PKC Isoenzymes in the Chicken Lens and TPA-Induced Effects on Intercellular Communication

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PURPOSE. Because lens connexins are phosphoproteins and intercellular communication between lens cells may be modulated by connexin phosphorylation, experiments were designed to characterize the expression of protein kinase C (PKC) isoenzymes in the chicken lens and in lentoid-containing cultures and to study the effects of 12-O-tetradecanoylphorbol-13-acetate (TPA) treatment on the distribution of PKC isoenzymes and intercellular communication.

METHODS. The presence and distribution of PKC isoenzymes were studied by immunoblot analysis and immunofluorescence in chicken lens sections and in cell cultures under control conditions and after treatment with TPA. Intercellular communication was assessed by transfer of microinjected Lucifer yellow.

RESULTS. PKC α, γ, ε, and μ were detected in lens homogenates by immunoblot analysis. The levels of PKC α, γ, ε, and μ decreased between the 7th and the 18th embryonic days. Levels of PKC ε remained relatively constant during the period of study. Similarly, lens cells in culture expressed isoenzymes α, γ, ε, τ, and μ. PKC β was not detected in lens or culture homogenates. In lens sections, all PKC isoenzymes analyzed were present in epithelial cells, in the annular pad region, and in the posterior aspect of fiber cells. The anti-PKC γ antibody also stained fiber cell membranes. Analysis of lentoid cultures by immunofluorescence revealed that PKC γ, ε, and τ and minimal amounts of PKC α were present in lentoid cells. Treatment with 200 nM TPA for 15 to 30 minutes induced translocation of PKC γ to the plasma membrane of lentoid cells and significantly reduced the transfer of microinjected Lucifer yellow.

CONCLUSIONS. Several PKC isoenzymes are expressed by lens cells in situ and in culture. The γ isoenzyme, present in lens fibers, was activated in lentoid cells by TPA, a known activator of PKC. We have previously demonstrated TPA-induced phosphorylation of the gap junction protein connexin56 (Cx56). The new data presented in the current study demonstrate that TPA treatment also decreased intercellular communication. Taken together, the results suggest that differential phosphorylation of Cx56 by PKCγ may induce a conformational change in the protein which, in turn, might lead to channel closure. (Invest Ophthalmol Vis Sci. 2000;41:850–858)

Protein kinase C (PKC) isoenzymes form a multigene family with subtle differences in their individual enzymologic characteristics.1–3 The patterns of distribution and intracellular localization differ among members of this family.1–3 Several members of the PKC family are activated by tumor promoter phorbol esters.4,5 Activation of PKC results in translocation of the protein to membranes.6–8 Gap junctional proteins are among the many targets of PKC activity.9–11

Gap junctions are aggregations of intercellular channels that allow intercellular passage of ions and molecules of up to 1000 Da. These channels are oligomeric assemblies of members of a family of related proteins called connexins.12 Most connexins are phosphoproteins, and phosphorylation has been implicated in trafficking, assembly, insertion into the plasma membrane, gating, internalization, and degradation of gap junctions.9,10,12–14 The effects of tumor promoter phorbol esters on intercellular communication have been extensively studied and differ depending on the cell and connexin type.9

We have been interested in the gap junction proteins in the lens and their regulation by phosphorylation. The lens is an avascular organ formed by an anterior epithelial layer and lens fibers that form the bulk of the organ. Gap junctions in the lens have been identified between epithelial cells and between lens fiber cells. The molecular components of lens gap junctions have been identified. Epithelial cells express connexin43 (Cx43),21 whereas lens fibers express lens-specific connexins. Several of these connexins have been cloned from different species, including rat Cx46, mouse Cx50, bovine Cx44, ovine Cx49, human Cx48, chicken Cx56, and chicken Cx45.6,22–28 Because of their permeability properties, gap junctions have been proposed as playing a pivotal role in the passage of nutrients and metabolites between lens cells, thus allowing maintenance of lens transparency.29

Lenses cells, when placed in culture, differentiate and form multicellular structures called lentoids that show a number of features similar to lens fiber cells.30–35 The lentoids contain...
fibertype gap junctions and permit intercellular passage of fluorescent dyes. In chicken lens cultures, expression and modification of Cx56 correlate with differentiation of the cells into lentoids. In homogenates prepared from lenses or from cultures, Cx56 exhibits several electroforetic forms that may result from phosphorylation at different residues. We have demonstrated constitutive phosphorylation of Ser-495 and a 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced increase in phosphorylation of Cx56 at Ser-118.

