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Connexin 35/36 is phosphorylated at regulatory sites in the retina

W. Wade Kothmann^{1,2}, Xiaofan Li¹, Gary S. Burr^{1,†}, and John O'Brien^{1,2}

¹ Department of Ophthalmology and Visual Science, University of Texas Health Science Center at Houston

² The Graduate School of Biomedical Sciences, University of Texas Health Science Center at Houston

Abstract

Connexin 35/36 is the most widespread neuronal gap junction protein in the retina and central nervous system. Electrical and/or tracer coupling in a number of neuronal circuits that express this connexin are regulated by light adaptation. In many cases the regulation of coupling depends on signaling pathways that activate protein kinases such as PKA, and Cx35 has been shown to be regulated by PKA phosphorylation in cell culture systems. To examine whether phosphorylation might regulate Cx35/36 in the retina we developed phospho-specific polyclonal antibodies against the two regulatory phosphorylation sites of Cx35 and examined the phosphorylation state of this connexin in the retina. Western blot analysis with hybrid bass retinal membrane preparations showed Cx35 to be phosphorylated at both the Ser110 and Ser276 sites, and this labeling was eliminated by alkaline phosphatase digestion. The homologous sites of mouse and rabbit Cx36 were also phosphorylated in retinal membrane preparations. Quantitative confocal immunofluorescence analysis showed gap junctions identified with a monoclonal anti-Cx35 antibody to have variable levels of phosphorylation at both the Ser110 and Ser276 sites. Unusual gap junctions that could be identified by their large size (up to 32 μm^2) and location in the IPL showed a prominent shift in phosphorylation state from heavily phosphorylated in nighttime, dark-adapted retina to weakly phosphorylated in daytime, light-adapted retina. Both Ser110 and Ser276 sites showed significant changes in this manner. Under both lighting conditions other gap junctions varied from non-phosphorylated to heavily phosphorylated. We predict that changes in the phosphorylation states of these sites correlate with changes in the degree of coupling through Cx35/36 gap junctions. This leads to the conclusion that connexin phosphorylation mediates changes in coupling in some retinal networks. However, these changes are not global and likely occur in a cell type-specific or possibly a gap junction-specific manner.

Keywords

Connexin36; phosphorylation; light adaptation; phospho-specific antibodies; gap junctions

INTRODUCTION

Gap junctions form a key component of retinal neural circuitry, and are present in every major class of retinal neuron (Cook & Becker, 1995). The identity of several connexins that form retinal gap junctions has been elucidated in the last decade. Connexin 35/36 (O'Brien *et al.*, 1996; Condorelli *et al.*, 1998; O'Brien *et al.*, 1998) was the first neuron-specific connexin identified. It has been found in a large number of neurons in the retina and central nervous system. In the retina this connexin is associated with AII amacrine cell gap junctions (Feigenspan *et al.*, 2001; Mills *et al.*, 2001), cone photoreceptor gap junctions (Feigenspan *et*

*Corresponding author: John O'Brien, Department of Ophthalmology and Visual Science, University of Texas Health Science Center at Houston, 6431 Fannin St., MSB 7.024, Houston, Texas 77030, Phone: (713) 500-5983, FAX: (713) 500-0682, e-mail: John.O'Brien@uth.tmc.edu.

[†]Present address: Department of Wildlife and Fisheries Sciences, Texas A&M University, College Station, TX.

et al., 2004; O'Brien *et al.*, 2004), and rod photoreceptor gap junctions in some species (Zhang & Wu, 2004). It is also apparent that a large number of other neurons use this connexin (O'Brien *et al.*, 2004). Disruption of the Cx36 gene in mice results in deficits in visual transmission through the rod pathway (Guldenagel *et al.*, 2001; Deans *et al.*, 2002), consistent with disruption of AII amacrine-cone ON bipolar cell and rod-cone gap junctions. Cx36-null mice also showed impaired firing synchrony (Deans *et al.*, 2001) and gamma-frequency oscillations (Hormuzdi *et al.*, 2001) in the neocortex, and anomalous patterns of sharp-wave bursts and ripple oscillations in the hippocampus (Maier *et al.*, 2002; Pais *et al.*, 2002).

Electrical coupling forms functional networks within which signals are shared, and can have many consequences. For example, gap junctions between photoreceptors spread responses through adjacent terminals, improving the signal:noise ratio under many conditions (Schwartz, 1975b; Lamb & Simon, 1976), and expanding the voltage range through which a synaptic response is possible (Attwell *et al.*, 1987). Rod-cone gap junctions also provide a pathway through which high-sensitivity rod signals may enter the cone pathways and chromatic or high frequency cone signals may enter the rod pathway (Schwartz, 1975a; Wu & Yang, 1988; Krizaj *et al.*, 1998).

During visual adaptation the extent of coupling between cells is modulated, which has significant effects on the sensitivity and resolution of the retina. Light adaptation has profound effects on receptive field size and tracer coupling in cone-driven horizontal cells (Baldridge & Ball, 1991; Lankheet *et al.*, 1993; Bloomfield *et al.*, 1995) and AII amacrine cells (Bloomfield *et al.*, 1997). Smaller but still significant changes are observed in some photoreceptors. Cone-cone coupling increases with light adaptation in the turtle (Copenhagen & Green, 1987), as does rod-cone coupling in salamander (Yang & Wu, 1989). These changes influence the functional efficacy of the output synapse (Smith *et al.*, 1986) as well as rod input into the cone system.

The effect of light on photoreceptors is partially mimicked by dopamine D2/D4-like receptor activation (Krizaj *et al.*, 1998), which results in reduction of adenylyl cyclase activity and cytoplasmic cAMP concentration (Cohen *et al.*, 1992; Nir *et al.*, 2002). In other retinal networks, the opposite effect is mediated by dopamine D1-type receptors. This effect results from elevation of cytoplasmic cAMP and is mediated by cAMP-dependent protein kinase (PKA). It uncouples gap junctions in horizontal cells (Lasater, 1987; DeVries & Schwartz, 1989) and AII amacrine cells (Hampson *et al.*, 1992).

