**Protein Phosphatase 2A Dephosphorylates CaBP4 and Regulates CaBP4 Function**

**Françoise Haeseleer,1 Izabela Sokal,1 Frederick D. Gregory,2 and Amy Lee2**

**PURPOSE.** CaBP4 is a neuronal Ca\(^{2+}\)-binding protein that is expressed in the retina and in the cochlea, and is essential for normal photoreceptor synaptic function. CaBP4 is phosphorylated by protein kinase C zeta (PKC\(_\alpha\)) in the retina at serine 37, which affects its interaction with and modulation of voltage-gated Ca\(_{1.1}\) Ca\(^{2+}\) channels. In this study, we investigated the potential role and functional significance of protein phosphatase 2A (PP2A) in CaBP4 dephosphorylation.

**METHODS.** The effect of protein phosphatase inhibitors, light, and overexpression of PP2A subunits on CaBP4 dephosphorylation was measured in vitro assays. Pull-down experiments using retinal or transfected HEK293 cell lysates were used to investigate the association between CaBP4 and PP2A subunits. Electrophysiologic recordings of cotransfected HEK293 cells were performed to analyze the effect of CaBP4 dephosphorylation in modulating Ca\(_{1.3}\) currents.

**RESULTS.** PP2A inhibitors, okadaic acid (OA), and fostriecin, but not PP1 selective inhibitors, NIPP-1, and inhibitor 2, block CaBP4 dephosphorylation in retinal lysates. Increased phosphatase activity in light-dependent conditions reverses phosphorylation of CaBP4 by PKC\(_\alpha\). In HEK293 cells, overexpression of PP2A enhances the rate of dephosphorylation of CaBP4. In addition, inhibition of protein phosphatase activity by OA increases CaBP4 phosphorylation and potentiates the modulatory effect of CaBP4 on Ca\(_{1.5}\) Ca\(^{2+}\) channels in HEK293T cells.

**CONCLUSIONS.** This study provides evidence that CaBP4 is dephosphorylated by PP2A in the retina. Our findings reveal a novel role for protein phosphatases in regulating CaBP4 function in the retina, which may fine tune presynaptic Ca\(^{2+}\) signals at the photoreceptor synapse. (Invest Ophthalmol Vis Sci. 2013;54:1214–1226) DOI:10.1167/iovs.12-11319

CaBP4 is a member of a subfamily of calmodulin-like neuronal Ca\(^{2+}\)-binding proteins (CaBP1-8). In addition to modulating voltage-gated Ca\(_{1.2}\), Ca\(^{2+}\) channels, PP2A family members modulate transient receptor potential (TRP) channels and inositol 1,4,5-trisphosphate (IP\(_3\)) receptors. CaBP4 is localized in photoreceptor synaptic terminals and in cochlear inner hair cells. CaBP4 is essential for photoreceptor synaptic function through enhanced activation of Ca\(_{1.4}\) L-type voltage-gated Ca\(^{2+}\) channels and transmitter release. CaBP4-knockout mice (CaBP4\(^{-/-}\)) possess morphologically and functionally deficient synapses, similar to that observed in mice lacking the pore-forming Ca\(_{1.4}\) subunit (\(\alpha_1\), 1.4) or the auxiliary Ca\(_{\beta_2}\) subunit. Moreover, human mutations in the CaBP4 gene are associated with autosomal recessive incomplete congenital stationary night blindness and cone-rod synaptic disorder.

Reversible phosphorylation of proteins is an essential mechanism regulating many cellular functions. Multiple photoreceptor proteins are regulated by light-dependent phosphorylation.57–84 Phosphorylation of Ca\(^{2+}\)-binding proteins, including calmodulin, can affect their Ca\(^{2+}\)-binding capability and interaction with target proteins.55,55–58 Similar to calmodulin, 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.13 We have shown that CaBP4 undergoes light-dependent phosphorylation by protein kinase C zeta (PKC\(_\alpha\)) at serine 37 both in vitro and in the retina.39 In electrophysiologic recordings of transfected HEK293 cells, phosphorylation of CaBP4 at serine 37 enhances the effect of CaBP4 in prolonging the opening of Ca\(_{1.3}\) Ca\(^{2+}\) channels, which have been reported to be present in photoreceptors.40–43 In contrast, Ca\(^{2+}\)-binding to CaBP4 weakens modulation of Ca\(_{1.3}\) channels.39 These findings suggest that phosphorylation as well as Ca\(^{2+}\) regulate the synaptic function of CaBP4 in the retina.

In the present study, we investigated the mechanisms underlying reversible phosphorylation of CaBP4. We identified protein phosphatase 2A (PP2A) as a phosphatase that dephosphorylates CaBP4 in the retina and in transfected cells. Although PKC\(_\alpha\) activity dictates the level of CaBP4 phosphorylation under light-adapted conditions, PP2A activity is also higher in light-adapted than dark-adapted retinas. In HEK293 cells, we characterized PP2A subunits involved in CaBP4 dephosphorylation and show that dephosphorylation of CaBP4 by PP2A inhibits its modulation of Ca\(_{1.3}\) activity.

**METHODS**

**Antibodies**

Commercially available antibodies were: alkaline phosphatase-conjugated anti-mouse and anti-rabbit (Promega Corp., Madison, WI); rabbit anti-PP2A sampler kit (anti-PP2A A [\(\beta_1\)], PP2A B [PR55, Bx], and PP2A C [\(\beta_2\)] subunits [for specificity, see #9780 online at Cell signaling technology, Danvers, MA]); anti-DYKDDDDK epitope (FLAG) (Sigma-Aldrich, St. Louis, MO); anti-c-myc, anti-influenza hemagglutinin epitope (HA) (Roche Applied Science, Indianapolis, IN). The specificity of anti-tag antibodies was confirmed by Western blot analysis using untransfected...
TABLE. PCR Primers Used to Amplify PP2A Subunits and PP1 Inhibitors by PCR

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<tr>
<th>mRNA / Gene Number</th>
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Epitope-Tagged PP2A Subunits

