Protein Kinase A-mediated Phosphorylation of Connexin36 in Mouse Retina Results in Decreased Gap Junctional Communication between All Amacrine Cells

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Gap junctions in all amacrine cells of mammalian retina participate in the coordination of the rod and cone signaling pathway involved in visual adaptation. Upon stimulation by light, released dopamine binds to D1 receptors on all amacrine cells leading to increased intracellular cAMP (cyclic adenosine monophosphate) levels. All amacrine cells express the gap junctional protein connexin36 (Cx36). Phosphorylation of Cx36 has been hypothesized to regulate gap junctional activity of all amacrine cells. However, until now in vivo phosphorylation of Cx36 has not been reported. Indeed, it had been concluded that Cx36 in bovine retina is not phosphorylated, but in vitro phosphorylation for Cx35, the bass ortholog of Cx36, had been shown. To clarify this experimental discrepancy, we examined protein kinase A (PKA)-induced phosphorylation of Cx36 in mouse retina as a possible mechanism to modulate the extent of gap junctional coupling. The cytoplasmic domains of Cx36 and the total Cx36 protein were phosphorylated in vitro by PKA. Mass spectroscopy revealed that all four possible PKA consensus motifs were phosphorylated; however, domains point mutated at the sites in question showed a prevalent usage of Ser-110 and Ser-293. Additionally, we demonstrated that Cx36 was phosphorylated in cultured mouse retina. Furthermore, activation of PKA increased the level of phosphorylation of Cx36. cAMP-stimulated, PKA-mediated phosphorylation of Cx36 protein was accompanied by a decrease of tracer coupling between all amacrine cells. Our results link increased phosphorylation of Cx36 to down-regulation of permeability through gap junction channels mediating light adaptation in the retina.

The mammalian retina is a structure with three neuronal layers and two synaptic layers in which retinal neurons are not only forming numerous chemical synaptic contacts but also electrically and chemically coupled networks via gap junction channels. Gap junctions were reported in rod and cone photoreceptor cells, cone bipolar cells, horizontal cells, various subtypes of amacrine cells, and ganglion cells (1). Interneuronal communication through gap junction channels is essential for the rod pathway under conditions of scotopic illumination. Visual information from the rods is carried via rod bipolar cells to all amacrine cells that form heterologous electrical synapses with ON cone bipolar cells, thus transferring rod signals to ganglion cells (2–4). Junctional hemichannels on the AII amacrine cell side consist of Cx36, whereas Cx36 and/or Cx45 protein form electrical synapses on the bipolar cell side (5–7). Additionally, all amacrine cells form homotypic gap junctions to neighboring AII amacrine cells, involving Cx36 (2, 3, 8–10). Under scotopic conditions, the AII network only shows weak coupling and thus supports optimal transfer of the rod signal to the ON cone bipolar cells. Under mesopic light conditions, all amacrine cells form an extensively coupled network. Pooling visual signals under this light condition increases sensitivity of all amacrine cells (11, 12). With increasing ambient light intensity, the AII amacrine network decouples (13). This decoupling of the AII amacrine cell network from bipolar cells under photopic conditions prevents attenuation of the ON cone bipolar cell signal by leaking into the AII network and therefore improves the sensitivity of the cone pathway. Thus, the highly dynamic conductance of gap junctions under different light conditions provides adjustment to ambient light conditions from the scotopic via the mesopic to photopic operating range, thereby maintaining the fidelity of both rod and cone mediated vision (11).

Modulation of gap junction-mediated intercellular communication among all amacrine cells and between all cells and cone bipolar cells is of physiological importance since it regulates the balance between sensitivity and spatial resolution in adjustment to the ambient light (14). Numerous studies have shown that ambient light conditions translate into a dopaminergic signal which acts at corresponding neurons affecting gap

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‡ The abbreviations used are: Cx36, connexin36; GST, glutathione S-transferase; CKII, casein kinase II; CAMKII, calmodulin-dependent protein kinase II; Bt2cAMP, dibutyryl cyclic AMP; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PKA, protein kinase A; PKC, protein kinase C.
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 Junctional coupling. Upon stimulation with light, dopamine is released in the retina, D$_1$ receptors are activated leading to elevated levels of cyclic adenosine monophosphate (cAMP) in neurons (15). Previous studies have shown that the activity of all amacrine gap junction channels can be regulated by dopamine and second messengers like cAMP (16, 17). Recent results (3) showed that dopamine and cAMP close Cx36 gap junction channels expressed by all amacrine cells, suggesting that the regulation of Cx36 is necessary for light adaptation of the retina. The question remained, however, what kind of molecular switch mediates the uncoupling of Cx36 gap junctions. Connexin proteins can be modified post-translationally by phosphorylation and it has been implicated that the closure of Cx36 containing gap junction channels could be involved (18, 19). Although it has been shown that glutathion S-transferase (GST) fusion proteins of Connexin35, the bass ortholog of Cx36, could be phosphorylated in vitro, Cx36 has not been demonstrated so far to be phosphorylated in vivo. On the contrary, when Sitaramayya et al. (20) addressed the issue of phosphorylation as possible means to regulate Cx36 mediated coupling in the bovine retina, they concluded that Cx36 is not directly phosphorylated and suggested that regulation of Cx36 channels takes place by phosphorylation of associated proteins.

