DEVELOPMENTAL BIOLOGY **191,** 88–102 (1997)
ARTICLE NO. DB978703

# The Differential Effects of 12-*O*-



# Junctions and Connexins of the Developing Mammalian Lens

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**Epithelial cells in primary ovine lens cultures express the gap junction proteins connexin43 (Cx43) and connexin49 (Cx49; a.k.a. MP70), a homologue of mouse connexin50. In contrast, lens cultures of differentiated, fiber-like cells (termed lentoid cells) express Cx49 and connexin46 (Cx46), but not Cx43. To investigate the regulation of lens cell gap junctions by protein kinase C (PKC), differentiating lens cultures were treated with the PKC activator 12-*O*-tetradecanoylphorbol-13-acetate (*β*-TPA). Within 10 min, *β*-TPA significantly inhibited the transfer of Lucifer Yellow dye between epithelial, but not lentoid, cells. This inhibition was correlated with the phosphorylation of Cx43 and was followed by the gradual disappearance of Cx43 from cell interfaces. The protein kinase inhibitor staurosporine prevented Cx43 phosphorylation and the loss of Cx43 from intercellular junctions. Following treatment of cultures with *β*-TPA for 2–6 hr, Cx49 disappeared from epithelial cell interfaces, and by 24 hr of *β*-TPA treatment, levels of Cx49 detected on immunoblots of purified epithelial membrane fractions had also diminished significantly. The *β*-TPA-induced loss of Cx49 both from regions of epithelial cell contact and from isolated membranes was correlated with the disappearance of Cx49 mRNA. In contrast to the epithelial connexins, the lentoid connexins Cx49 and Cx46 were unaffected by even extended *β*-TPA treatment. In spite of lentoid dye transfer being refractory to *β*-TPA, significant levels of PKC-*α* (a *β*-TPA-sensitive isoform) were detected in the lentoid cell. The response of lens gap junctions to *β*-TPA depends upon the stage of differentiation and the complement of connexins expressed. The contrasting effects of *β*-TPA on Cx43 and Cx49 in lens epithelial cells indicate a fundamental difference in the regulation of these connexin proteins in the developing mammalian lens.** © 1997 Academic Press

***Key Words:*** **Lens; cultures; gap junctions; connexins; *β*-TPA; communication; differentiation.**

## INTRODUCTION

Gap junction proteins (termed ''connexins'') comprise a large family of transmembrane proteins (Bennett *et al.*, 1991; Beyer, 1993) which assemble to form hemichannels (connexons) (Musil and Goodenough, 1993). Hemichannels in adjacent cell membranes dock with one and other to mediate the intercellular transfer of various ions and low-molecular-weight molecules (Beyer, 1993). A cluster of these channels at a cell interface, as revealed by freeze fracture electron microscopy, is referred to as a gap junction (Beyer, 1993). The various connexin genes are differentially expressed during development (Dermietzel *et al.*, 1989; Fromaget *et al.*, 1990; Kumar, 1991) and in adult tissues (Dermietzel *et al.*, 1989). Different connexin proteins can be expressed in the same tissue (Kumar, 1991), be present in the same gap junction (Traub *et al.*, 1989), or, as indicated by experiments using paired oocytes, form heterotypic channels with each hemichannel consisting of a distinct connexin (White *et al.*, 1995b). In addition, there is now strong evidence that connexin heterooligomers exist (Stauffer, 1995). The diversity and differential expression of connexins, together with differences in the permeability (Elfgang *et al.*, 1995) or regulatory characteristics of the assembled hemichannels (Bennett *et al.*, 1991), are likely important in the response of a tissue to the changing cellular environment associated with development, differentiation, or disease.

To study the regulation of mammalian lens gap junctions, we previously established and characterized primary cultures of differentiating ovine lens cells in which at least three different connexin proteins are expressed: connexin43

0012-1606/97 $25.00

(Cx43), connexin49 (Cx49 or MP70), a homologue of mouse connexin50, and connexin46 (Cx46) (TenBroek *et al.*, 1994). Cx43 has been localized to gap junctions of the anterior, epithelial layer of the lens (Musil *et al.*, 1990a; Paul *et al.*, 1991), while Cx49 and Cx46 have been colocalized to junctions of the differentiating fiber cells (Paul *et al.*, 1991; TenBroek *et al.*, 1992; White *et al.*, 1992), which constitute the bulk of the tissue. Lens epithelial cells in culture initially produce Cx43, but begin to also produce Cx49 after 5–7 days. As the epithelial cells continue to differentiate, they become enlarged and translucent and express both Cx49 and Cx46, but not Cx43. These highly differentiated lens cells are referred to as ''lentoid,'' or fiber-like, cells (TenBroek *et al.*, 1994). The progression of connexin expression and differentiation in culture is likely analogous to the differentiation of the epithelial cells in the equatorial zone of the intact lens. In the extensive gap junctions of the lentoid cells, immunofluorescently labeled Cx46 and Cx49 completely colocalize (TenBroek *et al.*, 1994), as is found in lens fiber cell membranes *in vivo* (Paul *et al.*, 1991; TenBroek *et al.*, 1992; White *et al.*, 1992). The dye coupling of cultured epithelial and lentoid cells can be inhibited by treatment with octanol (TenBroek *et al.*, 1994), which is characteristic of gap junctions in many other tissues (Bennett *et al.*, 1991).