The present study was designed to characterize the PKC isoenzymes expressed in the developing lens and in lentoid-containing cultures, their levels and distribution, and the effects induced by TPA on PKC isoenzyme distribution and gap junctional intercellular communication.

**Materials and Methods**

Unless otherwise stated, all reagents were obtained from Sigma (St. Louis, MO). Fertilized White Leghorn chicken eggs were obtained from SPAFAS (Norwich, CT). All investigations using animal materials adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Primary Cultures of Chicken Embryo Lens Cells**

Primary cultures of chicken embryo lens cells were prepared according to Menko et al. Briefly, lenses from 11-day-old embryos were collected in 140 mM NaCl, 5 mM KCl, 7 mM Na2HPO4, 5 mM glucose, and 250 mM Tris-HCl (pH 7.4) and incubated in Earle’s 199 containing 0.08% trypsin (Life Technologies, Grand Island, NY) for 30 minutes at 37°C. Cells were then triturated in Earle’s 199 containing 10% fetal bovine serum, 10 U/ml penicillin, 10 μg/ml streptomycin (Life Technologies) and plated on collagen-coated tissue culture dishes. Cells were fed every 2 days and allowed to differentiate. Experiments were performed on cultures that were 14 to 17 days old and contained several large lentoids.

**Antibodies**

Mouse monoclonal antibodies directed against different PKC isoenzymes were obtained from Transduction Laboratories (Cincinnati, OH). Horseradish peroxidase–conjugated donkey anti-mouse IgG antibodies and Cy3-conjugated goat anti-mouse IgG antibodies were obtained from Jackson ImmunoResearch (West Grove, PA).

**Sample Preparation for Immunoblot Analysis**

**PKC Expression in Whole Lenses.** Chicken lenses obtained at embryonic day 7, 8, 10, 12, 15, or 18 were homogenized in 4 mM EDTA, 2 mM phenylmethylsulfonyl fluoride (PMSF) in phosphate-buffered saline (PBS; pH 7.4) and stored at −80°C until analyzed.

**Expression of PKC in Epithelial- and Fiber-Enriched Fractions.** Samples enriched in lens epithelial cells or enriched in fiber cells were prepared taking advantage of the fact that most epithelial cells (and some cells from the annular pad) remain attached to the lens capsule when the latter is stripped off the lens; thus, lens capsule was stripped off using two pairs of tweezers, and the capsule (with the attached epithelium) and the remainder of the lens (mainly fiber cells) were collected in separate tubes. These samples were homogenized as described earlier.

**PKC Expression in Lens Cultures.** Lentoid-containing cultures were harvested in 4 mM EDTA and 2 mM PMSF in PBS and centrifuged at 14,000g for 7 minutes. The pellets were homogenized in PBS containing 4 mM EDTA and 2 mM PMSF.

**Activation of PKC Isoenzymes in Lens Cultures.** Demonstration of activation of PKC isoenzymes by translocation from a soluble compartment to a membrane-associated pool was assessed by an adaptation of the method of Oehrlein et al. Lentoid-containing cultures were left untreated, or they were treated with 200 nM TPA for 15 to 30 minutes. Cultures were rinsed with 2 mM EDTA and 2 mM PMSF in PBS, and cells were harvested in the same buffer and centrifuged at 350g for 10 minutes at 4°C. Cells were then homogenized in 10 mM HEPES, 2 mM EDTA, 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 1 mM PMSF, 1 μg/ml leupeptin, 1 μg/ml apro tin, 2 μg/ml chymostatin, and 1 μg/ml pepstatin (pH 7.5) with 15 to 20 strokes in a Potter-Elvehejm homogenizer (Fischer Scientific, Pittsburgh, PA). Homogenates were centrifuged at 100,000g for 1 hour, and the supernatants were separated from the pellets and analyzed by immunoblot analysis to detect soluble, non-membrane-bound PKC (termed the soluble fraction). The pellets were resuspended in homogenization buffer supplemented with 1% Triton X-100, sonicated, and centrifuged at 100,000g for 1 hour, and the supernatants were separated from the pellets. These supernatants, corresponding to the detergent-soluble fraction of the pellets, were analyzed by immunoblot analysis to detect PKC associated with membrane fractions (termed the membrane fraction).