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<td>HA-tagged PP2A A3 (+)</td>
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<tr>
<td>c-myc-tagged PP2A Cj (+)</td>
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<td>FLAG-tagged PP2A Bz (+)</td>
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Cloning, Bacterial Expression, and Purification of Active Nuclear Inhibitor of Protein Phosphatase-1 (NIPP-1) and Inhibitor2

Mouse NIPP-1 and inhibitor 2 were amplified by PCR with Plc polymerase (Life Technologies, Carlsbad, CA) from a mouse retina cDNA library with specific primers (Table) and cloned into pVEX3-D-TOPO vector (Life Technologies). After sequencing, the cDNAs were transferred by recombination into the pBluescript SKI+ vector using the Gateway Technology System (Life Technologies) for fusion to a His-tag and expression in bacteria. The His-fusion proteins were expressed in BL21(DE3)pLysS Escherichia coli after induction with 0.5 mM isopropyl β-D-thiogalactopyranoside (IPTG) and purified on Ni²⁺-NTA or glutathione column according to the manufacturer's protocol.

Protein concentration was determined by quantification of the corresponding band on Coomassie-Blue–stained SDS gels (Coomassie Blue R250; EM Science, Gibbstown, NJ) relative to known amounts of BSA. The intensity of each band on the scanned gels was quantitated using ImageJ (National Institutes of Health, Bethesda, MD). The concentration of purified NIPP-1 was estimated at 3.4 μM and 7.5 μM for inhibitor 2.

Phosphorylation/Dephosphorylation Assay

Mouse retinas were obtained from C57Bl/6J mice. Animals were treated in accordance with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. 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. When specified, dark-adapted retinas were obtained from mice kept in darkness for 4 hours and dissected under a dim red light. For all other experiments, retinas were obtained from light-adapted mice.

The assay was performed as described previously but with different phosphatase inhibitors. Briefly, retinas were dissected and homogenized in 100 μl of 10 mM bis-Tris-propane (BTP), pH 7.4 containing 150 mM NaCl and a mixture of proteinase inhibitors (Sigma-Aldrich). The absorbance at 280 nm of a 20 × dilution of our retinal homogenates was approximately 0.5/cm. Phosphorylation assays involved native CaBP4 in retinal lysates, recombinant glutathione S-transferase (GST)-tagged CaBP4 (2 μg) or GST-tagged CaBP4-S37A (2 μg), prepared as described previously, and were carried out in a 20 μl reaction using 5 μl of retinal homogenate and 3 μl of [γ-32P]ATP in 10 mM BTP, pH 7.4, 2 mM MgCl₂, 100 μM ATP and 0.5 mM dithiothreitol (DTT) for 10 minutes at 30°C. After incubation of the endogenous PKCβ with 10 μM Bisindolylmaleimide I (Calbiochem, La Jolla, CA), the reactions were incubated for another 45 minutes at 30°C with or without protein phosphatase inhibitors: 1 μM calyculin A (Calbiochem), 1 μM okadaic acid (OA; Sigma-Aldrich), 1 μM cyclosporin A (Calbiochem), or 300 nM NIPP-1. Reactions were terminated with the addition of SDS-PAGE sample buffer. The proteins were separated on SDS-PAGE gels followed by transfer onto immobilized P membrane. 32P-labeled CaBP4 was detected by autoradiography and equivalent input and transfer of CaBP4 were confirmed by red Poncsea staining of immobilized membrane (See Supplementary Material and Supplementary Fig. S1, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-11319/-/DSSupplemental) or Western blot analysis using anti-CaBP4 antibody. The contrast and brightness of the scanned images was increased equally for all lanes to improve band visibility.

For assays measuring dose-dependence of phosphatase inhibitors with respect to CaBP4 dephosphorylation and for the comparative assays involving recombinant PP1 or purified PP2A, recombinant CaBP4 was first phosphorylated with recombinant PKC (Calbiochem) in the presence of [γ-32P]ATP for 45 minutes at 30°C in a master mix. 32P-labeled (phosphorylated) CaBP4 was then added to 5 μl of mouse retinal homogenate along with 0.1 units of purified PP2A (Millipore, Billerica, MA) or 0.1 units of recombinant PP1 (New England Biolabs, Ipswich, MA) in a 15-μl reaction. The reactions were carried out further for 45 minutes at 30°C and analyzed on SDS-PAGE gels as described above. In experiments with NIPP-1, inhibitor 2, and fostriecin (Calbiochem), retinal lysate, rPP1, and purified PP2A were pre-incubated at 30°C for 10 minutes with the inhibitors.

For assays using HEK293 cell lysate, cells collected from a 10 cm³ plate of confluent HEK293 cells were sonicated in 10 mM BTP pH 7.2 (500 μl) containing 5 mM benzamidin. After centrifugation for 30
minutes at 10,000g, the supernatant was collected and processed as described above for mouse retinal homogenate.

**Analysis of Phosphorylated CaBP4 in Dark- and Light-Adapted Mouse Retina**

For those experiments repeated three times, native retinal CaBP4 from two dark- and two light-adapted mouse retinas was labeled with [γ-32P]ATP as described above in the presence or absence of OA. Reactions were then diluted twice with PBS and were cleared by incubation for 1 hour at 4°C with protein G-magnetic beads. After removal of the beads, samples were incubated for 1 hour at 4°C with affinity purified anti-CaBP4 polyclonal antibodies (10 μg). Protein G-magnetic beads were then added and incubated overnight at 4°C. Beads were washed 5 times with the same buffer, and proteins were eluted with 0.1% glycine, pH 2.8. Phosphorylated CaBP4 from dark/light-adapted retinas were resolved on SDS-PAGE and analyzed by autoradiography and Western blotting using anti-CaBP4 antibody. The intensity of each band was quantified using ImageJ.