Cx43, Cx49, and Cx46 of ovine lens cultures are all phosphoproteins (Arneson *et al.*, 1995; TenBroek *et al.*, 1994). In various tissues or cell types the phosphorylation of Cx43 following the activation of certain kinases has been temporally correlated with the inhibition of intercellular communication (Berthoud *et al.*, 1993; Brissette *et al.*, 1991; Lau *et al.*, 1992). Cx43 is detected as a phosphorylated species in both intact chick lens and in chick lens cultures (Musil *et al.*, 1990a; Musil and Goodenough, 1991), and the phosphorylation of Cx43 in cultures of bovine lens epithelial cells has been correlated with an inhibition of dye transfer following treatment with the protein kinase C (PKC) activator 12-*O*-tetradecanoylphorbol-13-acetate (β-TPA) (Reynhout *et al.*, 1992). β-TPA has also been found to inhibit *de novo* assembly of gap junctions, presumably through the activation of PKC (Lampe, 1994). Less is known about the phosphorylation of either Cx46 or Cx49, although the Cx46 homologues Cx56 (chick) and Cx44 (bovine) are both modified by phosphorylation by presently unidentified protein kinases (Berthoud *et al.*, 1994; Gupta *et al.*, 1994; Jiang *et al.*, 1993).

In the studies described here, we show that lens cell development in culture and the differential expression of Cx43, Cx49, and Cx46 affects the responsiveness of gap junctions to β-TPA. Although β-TPA significantly affects gap junction communication mediated by either Cx43 or Cx49 in the lens epithelial cell, the tumor promoter differentially affects these two connexins, indicating alternative regulatory effects following the activation of PKC. Unexpectedly, gap junctional communication between the more differentiated lentoid cells, which express Cx49 and Cx46, is not affected by β-TPA, thus indicating that differentiation in this instance leads to a loss in sensitivity of lens cell gap junctions to PKC.

## MATERIALS AND METHODS

***Materials.*** Sheep eyes were obtained from either Farmstead Foods (Albert Lea, MN) or John Morrell (Sioux Falls, SD). Hanks' balanced salts solution with calcium and magnesium (HBSS), trypsin, Medium 199 (M199), and penicillin–streptomycin were from Gibco (Grand Island, NY); fetal bovine serum (FBS) and Fetal Clone II from Hyclone (Salt Lake City, UT); formaldehyde from Polysciences (Warrington, PA); β-TPA; and 4-α-phorbol 12,13-diacetate (α-TPA) from LC Services (Woburn, MA); and rhodamine dextran (3000 $M_r$, anionic, lysine fixable) from Molecular Probes (Eugene, OR). Enhanced chemiluminescence solutions (ECL) were from Amersham (Arlington Heights, IL). Radiochemicals [α-$^{32}$P]dCTP and [$^{35}$S]methionine were obtained from ICN (Costa Mesa, CA); [$^{32}$P]-orthophosphate from NEN (Boston, MA). Nitrocellulose (0.2 μm) was from Schleicher & Schuell (Keene, NH), Zeta Probe nylon membrane from Bio-Rad (Hercules, CA). Paraphenylenediamine, poly-DL-ornithine, phenylmethylsulfonyl fluoride (PMSF), Protein A or G Sepharose 4B, x-IgM agarose, and all other chemicals, unless stated otherwise, from Sigma Chemical Co. (St. Louis, MO).

***Lens cultures and Cx50-transfected HeLa cells.*** Lens cultures were prepared as described previously (TenBroek *et al.*, 1994), except that in the present study a trypsin concentration of 0.25% was used in most experiments to avoid including any outer cortical fiber cells in the epithelial cultures. Cx50-transfected HeLa cells, generously provided by Dr. Klaus Willecke, were transfected as previously described for Cx45-transfected HeLa cells (Elfgang *et al.*, 1995).