In this study, we have investigated phosphorylation as a possible mechanism to regulate Cx36 channel activity in the mouse retina. We demonstrate that GST-Cx36 fusion proteins are phosphorylated in vitro by PKA and Cx36 is phosphorylated in cell lines as well as primary mouse retina cultures. Furthermore, activation of PKA by increased levels of cAMP results in increased phosphorylation of Cx36 in mouse retina suggesting that phosphorylation of Cx36 is responsible for the closure of the Cx36 channels during light adaptation. Tracer injections into all amacrine cells in the presence and absence of cAMP, PKA antagonist and D$_1$ receptor antagonist revealed modulation of electrical coupling as an important role for Cx36 phosphorylation during light adaptation.

EXPERIMENTAL PROCEDURES

Northern Blot Analysis—Twenty retinas were collected from 10 decapitated C57BL/6 mice (about 3 months old) at 4:00 p.m. under daylight conditions as well as from 10 adult C57BL/6 mice at 4:00 a.m. under dim red light conditions and immediately frozen in liquid nitrogen. Total RNA (20 μg) harvested from each pool of 20 retinas was prepared with TRIzol® reagent according to instructions provided by the manufacturer (Invitrogen). RNA was electrophoresed (21) and transferred to Hybond-N nylon membrane (Amersham Biosciences) by capillary diffusion in 20×SSC. Transcript sizes were determined by hybridization with a 32P-labeled 0.8-kb reverse transcription-PCR fragment of mouse Cx36 exon2 (22). Hybridization was carried out under high stringency conditions (55% formamide, 5× SSC, 5× Denhardt’s solution, and 0.5% SDS) in the presence of 60 μg/ml heat-denatured salmon sperm DNA for 12 to 24 h at 42 °C (21). After the last washing step with 0.1× SSC, 0.1% SDS for 10 min at 65 °C, the nylon membranes were sealed in plastic wrap and exposed to XAR x-ray film (Eastman Kodak Co.) with intensifying screen at −70 °C for 2 weeks. The amounts of total RNA on the Northern blot were standardized by hybridization to a probe of glyceraldehyde phosphate dehydrogenase (23). The densitometric analysis was carried out with the Scan Package (Version 4.0, Biometra, Göttingen, Germany).

Immunoprecipitation—Cells and tissue were lysed in RIPA buffer (10 mM phosphate buffer, 0.1% SDS, 1% Triton X-100, 0.1% sodium deoxycholate, 40 mM NaF) with 1× Complete Protease Inhibitor (Roche Applied Science), sonified, and cleared by centrifugation (13,000 rpm, 4 °C, 30 min). The supernatants were adjusted to equal protein concentrations of 1.5 mg/ml. For experiments with radioactive labeling, equal numbers of retinas were used and the protein amounts were quantified after waning of radioactivity. Protein concentrations varied at less than 5%. Equal volumes of lysates were precleared with 30-μl incubation with 60 μl of Sepharose (Amersham Biosciences) and equilibrated in RIPA buffer.

After transferring to fresh tubes, 4 μl of Cx36 antibodies (Zymed Laboratories Inc., catalog number 516300) immobilized to 30 μl protein A-Sepharose were added to the lysates and incubated at 4 °C overnight with end-over-end rotation. Afterward, the beads were washed three times with RIPA washing buffer (10 mM phosphate buffer, 1 M NaCl, 0.2% Triton X-100, 40 mM NaF, 10 mM EDTA) and once with double distilled H$_2$O. The samples were heated to 65 °C with 20 μl of 1× Laemmli sample buffer (62.5 mM Tris-HCl, 3% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromphenol blue, pH 7.4) and analyzed via SDS-PAGE or subjected to in vitro phosphorylation as described below.

Protein concentration was determined using the BCA protein assay (Sigma) and fatty acid-free bovine serum albumin standards.

SDS-PAGE and Immunoblot—Samples were analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis. For autoradiography, gel slabs were fixed in 50% methanol and 10% acetic acid, for 20 min at room temperature. 35S-labeled samples were incubated with Amplify Reagent (Amersham Biosciences) for 15 min, and gel slabs were vacuum-dried (Bio-Rad model 583) and subjected to autoradiography (Kodak Biomax).