The effect of PKA activity on coupling in AII amacrine cells has been duplicated with Cx35 stably expressed in a mammalian cell line (Ouyang *et al.*, 2005). Activation of PKA causes uncoupling while PKA inhibition causes an increase in coupling. Uncoupling requires phosphorylation at two sites on Cx35, Ser110 in the intracellular loop and Ser276 in the carboxyl terminal tail. These same two sites are also the target of phosphorylation by cGMP-dependent protein kinase (PKG) *in vitro*, suggesting that several signaling pathways may converge on these regulatory residues (Patel *et al.*, 2006). We reasoned that the phosphorylation state of Ser110 and Ser276 should be indicative of the degree of coupling possible through Cx35 gap junctions. In this study we made antibodies against the two regulatory phosphorylation sites of Cx35 and examined whether these sites are phosphorylated in the retina. A previous study has suggested that Cx36 is not phosphorylated in bovine retina (Sitaramayya *et al.*, 2003). However, our study finds that it is phosphorylated in both fish and mammalian retina, and that phosphorylation state varies with lighting conditions.

METHODS

Sequence analysis

DNA sequences obtained from GenBank were analyzed with GeneTool software (Biotools, Inc., Edmonton, AB) and translated to amino acid sequences. Amino acid sequences were analyzed with PepTool software (Biotools) to identify consensus phosphorylation sequences. Some sequence alignments were performed with Clustal W (Thompson *et al.*, 1994).

Development of phospho-specific antibodies

Phosphorylated synthetic peptides corresponding to the two major regulatory PKA phosphorylation sites of perch Cx35 (Ouyang *et al.*, 2005) were made at the M.D. Anderson Cancer Center Synthetic Antigen Laboratory. The sequence of the phosphorylated Ser110 peptide was CKERRY(S¹¹⁰PO₄)TVYLT and the sequence of the phosphorylated Ser276 peptide was CARRKS(S²⁷⁶PO₄)IYEIRN. The corresponding non-phosphorylated peptides were also synthesized. The phosphorylated peptides were crosslinked to *Limulus* hemocyanin (Sigma Chemical Co., St. Louis, MO) via the cysteine thiol group using N-[ε-Maleimidocaproyloxy]succinimide ester (EMCS; Pierce Chemical Co., Rockford, IL). Hemocyanin at 10 mg/ml in 83 mM NaH₂PO₄, 0.9 M NaCl, 10 mM EDTA, pH 7.2 was derivatized by incubation with 1.5 mg/ml EMCS for 2 hours at 4 °C, and desalted over Biogel P-10 resin (Bio-Rad Laboratories, Hercules, CA). For each reaction, 4.8 mg of phospho-peptide was added as dry solid to approximately 6 mg of derivatized hemocyanin and incubated 4 hrs at 4°C. Crosslinking efficiencies were approximately 50% for phospho-Ser110 peptide and 30% for phospho-Ser276 peptide, as determined by the concentration of free thiol measured by derivatization with dithionitrobenzoic acid (DTNB; Sigma) and measurement of absorbance at 412 nm. The hemocyanin-linked peptides were dialyzed for 20 hrs against PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH 7.4), sterile filtered, and sent to Spring Valley Laboratories (Sykesville, MD) for development of antisera in rabbits. Bleeds were screened for cross-reactivity with phosphorylated and non-phosphorylated Cx35 by western blot against *in vitro* phosphorylated Cx35 intracellular loop or carboxyl terminal domain fusion proteins (see below for methods).

Bleeds showing significant reactivity against phosphorylated Cx35 were affinity purified. To prepare affinity columns, approximately 3 mg of the phosphorylated and non-phosphorylated peptides were coupled to Sulfolink columns (Pierce) according to the manufacturer's instructions. Linking efficiencies ranged from 80% for non-phospho-Ser110 to 95% for phospho-Ser276. Antibodies were affinity purified by a modification of the method of Tsang & Wilkins (1991). 25–50 ml of serum were diluted with an equal volume of PBS and the following additions were made: 1% phosphatase inhibitor cocktail (Sigma), 4 µg/ml aprotinin, 4 µg/ml leupeptin, 10 mM benzamidine, 10 mM NaF, and 0.15% Igepal CA-630. The diluted sera were passed over the phosphopeptide columns twice, and the columns washed with 100 ml BBS (1 M NaCl, 100 mM boric acid, 20 mM Na borate, 0.1% Tween-20), followed by 6 ml HBS (25 mM Hepes, pH 7.4, 0.25 M NaCl, 0.01% NaN₃). The columns were then eluted with 15 ml EtMg (3 M MgCl₂, 25% ethylene glycol, 75 mM Hepes, pH 7.2), followed by 6 ml 0.1 M acetic acid, 0.1 M NaCl, 25% ethylene glycol. The acid eluate was immediately neutralized by addition of 1 M Tris base until the pH was >7. The eluates were combined and dialyzed for 20 hr against PBS plus 0.05% NaN₃ and 0.05% Igepal CA-630.

Dialyzed, affinity-purified antibodies were passed over the corresponding non-phosphopeptide columns twice and the flow-through fractions were concentrated using Centriprep centrifugal filtration devices (Millipore, Billerica, MA). Antibodies that bound to the non-phosphopeptide columns were washed, eluted, dialyzed, and concentrated as before to obtain “pan-specific” antibody fractions.

***In vitro* phosphorylation**

GST fusion proteins containing the cytoplasmic intracellular loop and carboxyl terminal domains of perch Cx35 (Burr *et al.*, 2005; Ouyang *et al.*, 2005) were expressed in *E. coli* strain BL21 and purified by binding to glutathione sepharose 4B (Amersham, Piscataway, NJ). Fusion proteins (0.5 to 1 µg protein per reaction) were incubated with 0.5 units cAMP-dependent protein kinase (PKA) catalytic subunit (mouse α isoform; New England Biolabs, Beverly, MA) for 90 min. at 37°C. The final solution contained 50 mM Tris-Cl (pH 7.5), 10 mM MgCl₂, 300 µM ATP, 27 mM NaCl, 0.5 mM KCl, 2 mM Na₂HPO₄, 0.3 mM KH₂PO₄, and 10% glycerol. The incubation mixtures were dissolved in an equal volume of 2x SDS sample buffer, electrophoresed in varying amounts, and blotted to nitrocellulose membranes for screening antisera and affinity-purified antibodies.