***Microinjection of cultured lens cells and transfected HeLa cells.*** Lens epithelial and transfected HeLa cells were microinjected iontophoretically with a 3.4% solution (wt/vol) of Lucifer Yellow CH either by repeated 0.1-sec pulses of a 1.1-nA current or by overcompensation of the negative capacitance circuit in the amplifier until the impaled cell was brightly fluorescent. Lentoid cells were similarly injected, but with a 2% Lucifer Yellow solution containing 5% rhodamine dextran. The fluorescent dextran served to mark these irregularly shaped, differentiated cells and allowed for a more accurate determination of dye transfer from the injected cell. A Zeiss IM35 fluorescence microscope with a mercury lamp and a DAGE SIT camera were used to monitor the movement of dye from injected to neighboring cells and the results were recorded on videotape for 3 min.

***Membrane preparation.*** Lens membranes were purified as described previously, through sucrose gradient fractionation following extraction with 4 and 7 *M* urea (Louis, 1989). The buffered solutions used included a protease inhibitor mixture of 0.5 m*M* diisopropylfluorophosphate, aprotinin (1 mg/L), pepstatin (1 mg/L), leupeptin (1 mg/L), 0.5 m*M* benzamidine, 1 mM EGTA, and 1 m*M* EDTA. Lens membrane preparations were frozen in 10 m*M* Hepes, 10% sucrose (pH 7.0) and kept at −70°C until use.

To isolate membranes from lens cultures, cells were scraped into 4°C Tris buffer (25 m*M* Tris, 100 m*M* NaCl, 10 m*M* EDTA, 0.02% sodium azide, 50 m*M* NaFl, 0.5 m*M* $NaVO_4$, pH 8.0) containing 2 m*M* PMSF, pepstatin (1 mg/L), leupeptin (1 mg/L), and aprotinin (1 mg/L), pelleted in a microfuge, and homogenized in fresh Tris buffer using a 27-gauge tuberculin syringe. Membranes were then centrifuged at 100,000*g*, resuspended in 10% sucrose, 10 m*M* Hepes buffer (pH 7.3), and either used immediately or stored at −70°C.

***Antibodies and cDNA probes.*** An IgM monoclonal antibody specific for sheep Cx49 (6-4-B2-C6) (Kistler *et al.*, 1986) was generously provided by Dr. Jorge Kistler (University of Auckland, N.Z.). Rabbit antibody to residues 360-382 of Cx43 (CT 360) was kindly supplied by Dr. Dale Laird, McGill University, Montreal, Canada.

PKC-$\alpha$ antibody was obtained from Transduction Laboratories, Inc. (Lexington, KY). Peroxidase-, FITC-, and rhodamine-labeled secondary antibodies were products of Boehringer Mannheim (Indianapolis, IN). Full-length cDNA probes to rat heart Cx43 and rat lens Cx46 were generously provided by Dr. Eric Beyer (Washington University, St. Louis, MO) and Dr. David Paul (Harvard University, Boston, MA), respectively. A 510-bp cDNA specific for the C-terminus of ovine Cx49 was kindly provided by Ding I. Yang (University of Minnesota, St. Paul, MN). A 2.0-kb chicken $\beta$-actin cDNA which cross-reacts with ovine $\beta$-actin was kindly provided by Jeff Essner (University of MN).

***Immunoblotting.*** Membrane fractions from lens cultures or whole lens membranes were analyzed by SDS–PAGE and Western immunoblotting as described previously (TenBroek *et al.*, 1994). Primary antibodies were diluted as described in the legends; secondary antibody conjugated to peroxidase was diluted 1:8000–1:10,000 and peroxidase activity was detected using enhanced chemiluminescence (ECL). Results were recorded on Kodak X-OMAT RP or AR film during the first few minutes of the reaction. Autoradiographs and stained SDS–PAGE gels were scanned in grayscale with 12-bit mode using a Umax Power Look Scanner. The images were then analyzed using NIH Image 2.5 software. Load variations were corrected by ratioing the band intensities of autoradiographs with those from the corresponding stained gel.

***Immunofluorescence.*** Cultures were fixed in 3.5% formaldehyde for 20 min at room temperature, rinsed with M199, and permeabilized with 0.1% Triton X-100 in HBSS for 30 min at 37°C. The cultures were then rinsed with HBSS and blocked with M199 + 20% goat serum for 1 hr prior to application of the primary antibody at specified dilutions in the same medium. The cells were immunolabeled for 1–2 hr at 37°C, washed three times with HBSS, and then incubated with a 1:500 dilution of secondary antibody conjugated to rhodamine or fluorescein in the goat serum-containing medium for an hour at 37°C. The culture dishes were finally washed with five changes of HBSS and then covered with a glass coverslip applied over a drop of 100 m*M* Tris buffer (pH 9.0), 90% glycerol containing 0.1% paraphenylenediamine to prevent fading of fluorescence. For most figures, immunofluorescently labeled cultures were viewed on a Zeiss photomicroscope Model 3 under epifluorescence, using a mercury lamp. For confocal microscopy (Fig. 3), a Bio-Rad MRC-600 was utilized which was equipped with a krypton/argon laser, a Nikon Diaphot inverted microscope, and a Nikon 60X Plan Apo objective (NA 1.4). The same brightness and contrast settings were used to collect 1.5- to 2.0-$\mu$m width images of the labeled samples.

