CTTAAGTTACTTTCTTCTCCTCTCCGA-3') that also includes a stop codon by PCR from mouse $\alpha_1.4$ plasmid (Haeseleer et al., 2004). The distal C-terminal downstream of the CT1 (fragment, CT2, was amplified with primers FH593 (5'-CACCGGGCTACTAGGAAGAGAGG-3') and FH556 (5'-GGATCCTTAGAGGGCATGGACACAGGC-3') that also introduces a *Bam*HI restriction site. The rat $\alpha_1.3$ CT1 (amino acids 1478–1644) was amplified with primers FH650 (5'-CACCTTGGACGAATCAAAAGAATATGG-3') and FH649 (5'-CTAGAGCATCCGTTCAAGCATCTG-3') by PCR from rat brain $\alpha_1.3$ (Xu and Lipscombe, 2001). All PCR fragments were subcloned in pENTR/D-TOPO vector (Invitrogen) and sequenced by dideoxyterminator sequencing (ABI-Prism; PerkinElmer, Boston, MA) and then transferred into pDest17 for fusion to a 6His tag using the Gateway Technology System as described by the manufacturer (Invitrogen).

Cloning of human PKC ζ and transfection in HEK293 cells. Full-length PKC ζ was amplified by PCR from human PKC ζ cDNA (clone 3835020; Open Biosystems, Huntsville, AL) using primers FH690 (5'-CACCA-TGCCAGCAGGAGG-3') and FH691 (5'-GCCTCACACCGAC-TCTCG-3'). The PCR products were cloned into the pENTR/D-TOPO vector (Invitrogen) and sequenced by dideoxyterminator sequencing (ABI-Prism; PerkinElmer) and then transferred into the pcDNA3.1(–) vector. For the pull-down assay, T-Rex-293 stable cell line expressing CaBP4 or CaBP4–S37A were transfected with human PKC ζ using Lipofectamine 2000 according to the instructions of the manufacturer (Invitrogen). Six hours after transfection, medium containing 1 μ g/ μ l doxycycline was added to the cells, and the cells were induced for 48 h. The cells were then washed with PBS and collected for additional analysis.

Reverse transcription-PCR assay. Total RNA (5 μ g) from mouse retina was used for an initial reverse transcription step to prepare cDNA. The gene-specific primers used to amplify PKC α (5'-CCAACGCTTCGCCCCGAAAG-3' and 5'-GCTTGGCTTTCTCAAACCTCTGCC3'-) or PKC ζ (5'-GCCTTCGCGCTGGTCTGTCAAG-3' and 5'-TGCAGC-CCCAGCCCCCTGAGAG-3') generated a fragment of ~900 and 550 bp, respectively, after an initial PCR step at 94°C for 5 min and 35 cycles at 94°C for 30 s, 60°C for 30 s and 72°C for 1.5 min with YieldAce DNA polymerase (Stratagene). Amplification with glyceraldehyde-3-phosphate dehydrogenase (G3PDH)-specific primers (5'-GAAGG-GCTAATGACACAGTCCAT-3' and 5'-TAGCATATTCGTTG-TCGATCCAGG-3') was performed as a positive control.

Gel overlay assay. Recombinant 6His-tagged purified proteins (2 μ g) were separated on SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) membranes. After overnight saturation at 4°C in PBS, 0.1% Tween 20, and 3% nonfat milk, the membrane were incubated in PBS, 0.1% Tween 20, and 2% nonfat milk (blotting buffer) containing 2 μ g/ml GST-tagged proteins for 1 h at room temperature. The blots were washed three times for 5 min with 10 ml of blot buffer and then incubated for 1 h with anti-GST antibody in blotting buffer at room temperature. After three washes of 5 min each, the blots were incubated with an anti-mouse antibody conjugated to alkaline phosphatase for 1 h at room temperature. The bound recombinant proteins were visualized by incubation with nitroblue-tetrazolium-chloride/5-bromo-4-chlor-indolyl-phosphate (Promega). The interactions were tested in the presence or absence of calcium. For the analysis in the absence of Ca^{2+} , 5 mM EGTA was added to the blotting buffer.

Immunohistochemistry. Mouse eyecups were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4 (PB), for 4 h. After fixation, tissues were incubated with a sucrose series to 20% sucrose in PB and then embedded in 33% OCT compound (Miles, Elkhart, NY) diluted with 20% sucrose in PB. Eye tissues were cut in 12 μ m sections. To block nonspecific labeling, retinal sections were incubated with 3% normal goat serum in PBST buffer (136 mM NaCl, 11.4 mM sodium phosphate, and 0.1% Triton X-100, pH 7.4) for 20 min at room temperature. Sec-

tions were incubated overnight at 4°C in a mix of diluted primary antibodies (1:400 for mouse anti-CaBP4; 1:250 for rabbit anti-PKC ζ). A mixture of Cy3-conjugated goat anti-rabbit IgG and Alexa 488-conjugated goat anti-mouse IgG was reacted with sections for 1 h at room temperature. Then, sections were rinsed in PBST and mounted with Prolong antifade reagent (Invitrogen) to retard photobleaching. Sections were analyzed under a confocal microscope (LSM510; Zeiss, Thornwood, NY). Immunofluorescent images (0.8 μ m thickness) were obtained with a Plan-Neofluar 40 $\times$ /1.3 numerical aperture (Zeiss) objective lens.

CaBP4 affinity chromatography. CaBP4 was coupled to CNBr-activated Sepharose (GE Healthcare, Piscataway, NJ) according to the protocol of the manufacturer. The CaBP4–Sepharose was either treated with rPKC ζ or left untreated as described above (kinase assay with recombinant PKC). For binding in the presence of Ca^{2+} , the CaBP4–Sepharose was equilibrated with 10 mM BTP, pH 8.0, 2 mM benzamidine, and 0.1 mM CaCl_2 . Purified His-tagged Ca $\alpha_1.4$ CT1 was incubated with the Sepharose at 4°C for 2 h. The CaBP4–Sepharose was then washed with the equilibration buffer (40 times the volume of the column) followed by the same buffer containing 150 mM NaCl. The elution was performed with 3 mM EGTA followed by 0.1 M Gly, pH 2.5. Fractions were collected and aliquots were analyzed by Western blot with an anti-6His tag antibody. For the binding in the presence of EGTA, the CaBP4–Sepharose was equilibrated with 10 mM BTP, pH 8.0, 2 mM benzamidine, and 0.1 mM EGTA and washed with this buffer followed by the same buffer containing 150 mM NaCl. The elution was performed with 5 mM CaCl_2 followed by 0.1 M Gly, pH 2.5.