Retinal membrane preparations

Hybrid striped bass (*Morone saxatilis*/*M. americana*) were obtained from the Texas A&M University Department of Wildlife and Fisheries Sciences and were maintained in circulating tanks in the laboratory on a 12hr light:12hr dark cycle. Fish were taken either 2 hr prior to the onset of light (dark-adapted) or 2 hr after the onset of light (light-adapted), anesthetized with 0.3% 2-aminobenzoic acid methyl ester (Sigma), and sacrificed. Retinas were dissected out and homogenized by sonication in ice-cold 0.32 M sucrose, 10 mM Tris-Cl, pH 7.2, 2 mM EGTA, 5 mM MgCl₂, 1 µM okadaic acid, 1% protease inhibitor cocktail (Sigma). For dark eyes, procedures were performed under dim red safelight illumination through the homogenization steps. The suspension was centrifuged for 5 min at 1000 × g to remove large particulates, and then the supernatant was centrifuged for 1 hr at 100,000 × g to pellet membranes. The membrane pellet was resuspended in the homogenization medium without protease or phosphatase inhibitors. Protein concentration was measured by the BCA technique (Pierce).

Daytime, light-adapted rabbit eyes were obtained from Dr. Michael Mauk (University of Texas Medical School at Houston) from animals sacrificed for other experiments. Retinas were dissected out and membrane preparations made as for hybrid bass. Daytime, light-adapted mouse eyes were obtained from Dr. Steven Wang (University of Texas Medical School at Houston) from animals sacrificed for other experiments. Membrane preparations were prepared in a similar manner except that high-speed centrifugation was performed in a Beckman airfuge for 30 min at approximately 100,000 × g. All procedures performed on animals were approved by the institutional animal care and use committee.

For each membrane preparation, a portion of the preparation was digested with bacterial alkaline phosphatase. The membrane preparations were diluted to 2 mg protein/ml with alkaline phosphatase assay buffer (final buffer composition 10 mM Tris-Cl, 10 mM MgCl₂, 0.5% Igepal CA-630, plus 15–28% of the membrane homogenization buffer), and digested with 17–25 units alkaline phosphatase (Fermentas, Hanover, MD) per mg protein for 30 min at 37°C plus an additional 2 hr at 50°C. The samples were then diluted to 1 mg protein/ml with 2x SDS sample buffer. Comparable non-digested samples were prepared at the same concentration with alkaline phosphatase buffer but no enzyme.

Western blot

Samples of *in vitro* phosphorylated fusion proteins or retinal membrane preparations were resolved on SDS-PAGE minigels by standard procedures. Proteins were transferred to nitrocellulose (for *in vitro* phosphorylated proteins) or PVDF (for retinal membrane preparations) membranes electrophoretically. For screening crude antisera, portions of the blots were incubated with dilutions of crude serum ranging from 1:500 to 1:50,000 in TBST (137 mM NaCl, 3 mM KCl, 25 mM Tris-Cl, pH 7.4, 0.1% Tween-20). Signals were developed

with Cy3-conjugated secondary antibodies (Jackson Immunoresearch, West Valley, PA) and viewed with a Typhoon imager (GE Healthcare Bio-Sciences, Piscataway, NJ). For retinal membrane preparations, blots were incubated with affinity purified phospho-specific antibodies diluted to 1 µg/ml in high-salt TBST (500 mM NaCl, 3 mM KCl, 25 mM Tris-Cl, pH 7.4, 0.1% Tween-20) plus 2% nonfat dry milk. Blots were also probed with a monoclonal anti-Cx35/36 antibody (Chemicon, Temecula, CA) that was developed against the perch Cx35 intracellular loop (O'Brien *et al.*, 2004) at 1 µg/ml in the same solution. Blots were developed with peroxidase-linked secondary antibodies (Pierce) and detected by chemiluminescence using x-ray film.

Immunoprecipitation

Daytime, light-adapted bass retinal membrane preparations (240 µg total protein) were diluted to 400 µl in modified PBST (137 mM NaCl, 3 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 2 mM EDTA, 1% Triton X-100, 0.5% Na deoxycholate, pH 7.4) with 1% protease inhibitor cocktail (Sigma) and incubated with 1 µg mouse anti-Cx36 antibody (Zymed Laboratories, San Francisco, CA) at 4°C for 4 hr. The protein-antibody mixture was further incubated with 20 µl of 50% slurry of protein G-agarose resin (Amersham) at 4°C for 1 hr. This mixture was centrifuged for 5 min at 1000 × g and the pellet was washed three times with 0.5 ml modified PBST and then resuspended in 2x SDS sample buffer for electrophoresis. The IP supernatant and a sample of the initial lysate were acetone precipitated to prepare samples for electrophoresis. Western blot of the samples was performed as above with this modification: the ReliaBlot kit (Bethyl Laboratories, Montgomery, TX) was utilized to mask the Ig signal from the immunoprecipitation antibody according to the manufacturer's instruction. Briefly, PVDF membrane with transferred protein samples was blocked in ReliaBlot blocking reagent and then probed with rabbit anti-phospho-S110 antibodies (1.6 µg/ml in ReliaBlot blocking reagent) overnight at 4°C. The blot was developed with peroxidase-linked secondary antibodies provided in the kit and detected by chemiluminescence using x-ray film.

Immunostaining

Eyecups were prepared from hybrid striped bass either 2 hr before the start of the light-cycle under infrared illumination (dark-adapted) or 2 hr after the start of the light-cycle under normal room lights (light-adapted). The eyecups were incubated in 2000 units/ml hyaluronidase (Sigma) in PBS for 20 min, and then washed twice for 15 min each in PBS. Subsequently all eyecups were fixed for 15 min in fresh 1% *N*-(3-Dimethylaminopropyl)-*N'*-ethylcarbodiimide hydrochloride (Sigma) in fixation buffer (116 mM Tris, 68 mM NaCl, 1.3 mM KCl, 52 mM Na₂HPO₄, 25 mM NaH₂PO₄, 0.9 mM KH₂PO₄, pH 7.5), and then washed three times for 30 min each in PBS. Eyecups were cryoprotected overnight at 4°C in PBS plus 30% sucrose, 0.25% NaN₃, embedded in Tissue-Tek O.C.T. compound (Sakura Finetek, Torrance, CA), and cryosectioned (20 or 40 µm sections). Sections were incubated in PBST (PBS plus 0.5% Triton X-100, 0.1% NaN₃) with 10% normal donkey serum (Jackson) for 5 hours to block nonspecific binding. Sections were then incubated with 2 µg/ml mouse anti-Cx35/36 (Chemicon) and either rabbit anti-phospho-Ser110 or rabbit anti-phospho-Ser276 (0.8 or 1 µg/ml, respectively) in PBST with 10% normal donkey serum overnight at 4°C. Sections were washed three times for 10 min each in PBST, and then incubated with Cy3-conjugated donkey anti-mouse IgG (Jackson; 1:250 dilution) and Alexa 488-conjugated donkey anti-rabbit IgG (Molecular Probes, Eugene, OR; 1:250 dilution) for 2.5 hours. Sections were then washed three times for 10 min each in PBST, mounted with Vectashield (Vector Laboratories, Burlingame, CA), and imaged on a Zeiss LSM 510 Meta confocal microscope using 12-bit data acquisition. All specimens were imaged using similar settings to allow comparison of phospho-antibody labeling in different parts of the retina and under different conditions. Images presented are stacks of three confocal slices totaling approximately 1 µm of focal depth. Image processing (Adobe Photoshop; Adobe Systems, San Jose, CA) was limited to applying a uniform threshold

(approximately 10%) to all images in all color channels to reduce background haze. One low-power overview image was brightened (figure 6, phospho-Ser276) to make small gap junction plaques visible.