***Northern blot analysis.*** Total RNA was isolated using a modification of Chomczynski and Sacchi (1987). Cultures were homogenized in ice-cold 5 *M* guanidine, 0.2 *M* NaOAc (pH 5.4), 0.5% Sarkosyl, 1 m*M* EDTA, and 0.5 *M* $\beta$-mercaptoethanol, extracted three times with 50% phenol:48% chloroform:2% isoamyl alcohol, and the aqueous phase precipitated using $\frac{1}{2}$ vol ice-cold 95% ethanol. RNA was resuspended in either 50 or 100% formamide and 10–15 $\mu$g electrophoresed on a 1% agarose–formaldehyde gel for transfer to a nylon membrane. Blots were prehybridized at 42°C in 50% formamide, 5× SSPE, 2× Denhardt's, 0.5% SDS, 0.1% *N*-lauryl sarcosine, 150 $\mu$g/ml of salmon sperm DNA, and 5% dextran sulfate and then hybridized for 12 hr at 42°C in the same solution containing the radiolabeled probe ($2–5 \times 10^9$ cpm/$\mu$g DNA). Cx49 and $\beta$-actin blots were washed progressively with: (1) 2× SSPE, 0.1% SDS for 20 min × 2 at 20°C; (2) 0.5× SSPE, 0.1% SDS for 20 min at 20°C; and (3) 0.2× SSPE, 0.1% SDS for 20 min ×2 at 50°C. Cx43 blots were washed similarly, except that the final washes were in 0.5× SSPE, 0.1% SDS at 55°C. Radiolabeled blots were exposed to Kodak X AR Omat film, and autoradiographs were analyzed (Molecular Dynamics SI Personal densitometer) using IP Lab Gel Densitometry and Gel Analysis software (Signal Analytics Corp., version 1.5).

***Statistics.*** When possible, data are expressed as a sample mean ± the standard error of the mean (SEM). Standard errors were calculated using the formula: standard deviation (SD)/$\sqrt{n}$. Comparisons of two sample means were made using the Student's *t* test. The two-tailed probability (*P*) values were considered significant when less than 0.05.

## RESULTS

### *β-TPA Inhibits Lens Epithelial, but Not Lentoid, Cell-to-Cell Communication*

To determine whether the tumor promoter and PKC activator $\beta$-TPA inhibits gap junction communication between lens cells, cultures of ovine lens epithelial and differentiating lentoid cells were treated with $\beta$-TPA (50 ng/ml) or the inactive isomer of TPA, $\alpha$-TPA (50 ng/ml), and their ability to transfer Lucifer Yellow dye was measured. $\beta$-TPA dramatically inhibited dye transfer between the epithelial-like cells by 1–2 hr (Fig. 1A). This suppression was significant as early as 10 min after the addition of $\beta$-TPA ($P < 0.03$) (Fig. 1B) and was maintained for the 48 hr during which the $\beta$-TPA was present (Fig. 1A). By 6–12 hr of treatment the originally confluent epithelial cells had acquired a fibroblast-like appearance with few intercellular contacts. Both the inhibition of gap junctional communication and the loss of cell contacts were reversible, but required more than 48 hr of incubation in control medium for a complete recovery (Fig. 1A).

In contrast to the epithelial cells, Lucifer Yellow dye transfer between the lentoid cells in the more differentiated cultures was not significantly attenuated following treatment with 50 ng/ml $\beta$-TPA for differing times up to 48 hr or with 100 ng/ml $\beta$-TPA for 3 hr (Table 1; Fig. 2). The data from 16 experiments were combined, as no differences were found when the results were grouped by the age of the lentoid cells or by the length of time that the cultures were exposed to $\beta$-TPA. The data are expressed as the percentage of injections which did or did not result in dye transfer. The number of cells to which Lucifer Yellow dye transfered was sometimes difficult to assess because of the transparency and the variable size of the lentoid cells. However, the boundaries of the injected cells were visualized by coinjection with rhodamine dextran, and the extent of dye transfer from these cells appeared to be identical in $\beta$-TPA treated, untreated, and $\alpha$-TPA-treated cultures. Treatment of the differentiating cultures with $\beta$-TPA failed to alter the shape of and degree of contact between the lentoid cells (Fig. 2).