Electrophysiological recordings. HEK293T cells were grown to 70–80% confluence and transfected using Gene Porter reagent (Gene Therapy Systems, San Diego, CA) according to the protocols of the manufacturer. Cells were transfected with ~5 μ g of total DNA [$\alpha_1.3$ (1.5 μ g), β_{2A} (0.8 μ g), $\alpha_2\delta$ (0.8 μ g) with or without CaBP4 (0.1 μ g)] and green fluorescent protein expression plasmid (0.01 μ g) for fluorescent detection of transfected cells. All electrophysiological data were acquired with EPC-9 patch-clamp amplifier driven by Pulse software (HEKA Elektronik, Lambrecht/Pfalz, Germany) and analyzed with Igor Pro software (WaveMetrics, Lake Oswego, OR). The time course of I_{Ca} decay was fit by $A_{\text{slow}}[\exp(-t/\tau_{\text{slow}})] + A_{\text{fast}}[\exp(-t/\tau_{\text{fast}})]$, where t is time, A_{slow} and A_{fast} are the amplitudes of the slow and fast exponentials, respectively, at $t = 0$, and τ_{slow} and τ_{fast} are the time constants of the decay of the two processes. Extracellular recording solutions contained the following (in mM): 150 Tris, 1 MgCl_2 , and 10 BaCl_2 or 10 CaCl_2 . Intracellular solutions consisted of the following (in mM): 140 N-methyl-D-glucamine, 10 HEPES, 2 MgCl_2 , 2 Mg-ATP , and 5 EGTA. The pH of intracellular and extracellular recording solutions was adjusted to 7.3 with methanesulfonic acid. Electrode resistances were typically 1–2 M Ω in the bath solution, and series resistance was ~2–4 M Ω , compensated up to 80%. All averaged data are presented as the mean $\pm$ SEM. Statistical significance of differences between two groups was determined by ANOVA or Student's t test as indicated (SigmaStat; SPSS, Chicago, IL).

Results

Phosphorylation of CaBP4 by an endogenous protein kinase C

Given the importance of light-dependent protein phosphorylation in regulating signal transduction in photoreceptors, we tested whether CaBP4 is phosphorylated in mouse retina by immunoprecipitation with anti-CaBP4 antibody (UW145) from retinal extracts in the presence of [γ - ^{32}P]ATP. Because massive rhodopsin phosphorylation in light-adapted retina extract obscures detection of other phosphorylated proteins that nearly comigrate with rhodopsin, the phosphorylation assay was performed in the dark with extracts of dark-adapted retinas unless specified. Under these conditions, a ^{32}P -labeled protein was immunoprecipitated, the identity of which was confirmed as CaBP4 by its presence in *Cabp4*^{+/+} (WT) but not *Cabp4*^{−/−} [knock-out (KO)] retinal extracts (Fig. 1A). In addition, GST-tagged CaBP4

was phosphorylated by the endogenous kinase when incubated with retinal extract (Fig. 1*B*). Because we used retinal extract of *CaBP4*^{−/−} mice in some assays, we also tested the kinase activity in *CaBP4*^{−/−} extract. Similar levels of phosphorylated GST–CaBP4 were also detected using retinal extract of *CaBP4*^{−/−} mice (Fig. 1*B*), indicating that the retinal kinase required for CaBP4 phosphorylation is not affected by loss of CaBP4. The endogenous kinase did not require Ca²⁺ in that CaBP4 phosphorylation was slightly higher in the absence than in the presence of Ca²⁺ (Fig. 1*B*).

We next undertook experiments to characterize the CaBP4 kinase in the retinal extract. First, we examined which subcellular fraction contained phosphorylated CaBP4. Greater levels of phosphorylated CaBP4 were detected in the soluble fraction than the membrane fraction, indicating that the endogenous kinase is primarily cytosolic rather than membrane associated (Fig. 1*C*). Because the activity of most protein kinases rely on Mg²⁺ (Hunter and Cooper, 1985; Edelman et al., 1987), we tested whether chelation of Mg²⁺ by EDTA prevented CaBP4 phosphorylation. Inclusion of EDTA prevented incorporation of ³²P into CaBP4 and phosphorylation was only partially restored by Mn²⁺ (Fig. 1*D*). This result confirms the Mg²⁺ dependence of CaBP4 phosphorylation by the retinal kinase. We also tested the effects of various protein kinase inhibitors on CaBP4 phosphorylation in retinal extracts. Whereas inhibitors of the Src family of tyrosine kinases (PP2) and casein kinase II (TBB) had no effect, CaBP4 phosphorylation was strongly decreased by the PKC inhibitor Bis and partially reduced by H-89, which inhibits PKA but also PKC at higher concentrations (Fig. 1*E,F*). These results indicated that CaBP4 was phosphorylated by a member of the PKC family.