Quantitative Image Analysis

Medium-power, high-resolution images that included the entire inner plexiform layer were collected for quantitative analysis using identical confocal microscope settings. For each condition 3–6 images were collected from each of 3 eyecups which were each from different fish. Images were analyzed with the same settings using SimplePCI software (Compix, Sewickley, PA) to automatically select regions of interest (ROIs) from the Cx35 plaques, as identified by the mouse anti-Cx35/36 (Chemicon) labeling. Briefly, regions of interest were defined as contiguous pixels with an intensity threshold greater than 20% of the total intensity range, and which covered a minimum area of 16 pixels ($\sim 0.1 \mu\text{m}^2$ or larger). Each image was then manually scanned for individual ROIs that included more than one plaque. These were eliminated from the analysis. Mean fluorescence intensity was measured in each channel (monoclonal Cx35 labeling; phospho-Cx35 labeling) for each ROI (hereafter called ‘plaques’). The intensity of phospho-Cx35 antibody labeling was then normalized to the intensity of the monoclonal Cx35 antibody labeling for each individual plaque. Cx35 plaques were separated into two subgroups for further analysis based on readily apparent differences between plaques of typical size and “giant plaques” that were distributed symmetrically in two bands in the IPL. Histograms of plaque size were generated in Matlab (The MathWorks, Natick, MA) using 250 pixel ($\sim 1.225 \mu\text{m}^2$) bins. A cutoff point (1000 pixels, $\sim 4.9 \mu\text{m}^2$) was empirically determined that distinguished the giant plaques apparent upon visual inspection from all other plaques. For each image the mean normalized phospho-Cx35 intensity of each population of plaques (“normal” and “giant”) was calculated. Mean values for 3–6 images per eyecup were averaged to yield a grand mean normalized phospho-Cx35 intensity for each population of plaques for each eyecup. Note that each eyecup represents one animal. Unpaired t-tests were performed to compare eyecups across conditions.

RESULTS

Cx35/36 is regulated by phosphorylation at conserved sites

Connexin 35/36 is the most widespread neuronal gap junction protein in the vertebrate central nervous system. A preponderance of evidence from retinal neurons suggests that gap junctional coupling through Cx35/36 can be regulated by environmental conditions such as light exposure and by neurotransmitter signaling pathways. Comparable regulation has been observed directly for Cx35 in cell culture expression systems and has been shown to depend on direct phosphorylation of the connexin (Ouyang *et al.*, 2005; Patel *et al.*, 2006). The outcomes of these studies indicate that two phosphorylation sites are critical for regulation of coupling by the cAMP/PKA pathway, and perhaps the cGMP/PKG pathway as well. Figure 1A shows these two sites, Ser110 in the intracellular loop and Ser276 in the C-terminal domain (Ser276 in teleost, amphibian, and avian Cx35 is equivalent to Ser293 of mammalian Cx36 and Ser273 of skate Cx35), and a putative model of regulation by PKA (adapted from Ouyang *et al.*, 2005). In this model, phosphorylation at both Ser110 and Ser276 is required to cause substantial uncoupling of Cx35 gap junctions; phosphorylation of either residue alone causes only very modest uncoupling (Ouyang *et al.*, 2005). Furthermore, this behavior can be reversed, i.e. phosphorylation can lead to increased coupling, under conditions that alter the interactions at the tip of the C-terminus. The molecular details of this “switch” are unknown.

Figure 1B shows an alignment of Cx35/36 sequences from several species in the regions surrounding these regulatory phosphorylation sites. Sequence analysis shows that these regions contain consensus phosphorylation sequences for a number of different protein kinases, listed

above the sequences. Those that have been verified experimentally (all through *in vitro* studies) are shown in bold. It is apparent that the previously identified regulatory phosphorylation sites are highly conserved across widely divergent vertebrate species. Furthermore, these sites are potential targets of several protein kinases, which in turn represent several different signaling pathways. These include cAMP-dependent protein kinase (PKA), a terminal effector of the dopamine signaling pathways, and cGMP-dependent protein kinase (PKG), a terminal effector of nitric oxide signaling, both of which are important in light adaptation in the retina.

Conserved regulatory phosphorylation sites are phosphorylated in the retina

The convergence of several different signaling pathways on common regulatory sites suggests that the phosphorylation status of these sites may be used to estimate the functional state of the gap junctions. To examine this hypothesis we developed phospho-specific antibodies against both regulatory sites of Cx35. We examined the specificity of these antibodies with western blots against bacterially-expressed GST fusion proteins of either the Cx35 intracellular loop (IL) domain, containing the Ser110 phosphorylation site, or the carboxyl terminal (CT) domain, containing the Ser276 phosphorylation site. Figure 2A shows a western blot with the phospho-Ser110 antibody. This antibody reacted strongly to the IL domain phosphorylated *in vitro* with PKA, but showed no measurable reaction to the non-phosphorylated IL domain. Furthermore, this antibody showed no cross-reaction with the *in vitro* phosphorylated Cx35 CT domain. Thus the antibody was specific for the phosphorylated form of the protein and did not cross-react with the other major PKA phosphorylation site. Likewise, figure 2B shows a similar western blot with the phospho-Ser276 antibody. As with the phospho-Ser110 antibody, this antibody was specific for the phosphorylated form of the protein and did not cross-react with the Ser110 site.