For immunoblotting, protein was blotted onto nitrocellulose membranes (Amersham Biosciences), blocked with 5% milk powder (Sucofin, Zeven, Germany) in TBS-T (10 mM Tris-HCl, 150 mM NaCl, 0.1% Tween 20) and probed with Cx36 antibodies (Zymed Laboratories Inc., catalog number 516300, 1:300), visualized after incubation with appropriate horseradish peroxidase-conjugated secondary antibodies (1:20,000), followed by chemiluminescence detection (Pierce).

Densitrometric analysis was conducted using the Image-Quant TL software (Amersham Biosciences).

Cloning of Fusion Proteins—DNA coding for the Cx36 COOH terminus (Cx36CT) or cytoplasmic loop (Cx36CL) was amplified via PCR from pCx36 (24), a vector containing the total coding region of mCx36 in a 10-kb genomic mouse fragment. Individual point mutations were introduced by overlap extension PCR. Then DNA molecules were amplified adding Smal and NotI (Cx36CT and its mutations) or BamHI and EcoRI restriction sites (Cx36CL and its mutations), and Smal/NotI- or, respectively, BamHI/EcoRI-digested, gel-purified,
and ligated in-frame into pGEX-GST expression vector (Amer-sham Biosciences). Inserted DNAs were sequence-verified (Agowa GmbH, Berlin, Germany) and transformed into Esche-ricchia coli BL21.

The following primers were used: Cx36CT-Smal-USP, 5'-TCC CCC GGG CTG ATT TAA ACC ATC TGG GAT GG-3'; Cx36CT-NotI-DSP, 5'-ATA AGA ATG CGG CCG CCC TTT CAC ACA TAG GCA GAG-3'; Cx36CL-BamHI-USP, 5'-CGG ATC CGC CAA GCA GCG AGA ACG-3'; Cx36CL-EcoRI-DSP, 5'-GGA ATT CCT CTG TAG ATA TCT TCC TGT CTG AGG-3'; Cx36S110A-USP, 5'-GAA AAC GCC GGT AGC CTA CTG TCT TCC TCC TTT CAC ACA TAG GCA GAG-3'; Cx36S110A-DSP, 5'-GGA AGA CAG TAG CGT ACC GGC GTT CTC-3'; Cx36S293A-USP, 5'-GCC AGG AGG GCA GTC TAT GAG ATA C-3'; Cx36S293A-DSP, 5'-GTA TCT CAT AGA CTG CCT GTC TGT CTT CGG AG-3'; Cx36S315A-USP, 5'-GGC AGG ACT CAG GCC AGT GAC TCT G-3'; Cx36S315A-DSP, 5'-CAG AGT CAC TGG CCT GAC TGC TGC C-3'.

Induction and Purification of GST-Cx36 Fusion Proteins—Transformed E. coli strains were grown in LB medium at 37 °C, expression of fusion proteins was induced at OD 0.6 using 1 mM isopropyl β-D-thiogalactopyranoside. Cells were harvested 4 h after induction and lysed by sonification. Lysates were cleared by centrifugation (13,000 rpm, 1 h), and GST fusion proteins after induction and lysed by sonification. Lysates were cleared by centrifugation (13,000 rpm, 1 h), and GST fusion proteins were affinity-purified from soluble bacterial protein by applying the supernatants onto 1-ml GSTrap columns (Amersham Biosciences), extensive washing with PBS (137 mM NaCl, 2.7 mM KCl, 8.1 mM Na2HPO4, 1.5 mM KH2PO4, pH 7.6) and sub-sequent elution with 10 ml of 10 mM reduced glutathione, 10 mM Tris-HCl, pH 8.3. Resulting eluates were concentrated and separated from glutathione by Millicon centrifugal devices (Millipore, Billerica, MA).

In Vitro Phosphorylation—Fifteen μg of purified GST-Cx36CT and Cx36CL-fusion proteins and their corresponding point mutations were immobilized on 15 μl of glutathione-coupled Sepharose beads (Pierce). Beads were washed three times with PBS and once with reaction buffer corresponding to the kinase used. After addition of γ-ATP and either PKA, PKC, CKII, or activated CAMKII (all from New England Biolabs), samples were incubated for 30 min at 30 °C and washed three times with PBS and once with double distilled water. Bound fusion protein was eluted by 20 μl of 1× Laemmli sample buffer.