**Immunoblot Analysis**

Proteins were separated on sodium dodecyl sulfate (SDS)–containing 8% polyacrylamide gels according to Laemmli. Proteins were electrotransferred onto membranes (Immobilon P; Millipore, Bedford, MA) using a semi-dry transfer apparatus (Bio-Rad, Richmond, CA), as previously described. After transfer, membranes were incubated in 5% nonfat dry milk in Tris-buffered saline (TBS; pH 7.4) for 1 hour at room temperature and then incubated in anti-PKC α antibody at 1:400, anti-PKC γ antibody at 1:500, anti-PKC ε antibody at 1:500, anti-PKC ι antibody at 1:500, or anti-PKC μ antibody at 1:1000 overnight at 4°C. Membranes were rinsed in TBS several times and incubated in a 1:4000 dilution of peroxidase-conjugated donkey anti-mouse IgG antibodies for 1 hour at room temperature. Membranes were then rinsed several times with TBS, and the immunoreactive complexes were detected using enhanced chemiluminescence (Amersham, Arlington Heights, IL), according to the manufacturer’s directions. All antibody dilutions were made in 5% nonfat dry milk in TBS. Densitometric analyses were performed using a flat-bed scanner (ScanJet 6100C/T; Hewlett Packard, Greeley, CO) and quantitation was performed using image analysis software (Photoshop 4.0; Adobe, San Jose, CA). For the studies on developmental variation of PKC levels, the density values obtained for each time point were expressed as a percentage of the value obtained for the sample taken on the seventh embryonic day. In the case of the anti-PKC μ antibody, only the band comigrating with that of the positive control (a Jurkat cell lysate or a rat brain lysate) was considered for these calculations. Data are presented as the mean ± SEM.
Immunofluorescence

Chicken lenses obtained at embryonic day 12 were fixed in 4% paraformaldehyde in PBS for 2 hours at room temperature and then transferred to 30% sucrose in PBS until they sank. Twelve-micrometer cryostat sections were obtained and stained for the different PKC isoenzymes. Chicken lens cells were plated on a four-well chamber, (Permanox slide; Nunc, Naperville, IL) and allowed to differentiate into lentoids for 14 to 17 days. After that time, cultures were left untreated or treated with 0.004% dimethyl sulfoxide (as a control for phorbol ester solvent) or 200 nM TPA for 15 to 30 minutes. Cells were then fixed in 2% formaldehyde in 100 mM lysine-HCl, 10 mM sodium m-periodate, and 50 mM sodium phosphate (pH 7.4) for 30 minutes at room temperature and rinsed with PBS. For confocal images, lentoids were transferred to glass slides after fixation. Fixed cells were permeabilized in 0.1% Triton X-100 in PBS for 30 minutes at room temperature. Fixed sections or cultures were incubated in 10% normal goat serum and 0.075% Triton X-100 in PBS (blocking solution) for 30 minutes at room temperature. They were then incubated overnight at 4°C in mouse monoclonal anti-PKC isoenzyme antibodies diluted in blocking solution (anti-PKCα, 1:100 for lens sections and 1:250 for lens cultures; anti-PKCγ, 1:100 for lens sections and 1:500 for lens cultures; anti-PKCε, 1:200; and anti-PKCι, 1:200). Specimens were rinsed several times in PBS and incubated in Cy3-conjugated goat anti-mouse IgG diluted in blocking solution (1:200 for lens sections and 1:750 for lens cultures) at room temperature. After 1.5 hours, specimens were rinsed in PBS as described. Coverslips were mounted using 2% n-propylgallate in PBS-glycerol (1:1). Slides were observed under a microscope (Axiophot; Carl Zeiss, Thornwood, NY) equipped with epifluorescence or a confocal microscope (LSM 410; Zeiss) equipped with an argon-krypton laser.