Because CaBP4 phosphorylation was not increased by Ca²⁺ (Fig. 1*B*), the retinal kinase was not likely to be a conventional PKC isoform (PKCα, PKCβ, and PKCγ), which would be activated by Ca²⁺ and phosphatidylserine and diacylglycerol. In addition, CaBP4 phosphorylation was strongly inhibited by Gö6983, an inhibitor of PKCα, PKCβ, and PKCδ that also inhibits PKCζ at higher concentrations (Fig. 1*F*) (Gschwendt et al., 1996) and was affected by the PKCα inhibitor Gö6976 only at high concentrations (100 μM) (Fig. 1*F*), at which this drug can also inhibit PKCζ (Martiny-Baron et al., 1993; Gschwendt et al., 1996). CaBP4 phosphorylation was also not altered by Rottlerin, an inhibitor of the novel PKC isoforms PKCδ and PKCθ (Fig. 1*F*) (Gschwendt et al., 1994). In contrast, a pseudosubstrate inhibitor of the atypical PKC isoform, PKCζ, nearly prevented CaBP4 phosphorylation (Fig. 1*F*). The involvement of PKCζ was further supported by the relative insensitivity of CaBP4

Figure 1. Phosphorylation of native and recombinant CaBP4 by PKCζ. **A**, Identification of native CaBP4 as a phosphorylated protein in retina. Retinal proteins were incubated in the presence of [γ -³²P]ATP and subject to immunoprecipitation with anti-CaBP4 antibody. Autoradiograph (top) shows a labeled band in *CaBP4*^{+/+} (WT) but not *CaBP4*^{−/−} (KO) retinal extract, which was also detected by immunoblotting with anti-CaBP4 antibody (bottom, top band). **B**, Ca²⁺-independent phosphorylation of recombinant CaBP4. GST–CaBP4 (1 μg) was incubated with a *CaBP4*^{+/+} (WT) or *CaBP4*^{−/−} (KO) retinal extract in the presence of [γ -³²P]ATP and Ca²⁺ (1 mM) or EGTA (1 mM). Phosphorylation of GST–CaBP4 is stronger without Ca²⁺ in extracts of *CaBP4*^{−/−} and *CaBP4*^{+/+} retina. Ponceau staining indicates equal levels of GST–CaBP4 between groups. **C–H**, Characterization of the CaBP4 kinase in retina as PKCζ. The same amount of bacterially expressed and purified 6His–CaBP4 (1 μg) was incubated with retinal extract in the presence of activators or inhibitors of protein kinases as indicated. Arrows indicate CaBP4, which was identified as the major protein of ~43 kDa by Ponceau staining. Assays contained Ca²⁺ (1 mM) or EGTA (1 mM) as indicated. **C**, The CaBP4 kinase is soluble. Phosphorylation assays were conducted with retinal extracts separated into soluble (S) and membrane (M) fractions and in the presence or absence of Ca²⁺. **D**, Mg²⁺ is required for kinase activity. CaBP4 phosphorylation (lane Mg²⁺) was prevented by inclusion of 10 mM EDTA (lane EDTA) and inhibited when Mn²⁺ was substituted for Mg²⁺ in the assay (lane Mn²⁺). **E**, The endogenous kinase is inhibited by PKC inhibitors. The kinase activity in the retinal extract (lane −, left) was not affected by PP2 or TBB but was inhibited partially by H-89 and completely inhibited by Bis, each at 10 μM. **F**, CaBP4 phosphorylation is inhibited by a PKCζ-specific inhibitor. CaBP4 phosphorylation was not affected by Rottlerin, partially inhibited by Gö6976 and Gö6983, and strongly inhibited by a PKCζ-specific inhibitor. The kinase activity in the retinal extract was tested in the presence of various concentrations of PKC inhibitors as indicated. **G, H**, Kinase activity is phospholipid independent. DAG (0.01 mg/ml), PS (0.1 mg/ml), and PMA (5 μM) did not affect CaBP4 phosphorylation in either the presence or absence of Ca²⁺. **I**, CaBP4 is phosphorylated by recombinant PKC isoforms *in vitro*. Phosphorylation of 6His–CaBP4 by recombinant PKCζ or PKCα was tested in the presence or absence of PKCζ inhibitor or Bis at the indicated concentrations.

phosphorylation to phospholipid PKC activators, which do not influence the activity of PKCζ. In these experiments, nearly equivalent levels of phosphorylated CaBP4 were detected in the presence and absence of DAG, PS (Fig. 1*G*), and the DAG analog PMA (Fig. 1*H*). Finally, in a cell-free assay using GST-tagged CaBP4 and recombinant PKC isoforms, CaBP4 was efficiently phosphorylated by PKCζ, which was strongly inhibited by the pseudosubstrate PKCζ inhibitor (Fig. 1*I*). PKCα also promoted CaBP4 phosphorylation, which was blocked by Bis but was essentially insensitive to high concentrations of the PKCζ inhibitor (Fig. 1*I*), in contrast to the strong effects of the PKCζ inhibitor on CaBP4 phosphorylation in the retinal extract

Figure 2. Expression of PKC ζ in the mouse retina. **A**, RT-PCR analysis of PKC ζ and PKC α expression in the retina. PKC ζ or PKC α cDNAs were amplified from total RNA from wild-type (*Cabp4*^{+/+}) or CaBP4 knock-out (*Cabp4*^{-/-}) mouse retina using isoform-specific primers or G3PDH-specific primers as positive controls. Standards, 1 kb DNA ladder. **B**, Specificity of the anti-PKC antibodies. Recombinant PKC α and PKC ζ were subjected to Western blot analysis with commercial anti-PKC isoform-specific antibodies. **C**, Western blot of mouse retina extract with anti-PKC α and anti-PKC ζ . A protein of ~80 kDa was detected in a mouse retina extract with the anti-PKC ζ (right), which was less intense than the ~85 kDa protein detected by the anti-PKC α antibodies (left). **D**, Colocalization of PKC ζ with CaBP4 in the mouse retina. Confocal micrographs of mouse retina sections double-immunostained for PKC ζ and CaBP4. The image overlay of labeling for PKC ζ (green, left, **A'**–**C'**) and CaBP4 (red, middle, **A''**–**C''**) is shown in the right panels (merge, **A'''**–**C'''**). A yellow color denotes colocalization of CaBP4 and PKC ζ at the photoreceptor synapse and inner segment. Scale bar: **A'**–**A'''**, 20 μ m. **B'**–**B'''**, Higher magnification of the photoreceptor layer; scale bar, 10 μ m. **C'**–**C'''**, Retina treated with secondary antibody alone; scale bar, 20 μ m. OS, Photoreceptor outer segment; IS, photoreceptor inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

(Fig. 1*F*). Collectively, these results implicate PKC ζ as the endogenous kinase that phosphorylates CaBP4 in the retina.