### *β-TPA Triggers the Disappearance of Connexins from Lens Epithelial, but Not Lentoid, Cell Interfaces*

To determine whether the observed reduction in gap junctional communication was correlated with a redistribution

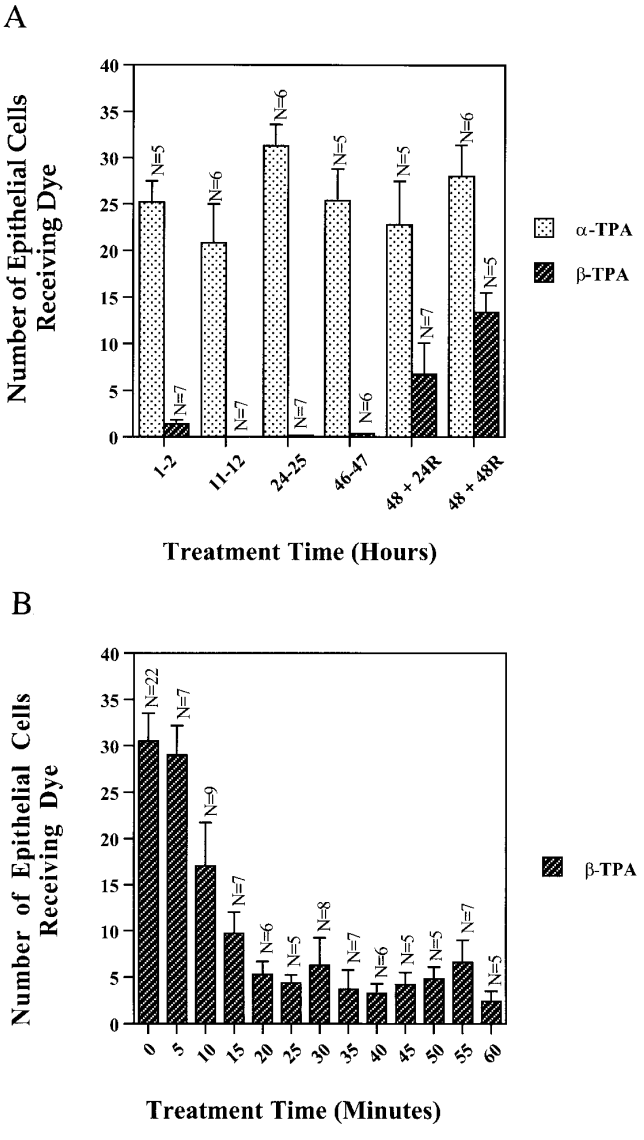


**FIG. 1.** Dye transfer between cultured ovine lens epithelial cells treated with β-TPA. (A) A representative experiment in which dye transfer was measured between the cells of 19-day-old epithelial cultures following treatment with β-TPA (50 ng/ml) (dark) or with the inactive isomer α-TPA (50 ng/ml) (light) for 1–2, 11–12, 24–25, 46–47, 71–72, or 48 hr plus an additional 48–49 hours of recovery time (similar results were obtained in 11 separate experiments). (B) Dye transfer was measured between the cells of 19- to 23-day-old epithelial cultures following treatment with β-TPA (50 ng/ml) for 0–60 min (seven different experiments). Data are expressed as the mean number of neighboring cells receiving dye ± SEM three minutes after microinjection of an epithelial cell with 4% Lucifer Yellow (as described under Materials and Methods).

of connexins within the cell, lens cultures of a similar age and level of differentiation (as assessed by the area of the culture dish covered by lentoid cells) were treated with β-TPA and immunolabeled for Cx43 and Cx49, the connexin proteins previously identified in lens epithelial cell cultures (TenBroek *et al.*, 1994). In 2- to 3-week-old epithelial cell cultures, treatment with β-TPA for 30–120 min resulted in a gradual disappearance of Cx43 from punctate foci at the epithelial cell interfaces (Fig. 3). In contrast, Cx49 did not disappear from epithelial interfaces until 2 hr of β-TPA treatment in 2-week-old cultures (data not shown) and 4–6 hr in 3-week-old cultures (Fig. 3). Cx43 and Cx49 immunofluorescence reappeared at cell interfaces within 48 hr after the removal of β-TPA (Fig. 3).

To examine the connexin distribution in the lentoid cell membrane following treatment with β-TPA, similar experiments were performed with more differentiated cultures. Consistent with the lack of an effect on dye transfer between the lentoid cells, the immunolocalization of both Cx49 and Cx46 in lentoid cell membranes was unaltered by treatment with β-TPA for up to 48 hr (Fig. 4B and data not shown).