Dye Coupling

Intercellular coupling between lentoid cells in untreated, 4-α-phorbol- or TPA-treated cultures was assessed by microinjection of Lucifer yellow (1% in water) into a lentoid cell using a picoinjector (Nikon, Garden City, NY). The transfer of dye to neighboring cells was observed using a Diaphot inverted microscope (Nikon) equipped with epifluorescence and Hoffman modulation contrast optics (Nikon). The number of coupled neighboring cells was quantitated 1 minute after injection of the dye.

Statistical analysis of dye coupling was performed using an unbalanced mixed-model analysis with a fixed effect for the treatment and random effects for the batch date and treatment–batch date interaction. The random effects were estimated and tested using least-squares estimation and Tukey's simultaneous tests. The software was used for analysis through the Division of Biostatistics at Washington University (SAS Proc Mixed; SAS Institute, Cary NC).

Measurement of Proteins

Proteins were measured by the method of Bradford using a commercial protein assay (Bio-Rad, Richmond, CA).
RESULTS

Characterization of PKC Isoenzymes Expressed in the Lens

To identify the PKC isoenzymes expressed in the lens and to study the variation of their relative levels during development, immunoblots of lens homogenates prepared from embryos of different ages were probed with antibodies specific for different PKC isoenzymes. PKC α, γ, ε, ι, and μ were detected in lens homogenates using isoenzyme-specific antibodies (Fig. 1). Specificity of the bands observed was confirmed by comigration with the specific PKC isoenzyme detected in a Jurkat cell lysate, used as a positive control. Two, or sometimes three, additional bands that did not comigrate with that present in the positive control were detected with the anti-PKC μ antibody. No immunopositive band was detected using the anti-PKC β antibody (not shown). Densitometric analysis performed on immunoblots from four different experiments demonstrated that the relative levels of PKC α, γ, ι, and μ decreased during development (Fig. 1). The extent of the decrease depended on the PKC isoenzyme. The relative levels of PKC α decreased on average by 58% between the 7th and the 18th embryonic days. During the same period, the relative levels of PKC γ, ε, and μ decreased 70%, 89%, and 82%, respectively. Thus, levels of PKC ι showed the most dramatic decrease between the 7th and the 18th embryonic days. Levels of PKC ε remained relatively constant during the developmental period studied.

The distribution of the PKC isoenzymes expressed in the lens was studied by immunofluorescence on lens sections using isoenzyme-specific anti-PKC antibodies. Because the anti-PKC μ antibody recognized more than one band in immunoblots, no results of localization of immunoreactivity are presented for this antibody. Immunopositive staining with anti-PKC α, γ, ε, and ι antibodies was observed in the epithelium...
Some positive immunoreactivity was also observed along the posterior aspect of the fiber cells (Fig. 2F). The immunoreactivity observed in these areas was rather diffuse and had a cytoplasmic localization; its intensity was much higher in the epithelium and annular pad than along the posterior aspect of the fiber cells. Immunopositive staining at the plasma membrane of fiber cells was observed with the anti-PKC antibody (Fig. 2C, inset), but its intensity was much lower than that observed in the epithelium (compare the inset in Fig. 2C with Fig. 2C).

The differential localization of PKC isoenzymes in the lens was also examined by immunoblot analysis of samples enriched in epithelium or samples containing primarily fiber cells. When these samples were analyzed by immunoblot analysis using anti-PKC α, γ, ε, δ, or µ antibodies, a distinct band, comigrating with that of a positive control, was detected in the epithelium sample and an extremely light band was observed in the fiber cell sample (Figs. 3A, 3D, 3E). When anti-PKC γ or ε antibodies were used, distinct bands were observed in both samples with levels being higher in the epithelium-enriched sample (Figs. 3B, 3C). These results are in agreement with the distribution of PKC isoenzymes observed by immunofluorescence, although the differences in PKC γ and ε levels between the two samples were not as pronounced as those expected from the immunofluorescence results.

Characterization of PKC Isoenzymes Expressed in Lentoid-Containing Cultures

Identification of the PKC isoenzymes expressed in lentoid-containing cultures prepared from embryonic chicken lenses was performed by immunoblot analysis. A band that comigrated with that detected in samples from lens homogenates was observed when using anti-PKC α, γ, ε, δ, or µ antibodies (Fig. 4). No PKC β was detected (not shown).