**Analysis of PP2A Activity in Dark- and Light-Adapted Mouse Retina Using Recombinant Phosphorylated CaBP4**

Recombinant CaBP4 was phosphorylated with recombinant PKCδ in the presence of [γ-32P]ATP for 45 minutes at 30°C in a master mix as described above. After inactivation of PKCδ by addition of Bis (10 μM), aliquots containing 1 μg of labeled CaBP4 were then used as substrate for phosphatase. Phosphorylated proteins were added to 10 μL of dark- or light-adapted retinas homogenized in 10 mM BTP pH 7.4 and incubated further for 15 or 45 minutes at 30°C. An aliquot incubated for 45 minutes in the presence of OA (5 μM) was used as an internal control wherein both the kinase and phosphatase activity should be inhibited and, therefore, should be similar to results obtained immediately after addition of retinal extract (at time [T] = 0). Reactions were stopped by addition of SDS-PAGE sample buffer, and proteins were separated on SDS-PAGE followed by transfer onto immobilization membrane. 32P-Labeled CaBP4 was detected by autoradiography and amount of CaBP4 was confirmed by Ponceau staining. The intensity of each band on the autoradiography was quantified using ImageJ.

**Analysis of PP2A Expression Using Western Blot Analysis**

Mouse retina or HEK293 cells were homogenized in 100 μL of PBS. Homogenate was centrifuged at 100,000g for 1 hour. After centrifugation, the supernatant was collected and the pellet was resuspended in 100 μL of PBS. Both samples were mixed with SDS-PAGE sample buffer and proteins were separated by SDS-PAGE follow by transfer onto immobilization membrane. Membranes saturated with 5% milk in Phosphate buffer saline containing 0.1% Triton X-100 (PBST) were incubated for 1 hour at room temperature with polyclonal antibodies raised against PP2A A, PP2A B, or PP2A C. After extensive washing with PBST and 1 hour incubation with alkaline phosphatase-conjugated secondary antibodies, the labeled bands were detected colorimetrically by developing in 5-bromo-4-chloro-3-indolyl-phosphatase-conjugated secondary antibodies, the labeled bands were detected colorimetrically by developing in 5-bromo-4-chloro-3-indolyl-phosphatase (BCIP)/Nitro Blue Tetrazolium Chloride (NBT) solution.

**Cloning of Retina PP2A Subunits**

Mouse PP2A subunits (Aα, Cα, Cβ, and B9) were cloned after amplification by PCR with Pfx polymerase from a mouse retina cDNA library with primers listed in the Table. Epitope tags were added by PCR to the cloned PP2A subunits using primers containing the HA, c-myc, or FLAG tag (Table). After sequencing of all cloned subunits, the tagged-subunits were subcloned into the pcDNA3.1 (+) vector for expression in mammalian cells.

**GST Pull-Down Assay**

HEK293 cells were cotransfected with equal amounts of pcDNA-PP2A Aα, pcDNA-PP2A Cα, or Cβ and pcDNA-PP2A B9 plasmids using Lipofectamine (Life Technologies) and collected 2 days after transfection. Cell lysates (~5–7 mg/mL) were prepared by sonication of a 10 cm² plate of HEK293 cells in 1 mL of PBS containing 0.1% Triton X-100 and 5 mM benzamidin, and centrifuged for 30 minutes at 10,000g. GST (negative control) and GST-CaBP4 (5 μg), immobilized on glutathione magnetic beads, were incubated for 3 hours at 4°C with 0.5 mL of cell lysate. The beads were washed five times with the same buffer and the proteins were eluted by boiling in 100 μL SDS-PAGE sample buffer. Samples were probed by Western blot analysis using anti-tag or anti-PP2A subunit antibodies. All samples were loaded on the same gel. When indicated, the development of the Western blot with NBT/BCIP colorimetric reagent was stopped after 1 minute for lanes loaded with the cell lysate compared with 5 minutes for the eluted proteins of the pull-down assay; a black line was added to the Western blot to delineate the cut membrane. The contrast of the Western blot images was increased equally across the whole image in Photoshop CS3 (Adobe Systems, San Jose, CA) to improve band visibility. For pull-down assays using retinas, we used bovine retinas, which provided a larger amount of tissue than is possible with mouse retina. Two bovine retinas (In Vision BioResources, Seattle, WA) were homogenized and sonicated in 4 mL of PBS containing 0.1% Triton X-100 and 5 mM benzamidin and centrifuged for 30 minutes at 10,000g. Retina supernatant was used in pull-down assays as described above.

**Analysis of Native and Recombinant PP2A Activity in HEK293 Cell Lysates**

Cells collected from a 10 cm² plate of nontransfected cells or cells transfected with PP2A A, B, and C were sonicated in 500 μL of 10 mM BTP pH 7.2 containing 5 mM benzamidin. After centrifugation for 30 minutes at 10,000g, the total protein concentration of the supernatants was determined and equal amounts of proteins (~25 μg) were used in the assay. A master mix for eight samples containing recombinant CaBP4 (8 × 2 μg), was added to 40 μL (8 × 5 μL) of cell extracts in the presence of [γ-32P]ATP and 10 mM BTP pH7.2, 2 mM MgCl2, 100 μM cold ATP, 0.5 mM DTT, 5 mM benzamidin in a 160 μL (8 × 20 μL) volume, and incubated for 15 minutes at 30°C. OA (5 μM) was added to one aliquot and further incubated for 10 minutes at 30°C. One aliquot was taken and the reaction stopped as T equals 0 by addition of SDS-PAGE sample buffer. Bis (10 μM) was added to the rest of the reaction and further incubated at 30°C. Aliquots (20 μL) were taken after 5, 15, 30, 60, or 90 minutes and stopped by addition of SDS-PAGE sample buffer. The reactions were loaded on SDS-PAGE and transferred to immobilom membrane followed by Ponceau staining and autoradiography. The intensity of each band on the autoradiography was quantified using ImageJ.