If phosphorylation of Cx35 is involved in regulation of coupling in retinal neurons, we would predict that both Ser110 and Ser276 sites would be phosphorylated to some extent in Cx35 derived from the retina. To test this prediction we performed western blots on membrane preparations from hybrid bass retina taken in the dark and light parts of their diurnal cycle. We treated half of each membrane preparation with alkaline phosphatase (AP) to remove phosphate groups from the protein. A monoclonal antibody against Cx35 (figure 3, panel A) identified Cx35 as a diffuse band running around 30–32 kDa, in keeping with previous reports (O'Brien *et al.*, 1998; Pereda *et al.*, 2003; O'Brien *et al.*, 2004). The phospho-Ser110 antibody (panel B) labeled the same band in untreated retinal membranes. The labeling was lost in AP digested membranes. Note that this antibody also labeled several high molecular weight bands in retinal membranes. That labeling was also lost upon AP digestion, suggesting that these were phosphorylated proteins with PKA phosphorylation sites similar to that of Cx35's Ser110 site. There was little difference in the intensity of phospho-Ser110 labeling on the Cx35 band between dark and light conditions, nor in the total amount of Cx35 between dark and light conditions. The phospho-Ser276 antibody also recognized the Cx35 band in retinal membranes (panel C), and this labeling was lost upon AP digestion. No non-specific bands were detected with this antibody. Again, the difference between dark and light conditions was minimal. These results suggest that Cx35 is indeed phosphorylated in bass retina at both regulatory sites.

The regulatory phosphorylation sites of Cx35 are highly conserved among diverse vertebrate species (e.g. figure 1B), which suggests that regulation by phosphorylation at these two sites is likely also to be conserved. To test whether these sites were phosphorylated in other species we examined retinal membranes from two mammals, mouse and rabbit, for phosphorylation of Cx36 on Ser110 and Ser293 (= Ser276 of teleost Cx35). Figure 4 shows that both phospho-specific antibodies labeled Cx36 in daytime, light-adapted mouse and rabbit retinal membranes. This labeling was destroyed by digestion of the membranes with AP. Although

not shown in these blots, labeling of phosphorylated high molecular weight bands in mouse and rabbit retina was again evident with the phospho-Ser110 antibody.

Labeling of phospho-proteins other than Cx35 in western blots using the phospho-Ser110 antibody raises the question of whether this antibody can be used to examine phosphorylation of Cx35 in intact tissues. It is possible that labeling of Cx35 gap junctions by the antibody may represent phosphorylation of proteins associated with the Cx35 gap junctions rather than actual phosphorylation of Cx35. To examine this question we performed immunoprecipitation experiments with the monoclonal anti-Cx35 antibody and probed the precipitates with the phospho-Ser110 antibody. Figure 5 shows that the immunoprecipitate containing phosphorylated Cx35 did not contain any of the high molecular weight phospho-proteins found in the original retinal membrane preparation. In contrast, the supernatant from the experiment contained the same complement of high molecular weight phospho-proteins as did the crude membranes. This implies that the phosphorylated proteins recognized by the phospho-Ser110 antibody are not associated with Cx35 in a complex, and thus are not predicted to interfere with analysis of Cx35 gap junctions.

Phosphorylation of Cx35 is controlled at the level of individual gap junctions

We did not see gross changes in Cx35 phosphorylation between dark and light conditions by western blot analysis. However, it is possible that the phosphorylation state, and by inference the coupled state, of Cx35 gap junctions may be regulated at the level of individual gap junctions. To test this hypothesis we examined hybrid bass retina by confocal immunofluorescence techniques using the two phospho-specific Cx35 antibodies. Figure 6 shows the pattern of labeling by the Cx35 phospho-Ser110 antibody (A–C) and the Cx35 phospho-Ser276 antibody (D–F) compared to labeling with a Cx35 monoclonal antibody. Both phospho-Ser110 (A) and phospho-Ser276 (D) antibodies labeled abundant punctate structures in both inner and outer plexiform layers in a pattern similar to that of Cx35 labeling (B and E). In the merged images it is evident that many Cx35 plaques were also labeled for phospho-Ser110 (C) or phospho-Ser276 (F). The phospho-Ser110 antibody also showed strong non-specific labeling of Müller cells (A and C). This labeling was much less apparent, though present to some extent, in retina from other species (authors' unpublished observations). The phospho-Ser276 antibody showed only minor non-specific labeling of blood vessels in the retina.

Closer examination of the inner plexiform layer revealed that individual gap junctions labeled with the Cx35 monoclonal antibody (figure 7 B and C; shown in red) had highly variable labeling for phospho-Ser276 (figure 7 A and C; green). Some Cx35 plaques showed extensive, bright phospho-Ser276 labeling while others had essentially none (arrow). One readily identifiable group of Cx35 gap junctions were the extremely large (up to 32 μm^2) plaques distributed in two bands, one each in sublamina a and sublamina b, that we have previously reported (O'Brien *et al.*, 2004). The cell type harboring these gap junctions is unknown, although they fit the description of the large gap junctions between large-caliber amacrine cell processes identified by Marc *et al.* 1988 in TEM studies of goldfish retina. In nighttime, dark-adapted retina these gap junctions were highly phosphorylated at Ser276 (figure 7 A and C, triple arrowhead). However, in daytime, light-adapted retina there was much less immunostaining for phospho-Ser276 on these gap junctions (figure 7 D and F, triple arrowhead). At the same time one population of medium-sized Cx35 gap junctions still showed substantial phosphorylation on the Ser276 site (single arrowheads), while again others had none (arrow). We could not determine from these data whether the medium-sized Cx35 gap junctions, some of which were highly phosphorylated and some non-phosphorylated under both dark and light conditions, belonged to the same population or to different ones.

Similar results were observed with the phospho-Ser110 antibody (figure 7 G–L). Again, there was substantially more Ser110 phosphorylation on the giant plaques in dark conditions than in the light. The small to medium sized Cx35 gap junctions showed variable levels of phosphorylation in both light and dark conditions, with some gap junctions showing no phosphorylation (arrow).