Cell and Retina Culture—Mice were bred in local animal facilities and maintained at a 12 h dark/light cycle (6 a.m./6 p.m.) with food and water ad libitum. All protocols were carried out in accordance with German standard ethical guidelines for laboratory animals. Eyes of 6–8-week-old female C57/BL6 mice were enucleated and dissected in PBS (4 °C), using microscissors and forceps to extract intact retinae. Retinæ were plated with the photoreceptor cell layer facing down on Millicell-CM membrane inserts (Millipore) in 35 mm cell culture dishes and cultivated for 7.5 h at 37 °C, 10% CO2 in 1 ml of Dulbecco’s modified Eagle’s medium, 10% fetal calf serum, without methionine or ortho-phosphate (CCPro, Neustadt, Germany). In some experiments, 1 mM membrane-permeable cAMP, Bt2cAMP (Sigma), was added to the medium for the last 20 min of cultivation. HeLa-Cx36 and RT4-AC-Cx36eGFP transfectants were cultivated as described by Teubner et al. (24) and Zoidl et al. (25), respectively.

Metabolic Labeling—Cultured HeLa-cells, RT4AC-cells or retinae were fed for one hour with medium free of methionine or orthophosphate. Subsequently, 200 μCi/ml [35S] methionine (Amersham Biosciences) or 300 μCi/ml [32P] orthophosphate (Amersham Biosciences) were added to cells for 4 h. Then the cells were harvested in RIPA buffer and subjected to immunoprecipitation.

Mass Spectrometric Phosphosite Analysis—Phosphorylated Connexin36 GST fusion proteins covering the cytoplasmic loop and the carboxyl terminus as well as the appropriate controls without kinase incubation were excised from Coomassie Brilliant Blue™- and/or zinc-stained (GelCode E-Zink Reversible Stain Kit; Pierce) SDS-PAGE gels and subjected to tryptic in-gel digestion followed by MALDI-TOF analysis (carried out at the Center for Molecular Medicine, Cologne, Germany) and electrospray ionization-MS mass spectrometry-based identification of the phosphorylation sites (carried out at the Mass Spectrometry Analysis Subunit of the RIKEN Center for Developmental Biology).

Phosphoprotein mapping was performed using a combined setup consisting of the HTS-PAL autosampler (CTC Analyt-ics), Paradigm MS4 nanoHPLC (Michrom BioResources), and the Thermo Finnigan LTQ linear ion trap mass spectrometer, operated in the data-dependent neutral loss MS3 scanning mode. The MS3 data obtained for each of the four tryptic digests analyzed (phosphorylated as well as non-phosphoryla-ted GST-Cx36 CT and CL fusion proteins) were searched using TurboSEQUEST within Bioworks as well as MASCOT-Cluster analysis. Predicted phosphorylation sites were validated manu-ally and via SEQUEST using the MS2 and MS3 raw data.

Neuroubiotin Injections in All Amacrine Cells—In C57Bl/6N mice, eyes were enucleated and retinae dissected under deep anesthesia after intraperitoneal injections of xylazine (70 mg/kg) and ketamine (40 mg/kg). Subsequently the mice were killed by cervical dislocation. Isolated retinae were cut in four pieces and incubated in carboxygenated Ames Ringer solution, pH 7.4, containing 10 μM 4,6-diamidino-2-phenylindole (Sigma), for 60 min at room temperature. In different sets of experiments, this solution additionally contained 1 mM Bt2cAMP (Sigma), 1 μM of the PKA antagonist H-89 (Sigma) or 10 μM of the D1 receptor antagonist R(+)-SCH23390 (Sigma). Moreover, in the latter case, R(+)-SCH23390 and H-89 were also permanently applied during the injection procedure. The retina pieces were then mounted on filter paper (Millipore) and placed in the recording chamber. The exchange flow rate with Ames Ringer solution in the recording chamber was 2 ml/min. Putative All amacrine cells were identified by their small 4,6-diamidino-2-phenylindole-stained nuclei located in the inner nuclear layer in direct proximity to the inner plexiform layer. Intracellular injections were carried out with borosilicate glass electrodes (170–200 MΩ) that were pulled with a Sutter puller (Sutter P-97, Brown and Flaming Micropipette Puller, Novato, CA) and filled with a solution containing 1% Lucifer Yellow.
and 4% neurobiotin (Vector Laboratories) dissolved in 200 mM KCl buffer, pH 7.3. After impaling the cell under a 63× water-immersion objective (Zeiss Achromat, NA 0.6), Lucifer Yellow was iontophoresed with negative current of −1 nA (750 ms at 1 Hz). When the bistratified morphology of the AII amacrine cell could be recognized, the direction of the current was reversed for 4 min to inject positively charged neurobiotin molecules. Two to three AII amacrine cells were injected per retinal piece. Following the last injection, the retinal piece remained for 20 min in the recording chamber, resulting in diffusion times of 20–35 min depending on the order of the injections. Then the retina pieces were transferred into fixation solution (4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.4) for 10 min and washed several times. Intercellular spreading of neurobiotin was visualized by incubation with streptavidin-Indocarbocyanine (Cy3, Jackson Immunoresearch, dilution 1:500) in 0.3% Triton X-100 (Sigma) and 0.1% sodium azide (Sigma) dissolved in 0.1 M phosphate buffer. The extent of neurobiotin diffusion through cells of the retina were documented using the 568 nm line of a krypton-argon laser in a confocal microscope (Leica). Images were subsequently processed using Adobe Photoshop 7. Statistical calculations were performed with Origin 7.0 software packages (Microcal, Natick, MA).