PKC ζ is expressed in photoreceptors and colocalizes with CaBP4

If PKC ζ is physiologically relevant as the CaBP4 kinase, it should be coexpressed and colocalized with CaBP4 in the retina. To test this, we first confirmed the expression of the PKC ζ isoform in the retina by reverse transcription (RT)-PCR (Fig. 2*A*). As expected, PKC α was also amplified because it is primarily expressed in rod bipolar cells (Greferath et al., 1990). We confirmed the expression of PKC α and PKC ζ in the retina by immunoblot. The specificity of the isoform-specific antibodies was first confirmed using recombinant proteins. The anti-PKC ζ antibody recognized specifically PKC ζ and not PKC α , whereas the anti-PKC α antibody strongly detected PKC α and only very weakly PKC ζ (Fig. 2*B*). In Western blot analyses of retinal lysates, the anti-PKC α and anti-PKC ζ antibodies detected proteins of ~85 and ~80 kDa, respectively, which were consistent in size with the corresponding recombinant proteins (Fig. 2*B,C*). These results confirmed that PKC ζ is expressed at both the mRNA and protein level in the retina.

Next, we determined whether PKC ζ is colocalized with CaBP4 in the retina by confocal microscopy. In retina sections double-labeled with antibodies against PKC ζ and CaBP4, PKC ζ was localized to the photoreceptor inner segment, and weaker staining was also observed at the photoreceptor synapse in the outer plexiform layer (Fig. 2*D*, OPL). As reported previously, CaBP4 was localized to the photoreceptor synapse, and weak staining was

also detected in the inner segment. Overlay of the two single-labeled images showed strong overlap of immunofluorescence for CaBP4 and PKC ζ in the synaptic and inner segment layers (Fig. 2*D*), which is consistent with a role for PKC ζ in regulating CaBP4.

Serine 37 is the major site of phosphorylation of mouse CaBP4 by PKC ζ

To define potential sites in CaBP4 for PKC ζ phosphorylation, we compared phosphorylation of truncated GST-tagged CaBP4 fragments in retinal extracts in the presence of [γ -³²P]ATP (Fig. 3*A,B*). Whereas GST–CaBP4 fragments containing amino acids 1–34 and 60–271 showed no incorporation of ³²P, the fragments containing amino acids 1–62 and 34–271 were efficiently phosphorylated (Fig. 3*B*), indicating phosphorylation site(s) between amino acids 34–62. Analysis of this region using the NetPhos 2.0 server (Technical University of Denmark) identified serine residues 37, 46, 50, and 51 as putative phosphorylation sites that are also conserved or show conserved substitutions (S/T) in CaBP4 orthologs (Fig. 3*A*) and so may be physiologically important sites of phosphorylation. If so, then substitution of these serine residues with alanine should inhibit the incorporation of ³²P into the GST–CaBP4 fragment containing amino acids 1–61. This result was obtained with

fragments containing a serine 37 to alanine substitution (S37A), whereas serine to alanine substitution at residues 46, 50/51 did not affect levels of ³²P incorporation compared with the wild-type CaBP4 fragment (Fig. 3*C*). When S37A was introduced into the full-length CaBP4, phosphorylation was almost completely abolished (Fig. 3*D*). The S37A mutation in both GST–CaBP4 (amino acids 1–61) and full-length CaBP4 also prevented phosphorylation by recombinant PKC ζ (Fig. 3*E*), indicating that S37 is the major phosphorylation site for recombinant PKC ζ . Whereas the S37A mutation also blocked phosphorylation of GST–CaBP4 (amino acids 1–61) by PKC α , ³²P incorporation was still observed in the full-length CaBP4 containing S37A (Fig. 3*E*), suggesting that there is an additional site phosphorylated by PKC α in the cell-free assay.

To test whether phosphorylation at S37 might affect Ca²⁺ binding, we compared the Ca²⁺-induced mobility shift of the S37A mutant and phosphorylated CaBP4 in SDS-PAGE gels. A similar Ca²⁺ shift was found for both CaBP4 and S37A mutant. No Ca²⁺ shift was observed for a mutant of CaBP4 unable to bind Ca²⁺ (CaBP4–EF; Ponceau-stained blot) (Fig. 3*F*), in which the glutamic acid in position 12 of the putative functional EF-hands 1, 3, and 4 was substituted with glutamine, which should disrupt Ca²⁺ coordination by these regions. This finding demonstrates that phosphorylation does not grossly influence the Ca²⁺ binding properties of CaBP4. In a reverse approach, we tested whether Ca²⁺ binding to CaBP4 might affect CaBP4 phosphorylation by comparing the phosphorylation of the wild-type CaBP4 with that of CaBP4–EF. The level of phosphorylation of CaBP4–EF was not different from that of wild-type CaBP4 in either the presence

To further test this, we compared the ability of CaBP4 and S37A-CaBP4 to modulate Ca_v1 channels in electrophysiological recordings of transfected HEK293T cells. Although we initially analyzed how phosphorylated CaBP4 influenced Ca_v1.4 channels, these experiments were hindered by the low expression lev-

As shown previously (Yang et al., 2006), $\text{Ca}_v1.3$ channels undergo considerably faster decay (inactivation) when Ca^{2+} rather than Ba^{2+} is used as the charge carrier (Fig. 5*A, B*). This Ca^{2+} -dependent inactivation is because of to CaM and is opposed by CaBP4 binding to $\alpha_11.3$ (Yang et al., 2006). In our experiments, inactivation of Ca^{2+} and Ba^{2+} currents (I_{Ca} , I_{Ba}) was measured as $I_{\text{res}}/I_{\text{pk}}$, which was the current amplitude at the end of a 1 s depolarizing pulse normalized to the peak current amplitude. We found CaBP4 to have a significant effect in suppressing inactivation of I_{Ca} ($\sim 70\%$; $p < 0.05$), which was not observed with S37A–CaBP4 (Fig. 5*A*). Neither CaBP4 nor S37A–CaBP4 influenced inactivation of I_{Ba} (Fig. 5*B*), indicating a selective effect of CaBP4 on Ca^{2+} -dependent inactivation. CaBP4 but not S37A–CaBP4 significantly inhibited inactivation of I_{Ca} for various test voltages (-20 to 0 mV) (Fig. 5*C*). Kinetic analyses re-