**Electrophysiologic Recordings**

Patch clamp recordings were performed as described previously. Briefly, HEK293T cells were plated on 35-mm culture dishes and transiently transfected with approximately 3-μg total DNA (α1,1.3, β, αβδ subunits ± CaBP4) using Fugene transfection reagent according to manufacturer instructions. The plasmid pEGFP-N1 (0.1 μg) was included for fluorescent detection of transfected cells. Ca2⁺ currents were recorded at room temperature using the whole cell patch clamp technique 48 to 72 hours after transfection. The internal recording solution contained (in mM): 140 N-methyl-D-glucamine (NMDG), 5 EGTA, 10 HEPES, 2 MgCl2, 2 Mg-ATP, pH 7.3 (adjusted with methanesulfonate), and 290 milliosmole (mOsmol). The external solution contained the following (in mM): 130 Tris, 2 MgCl2, 20 CaCl2, pH 7.3 (adjusted with methanesulfonate), and 310 mOsmol. Dimethyl sulfoxide (DMSO), (0.04% final concentration) or OA (200 nM final concentration) was included for fluorescent detection of transfected cells. Ca2⁺ currents were recorded at room temperature using the whole cell patch clamp technique 48 to 72 hours after transfection. The internal recording solution contained (in mM): 140 N-methyl-D-glucamine (NMDG), 5 EGTA, 10 HEPES, 2 MgCl2, 2 Mg-ATP, pH 7.3 (adjusted with methanesulfonate), and 290 milliosmole (mOsmol). The external solution contained the following (in mM): 130 Tris, 2 MgCl2, 20 CaCl2, pH 7.3 (adjusted with methanesulfonate), and 310 mOsmol. Dimethyl sulfoxide (DMSO), (0.04% final concentration) or OA (200 nM final concentration) was included in the internal solution. Electrode
The results showed that the ability of PP1 to dephosphorylate CaBP4 (see Supplementary Material and Supplementary Figs. S2C, S2D, http://www iovs.org/lookup/suppl/doi:10.1167/ iovs.12-11319/-/DCSupplemental). In addition, OA fully inhibited CaBP4 retinal phosphatase at a concentration (300 nM) that did not affect PP1 dephosphorylation of CaBP4, and there were no additive effects of inhibitor 2 and NIPP-1 to those of OA (Fig. 1D). Because OA inhibits not only PP2A but also PP4, PP5, and PP6, we also analyzed whether CaBP4 phosphatase is promoted by fostriecin, which is a more selective inhibitor of PP2A. CaBP4 dephosphorylation by retinal extracts was strongly inhibited by fostriecin concentrations that do not inhibit CaBP4 dephosphorylation by rPP1 (Fig. 1E). Taken together, these results suggest that the properties of the phosphatase that dephosphorylates CaBP4 in the retina are consistent with PP2A.

We next tested the involvement of serine 37 as the residue in mouse CaBP4 that is dephosphorylated by PP2A. This residue was shown to be phosphorylated by PKC in HEK293 cells and by PKCζ in the retina, which is important for Ca2+ channel modulation.59 If PP2A dephosphorylates serine 37, inhibiting PP2A should not affect the phosphorylation status of CaBP4 lacking this phosphorylation site. Consistent with this prediction, phosphorylation of wild-type (WT) CaBP4 but not CaBP4-S37A was increased with OA (Fig. 1F). These results suggest that PP2A can dephosphorylate CaBP4 at serine 37.

**Interaction of CaBP4 with PP2A Subunits**

Two structural (Aα, Bβ, and Cγ) and 2 catalytic (Cx, β) subunits of PP2A have been characterized. In addition, there are 16 PP2B regulatory (Bβ) subunits divided across four gene families (Bβ, Bγ, Bδ, and Bζ), which help define substrate specificity. To gain insights as to which PP2A subunits may regulate CaBP4 in the retina, we performed pull-down assays using GST-tagged CaBP4 (GST-CaBP4) and lysate from bovine retinas or HEK293 cells. Unlike for the CaBP4 phosphorylation assays, in which mouse retinal lysate was used, larger amounts of starting material are required for pull-down assays, which is why we turn to lysates from bovine retinas and HEK293 cells. Consistent with previous results, the Aα, Bβ, and Cγ subunits of PP2A were detected in extracts of retina by Western blotting with anti-PP2A (Aα, Bβ, and Cγ) antibodies (Fig. 2A). In pull-down assays, GST-CaBP4 interacted with Aα, Bβ, and Cγ subunits that were endogenously expressed in retina (Fig. 2B). By affinity chromatography and mass spectrometry of bovine retinal lysates, peptide sequences corresponding to PP2A Aα and common to particular PP2A subunits (Cγ, Cδ, and Cε) were identified among the proteins interacting with CaBP4 (data not shown). In addition, each of these PP2A subunits (Table) found to interact with CaBP4 could be amplified by PCR from mouse retina cDNA, consistent with their potential role in regulating CaBP4 phosphorylation in the retina. These results provide further support for a role of PP2A in CaBP4 dephosphorylation in the retina.