To determine if the changes in Cx35 phosphorylation that were observed empirically represent consistent changes we performed quantitative analysis of the phospho-specific antibody labeling on giant Cx35 plaques in the inner plexiform layer. The large size of these giant plaques relative to others in the hybrid bass retina, their typical oblong shape, and their unique distribution were used to segregate them from the total population of plaques for analysis. Figure 8A shows a histogram of the distribution of Cx35 plaques by size in the inner plexiform layer. Plaque abundance decreased steadily with increasing area, and the vast majority of plaques were included in bins containing areas less than $2.5 \mu\text{m}^2$. The number of plaques per bin became relatively stable in bins containing areas greater than $4.9 \mu\text{m}^2$, and this was chosen as the cutoff point to segregate giant plaques from the total population. Visual inspection of the image data confirmed that all plaques above the cutoff point were of the characteristic size, shape, and distribution described above. Phospho-specific antibody labeling normalized to the monoclonal Cx35 antibody labeling was calculated for each plaque in 3–6 images per animal (see methods). Figure 8B shows the effect of background illumination on Cx35 phosphorylation at Ser110 and Ser276 ($n = 3$ animals per condition). Giant plaques (figure 8B, right two pairs) showed significantly less phosphorylation at Ser110 and Ser276 in light-adapted retina (unpaired t-test, $p < 0.05$ for each). The remaining smaller plaques (figure 8B, left two pairs) did not show any significant difference phosphorylation at either Ser110 or Ser276 between dark- and light-adapted retinas.

In the outer plexiform layer, where Cx35 is present in both cone and bipolar cell gap junctions (O'Brien *et al.*, 2004), the phospho-Ser276 antibody labeled several morphologically distinct populations of Cx35 gap junctions (figure 9). Large plaques that varied from round to ellipsoid (arrows), and which may represent bipolar cell gap junctions, showed strong phospho-Ser276 labeling in both dark- and light-adapted retina. Narrow, string-like gap junctions (double arrowheads), which likely represent those found on cone telodendria (O'Brien *et al.*, 2004), were also well-labeled by the phospho-Ser276 antibody in both conditions. Small, round plaques (arrowheads) showed lesser phospho-Ser276 labeling than the other two populations in the dark-adapted retina and almost no labeling in the light-adapted retina.

DISCUSSION

The regulation of coupling through Cx35/36 gap junctions by phosphorylation has now been well described. Mitropoulou & Bruzzone (2003) found that a consensus PKA recognition sequence in the intracellular loop of perch Cx35 was essential for the 8-Br-cAMP-induced reduction in hemichannel currents observed in *Xenopus* oocytes. They further found that recreating this recognition sequence in skate Cx35, which was unresponsive to 8-Br-cAMP in its wild-type form in *Xenopus* oocytes, conferred the same behavior to this connexin. This strongly suggested that phosphorylation regulates current flow through Cx35. We subsequently showed that phosphorylation by PKA at Ser110, the target of this PKA recognition sequence, was responsible for reduction of tracer coupling in perch Cx35 expressed in HeLa cells. However, full regulation required concomitant phosphorylation at a second site, Ser276, in addition to Ser110 (Ouyang *et al.*, 2005). Phosphorylation at either site alone caused only a small degree of uncoupling: a 20% reduction in diffusion coefficient in mutants lacking one phosphorylation site compared to a 56% reduction in diffusion coefficient in the wild-type.

PKA phosphorylation at the homologous sites has been confirmed for mouse Cx36 and parallel regulation by PKA phosphorylation has been proposed (Urschel *et al.*, 2006), although direct regulation by phosphorylation was not tested. Nonetheless, the high degree of sequence homology between these connexin gene homologues suggests that the mammalian Cx36 is regulated in the same fashion as fish Cx35. Our finding that both mouse and rabbit Cx36 were phosphorylated at both Ser110 and Ser293 (homologous to Ser276 in fish Cx35) in the retina supports this contention.

It is not yet clear how phosphorylation regulates coupling in Cx35/36 gap junctions. Phosphorylation could have a direct effect on channel properties of Cx35/36 gap junctions, as has been seen for a number of other connexin channels. For example, Cx43 channels expressed in SkHep1 cells show a reversible shift from a 90–100 pS main state to a 60–70 pS subconductance state with activation of PKC (Moreno *et al.*, 1994; Kwak *et al.*, 1995). These effects are mediated by phosphorylation of Ser368 by PKC (Lampe *et al.*, 2000). Macroscopic coupling through Cx45 was also found to be influenced by phosphorylating conditions: PKC phosphorylation increased conductance, while PKA or tyrosine phosphorylation decreased conductance (van Veen *et al.*, 2000). However, in this case subconductance states were not observed to change; changes in the open probability of the channels were responsible for the changes in macroscopic conductance. Thus phosphorylation can affect several important characteristics of gap junction channels that influence functional coupling.

Phosphorylation may also affect assembly or disassembly of gap junctions. Treatment with phorbol esters increased Cx43 phosphorylation and inhibited assembly of plasma membrane Cx43 into gap junctions in Novikoff hepatoma cells (Lampe, 1994). This treatment did not appear to influence stability of pre-existing gap junctions. In lens fiber cells, Cx50 and Cx46 were phosphorylated and gap junctions partially disassembled by similar phorbol ester treatments (Zampighi *et al.*, 2005). Such treatments also resulted in a substantial decrease in dye coupling, although it is not clear whether the reduction in dye coupling is a result of gap junction disassembly or changes in channel properties. Studies of Cx36 have revealed that mutation of the major consensus phosphorylation sites did not affect trafficking of Cx36 to the plasma membrane or the formation of gap junctions (Zoidl *et al.*, 2002). The latter suggests that regulation of Cx35/36 by phosphorylation on the identified regulatory sites is more likely due to modification of channel properties than of gap junction assembly.

The requirement for phosphorylation of two separate sites to regulate Cx35/36-mediated coupling imparts significant flexibility. First, these two phosphorylation sites have substantially different sequences that may have different affinities for PKA or the phosphatases that de-phosphorylate them. One of the sites, Ser276, is also known to overlap with a calmodulin binding site (Burr *et al.*, 2005). Hence the sites can be considered independent. Second, the two identified regulatory phosphorylation sites appear to be targets for a number of signaling pathways in addition to the cAMP/PKA pathway. We have found that PKG also phosphorylates both of these sites *in vitro* (Patel *et al.*, 2006). These sites are also consensus phosphorylation sequences for Casein Kinase II, Ca²⁺-Calmodulin-dependent kinases, and S6 Kinase II (summarized in figure 1; see also Sohl *et al.*, 1998; Urschel *et al.*, 2006). Not all of these kinases are predicted to phosphorylate both sites, which emphasizes that the sites are not equivalent. It is possible to construct a number of regulatory schemes that rely on two or more independent signaling pathways to control the coupling through Cx35/36 gap junctions. Two such pathways, the dopamine pathway routed through cAMP and the nitric oxide pathway routed through cGMP, are both important in light adaptation and known to influence gap junction coupling.