Figure 4. Interaction of CaBP4 and S37A mutant with Ca_v1 channels. **A**, Gel overlay assay of recombinant CaBP4 with $\alpha_1.4$ cytoplasmic domains. His-tagged $\alpha_1.4$ C-terminal domain with (CT1) and without (CT2) CaM-binding sequences were separated on SDS-PAGE and transferred to PVDF membranes, which were incubated in the presence or absence of EGTA with GST or GST-tagged CaBP4, phosphorylated (P-CaBP4) or not (CaBP4) with rPKC ζ . Bound proteins were detected with an anti-GST antibody. Ponceau staining (left) shows the relative amount of purified $\alpha_1.4$ proteins in the assay. **B–E**, Binding of $\alpha_1.4$ and $\alpha_1.3$ CT1 to CaBP4 by affinity chromatography. His-tagged $\alpha_1.4$ CT1 (**B**, **C**) or $\alpha_1.3$ CT1 (**D**, **E**) was incubated with Sepharose coupled with nonphosphorylated CaBP4 (CaBP4-Seph) or phosphorylated CaBP4 (P-CaBP4-Seph) in the presence of Ca^{2+} (**B**, **D**) or EGTA (**C**, **E**). After washes with buffer containing 150 mM NaCl, the proteins were eluted with 3 mM EGTA (**B**, **D**) or 5 mM CaCl_2 (**C**, **E**), followed by 0.2 M glycine buffer, pH 2.1. The fractions were then analyzed by Western blot with anti-His tag antibody. Lane 1, Flow-through; lane 2, last wash; lanes 3–11, elution with Ca^{2+} or EGTA; lanes 12–17, elution with 0.1 M glycine.

vealed that CaBP4 suppressed inactivation of I_{Ca} primarily by increasing the time constant for slow (τ_{slow}) but not fast (τ_{fast}) inactivation (Fig. 5D). In contrast, τ_{slow} and τ_{fast} were not different in cells transfected with $\text{Ca}_v1.3$ alone and those cotransfected with S37A–CaBP4 (Fig. 5C).

The ineffectiveness of S37A–CaBP4 suggests the importance of CaBP4 phosphorylation for modulating inactivation of $\text{Ca}_v1.3$ I_{Ca} . Alternatively, lower expression levels of S37A–CaBP4 compared with CaBP4 could also account for our results. However, Western blots with CaBP4 antibodies showed strong levels of both S37A–CaBP4 and CaBP4 in lysates of HEK293T cells transfected as for electrophysiological experiments (Fig. 5E). To confirm that CaBP4 in transfected cells was subject to phosphorylation at S37 as we found in the retina (Fig. 3C–F), we compared ^{32}P incorporation into GST–CaBP4 and GST–S37A–CaBP4 during incubation with cell lysates. In these experiments, GST–CaBP4 but not S37A–CaBP4 was efficiently phosphorylated, which was inhibited by Bis (Fig. 6A).

To further confirm the importance of serine 37 for CaBP4 phosphorylation in transfected cells and in the retina, we developed and characterized an antibody that specifically recognizes

Figure 5. CaBP4 phosphorylation is required for modulation of $\text{Ca}_v1.3$. **A**, I_{Ca} and I_{Ba} were evoked by 1 s pulses from -90 to -10 mV (I_{Ca}) or -20 mV (I_{Ba}) in HEK293T cells transfected with $\text{Ca}_v1.3$ ($\alpha_1.3$, β_{2A} , $\alpha_2\delta$) alone or cotransfected with CaBP4 or CaBP4-S37A. $I_{\text{res}}/I_{\text{pk}}$ represents amplitude of the current at the end of the pulse normalized to the peak current amplitude. Numbers of cells are indicated in parentheses. * $p < 0.05$. Representative current traces are shown above. Vertical calibration bars: **A**, 0.2 nA; **B**, 0.4 nA. **C**, For cells transfected as in **A**, $I_{\text{res}}/I_{\text{pk}}$ was measured for I_{Ca} during 1 s pulses from -90 mV to various voltages and plotted against test voltage. Asterisks indicate voltages at which values for with CaBP4 significantly differ from $\text{Ca}_v1.3$ alone ($p < 0.05$). Points represent mean $\pm$ SE of 15–21 cells. **D**, Fast and slow time constants for inactivation (τ_{fast} , τ_{slow}) obtained from double-exponential fits of I_{Ca} evoked as in **A** for cells transfected with $\text{Ca}_v1.3$ alone ($n = 8$), with CaBP4 ($n = 9$), or with S37A ($n = 9$). * $p < 0.05$. **E**, Western blot of lysates from cells transfected as in **A** probed with anti-CaBP4 antibodies.

the sequence including phosphorylated S37 (anti-phospho-S37). In Western blots, anti-phospho-S37 recognized recombinant CaBP4 that was phosphorylated by recombinant PKC ζ and not unphosphorylated CaBP4 (Fig. 6B). Moreover, anti-phospho-S37 did not detect S37A–CaBP4 incubated with or without PKC ζ in the cell-free assay (Fig. 6B), which further validated the specificity of this antibody for the phospho-S37. The anti-phospho-S37 antibodies detected a band in HEK293T cells transfected with CaBP4 but not S37A–CaBP4 (Fig. 6C). The identity of this band as CaBP4 was confirmed by Western blotting with CaBP4 antibodies that recognize both wild-type and mutant forms. To determine which PKC isoform in HEK293T cells may mediate phosphorylation of CaBP4, we performed Western blots with antibodies against PKC antibodies. These experiments showed that PKC α but not PKC ζ was expressed endogenously in HEK293T cells (Fig. 6D), which suggested that, unlike in the retina, phosphorylation of S37 in CaBP4 in these cells is performed by PKC α .