Effect of TPA Treatment on Translocation of PKC Isoenzymes

Activation of PKC is associated with translocation of the isoenzyme(s) from a cytosolic compartment to a membrane compartment.
To study which PKC isoenzyme(s) expressed in lentoid cells might be activated by TPA treatment, lens cultures were treated with TPA for 15 to 30 minutes, and translocation of PKC isoenzymes was studied by immunoblot analysis after separation of homogenates into soluble and membrane fractions. Under control conditions, all the PKC isoenzymes expressed in lens cultures were detected in the soluble fraction (Figs. 5A through 5E, lanes 1) with no significant PKC levels observed in the membrane fraction (Figs. 5A through 5E, lanes 3). After treatment with TPA, a substantial amount of PKC \( \gamma \) (Fig. 5B, lane 4) was detected in the membrane fraction, whereas its levels in the soluble fraction showed a concomitant decrease (Fig. 5B, lane 2). Levels of PKC \( \alpha \) and \( \mu \) in the soluble fraction also decreased after TPA treatment (Figs. 5A and 5E, lanes 2), but only a minor proportion of these isoenzymes was detected in the membrane fraction (Figs. 5A and 5E, lanes 4). TPA treatment induced no redistribution of PKC \( \epsilon \) or \( \iota \) between the soluble and membrane fractions—that is, these isoenzymes remained in the soluble fraction (Figs. 5C, 5D).

**Effect of TPA Treatment on the Cellular Distribution of PKC Isoenzymes**

The experiments of translocation of PKC isoenzymes indicated that PKC \( \alpha \), \( \gamma \), and \( \mu \) were activated by TPA treatment. Because lens cultures contain epithelial-like (cobblestone) cells and lentoid cells, it was of interest to study the distribution of the different PKC isoenzymes in these cultures under control conditions and after TPA treatment. Immunofluorescence studies on lentoid-containing cultures demonstrated the presence of weak diffuse cytoplasmic staining with the anti-PKC \( \alpha \) antibody in epithelial-like and lentoid cells (Fig. 6A) under control conditions. A more intense diffuse cytoplasmic immunoreactivity was observed in lentoid cells with the anti-PKC \( \gamma \) antibody (Fig. 6C), whereas epithelial-like cells were devoid of positive immunoreactivity. After treatment with TPA, the distribution of immunoreactivity observed with the anti-PKC \( \alpha \) antibody seemed localized to a more perinuclear region in epithelial-like cells (not shown), whereas no significant changes in the distribution of PKC \( \gamma \) in lentoid cells were observed (Fig. 6C). In contrast, a marked difference in the distribution of the immunopositive staining obtained with the anti-PKC \( \gamma \) antibody was observed in lentoid cells after treatment with the phorbol ester. The immunopositive staining localized to the plasma membrane (Fig. 6D).

**Effect of TPA Treatment on Intercellular Communication between Lentoid Cells**

To examine whether TPA induced any changes in function of lentoid gap junctions, dye coupling between lentoid cells was evaluated in lens cultures left untreated (control) or treated with TPA or its inactive analogue, 4-\( \alpha \)-phorbol. Extensive transfer of Lucifer yellow from an injected lentoid cell to its neigh-
bors was observed under control conditions (Figs. 7A, 7C), in agreement with previously published data. Treatment of lens cultures with 200 nM TPA for 30 minutes (Figs. 7B, 7D) induced a dramatic and statistically significant decrease in dye transfer. Dye passed from the microinjected cell to an average of 19 ± 2 neighboring cells in control cultures (n = 130), but only to 8 ± 3 (n = 65) neighbors in cultures treated with TPA (P < 0.05). Treatment of cultures with 200 nM 4-α-phorbol had no significant effect on dye coupling.

**DISCUSSION**

In this article, we have provided the first documentation of distribution and levels of PKC isoenzymes in the lens: multiple PKC isoenzymes were expressed in the embryonic chicken lens, and isoenzyme-specific variations in their levels occurred during development. We found that most isoenzymes were present in the cytoplasm and were especially concentrated in epithelial cells, whereas a minor proportion of PKC γ was present at fiber cell membranes. The specificity of PKC isoenzyme expression was retained by lens cells in culture. Moreover, activation of PKC γ correlated with a decrease in the intercellular communication between lentoid cells.