Connexins have been found to vary in their permeability to low-molecular-weight dyes (Elfgang *et al.*, 1995), and the permeability characteristics of Cx49 or its homologue, Cx50, are not yet characterized. Thus, to establish whether Cx49 (Cx50) channels could mediate Lucifer Yellow dye transfer, HeLa cells transfected with the mouse homologue of Cx49 (Cx50) were examined by microinjection. Cx50-HeLa cells, grown to 90% confluency, transfered dye to an average of 6.5 ± 1.1 cells 3 min following injection of one cell ($n = 8$). Untransfected HeLa cells showed no dye transfer under identical conditions ($n = 7$). To determine whether Cx50-mediated dye transfer was affected by β-TPA treatment in this cell type, Cx50-HeLa cells were treated with β-TPA (50 ng/ml) for 0–24 hr and dye transfer was measured. As was found with the lentoid cells, β-TPA had no effect on dye transfer between the Cx50-HeLa cells during this time period (not shown).

### *Altered Expression of Cx43 and Cx49 during Differentiation of Lens Cultures*

Immunofluorescent antibody labeling of ovine lens cultures indicates that the epithelial cells initially express

**TABLE 1**

Dye Transfer between Lentoid Cells Following TPA Treatment

| | β-TPA (50 ng/ml) | β-TPA (100 ng/ml) | α-TPA (50 ng/ml) | No treatment |
|---|---|---|---|---|
| $n$ = | 78 | 6 | 24 | 62 |
| Transfer[a] | 91 | 100 | 88 | 94 |
| No transfer | 9 | 0 | 13 | 7 |

[a] Dye transfer between lentoid cells in ovine lens cultures treated with either β-TPA or its inactive isomer α-TPA. Differentiating 1- to 8-week-old lens cultures containing lentoids were treated with β-TPA (50 ng/ml) or α-TPA (50 ng/ml) for 1–48 hr or with β-TPA (100 ng/ml) for 3 hr, and dye transfer was assessed by microinjection of 4% Lucifer Yellow (as described under Materials and Methods). The results represent the compilation of 16 separate experiments, and the data are expressed as the percentage of injections which did or did not result in dye transfer. Rhodamine dextran was used to facilitate the identification of the injected cells.

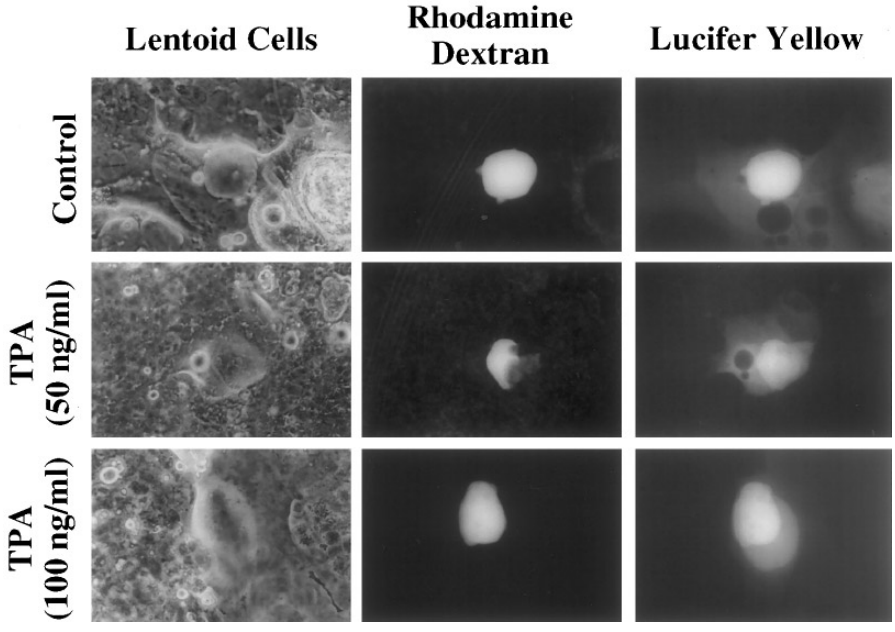


**FIG. 2.** Dye transfer between lentoid cells treated with $\beta$-TPA. Dye transfer was measured between the cells of 14-day-old lens cultures containing lentoids and again following treatment with $\beta$-TPA (50 or 100 ng/ml). The injected cell was ''marked'' with rhodamine dextran.