One significant prediction of the requirement for phosphorylation at two sites to regulate coupling is that gap junctions in different cell types could be regulated in different ways. Our

phosphorylation data provide evidence that this is the case in the fish retina. The giant Cx35 gap junctions in the IPL showed prominent shifts in phosphorylation state at both Ser110 and Ser276 sites between dark-adapted and light-adapted conditions. Our model predicts that the weakly phosphorylated state of these gap junctions in the light would correlate with strong coupling, while the heavily phosphorylated gap junctions in the dark-adapted state would support reduced coupling. However, the changes in phosphorylation state seen in the very large gap junctions were clearly not global. Many medium sized Cx35 gap junctions in the vicinity of these large plaques were well phosphorylated under both dark- and light-adapted conditions, and a few were virtually non-phosphorylated under both conditions. Since we could not identify the cell types in which these gap junctions reside, we could not determine if they represent relatively invariant gap junctions or rather a shift in phosphorylation status between light- and dark-adapted states in particular cell populations. In either case, the presence of strongly and weakly phosphorylated gap junctions in close proximity indicates that the phosphorylation state of the gap junction is under local control. This implies that adjacent neural circuits regulate their coupled states independently, even though they use the same connexin.

The abundant Cx35 gap junctions in fish retina belong to many cell types. Although we have found them to be present in cone photoreceptor and Mb1 bipolar cell gap junctions, the majority are likely to be in amacrine cells, as revealed by *in situ* hybridization labeling of many cell types in the proximal inner nuclear layer (O'Brien *et al.*, 2004). Tracer coupling and the ultrastructural presence of gap junctions has been described in several types of amacrine cells in the fish retina (Teranishi *et al.*, 1984; Marc *et al.*, 1988; Hidaka *et al.*, 1993; Teranishi & Negishi, 1994; Hidaka *et al.*, 2005). These gap junctions allow expansion of receptive fields beyond the dendritic spread, spike synchronization, and summation of responses (Hidaka *et al.*, 1993; Hidaka *et al.*, 2005). In mammalian AII amacrine cells, homologous coupling provides for summation of rod signals, in part setting the sensitivity of the rod pathway. Regulation of coupling preserves the fidelity of rod signaling through the broad scotopic operating range (Bloomfield & Volgyi, 2004). Unlike the very well studied mammalian AII amacrine cell, very little has been reported regarding changes in fish amacrine cell receptive fields in response to light adaptation.

The striking change in phosphorylation of Cx35 in the giant gap junctions with light adaptation suggests that the cell type harboring these gap junctions re-configures its receptive field during adaptation. Based upon the previous results reported in cell culture systems (Ouyang *et al.*, 2005), we predict that the decrease in phosphorylation at both Ser110 and Ser276 on giant gap junctions in light-adapted retina will result in increased coupling, which in turn may influence receptive field size and signaling range in the cell type harboring them. Note that this change in coupling in response to light-adaptation would be opposite to that which is observed in the AII amacrine cell (Bloomfield & Xin, 1997). Dopamine signaling is known to increase in light-adapted retina (Kramer, 1971; Iuvone *et al.*, 1978; Dearry & Burnside, 1986) and to cause uncoupling in the AII amacrine cell via D1-type receptors (Hampson *et al.*, 1992). If dopamine also influences the phosphorylation of the Cx35 giant gap junctions we predict that it would do so via D2/D4-type dopamine receptors, such as those found in photoreceptors (Cohen *et al.*, 1992) and dopaminergic interplexiform cells (Harsanyi & Mangel, 1992) and which cause a decrease in cAMP signaling through a g_i protein. The nitric oxide/cGMP/PKG pathway has also been shown to affect Cx35 phosphorylation, and several other signaling pathways are predicted to (Patel *et al.*, 2006). This raises the possibility that phosphorylation of the giant gap junctions is influenced by transmitters other than dopamine. In the absence of physiological data we cannot at present speculate further about the consequences of decreased phosphorylation on giant Cx35 gap junctions in hybrid bass, but presumably such a change would contribute to optimization of signal processing during light adaptation.

The diversity of neurotransmitter receptors and signaling pathways in retinal neurons allows for vastly different but finely-tuned responses specific to individual cell types. Cx35 gap junctions are a key component of some neural circuits and have the potential to remodel circuit properties in response to signaling. The convergence of multiple signaling pathways at regulatory phosphorylation sites on Cx35 (see figure 1) implies that the coupling state of Cx35-expressing cells can be controlled with a high degree of precision, and our results suggest that this happens at a very local level in retinal neurons.

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Figure 2. Specificity of the phospho-Cx35 antibodies

Western blot analysis of GST-fusion proteins phosphorylated *in vitro* with PKA using two phospho-specific anti-Cx35 antibodies. A. The blot was probed with polyclonal anti-Cx35 phospho-Ser110. The antibody labeled the Cx35 intracellular loop domain (IL) phosphorylated by PKA (middle lane), but not the non-phosphorylated IL domain (left lane). The IL domain contains the Ser110 site targeted by this antibody. The antibody also failed to recognize the phosphorylated Cx35 C-terminus (CT; right lane), which contains the Ser276 site. B. The blot was probed with polyclonal anti-Cx35 phospho-Ser276 antibody. This antibody labeled the *in vitro* phosphorylated Cx35 CT (middle lane), but did not label the non-phosphorylated CT (left lane). Phosphorylated Cx35 IL (right lane) was not labeled. Each lane contains 200 ng GST fusion protein.

Figure 3. Phospho-Cx35 antibodies recognize Cx35 in hybrid bass retinal membrane preparations

A. Western blot of membrane preparations from dark-adapted retina (D, left two lanes) and light-adapted retina (L, right two lanes). A monoclonal antibody against Cx35 recognized the appropriate band between 30–32 kDa. Digestion of the membranes with alkaline phosphatase (AP; right lane of each pair) had no effect on this labeling. **B.** Western blot of retinal membranes probed with the phospho-Ser110 antibody. The phospho-Ser110 antibody recognized the same band identified by the monoclonal antibody in preparations from both dark- and light-adapted retinas. Labeling by the phospho-Ser110 antibody was lost in membranes digested with AP. The phospho-Ser110 antibody also recognized high molecular weight bands in the blot, but labeling was lost with AP digestion indicating that these are phospho-proteins. **C.** Western blot of retinal membranes probed with the phospho-Ser276 antibody. The phospho-Ser276 antibody also recognized the Cx35 band in both dark- and light-adapted retinal membranes. Labeling by the phospho-Ser276 antibody was lost in membranes digested with AP. Each lane contains 40 µg retinal membrane protein.