To confirm the interaction of CaBP4 with these specific PP2A subunits, epitope-tagged PP2A subunits (Aα, Cx, Cβ, and Bz) were expressed in HEK293 cells and tested for binding to GST-CaBP4. Cx, Cβ, and Bz, but not Aα, subunits interacted specifically with GST-CaBP4 as shown by Western blot analysis using anti-tag antibodies (Figs. 3A–D, top panels). Western blotting using antibodies against Aα, Bβ, and Cγ subunits detected two bands corresponding to the epitope-tagged PP2A Cx, Cβ, and Bz (Figs. 3B–D, lower panels, upper band) and the endogenous Bβ and Cγ subunits, which are expressed in HEK293 cells (Figs. 3B–D, lower panels, lower band). Our inability to detect interaction of PP2A Aα subunit with GST-CaBP4 in these experiments with HEK293 cell lysates suggested interactions...
**Figure 1.** CaBP4 phosphorylation by mouse retinal lysates is inhibited by PP2A inhibitors. Dephosphorylation of GST-CaBP4 due to the endogenous phosphatase in lysates from light-adapted mouse retinas (A–F) or HEK293 cells (F) was determined by autoradiography (upper panel) and equivalent input and transfer of CaBP4 in all reactions was confirmed by Ponceau staining (lower panel). The data are representative results of three separate experiments. Arrowheads indicate the band corresponding to GST-CaBP4. **(A)** Identification of the serine/threonine phosphatase involved in dephosphorylation of CaBP4 in retinal extracts. GST-CaBP4 was phosphorylated with the endogenous kinase in mouse retinal extracts in the presence of [γ-32P]ATP. After quenching of kinase activity, the reaction was further incubated with or without calyculin A (CalA, 1 μM), OA (1 μM), cyclosporin A (CsA, 1 μM), or NIPP-1 (300 nM). **(B)** Effect of Ca2+ on retinal phosphatase activity. GST-CaBP4 was first phosphorylated with recombinant PKCf in the presence of [γ-32P]ATP. After inactivation of PKCf, 32P-CaBP4 was added to an extract of mouse retinas without or with OA (5 μM), 5 mM CaCl2, 5 mM EGTA, or 5 mM EDTA as indicated and the incubation was carried on for 45 minutes at 30°C. **(C)** Dose-dependent inhibition of CaBP4 dephosphorylation by calyculin A (upper panel) and OA (lower panel) in mouse retina extract. CaBP4 dephosphorylation was analyzed in retinal extracts as described in (A) with or without inhibitors as indicated. **(D)** Comparison of retinal phosphatases and rPP1 activities on 32P-labeled CaBP4 in the presence or absence of phosphatase inhibitors. Phosphatase assays were carried out with radioactively labeled CaBP4 with or without 500 nM OA, 500 nM inhibitor 2 or 300 nM NIPP-1, or a combination of OA and inhibitor-2 or NIPP-1 (300 nM each). **(E)** Dose-dependent inhibition of CaBP4 dephosphorylation by fostriecin. GST-CaBP4 dephosphorylation assays were carried out as in (D) using mouse retinal lysate, rPP1, or purified PP2A, but with various concentrations of fostriecin or OA (1 μM). **(F)** Phosphorylated CaBP4-Ser37 is the substrate for the endogenous phosphatase in retina and HEK293 cells. GST-CaBP4 or GST-CaBP4-S37A mutant were incubated with equivalent amounts of retina or HEK293 extract in the presence of [γ-32P]ATP. After quenching of PKC activity with Bis, reactions were incubated further in the presence or absence of OA (1 μM).
through other retina-specific proteins, which could account for the pull-down of PP2A A subunit by GST-CaBP4 from retinal lysates (Fig. 2B). Thus, we conclude that CaBP4 interacts, either directly or indirectly, with PP2A subunits α, β, γ, and δβ in the retina.

**Overexpression of PP2A Enhances the Rate of Dephosphorylation of CaBP4 in Transfected HEK293 Cells**

To verify the importance of PP2A in regulating CaBP4 dephosphorylation, we tested the consequences of varying expression levels of PP2A on CaBP4 phosphorylation in HEK293 cells. Because we were unable to achieve knockdown of PP2A subunit expression in HEK293 cells with siRNA, we assessed instead the effect of increasing PP2A by overexpression on CaBP4 dephosphorylation in HEK293 cells. For these experiments, we cotransfected HEK293 cells with PP2A subunits (α, β, δβ, γβ), which interacted with CaBP4 (Figs. 2, 3), and compared the effects of lysates from untransfected and PP2A-transfected cells on CaBP4 dephosphorylation. Recombinant GST-CaBP4 was first prephosphorylated by the endogenous kinase in HEK293 cells and similar levels of kinase activity were confirmed in untransfected and PP2A-transfected cells upon phosphatase inhibition with OA (Fig. 4B, lane 1). We then analyzed the time course of CaBP4 dephosphorylation by PP2A after addition of the PKC inhibitor, Bis. The rate of CaBP4 dephosphorylation for PP2A-transfected cells (11.8 ± 2.5%/min, *P* = 0.009 by *t*-test) was significantly greater than that in untransfected cells (1.8 ± 0.7%/min). These findings confirm that PP2A can efficiently dephosphorylate CaBP4.

**CaBP4 Dephosphorylation by PP2A Inhibits Modulation of Ca1.3**

To address the functional consequences of CaBP4 dephosphorylation by PP2A, we used an assay that measures the modulation of Ca1.3 channels by CaBP4 in transfected HEK293T cells.69 While we have shown that CaBP4 enhances voltage-dependent activation of Ca1.4 channels,8 Ca1.4 Ca2+ currents (I_{Ca}) are relatively small in amplitude compared with Ca1.3 currents, which complicates analysis of CaBP4 modulation. Therefore, we have used Ca1.3 channels to characterize CaBP4 modulation by phosphorylation. CaBP4 has a modest effect on slowing inactivation of I_{Ca} in HEK293T cells cotransfected with Ca1.3, which is prevented by the S57A mutation that prevents phosphorylation of CaBP4.39 If PP2A dephosphorylation of CaBP4 affects the function of CaBP4, then OA should enhance the effect of CaBP4 on slowing Ca1.3 inactivation. To test this hypothesis, we compared I_{Ca} in HEK293T cells cotransfected with Ca1.3 and CaBP4 (Fig. 5). Inactivation was measured as the ratio of the current amplitude at the end of a 300 ms test pulse and the peak current amplitude (r_{500}, Figs. 5A, B). In cells cotransfected with CaBP4 and Ca1.3, I_{Ca} inactivation was significantly weaker (~22%–43% across the voltage range tested, Fig. 5B) when exposed to OA compared with control solution (*P* = 0.02, by two-way ANOVA). By contrast, OA had no effect on I_{Ca} inactivation in cells transfected with Ca1.3 alone (*P* = 0.58, Fig. 5A). These results confirm that CaBP4 dephosphorylation can negatively regulate functional interactions with targets such as Ca1.3. Although PP2A inhibition can directly affect activation of Ca1.1 currents,36 we found no effect of OA on the voltage-dependent activation in cells transfected with Ca1.3 alone (Fig. 5C) or cotransfected with Ca1.3 and CaBP4 (Fig. 5D). Together with our biochemical data, these results suggest that CaBP4 dephosphorylation by PP2A can inhibit CaBP4 modulation of Ca1.3 inactivation.