Cx43 but not Cx49 (data not shown), but that by 5 days in culture, Cx49 is expressed and localized to epithelial cell interfaces (TenBroek *et al.*, 1994). To determine the appropriate stage of lens epithelial development for studying the regulation of both connexins, Cx43 and Cx49 levels were examined by Western immunoblotting of membranes purified from 9-, 16-, and 28-day-old cultures. Densitometric values were obtained from autoradiographs of Western immunoblots and corresponding SDS–PAGE gels to adjust for loading differences. Membranes isolated from newly confluent 9-day-old lens cultures were found to possess significantly more Cx43/mg protein than membranes prepared from 16-day-old cultures, and 16-day-old cultures possessed significantly more Cx43 than did 28-day-old cultures. In contrast, membranes from 9-day-old cultures analyzed by immunoblotting had significantly less Cx49 than 16- or 28-day-old cultures. There was no change in Cx49 levels from 16 to 28 days. Outer cortical cell membranes prepared from sheep lens were included as a negative control for Cx43 and a positive control for Cx49 (Figs. 5A and 5B). These findings were in agreement with results obtained by immunofluorescent antibody labeling of similar cultures (data not shown) and indicated that approximately 2- to 3-week-old cultures would possess sufficient levels of both connexins for further studies.

### *β-TPA Treatment Results in Cx43 Phosphorylation in Lens Epithelial Cultures*

Differentially phosphorylated forms of Cx43 are detected on Western immunoblots as three to four different species with apparent molecular weights of 39 to 47 kDa (Kadle *et al.*, 1991; Musil *et al.*, 1990b). Both a phosphatase-sensitive 45-kDa and an insensitive 42-kDa species of Cx43 were detected by Western immunoblotting of urea-washed membranes isolated from ovine lens epithelial cell cultures (TenBroek *et al.*, 1994). Two additional phosphatase-sensitive Cx43 species of 44 and 47 kDa are resolved on immunoblots of buffer-washed lens epithelial cell membranes (data not shown and Fig. 5A). All three phosphatase-sensitive species are phosphorylated, as indicated by their immunoprecipitation from [$^{32}$P]ATP-labeled ovine lens cultures using Cx43 antibodies (data not shown). The different phosphorylated forms of Cx43 will be referred to here as $P_1$, $P_2$, and $P_3$ (of increasing apparent molecular weight) and the fastest migrating, nonphosphorylated, form as NP.

To determine whether the $\beta$-TPA-induced changes in intercellular communication and Cx43 localization correlate with Cx43 phosphorylation, membranes isolated from $\beta$-TPA-treated lens cultures were analyzed by immunoblotting. An increase in the phosphorylation of Cx43 was observed following $\beta$-TPA treatment, where a decrease in the relative amount of Cx43-NP was accompanied by an increase in the relative amount of Cx43-$P_3$ (Figs. 5A and 6A). The shift lasted from 1–2 hr and could be detected as early as 15 min following $\beta$-TPA treatment (Fig. 6A). While the time at which there was a shift from the NP to the $P_3$ form varied slightly from experiment to experiment, the amount of Cx43-$P_3$ was on average maximal by 30 min following the addition of $\beta$-TPA, while the amount of Cx43-$P_2$ was maximal by 15 min. In all cases the shift preceded the loss of Cx43 from the plasma membrane (Fig. 3) and was coincident