Chemicals and enzymes were purchased from Merck, New England Biolabs, or Sigma, if not mentioned otherwise.

RESULTS

The Amounts of Cx36 Transcript and Protein Are Not Changed during Adaptation to Daylight or Darkness—To access whether the abundance of Cx36 was altered under photopic/scotopic conditions, we performed Northern and immunoblot analyses. We wanted to exclude the possibility that effects of the circadian clock induced alterations in the retina could counteract changes by dark/light adaptation by using mice adjusted to the normal day/night cycle. The amount of Cx36 transcript abundance was not changed during day/night cycles (Fig. 1), thus the altered dopamine level is likely not to influence the transcription of Cx36.

In addition, immunoblot analyses of Cx36 from retinae, prepared at different time points, confirmed this result (data not shown). Indeed, retinae harvested at different time points during a day/night cycle showed no differences in the amount of Cx36 protein. We conclude that expression of Cx36 mRNA and protein are not changed during dark/light adaptation.

Cx36 Is Phosphorylated in Retina and in Transfected Cell Lines—We metabolically labeled HeLa-Cx36 and RT4-AC-Cx36eGFP transfectants with [35S]methionine or [32P]orthophosphate and immunoprecipitated Cx36 from the lysates (Fig. 2). Phosphorylated bands were detected at the same height as bands labeled with 35S. Phosphorylated Cx36/Cx36eGFP was only detected as a faint band suggesting that a small amount of Cx36 is phosphorylated in both transfected cell lines. Metabolical labeling of primary retina cultures was established, retinae were prepared in media free of methionine or orthophosphate and cultivated in the presence of radioactively labeled reagents in the dark. Retinal 35S- and 32P-labeled Cx36 was immunoprecipitated and analyzed via SDS-PAGE (Fig. 2). A comparison of the ratio of signal intensities of 35S-labeled Cx36 and 32P-labeled Cx36 shows that phosphorylation of Cx36 in cultured retina is more abundant than in transfected cells.

The Cytoplasmic Domains of Cx36 Are Phosphorylated in Vitro by PKA—During light adaptation, the neurotransmitter dopamine is increasingly released by the elevation of ambient...
light (26). It then binds to D₁ receptors on horizontal and All amacrine cells leading to an increase of cAMP concentration and thus inducing PKA activity.

The cytoplasmic loop and the COOH terminus of mouse Cx36 protein contain several putative consensus motifs for PKA as well as for other kinases (22). To determine whether PKA consensus motifs are accessible for phosphorylation, we fused the cytoplasmic loop (Cx36CL) and the COOH terminus of Cx36 (Cx36CT) to GST, immobilized the proteins to glutathione beads, and performed in vitro phosphorylations. PKA phosphorylates both cytoplasmic domains of Cx36 (Fig. 3A).

Additionally, mass spectroscopic analyses of Cx36CL and Cx36CT, in vitro phosphorylated by PKA, were conducted in parallel to non-phosphorylated controls. There are four potentially phosphorylated amino acid residues within four PKA consensus motifs. Phosphorylated peptide fragments representing all consensus sequences were identified by MALDI-TOF analysis of Cx36 CL and COOH terminus (Fig. 3C). For MS/MS analysis four different samples were processed in parallel. Peptide RRSYTV (Ser-110) was found in all the four samples and peptide RRSYVEIR (Ser-293) was found in three samples. The cytoplasmic loop peptide RYSTVFLALDR.D (Ser-110) was found in two samples. Furthermore, phosphorylated peptides, also containing Ser-315, have been identified by MALDI-TOF studies. Spectra consensus motifs for PKA are used; seven phosphorylated peptides containing all the PKA consensus sequences were found; consensus sequences are depicted in bold letters (C).

In vitro phosphorylation of Cx36 also occurs in full-length Cx36 protein, we immunoprecipitated Cx36 from mouse retinae and phosphorylated the protein by PKA (Fig. 3B). To elucidate the usage of the individual PKA consensus motifs, GST fusion proteins carrying a point mutation of serine were processed in parallel with mutated and unmutated fusion protein, which was set to 100%.