**Dephosphorylation of CaBP4 by PP2A is Greater in Light-Adapted than Dark-Adapted Retina**

CaBP4 phosphorylation by the atypical Ca2+-independent PKCζ is greater in light-adapted conditions when Ca2+ levels are low than in dark-adapted mouse retinas when Ca2+ levels are comparatively high.37,57,59 Therefore, the phosphorylation state of CaBP4 could be determined by Ca2+-dependent dephosphorylation in darkness or Ca2+-independent enhancement of CaBP4 phosphorylation in light-adapted retina. The former possibility is unlikely since CaBP4 dephosphorylation by PP2A is unaffected by Ca2+ (Fig. 1B). Nevertheless, we compared the effects of PP2A inhibition on CaBP4 phosphorylation in light- and dark-adapted retina. In these experiments, PP2A inhibition by OA only slightly increased CaBP4 phosphorylation in retina extract from dark-adapted mice (Figs. 6A, B). Since an effect of PP2A may have been missed due to the low level of CaBP4 phosphorylation under dark-adapted conditions, we compared the effect of OA on recombinant CaBP4 that was prephosphorylated with recombinant PKCζ to the same levels prior to the addition of extract of dark- or light-adapted mouse retinas. Under these conditions, the same initial level of phosphorylated CaBP4 should be then regulated by the endogenous phosphatase activity in light- or dark-adapted retinal lysates. By analyzing samples taken at different time.
points, we found that dephosphorylation of CaBP4 was faster in light-compared with dark-adapted retinas in that the level of phosphorylated CaBP4 was significantly lower in light-adapted than in dark-adapted retinas (Figs. 6C, D; $T = 15$ minutes, $P < 0.015$, $T = 45$ minutes, $P < 0.005$, $t$-test). Thus, PP2A activity is actually greater under light-adapted than dark-adapted conditions. These results were not due to a light-dependent alteration in the subcellular distribution of PP2A subunits, which was confirmed by Western blot analysis of soluble and membrane fractions of light- and dark-adapted retinal extracts (data not shown). While $\text{Ca}^{2+}$-independent activity of PKC$_\varepsilon$ may dictate the level of light-dependent CaBP4 phosphorylation, activation of PP2A in light conditions may be necessary to balance the activity of PKC$_\varepsilon$ in controlling the phosphorylation state of CaBP4.

**DISCUSSION**

Reversible phosphorylation of $\text{Ca}^{2+}$-binding proteins such as calmodulin (CaM) has emerged as an important mechanism for the dynamic regulation of effectors. Small-conductance $\text{Ca}^{2+}$-activated potassium channels (SK channels) are gated by $\text{Ca}^{2+}$ ions through the association of CaM directly with the SK channel protein. Casein kinase 2 phosphorylation of CaM at threonine 80 inhibits $\text{Ca}^{2+}$ activation of SK channels, which may be reversed by PP2A dephosphorylation. In this study, we demonstrate that PP2A dephosphorylates CaBP4 in the retina. CaBP4 dephosphorylation is enhanced by light in the retina and limits CaBP4 potentiation of $\text{Ca}_{\text{v1.3}}$ activity in transfected HEK293T cells.

Although multiple serine/threonine protein phosphatases have been identified in the retina, PP2A inhibitors, OA, and fostriecin, but not PP2B or PP1 selective inhibitors, block CaBP4 dephosphorylation in retinal lysates (Fig. 1). Higher concentrations of fostriecin and OA (hundreds of nanomolar) were required to fully inhibit CaBP4 dephosphorylation both in the retinal extract and by purified PP2A than would be predicted based on previous reports (low nanomolar concentrations) (Figs. 1C, 1D, 1F and see Supplementary Material and Supplementary Fig. S2, http://www.iovs.org/lookup/suppl/doi:10.1167/iovs.12-11319/-/DCSupplemental). However, variation among IC50 values of OA and fostriecin in...
Figure 4. Recombinant PP2A subunits enhance CaBP4 dephosphorylation in HEK293 cells. HEK293 cells were not transfected (lanes 1) or cotransfected with rPP2A A, Bα, and Cβ (lanes 2). (A) Western blot analysis of the recombinant epitope-tagged (black arrowhead) or endogenous untagged (grey arrowhead) PP2A subunits in HEK293 cell lysates used in (B) using anti-PP2A subunit antibodies. (B) Time course of CaBP4 dephosphorylation by PP2A subunits. Lysates of HEK293 cells were incubated with GST-CaBP4 in the presence of \([\gamma-32P]ATP\). Similar levels of kinase activity between groups was confirmed upon further incubation with OA (5 \(\mu\)M) for 10 minutes to inhibit PP2A activity. Dephosphorylation of radiolabeled CaBP4 by PP2A was analyzed between 0 and 90 minutes after addition of Bis (10 \(\mu\)M) to inhibit PKC activity. Autoradiograph (left) and Ponceau staining (right) shows levels of phosphorylated CaBP4 and equivalent amounts of CaBP4 in each reaction, respectively. (C) Quantitative analysis of time course of CaBP4 dephosphorylation by PP2A subunits. The intensity of each band of the autoradiography as shown in (B) was quantified using ImageJ and normalized to that of the reaction at \(T=0\) minutes after quenching of PKC activity. Points represent mean \(\pm\) SEM, \(n=4\). The data were fit with a single exponential function. The rate for untransfected cells (1.8 \(\pm\) 0.7%/min) is significantly slower than that for PP2A-transfected cells (11.8 \(\pm\) 2.5%/min, \(P=0.009\) by \(t\)-test).
cell-free assays may depend on the amount of phosphatase as well as the substrate employed. Although inhibition of PP1 is independent of phosphatase concentration, the concentration of OA required for inhibition of PP2A increases with increasing phosphatase levels. Therefore, it is possible that CaBP4 as a PP2A substrate and/or high phosphatase levels in our assay account for the higher concentration of OA and fostriecin needed to inhibit fully CaBP4 dephosphorylation.