Our findings demonstrate that phosphorylation of CaBP4 is required to maintain functional interactions with $\alpha_1.3$, somewhat at odds with our biochemical data indicating weaker binding of P-CaBP4 to $\alpha_1.3$ (Fig. 4D,E). One mechanism to reconcile these results would be if low-affinity interactions of P-CaBP4 with $\alpha_1.3$ were important for the net effect of suppressing I_{Ca} inactivation. Because we also found that P-CaBP4 and CaBP4 binding to $\alpha_1\text{CT1}$ was weaker in the absence of Ca^{2+} (Fig. 4), one possibility is that CaBP4 modulates the channel more strongly when it is not associated with Ca^{2+} . To test this, we analyzed the effects of the mutant of CaBP4 that can no longer bind Ca^{2+}

Figure 6. CaBP4 is phosphorylated at S37 in transfected HEK293 cells. **A**, CaBP4 is phosphorylated by the endogenous PKC in HEK293 cells. Autoradiograph and Ponceau staining of recombinant GST-tagged CaBP4 (1 μ g) or GST-tagged S37A mutant (1 μ g) incubated with an equivalent amount of cell lysate from HEK293 cells in the presence of [γ - 32 P]ATP (left). The kinase activity in the cell lysate was tested with GST-tagged CaBP4 in the presence or absence of 10 μ M Bisindolymaleimide (right). **B**, **C**, Detection of phosphorylated CaBP4 by a phospho-specific antibody. In **B**, anti-phospho-Ser37–CaBP4 antibody detected GST-tagged CaBP4 (0.2 μ g) but not GST-tagged S37A mutant (0.2 μ g) only when phosphorylated by recombinant PKC ζ (left). Labeling with anti-CaBP4 (UW145, right) confirms equal levels of GST–CaBP4 proteins in each lane. In **C**, 6His-tagged CaBP4 or 6His-tagged S37A mutant were purified from stably transfected HEK293 cells and analyzed by Western blotting with anti-phospho-Ser37–CaBP4 antibody (left) or with anti-CaBP4 as a positive control (right). CaBP4 but not S37A mutant immunoreacted with anti-phospho-Ser37–CaBP4. **D**, PKC α is expressed in HEK293 cells. Equivalent amount of total proteins from lysates of HEK293 cells transfected or not with PKC ζ were analyzed for PKC α and PKC ζ expression by Western blot (lanes 3, 4). rPKC α or rPKC ζ (10 ng each) were also loaded as positive controls (lanes 1, 2).

(CaBP4–EF) but was still efficiently phosphorylated by the retinal extract (Fig. 3F). As predicted, CaBP4–EF robustly inhibited I_{Ca} inactivation ($\sim 113\%$) (Fig. 7A) across a larger range of test voltages than CaBP4 (-30 to $+10$ mV) (Fig. 7B). Moreover,

Figure 7. Modulation of Ca $_v$ 1.3 does not require Ca $^{2+}$ binding to CaBP4. **A**, I_{res}/I_{pk} was determined for I_{Ca} and I_{Ba} as in Figure 5, **A** and **B**, in cells transfected with Ca $_v$ 1.3 alone or cotransfected with CaBP4–EF (+EF). Numbers of cells are indicated in parentheses. Representative current traces are shown above. Vertical calibration bars, 0.3 nA. **B**, I_{res}/I_{pk} plotted against test voltage as in Figure 5C. Points represent mean $\pm$ SE of 12–21 cells. **C**, τ_{fast} and τ_{slow} for cells transfected with Ca $_v$ 1.3 with or without EF were determined as in Figure 5D for cells transfected with Ca $_v$ 1.3 alone ($n = 8$) or with EF ($n = 9$). * $p < 0.01$.

unlike CaBP4, CaBP4–EF inhibited both fast and slow time constants for inactivation (Fig. 7C), which suggests that Ca $^{2+}$ binding to CaBP4 may have additional effects on I_{Ca} inactivation compared with CaBP4 phosphorylation. Interestingly, CaBP4–EF also significantly increased inactivation of I_{Ba} (Fig. 7A), suggesting additional mechanistic differences in the roles of Ca $^{2+}$ and phosphorylation in CaBP4 modulation of Ca $_v$ 1.3 function.

Light-dependent phosphorylation of CaBP4 in mouse retina

Our results illustrate that phosphorylation of CaBP4 can significantly alter its interactions with target molecules. Light-dependent alterations in CaBP4 phosphorylation could therefore significantly impact the consequence of the light signal in photoreceptors. To test whether phosphorylation of CaBP4 changes with the light conditions, we compared the level of CaBP4 phosphorylation in dark-adapted or light-adapted mouse retina. In Western blots, the anti-phospho-S37 antibody detected a protein

Figure 8. CaBP4 phosphorylation in the retina increases in response to light. **A**, Western blot analysis of native CaBP4 in wild-type mouse retina using the phospho-S37 antibody. Phosphorylation of CaBP4 was analyzed by Western blot with the phospho-Ser37-specific antibody in three groups (1–3; 2 retinas per group) of dark-adapted (WT, Dark) or light-adapted (WT, Light) mouse retina (top). Bottom shows equivalent levels of CaBP4 detected by the anti-CaBP4 antibody. Lysate of light-adapted retina from *Cabp4*^{−/−} mice (*Cabp4*^{−/−}, Light; right) was used as a negative control. **B**, Western blot and autoradiograph of immunoprecipitated ³²P-labeled phosphorylated CaBP4 from dark-adapted and light-adapted mouse retinas. Retinal proteins from dark-adapted (WT, Dark) or from light-adapted *Cabp4*^{+/+} (WT, Light) retinas were incubated with [γ -³²P]ATP under dim red light or in light, respectively, and subject to immunoprecipitation with anti-CaBP4 antibody. Incorporation of ³²P in CaBP4 is indicated in autoradiograph (top). Western blot with anti-CaBP4 antibody (bottom) shows levels of immunoprecipitated CaBP4. Reactions with retina from light-adapted *Cabp4*^{−/−} (*Cabp4*^{−/−}, Light) mice was performed in parallel as a negative control. **C**, **D**, Absence of light-dependent CaBP4 phosphorylation in *Gnat1*^{−/−} mouse retina. **C** shows similar experiment as in **A** except that retina was from *Gnat1*^{−/−} mouse. Retina from mice lacking both CaBP4 and Gnat 1 (*Cabp4*^{−/−}/*Gnat1*^{−/−}) was used as a negative control. **D** shows similar experiment as in **B** except with retina from WT, *Gnat1*^{−/−}, and *Cabp4*^{−/−}/*Gnat1*^{−/−} mice.