Figure 4. Phospho-Cx35 antibodies recognize Cx36 in mammalian retinal membranes

Cx36 was labeled by the Cx35 monoclonal antibody (bottom panel) in western blots of membrane preparations from light-adapted mouse (left two lanes) and rabbit (right two lanes) retina. Both the phospho-Ser110 (top panel) antibody and the phospho-Ser276 antibody (middle panel) recognized the same band identified by the monoclonal antibody in both species. This labeling was largely lost in membranes digested with alkaline phosphatase (AP). Each lane contains 40 µg retinal membrane protein.

Figure 5. Phospho-proteins non-specifically recognized by the phospho-Ser110 antibody are not associated with Cx35

Immunoprecipitation of Cx35 from hybrid bass retinal membrane preparations using monoclonal anti-Cx35 and probed with anti-phospho-Ser110. The immunoprecipitate lane (IP) contained phosphorylated Cx35 but none of the high molecular weight phospho-proteins detected in western blots of retinal membranes (lysate lane; see also figure 3B). In contrast, the IP supernatant (super.) contained the same suite of phospho-proteins as found in the lysate. Each lane contains the equivalent of 90 μ g retinal membrane protein.

Figure 6. Phospho-Cx35 antibody labeling patterns in hybrid bass retina

A. Labeling pattern of phospho-Ser110 antibody in light-adapted hybrid bass retina. The phospho-Ser110 antibody labeled abundant punctate structures in the inner and outer plexiform layers (IPL and OPL, respectively). It also strongly labeled Müller cells, as illustrated here by the bright labeling of their somas above the IPL and long trunks descending through the IPL. Scale bar is 20 μm ; scale is the same for each image. **B.** Labeling with the monoclonal Cx35 antibody in the same section reveals the distribution of Cx35 in the OPL and IPL. **C.** Merged image of **A** and **B**. Many of the plaques identified as Cx35 by the monoclonal antibody were also labeled by the phospho-Ser110 antibody (co-localization shown in yellow). **D.** Labeling pattern of phospho-Ser276 antibody in light-adapted hybrid bass retina. The phospho-Ser276 antibody also labeled abundant punctate structures in the IPL and OPL. Minor non-specific labeling resembled blood vessels in the retina. **E.** Cx35 monoclonal antibody staining in the same section shows a largely overlapping distribution of signals. **F.** Merged image of **D** and **E**. Many of the plaques identified as Cx35 by the monoclonal antibody were also labeled by the phospho-Ser276 antibody (co-localization shown in yellow).

Figure 7. Cx35 phosphorylation varies across gap junction type and with light-adaptation state
 All images are from sublamina b of the inner plexiform layer of hybrid bass retina. *A–C*. Double labeling for phospho-Ser276 (*A*) and monoclonal anti-Cx35 (*B*) in dark-adapted retina. Scale bar is 5 μ m; scale is the same for each image. *C*. Merged image of *A* and *B*. Many Cx35 plaques were strongly labeled by the phospho-Ser276 antibody in dark-adapted retina, including the giant plaques described (triple arrowhead), while other plaques were devoid of labeling (arrow). *D–F*. Double labeling for phospho-Ser276 (*D*) and monoclonal anti-Cx35 (*E*) in light-adapted retina. *F*. Merged image of *D* and *E*. Phospho-Ser276 antibody labeling was markedly reduced on Cx35 giant plaques in light-adapted retina (triple arrowhead), and again some plaques showed no labeling at all (arrow). A population of medium-sized plaques continued to show strong labeling in light-adapted retina (single arrowheads). *G–I*. Double labeling for phospho-Ser110 (*G*) and monoclonal anti-Cx35 (*H*) in dark-adapted retina. *I*. Merged image of *G* and *H*. Numerous Cx35 plaques were labeled by the phospho-Ser110 antibody in dark-adapted retina, including strong labeling on the giant plaques. *J–L*. Double labeling for phospho-Ser110 (*J*) and monoclonal anti-Cx35 (*K*) in light-adapted retina. *L*. Merged image of *J* and *K*. Phospho-Ser110 labeling was reduced on Cx35 giant plaques in light-adapted retina. Labeling on other plaques was variable, with most Cx35 gap junctions showing some labeling. However, a few showed no labeling (arrow). The descending process of a Müller cell non-specifically labeled by the phospho-Ser110 antibody can be seen on the right side of the image.

Figure 8. Quantitative analysis of phosphorylation on Cx35 plaques in the inner plexiform layer
A. Histogram of Cx35 plaque size in the IPL. The arrow indicates the empirically determined cutoff point used to distinguish the giant plaques distributed in two symmetric bands in the IPL from the total population of Cx35 plaques (see methods). Each bin is 250 pixels ($\sim 1.225 \mu\text{m}^2$). For visual clarity, only every other bin is labeled on the x-axis. Note that scaling on the y-axis switches to logarithmic after the break. Data from six animals are shown (3–6 images per animal). **B.** Effects of background illumination on Cx35 gap junction phosphorylation. Giant plaques (right two pairs) exhibited significantly less phosphorylation at both Ser110 and Ser276 in light-adapted retina than in dark-adapted retina. The remaining smaller plaques (left two pairs) did not show a significant difference in phosphorylation at Ser110 or Ser276 between

dark- and light-adapted retina. n = 3 animals per condition. * designates significance at $p < 0.05$ level.

Figure 9. Phosphorylated Cx35 in the outer plexiform layer

A–C. Double labeling for phospho-Ser276 (*A*) and monoclonal anti-Cx35 (*B*) in dark-adapted OPL. Scale bar is 5 μm ; scale is the same for each image. *C.* Merged image of *A* and *B*. Large, round-ellipsoid (arrows) and string-like (double arrowheads) Cx35 plaques were well-labeled by phospho-Ser276 in dark-adapted OPL. Small, round plaques (arrowheads) showed lesser labeling. *D–F.* Double labeling for phospho-Ser276 (*D*) and monoclonal anti-Cx35 (*E*) in light-adapted OPL. *F.* Merged image of *D* and *E*. Phospho-Ser276 antibody labeling on light-adapted OPL was similar to dark-adapted OPL for large, round-ellipsoid (arrows) and string-like (double arrowheads) Cx35 plaques, while small round plaques (arrowheads) showed almost no labeling.