A few publications have reported the presence of PKC isoenzymes in lens epithelial cells in culture. Epithelial cells, as well as lentoids, contain PKC α in cultures derived from sheep lenses. PKC α and γ have been detected in cultured bovine lens epithelial cells, in which PKC α is the major isoenzyme. In agreement with these in vitro studies, we also detected PKC α and γ in epithelial cells by immunofluorescence staining of sections of chicken lenses. Because PKC isoenzymes in the lens were expressed at higher levels in epithelial cells than in fiber cells, a change in expression of PKC isoenzymes must take place during differentiation of bow region cells into fiber cells. This change in expression of PKC isoenzymes during differentiation and the increased concentration of crystallins in lens fiber cells may explain in part the observed decrease in the relative levels of several PKC isoenzymes with increasing embryonic age. The less pronounced difference in levels of PKC γ observed in immunoblots of epithelium- or fiber cell–enriched samples are in concordance with its presence in fiber cells as detected by immunofluorescence. Similar results were obtained by immunoblot analysis for PKC ε; however, we could not detect immunopositive staining for this isoenzyme in fiber cells, except in their posterior aspect, nor could we detect changes in its relative levels during development. It is possible that accessibility of the monoclonal anti-PKC ε antibody to its epitope might have been hindered because of the increased concentration of crystallins in fiber cells; a phenomenon that did not affect the other isoenzymes. Alternatively, because fiber cells are so large compared with epit-
The association of PKC isoenzymes with different cellular compartments is dependent on expression and subcellular localization of anchoring proteins for PKC such as receptors for activated PKC (RACKs) and receptors for inactive PKC isoenzymes (RICKs). We speculate that there may be a change in expression of RACKs and RICKs for PKCγ during differentiation, because this isoenzyme was localized in the cytoplasm of epithelial cells, but at least in part at the plasma membrane in fiber cells.

It has been reported that TPA treatment had no effect on dye coupling between lentoid cells in ovine cultures. In contrast, we found that TPA induced a decrease in intercellular communication between lentoid cells in chicken cultures. The explanation for this difference is not clear, but it may reflect a species difference or a difference in the initial state of phosphorylation of the connexins involved. In this respect, a TPA-induced effect on gap junctional intercellular communication in rat cardiac myocytes is only detectable after prior reduction of general protein kinase activity. The PKC isoenzymes involved in the TPA-induced effects could include PKCα, γ, or μ, because all three isoenzymes translocated to a membrane compartment after treatment of chicken lentoid cultures with TPA; however, the immunofluorescence data strongly suggest that PKCγ, which translocated to the plasma membrane of lentoid cells, is the isoenzyme responsible for the TPA-induced effects on intercellular communication. These experiments do not discriminate between a direct effect of PKCγ on lens connexins or an indirect effect through activation of a PKC-γ-dependent pathway. Nevertheless, our previous data argue in favor of a direct effect, because the pattern of Cx56 tryptic phosphopeptides derived from these cultures is changed by TPA treatment, and bacterially expressed Cx56 fusion proteins are phosphorylated in vitro by a preparation of rat brain PKC (a mixture of PKCα, β, and γ). Thus, Cx56 may be a substrate for direct phosphorylation by PKCγ in vivo.

Previous characterization of these cultures showed that lentoid cells mimic differentiating fibers in the lens. Thus, we speculate that the TPA-induced decrease in intercellular communication between lentoid cells may reflect effects that occur after activation of PKC isoenzymes in differentiating fibers in vivo. Measurement of coupling in mature and differentiating fibers of frog and rat lenses has demonstrated that coupling in differentiating fibers is two to four times greater than that measured in mature fibers. Thus, Cx56 tryptic phosphopeptides derived from these cultures is changed by TPA treatment, and bacterially expressed Cx56 fusion proteins are phosphorylated in vitro by a preparation of rat brain PKC (a mixture of PKCα, β, and γ). Thus, Cx56 may be a substrate for direct phosphorylation by PKCγ in vivo.

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