consistent in size with CaBP4 (~35 kDa) more strongly in light-adapted than dark-adapted retina (Fig. 8A). This result was not attributable to alterations in CaBP4 expression because an equal level of CaBP4 was detected in light- and dark-adapted retina with anti-CaBP4 antibodies. Neither of the antibodies detected the 34 kDa band in light-adapted retina of *Cabp4*^{−/−} mice, which verified the identity of this protein as CaBP4. Light-dependent phosphorylation of CaBP4 was also observed by immunoprecipitation from dark-adapted or light-adapted retinal extracts preincubated with [γ -³²P]ATP. Anti-CaBP4 antibodies immunoprecipitated a ³²P-labeled band corresponding to the phosphorylated CaBP4

much more strongly in light-adapted than dark-adapted conditions (Fig. 8B), whereas no signal was detected in parallel experiments with retinal extracts of *Cabp4*^{−/−} mice. Light-dependent changes in CaBP4 phosphorylation required activation of the phototransduction cascade in rods, because no changes in the levels of phosphorylated CaBP4 were detected between light-adapted and dark-adapted retina from mice lacking transducin (*Gnat1*^{−/−}) (Fig. 8C,D), the G-protein that couples rhodopsin to the electrical photoresponse (Calvert et al., 2000).

Together, our results indicate that CaBP4 phosphorylation is favored in the light, which may significantly influence the role of this protein in regulating photoreceptor synaptic transmission.

Discussion

CaM-like CaBPs have emerged as essential regulators of Ca²⁺ signaling in neurons (Lee et al., 2002; Haeseleer et al., 2004; Zhou et al., 2004, 2005). Our study provides the first evidence that CaBP4, one CaBP family member, is subject to phosphorylation by PKC and that phosphorylation can modulate interactions of CaBP4 with Ca_v1.3 Ca²⁺ channels. Light-stimulated phosphorylation of CaBP4 in the retina suggests a mechanism by which the activity of CaBP4 may be upregulated or downregulated with respect to photoreceptor light signal transmission.

CaBP4 is phosphorylated by PKC ζ

PKCs are serine/threonine kinases that are classified according to their dependence on Ca²⁺, phospholipid, and DAG (Mellor and Parker, 1998). The classical PKCs (α , β , β , and γ) are Ca²⁺ and DAG dependent, whereas the novel PKCs (δ , ϵ , η , and θ) require DAG but not Ca²⁺. Atypical PKCs (ζ , δ , ϵ , η , and θ) are insensitive to both Ca²⁺ and DAG. Our conclusion that PKC ζ phosphorylates CaBP4 in the retina is based on our findings that CaBP4 phosphorylation does not require Ca²⁺ or DAG and is inhibited by the specific pseudosubstrate inhibitor for PKC ζ and Bisindolymaleimide I, a common PKC inhibitor (Fig. 1). This conclusion is further supported by the expression of PKC ζ in the retina and colocalization with CaBP4 in inner segments and at the photoreceptor synapse in the outer plexiform layer (Fig. 2D). This result is consistent with other studies that have described expression of PKC ζ in the retina and in photoreceptors (Fujisawa et al., 1992; Ghalayini et al., 1994). Our results also indicate that CaBP4 can be phosphorylated by PKC α *in vitro* and in transfected HEK293T cells (Figs. 3E, 6). However, in the retina, PKC α is primarily expressed in bipolar cells and not at the photoreceptors synapse. Although biochemical evidence of PKC α expression in rod outer segments have been reported, PKC α is unlikely to participate in the regulation of CaBP4 *in vivo* because CaBP4 is not localized in photoreceptor outer segments (Williams et al., 1997; Sokal et al., 2003). Although our results indicate S37 to be the major site targeted by PKC ζ , CaBP4 contains additional consensus sites for phosphorylation by various protein kinases. One of these sites, threonine 223, is analogous to serine 120 of CaBP1 and serine 101 of CaM, which are phosphorylated by CK2 (Nakajo et al., 1988; Kasri et al., 2004). Although CaBP4 can be phosphorylated by recombinant CK2 *in vitro* (data not shown), CaBP4 phosphorylation in the retinal extract was not affected by the CK2 inhibitor (Fig. 1E) or by mutation of threonine 223 to alanine in CaBP4 (data not shown). Our findings that the S37A mutation in CaBP4 completely eliminates phosphorylation of CaBP4, by both recombinant and native PKC (Figs. 3, 6), demonstrates that S37 is the primary site of phosphorylation in CaBP4 and that PKC ζ is the physiologically relevant kinase for CaBP4 in the retina.

Multiple lines of evidence indicate that CaBP4 is phosphorylated under conditions when intracellular Ca^{2+} is minimal. First, CaBP4 phosphorylation is favored in the presence of EGTA, which chelates Ca^{2+} , and under light-adapted conditions when Ca^{2+} concentrations are low in photoreceptors compared with in darkness (Rieke and Schwartz, 1996). Second, the CaBP4 EF-hand mutant unable to bind Ca^{2+} was phosphorylated as efficiently as the wild-type CaBP4. This result also shows that Ca^{2+} -induced conformational change does not mediate the decreased phosphorylation of CaBP4 in the presence of Ca^{2+} . More likely, dephosphorylation of CaBP4 in the dark may be mediated by a Ca^{2+} -dependent phosphatase (Kutuzov and Bennett, 1996; Sherman et al., 1997) and its phosphorylation promoted by light-dependent activation of PKC ζ .