FIGURE 4. PKA predominantly phosphorylates serines 110 and 293. Equal amounts of fusions proteins with GST and mutated as well as non-mutated cytoplasmic domains of Cx36 were phosphorylated by PKA in vitro. Fusion proteins of the cytoplasmic loop (A), the COOH terminus (B), and point-mutated fusion proteins of Cx36 cytoplasmic domains with altered PKA consensus motifs (S110A and S293A, S306A, and S315A) were analyzed. Densitometric quantification of the signal intensity of phosphorylated fusion proteins showed a weak signal for S110A (2.9%, S.E. 1.21) and a severe reduction of intensity for S293A (13.6%, S.E. 1.23). A slight reduction was observed for S315A (80.4%, S.E. 10.64), while S306A showed a minor increase (106.5%, S.E. 7.05). Percentages refer to the signal intensity of phosphorylated non-mutated fusion protein, which was set to 100%.

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Tracer Coupling of All Amacrine Cells is Down-regulated by cAMP—Retinal dark to light adaptation is at least partially mediated by CAMP and further downstream by elevated levels of the second messenger CAMP and activated PKA. Gap junctional channel activity in All amacrine cells of rabbit retina is regulated by CAMP (3). We have studied the modulation of tracer coupling of mouse All amacrine cells by Bt2cAMP and D₁ receptor antagonists to analyze the influence of the dopamine/cAMP pathway on Cx36-mediated gap junctional communication in the mouse retina.

In the ON sublamina of the IPL, All amacrine cells receive glutamatergic input through their distal appendages with which they also form electrical synapses to ON cone bipolar
cells. Additionally, with their lobular appendages they provide glycinergic synaptic output to OFF cone bipolar cells in the OFF sublamina (8). Neurobiotin injections revealed gap junctional coupling of all amacrine cells to other all amacrine cells and bipolar cells. Coupled all amacrine cells were detected around the injected cell (Fig. 5A). The average number of coupled all amacrine cells was 7.5 (S.E. 0.6, n = 10) (Fig. 5B).

Additionally, smaller soma with locations in different depths more centrally in the INL showed tracer signals indicating that these cells were coupled bipolar cells. Axons descending to the ON sublamina of the IPL and dendritic processes ascending to the outer plexiform layer suggested that these neurons were ON cone bipolar cells (data not shown). The average number of coupled bipolar cells was 10.8 (S.E. 0.6, n = 10) (Fig. 5C). The majority of bipolar cells was intensively labeled, whereas some others were only faintly stained. This dichotomy of staining was not correlated to the distance of the bipolar cells from the injected ALL cell (Fig. 5A). Almost all coupled ON-cone bipolar cells were positioned within the edges of distal dendritic arbor of the injected all amacrine cell, thus indicating a direct tracer coupling from the injected neuron to the bipolar cells rather than an indirect coupling via neighboring all amacrine cells (data not shown).

Incubation with the membrane-permeable cAMP analog Bt2cAMP (1 mM) led to a significant reduction of the coupling amount for both all amacrine cells and bipolar cells. The average number of coupled neurons was 4.1 (S.E. 0.8) and 3.8 (S.E. 0.9), respectively (significance for all cells p ≤ 0.01, significance for bipolar cells p ≤ 0.001, n = 20) (Fig. 5, B and C). The effect of Bt2cAMP could be antagonized by the simultaneous application of H-89 (1 μM). The extent of coupling to both other all amacrine cells and bipolar cells was enhanced (average 8.4 (S.E. 1.0), significance p ≤ 0.01 and 12.7 (S.E. 0.7), significance p ≤ 0.001, respectively, n = 10). Application of H-89 alone did not have any impact on tracer spread, which resembled the control condition. In average, 7.5 (S.E. 0.7) all amacrine cells and 11 (S.E. 1.2) bipolar cells revealed tracer spread (n = 4, data not shown).

In contrast, application of the D1 receptor antagonist R(+) SCH23390 before and during injection procedure resulted in a larger number of coupled neurons. On average, 8.1 (S.E. 0.6) all amacrine cells showed tracer coupling. In addition, 14.6 (S.E. 1.6) bipolar cells were stained (significance p ≤ 0.05, n = 11) (Fig. 5, B and C). Co-application of R(+) SCH23390 with Bt2cAMP reversed the modulation of neurobiotin spread and reduced the number of coupled cells to 4.6 (S.E. 0.8) for all amacrine cells and 4.5 (S.E. 1.0) for bipolar cells (significance for all cells p ≤ 0.01, significance for bipolar cells p ≤ 0.001, n = 18) (Fig. 5, B and C).