**FIGURE 5.** OA potentiates modulation of Cav1.3 channels by CaBP4. (A, B) Left panels: Representative traces showing Ca\(^{2+}\) currents (I\(_{Ca}\)) evoked by a 300 ms test pulse from \(-90\) mV to \(-20\) mV for cells with Cav1.3 alone (A) or cotransfected with CaBP4 (B). Recordings were with 0.04% DMSO (control; black) or with 200 nM OA included in the patch pipette (+OA, grey). Control and OA traces were normalized for comparison. Right panels: Inactivation was measured as the amplitude of I\(_{Ca}\) at the end of the 300 ms depolarization normalized to the peak current amplitude (r300) and plotted against test voltage for Cav1.3 alone (n = 5 for control, n = 4 for +OA) or for Cav1.3 + CaBP4 (n = 4 for control, n = 4 for +OA). P values were based on two-way ANOVA. (C, D) Current-voltage (I-V, left panels) relationships for cells transfected and recorded as in (A, B). Currents were evoked by 20 ms test pulses from \(-90\) mV to various test potentials ranging from \(-70\) mV to \(+40\) mV for cells with Cav1.3 alone (C) or cotransfected with CaBP4 (D). Peak currents were normalized to I\(_{Ca}\) for each cell (I/I\(_{max}\)) and plotted against voltage. Parameters from Boltzmann fits of IV curves in (C, D) were: for Cav1.3 alone (control: \(V_{1/2} = -15.3 \pm 1.4\) mV; \(k = -7.5 \pm 0.3\); n = 6; +OA: \(V_{1/2} = -18.4 \pm 1.6\) mV; \(k = -7.4 \pm 0.5\); n = 6), and for Cav1.3 + CaBP4 (control: \(V_{1/2} = -21.1 \pm 1.1\) mV; \(k = -6.6 \pm 0.4\); n = 7; +OA: \(V_{1/2} = -17.9 \pm 1.3\) mV; \(k = -7.4 \pm 0.6\); n = 7). OA had no significant effect on any of the parameters (Cav1.3 alone: \(V_{1/2}: P = 0.17; k: P = 0.76; V_{rev}: P = 0.90\), by \(t\)-test; Cav1.3 + CaBP4: \(V_{1/2}: P = 0.10; k: P = 0.17, V_{rev}: P = 0.27\).
The PP2A-like phosphatases, PP4, PP5, and PP6, are also inhibited by OA concentrations close to those that inhibit PP2A. However, fostriecin inhibited CaBP4 dephosphorylation at concentrations that inhibit PP2A, but not PP1 and PP5 (Fig. 1E), arguing against a role for PP1 or PP5 in CaBP4 dephosphorylation in retinal extracts. These data alone cannot rule out the involvement of PP4, since there are currently no selective inhibitors, including fostriecin, that distinguish between PP2A and PP4. The sensitivity of PP6 to fostriecin has not been characterized, although predicted to be comparable to that of PP2A based on conserved amino acids between the PP2A and PP6 fostriecin-binding domain. However, PP6 is inhibited by 10 times higher concentration of OA than PP2A. Therefore, PP6 is unlikely to be involved in CaBP4 dephosphorylation in the retina.

As expected for PP2A, phosphatase activity was not changed by addition of Ca\(^{2+}\) or EGTA; however, a small decrease of CaBP4 dephosphorylation was observed in the presence of EDTA compared with EGTA (Fig. 1B). In contrast to EGTA, which preferentially binds Ca\(^{2+}\), EDTA chelates all released cations. PP2A activity does not require cations but can be stimulated by cations including Mn\(^{2+}\). Reversible methylation of PP2A is also significantly stimulated by divalent cations, especially Mn\(^{2+}\). Methylation of PP2A C is important for regulating the formation of PP2A heterotrimers containing B-type subunits and, therefore, may indirectly regulate PP2A activity.

Considering the role of PP2A B subunits in regulating various aspects of substrate specificity, the association of the PP2A B\(_x\) subunit with CaBP4 (Figs. 2B, 3C) may mediate specific dephosphorylation by the heterotrimeric PP2A holoenzyme. PP2A B subunits have been shown to recruit PP2A to substrates such as calcium/calmodulin-dependent protein kinase IV, and may similarly facilitate CaBP4 dephosphorylation at photoreceptor synapses by the PP2A holoenzyme. Crystallization of a PP2A holoenzyme including the B\(_x\) subunit revealed that a highly acidic groove contributes to the putative substrate binding site that associates with the cytoskeletal tau protein. The two peptide sequences in tau that were identified as interacting with B\(_x\) were highly basic, with 11 lysine residues. The 40 amino acids surrounding the CaBP4 phosphorylation site (serine 37) are also highly basic, with nine positively charged residues, which may similarly contribute to a PP2A B\(_x\)-binding region. The faster CaBP4 dephosphorylation in HEK293 cells overexpressing PP2A B\(_x\), as well as Az and CB (Figs. 4B, 4C), support the participation of PP2A B\(_x\) in PP2A complexes involved in CaBP4 dephosphorylation. However, it is possible that other regulatory B subunits in addition to B\(_x\) contribute to heterotrimeric PP2A complexes that recognize CaBP4 as a substrate.