Functional significance of CaBP4 phosphorylation

Phosphorylation of S37 did not affect binding of Ca^{2+} to CaBP4 (Fig. 3E) and therefore is unlikely to influence Ca^{2+} -dependent activities of CaBP4. However, CaBPs, including CaBP4, associate with Ca_v voltage-gated Ca^{2+} channels independent of Ca^{2+} (Lee et al., 2002; Haeseleer et al., 2004; Zhou et al., 2004, 2005; Cui et al., 2006). CaBP1 and CaBP4 compete with CaM in binding to the C-terminal IQ motif in the $\text{Ca}_v1.2$ and $\text{Ca}_v1.3$ α_1 subunit (Zhou et al., 2004, 2005; Cui et al., 2006; Yang et al., 2006). CaM binding to this site mediates Ca^{2+} -dependent inactivation of Ca_v1 channels (Peterson et al., 1999; Zuhlke et al., 1999). CaBP1 binding to this site, as well as to a modulatory site in the N-terminal domain of the $\text{Ca}_v1.2$ α_1 subunit ($\alpha_11.2$), opposes the actions of CaM and causes prolonged activation of $\text{Ca}_v1.2$ Ca^{2+} currents (Zhou et al., 2004, 2005). CaBP4 also associates with the IQ domain of $\alpha_11.3$ but causes a more modest suppression of Ca^{2+} -dependent inactivation than CaBP1 (Cui et al., 2006; Yang et al., 2006). Whereas CaBP4 also interacts with the C-terminal domain of $\alpha_11.4$ (Haeseleer et al., 2004), $\text{Ca}_v1.4$ channels alone show no Ca^{2+} -dependent inactivation attributable to an autoinhibitory module that inhibits this process located in the distal C-terminal domain of $\alpha_11.4$ (Singh et al., 2006; Wahl-Schott et al., 2006). However, the presence of this autoinhibitory module does not interfere with CaBP4 binding to or modulation of $\text{Ca}_v1.4$ (Haeseleer et al., 2004).

Perhaps because of distinct sequence elements in their C-terminal domains, $\alpha_11.4$ and $\alpha_11.3$ interacted differentially with phosphorylated CaBP4 (Fig. 4). CaBP4 phosphorylation strengthened Ca^{2+} -independent interactions with $\alpha_11.4$ (Fig. 4B,C) but slightly weakened binding to $\alpha_11.3$ both with and without Ca^{2+} (Fig. 4D,E). For $\text{Ca}_v1.3$, phosphorylation of S37 in CaBP4 may facilitate low-affinity interactions that transduce inhibitory modulation of $\text{Ca}_v1.3$ inactivation. Phosphorylation-induced conformational changes would be prevented by the S37A mutation in CaBP4, as shown by the negative effects on $\text{Ca}_v1.3$ inactivation (Fig. 5). The importance of low-affinity interactions of CaBP4 with $\alpha_11.3$ for channel modulation is further supported by the stronger effects of the CaBP4 EF-hand mutant (CaBP4-EF) on suppressing $\text{Ca}_v1.3$ inactivation (Fig. 7). Because CaBP4 binding to $\alpha_11.3$ was weaker in the absence of Ca^{2+} (Fig. 4), it is likely that CaBP4-EF maintains the low-affinity interaction required for channel modulation (Fig. 7). Light-dependent decreases in the photoreceptor Ca^{2+} concentration may increase CaBP4 phosphorylation. Physiologically, we would thus predict that phosphorylation of CaBP4 may have an opposite effect on CaBP4 activity than Ca^{2+} binding, which is consistent with our biochemical and electrophysiological analyses.

Light-dependent protein phosphorylation is a common regu-

latory mechanism in photoreceptors (Kuhn and Dreyer, 1972; Lee et al., 1984; Boesze-Battaglia et al., 1997; Roof et al., 1997; Hayashi et al., 2000; Hu et al., 2001; Rajala et al., 2002; Trojan et al., 2003). Because CaBP4 phosphorylation is increased under light conditions, CaBP4 may therefore adjust $\text{Ca}_v1.3$ and $\text{Ca}_v1.4$ Ca^{2+} signals as a function of light intensity. $\text{Ca}_v1.3$ channels are expressed primarily in cones (Morgans et al., 1998; Morgans, 1999; Barnes and Kelly, 2002), which show faster rates of neurotransmitter release compared with rods in darkness (Choi et al., 2005; Sheng et al., 2007). This difference in transmission may result from a higher intraterminal Ca^{2+} concentration in cones compared with rods in the dark (Sheng et al., 2007). We have shown previously that CaBP4 is required for normal cone to bipolar cell transmission (Maeda et al., 2005). For $\text{Ca}_v1.3$, light-dependent phosphorylation of CaBP4 may maintain activation of the presynaptic Ca^{2+} current at various light intensities, thus ensuring high release rates even at more hyperpolarized voltages. $\text{Ca}_v1.4$ channels are expressed primarily in rods (Morgans, 2001; Morgans et al., 2001; Barnes and Kelly, 2002), which are biochemically specialized for responses to dim illumination. Phosphorylation of CaBP4 during a light stimulus may help regulate $\text{Ca}_v1.4$ Ca^{2+} signals, thus increasing the signal-to-noise ratio and reliability of transmission at the rod synapse.

Although the upstream signaling events that ultimately lead to CaBP4 phosphorylation by PKC ζ remain to be addressed, the activation of the rod phototransduction cascade is required because light-dependent phosphorylation of CaBP4 was not observed in retina from *Gnat1*^{−/−} mice (Fig. 8C,D), which lack functional rods (Calvert et al., 2000). Although CaBP4 phosphorylation might still occur in cones of *Gnat1*^{−/−} mice, cones constitute only 3% of the photoreceptors in mouse retina (Carter-Dawson and Lavail, 1979) such that this small fraction of phosphorylated CaBP4 may not have been detected in our assay.

Although we have identified Ca_v1 channels as important targets for CaBP4 modulation, we cannot exclude that CaBP4 also interacts with and/or regulates other synaptic proteins. Phosphorylation of CaBP4 may differentially regulate the function of a variety of effectors, helping to shape presynaptic Ca^{2+} signals in photoreceptors and their neurotransmitter release properties in response to light.

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