**Activation of PKA after Incubation with Bt2cAMP Increases Phosphorylation of Retinal Cx36**—Recently, Xia and Mills (3) reported down-regulation of coupling between Cx36 expressing all amacrine cells in rabbit retina. Although they found Cx36 channels to be sensitive to increased CAMP levels, the phosphorylation of Cx36 as a likely candidate for altered gap junction channel permeability was not explored. Therefore, in primary retina cultures harvested from mice, the abundance of phosphorylated Cx36 protein in the absence and presence of CAMP has been determined in this study. Dark-adapted retinnae were metabolically labeled with [32P] ortho-phosphate, and Cx36 was immunoprecipitated. Signals reflecting phosphorylated Cx36 were more prominent in CAMP treated compared with control retinnae, an average increase of phosphorylation by 1.8-fold (S.D.: 0.31) was observed (Fig. 6B). We demonstrated that CAMP mediated activation of PKA increased phosphorylation of Cx36 in vivo (Fig. 6A). Furthermore, when we exposed...
dark-adapted retinae to daylight (10,000 lux) for 20 min, no significant change in phosphorylation was observed (data not shown). Immunoprecipitation of Cx36 performed in parallel with three untreated control samples showed that the signal intensity of controls varied about 20% (signal intensities 70, 110, and 100%; S.D.: 20.8, data not shown) due to the experimental set-up. Thus it is not possible to monitor changes in the range of 20% using this set-up.

**Cx36 Is a Target for Multiple Kinases** —Cx36 contains several consensus motifs for several kinases, i.e. CAMKII, CKII, and PKC (22). After showing that Cx36 is phosphorylated by PKA we checked whether the consensus motifs on Cx36CT and Cx36CL for CAMKII, CKII and PKC could also be phosphorylated. Our results indicate that Cx36 could be phosphorylated by each of the kinases analyzed in vitro (Fig. 7A).

Most connexins are targeted by more than one kinase. Phosphorylation of one kinase can cause down-regulation of gap junctional coupling and another can up-regulate coupling (19). To evaluate the role of Cx36 in regulating gap junctional communication, we accessed the influence of initial phosphorylation by kinases other than PKA on subsequent phosphorylation efficacy by PKA (Fig. 7B). The in vitro phosphorylation efficacy of the cytoplasmic domains of Cx36 by PKA depends on the prior phosphorylation by other kinases. Phosphorylation with other kinases prior to PKA resulted in decreased phosphorylation of Cx36CT and CL. However, phosphorylation of Cx36CL by PKA increased after prior phosphorylation with CKII.

**DISCUSSION**

All amacrine interneurons transfer rod signals to ganglion cells via the cone pathway through abundant Cx36 containing gap junctions to ON cone bipolar cells (2, 9). When Cx36 containing electrical synapses are lacking between AII amacrine cells and ON cone bipolar cells in Cx36 deficient mice, scotopic vision is strongly impaired (10, 29, 30). Homotypic gap junctions between AII amacrine cells are important for adjustment to different light conditions. Under scotopic and photopic light conditions, the AII amacrine cells are uncoupled, providing optimal signal transfer from rods or cones to ganglion cells, respectively. In contrast, under mesopic illumination AII amacrine cells form an extensively coupled network which promotes signal pooling and subsequent increase of sensitivity (11).

Obviously, one of the crucial features of electrical synapses between retinal neurons is that their conductance can be modulated as a result of light adaption (13). In this study, we have investigated the regulatory mechanisms of Cx36 channel activity, especially during light/dark adaption in the retina. Cx36 channels display the least voltage dependence of all connexin channels (31). Thus, it seems unlikely that these pores are voltage-gated under physiological conditions. On the other hand, chemical modulators such as do-
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Dopamine and cAMP exert strong uncoupling effects on gap junctions between AII amacrine cells (16, 17, 32). Xia and Mills (3) performed coupling studies with rabbit retina and showed that activation of PKA modulated Cx36 mediated AII coupling in vivo, thereby suggesting that Cx36 is a major candidate for phosphorylation. Using neurobiotin injections in AII amacrine cells of the mouse retina, we have now demonstrated that coupling among AII amacrine cells as well as between AII amacrine cells and bipolar cells might be modulated by activation of dopaminergic D1 receptors leading to elevation of intracellular cAMP, activation of PKA, and subsequent phosphorylation of Cx36. Blockade of the D1 receptor resulted in a significant increase of coupling between AII amacrine cells and bipolar cells. In accordance with the experiments of Hampson et al. (16), no significant effect was found for the coupling among AII amacrine cells. This might be explained by the fact that AII amacrine cells have a lower membrane input resistance than bipolar cells (14). Therefore, the diffusion of neurobiotin into all amacrine cells is more difficult to detect than into bipolar cells. Alternatively, a stronger expression of D1 receptors on bipolar cells and therefore a higher sensitivity for dopamine could occur in mouse retina. The R(+-)-SCH23390 effect was reversed by application of the membrane permeable Bt2cAMP indicating the elevation of cAMP downstream to the D1 receptor activation. The decrease of AII amacrine cell and bipolar cell coupling by application of Bt2cAMP could be prevented by the PKA inactivating drug H-89. This clearly shows that D1 receptor activation is followed by an intracellular increase of cAMP resulting in diminished coupling. Whereas the coupling between AII cells depends directly on the modulation of the Cx36 containing gap junctions between the amacrine cells, the regulation of coupling between AIIIs and bipolar cells might be more complex. The drugs applied might directly affect gap junctions between AII and ON bipolar cells containing Cx36. As an alternative, we cannot exclude the possibility that tracer spread to bipolar cells might be indirectly modulated by gap junctions between all amacrine cells. However, since most coupled ON cone bipolar cells spatially match with the distal dendritic arbor of the injected AII amacrine cell, an indirect tracer diffusion to bipolar cells via other AII amacrine cells is hardly conceivable. Furthermore, a recent report showed in rabbit retina that dopaminergic modulation of electrical synapses formed by Cx36 occurred between both AIIIs/AIIIs and AIIIs/ON cone bipolar cells (3). Since ON cone bipolar cells do not only express Cx36 but also Cx45 (3) and PKA also phosphorylates Cx45 in vitro, a stronger regulating effect of PKA mediated phosphorylation on Cx45 than on Cx36 may have caused the observed results.