PP2A complexes containing the B\(_x\) subunit, B56 epsilon, translocate to the cytosolic fraction upon exposure to light, which stimulates light-dependent dephosphorylation of phosphoducin and downregulation of visual transduction. However, minutes and 45 minutes at 20°C. A reaction carried out with OA provided a negative control in which both the native kinase and phosphatase activity should be inhibited. The level of \(^{32}\)P-CaBP4 is revealed by autoradiography (upper panel) and amount of immunoprecipitated CaBP4 in each sample was detected by Western blot analysis (WB, bottom panel) with anti-CaBP4 antibodies. CaBP4 knockout mouse retinas were used as a negative control (lanes 1, 2). The data are representative results of three independent experiments. Arrowheads indicate the band corresponding to CaBP4. (B) Quantitative analysis of \(^{32}\)P-CaBP4 in dark- and light-adapted mouse retina in the presence and absence of OA as shown in (A). The intensity of each band of the autoradiograph was quantified and normalized to the intensity of each lane. Data represent mean ± SEM (n = 3, \(P < 0.05\), \(**P < 0.001\), t-test). (C) Recombinant CaBP4 was phosphorylated with recombinant PKC\(_{\gamma}\) in the presence of \(^{32}\)P-ATP. After inactivation of PKC\(_{\gamma}\) with Bis (T = 0 minutes), radioactively labeled CaBP4 was added to an extract of dark- or light-adapted mouse retinas and the incubation was carried out for 15 minutes and 45 minutes at 20°C. A reaction carried out with OA provided a negative control in which both the native kinase and phosphatase activity should be inhibited. The level of \(^{32}\)P-CaBP4 is revealed by autoradiography (upper panel) and amount of immunoprecipitated CaBP4 in each sample was detected by Western blot analysis (WB, bottom panel) with anti-CaBP4 antibodies. CaBP4 knockout mouse retinas were used as a negative control (lanes 1, 2). The data are representative results of three independent experiments. Arrowheads indicate the band corresponding to CaBP4. (B) Quantitative analysis of \(^{32}\)P-CaBP4 in dark- and light-adapted mouse retina in the presence and absence of OA as shown in (A). The intensity of each band of the autoradiograph was quantified and normalized to the intensity of each lane. Data represent mean ± SEM (n = 3, \(P < 0.05\), \(**P < 0.001\), t-test). (C) Recombinant CaBP4 was phosphorylated with recombinant PKC\(_{\gamma}\) in the presence of \(^{32}\)P-ATP. After inactivation of PKC\(_{\gamma}\) with Bis (T = 0 minutes), radioactively labeled CaBP4 was added to an extract of dark- or light-adapted mouse retinas and the incubation was carried out for 15
light-dependent translocation of the B2 subunit was not observed (and data not shown). Therefore, formation of PP2A A, B2, C heterotrimerers that may interact with CaBP4 most likely occurs at the membrane and does not require light-dependent redistribution. In PP2A A, B2, C, the B2 subunit was also shown to promote targeting of PP2A to the microtubule cytoskeleton. 55,56 It is possible that light-driven changes in PP2A AB2C association with substrates, such as CaBP4, may underlie enhanced PP2A activity in light-adapted retinas (Fig. 6).

In photoreceptors, activation of the phototransduction cascade by light results in hyperpolarization of the membrane potential that is transmitted from the outer segment to the synaptic terminals and causes closure of synaptic Ca2+ voltage-gated Ca2+ channels. Because neurotransmitter release is proportional to Ca2+ influx, Ca1 Ca2+ channels are key players in transforming the change in membrane potential into a change in neurotransmitter release. 58,59,77 In photoreceptor terminals, CaBP4 interacts with and potentiates the activity of Ca1,4 Ca2+ channels, primarily by enhancing voltage-dependent activation. 18 The analysis of CaBP4 modulation of Ca1,4 in transfected HEK293 cells is greatly hindered by the low open probability and unitary conductance of Ca1,4, 78 and the inhibitory effects of CaBP4 on Ca1 channel expression. 79 Therefore, we have used the prolongation of Ca1,3 Ca2+ currents due to effects of CaBP4 in antagonizing inactivation, as a metric for CaBP4 modulation. Using this approach, we showed that phosphorylation of serine 37 in CaBP4 facilitates low affinity interactions with Ca1,3 and enhances the effect of CaBP4 in prolonging Ca1,3 Ca2+ currents in HEK293 cells. 39 The functional importance of CaBP4 reversible phosphorylation is supported by our present data showing that inhibition of PP2A by OA enhances the effect of CaBP4 on Ca1,3 inactivation in HEK293 cells (Fig. 5).

While CaBP4 does not affect voltage-dependent activation of Ca1,3, it strongly suppresses Ca2+ dependent inactivation (CDI) of Ca1,3. 39,59,77 Ca1,4 channels show low CDI independent of CaBP4 due to an inhibitory C-terminal domain (CTD) in the pore-forming Ca1,4 α2 subunit. 79,80 Deletion of the CTD restores CDI to Ca1,4, which can then be suppressed by CaBP4. 13 Splice variants of the Ca1,4 α2 subunit lacking the CTD have been detected in human retina. 81 Therefore, while our present data indicate effects of CaBP4 dephosphorylation on Ca1,3 CDI, they are likely relevant for regulation of CDI of these Ca1,4 splice variants. It was previously reported that in retinal explants OA increases Ca2+ influx in a time- and dose-dependent manner, which is prevented by inhibitors of Ca1,4 Ca2+ channels and PKC. 82 These findings are consistent with our results that OA inhibits inactivation of Ca1,3 channels by enhancing the modulatory effects of phosphorylated CaBP4.

Photoreceptors are able to respond to a wide range of light intensity. Thus, Ca1 channel activity must be highly regulated to transform any change in light intensity into a precise change in the rate of transmitter release. In addition to Ca2+ binding, reversible phosphorylation provides an additional mechanism to control the function of CaBP4 and consequently Ca1 activity. In light conditions, CaBP4 phosphorylation may maintain Ca1-mediated Ca2+ influx to sustain neurotransmitter release at various light intensities. However, prolonged CaBP4 phosphorylation would cause excessive Ca2+ influx and neurotransmitter release, which would likely degrade visual signal transmission. Therefore, the light-dependent enhancement in PP2A activity may be important for terminating the PKC-dependent boost in Ca1 Ca2+ signaling, so as to preserve the fidelity of photoreceptor responses to light stimuli.

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