In contrast to the findings of Sitaramayya et al. (20), whose immunoprecipitation studies indicated that Cx36 was not phosphorylated in bovine retina, we could show that Cx36 immunoprecipitated from mouse retina is phosphorylated by PKA in vitro. Additionally, phosphorylation and mass spectroscopic analyses of GST-Cx36 fusion proteins revealed that all four PKA consensus sequences of Cx36 are used in vitro by PKA suggesting that phosphorylation of Cx36 also occurs in vivo. These findings are consistent with results obtained with Cx35, the fish ortholog of Cx36 (33, 34). To further analyze the significance of the different PKA motifs, we performed in vitro phosphorylation of point mutated GST fusions proteins. The results showed that the amino acid residues Ser-110 and Ser-293 were predominately phosphorylated by PKA. Since phosphorylation of Ser-110 and Ser-276 (ortholog site to Ser-293 in Cx36) in the fish ortholog Cx35 influences Cx35 regulation in HeLa cells (27), that might as well be the case with Cx36 in AII amacrine cells during light adaption.

In this study we have shown for the first time that Cx36 is phosphorylated in cultured mouse retina and transfected cell lines. Furthermore, we found that Cx36 is phosphorylated upon cAMP induced activation of PKA in the mouse retina. The observation that retinal Cx36 is phosphorylated shortly after activation of PKA is consistent with down-regulation of gap junctional coupling among AII amacrine cells as well as between AII amacrine and ON cone bipolar cells after incubation with dopamine or membrane permeable cAMP. Furthermore, treatment with Bt2cAMP also reduces coupling between RT4AC-Cx36 transfectants. However, dark-adapted retina cultures submitted to day light conditions (10,000 lux) for 20 min showed no effect on Cx36 phosphorylation. This might be due to disturbing proper in vivo retinal physiology in this retinal culture model. It is likely that sufficient dopamine was produced upon light stimulation by the dopaminergic interplexiform cells but that this dopamine was diluted too fast into the surrounding culture medium. Therefore, dopamine concentration seemed to be threshold to trigger a detectable dopamine D1 receptor, cAMP, PKA phosphorylation response on Cx36 protein.

Our results highlight the correlation between down-regulation of gap junctional permeability and phosphorylation of Cx36, thus providing a molecular switch to decrease coupling of Cx36 gap junctional channels.

In addition, we have shown that Cx36 is a target of not only PKA but for also of four other kinases in vitro. Thus multiple signal transduction pathways could modulate its activity. Possibly not only PKA is involved in the regulation of Cx36 gap junction channels, but also CAMKII, CKII, and PKC. Cx36 could be regulated by phosphorylation through a complex interplay of different kinases. In this aspect, Cx36 appears to be similar to other proteins involved in synaptic plasticity, for example N-methyl-D-aspartate-type glutamate receptors (35). The enhancement of their function via increased phosphorylation of PKA is a prerequisite of pain related plasticity in the amygdala (36). Function of N-methyl-D-aspartate-type glutamate receptors and thereby synaptic plasticity is also modulated by other kinases, like CKII (37) and CAMKII (28).

Taken together, we have shown that phosphorylation of Cx36 by PKA is a molecular switch that regulates coupling via Cx36 gap junction channels in all amacrine cells, thus modulating synaptic plasticity in the retina during adaptation to ambient light conditions. This is in accordance with the concept that the activity of chemical synapses (i.e. the release of

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3 T. Höher, unpublished results.
dopamine) leads to down-regulation of Cx36 containing electrical synapses in the retina.

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