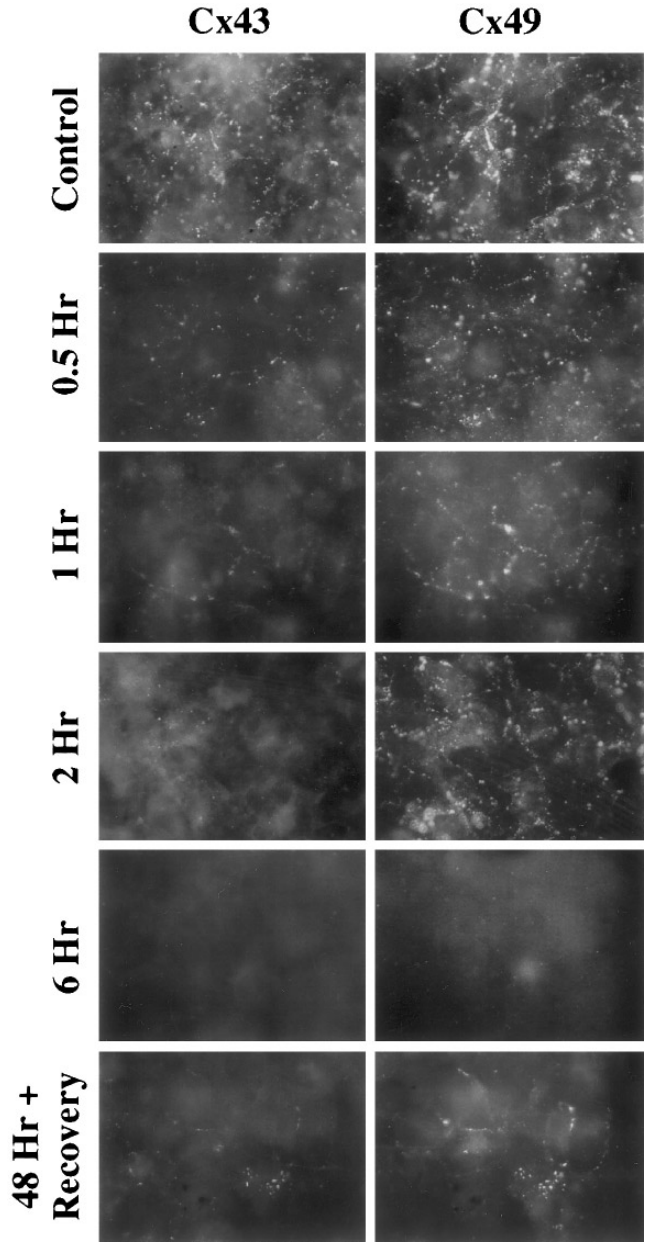


**FIG. 3.** Immunolocalization of Cx43 and Cx49 in 22-day-old β-TPA-treated ovine lens epithelial cells. Cells were incubated with 50 ng/ml β-TPA for 0, 0.5, 1, 2, 6, or 48 hr followed by 48 hr in medium lacking β-TPA or for 3 hr in 50 ng/ml α-TPA and were labeled with both anti-Cx43 (1:500) detected with rhodamine-conjugated secondary antibody (1:500) and anti-Cx49 (1:100) detected with fluorescein-conjugated secondary antibody (1:200), as described under Materials and Methods. The control panel shown is a representative image from the culture treated with α-TPA.

with an inhibition of dye transfer (Figs. 1A and 1B). Treatment of the lens cultures with the inactive isomer α-TPA (50 ng/ml) did not affect the mobility of Cx43 (data not shown). Cx43 $P_1$ recovered to control levels after 4–6 hr of β-TPA treatment (Fig. 6A). When adjusted for protein load, the amount of Cx43 and its phosphorylation pattern following 48 hr of recovery in the absence of β-TPA were always similar to controls (Figs. 5A and 6A).

A reproducible 60% decrease in total Cx43 detected on immunoblots was observed within 15 min of β-TPA treatment ($P < 0.0001$) (Fig. 5B), and the decrease was correlated with the increase in the $P_3$ form of Cx43 (Fig. 6A). Total Cx43 protein recovered to control levels by 60 min of β-TPA treatment (Fig. 6B). Immunocytochemistry revealed no obvious changes in the distribution or intensity of fluorescent labeling of plasma membrane Cx43 at 15–20 min. Although the decrease in total Cx43 protein measured on Western immunoblot could be explained by a rapid degradation of Cx43 from either or both intracellular and plasma membrane fractions, it is more likely that a transient modification of Cx43 affects its antigenicity.

### *Staurosporine Inhibits the Early Effects of β-TPA on Culture Gap Junctions*

Staurosporine is an inhibitor of PKC which has been shown to block the β-TPA-mediated phosphorylation of Cx43 (Berthoud *et al.*, 1992). To determine whether an increase in protein kinase activity was responsible for the β-TPA-mediated changes in gap junction communication and connexin localization in the ovine lens cultures, the effects of β-TPA on dye transfer, Cx43 phosphorylation, and the localization of Cx43 were measured in the presence of different concentrations of staurosporine.

Although 100 n*M* staurosporine was unable to prevent the β-TPA-induced inhibition of dye transfer or to consistently prevent the shift in Cx43 mobility detected by Western immunoblotting, it did significantly inhibit the β-TPA-dependent loss of Cx43 from cell interfaces for up to 2 hr (data not shown). In contrast, 250 n*M* staurosporine relieved the β-TPA-dependent inhibition of dye transfer between the lens epithelial cells by 65% (Fig. 7A); 250 and 400 n*M* staurosporine also inhibited the decrease in Cx43 detected on immunoblots following 30 min of β-TPA treatment (Fig. 7B and data not shown), the shift in Cx43 phosphoforms (Fig. 7B), and the loss of Cx43 from cell interfaces (data not shown). Treatment with the protein kinase inhibitor in the absence of β-TPA had no effect on dye transfer when compared to untreated controls (Fig. 7A) and also did not affect the relative amounts of the different Cx43 forms as determined by SDS–PAGE (Fig. 7B). As measured by trypan blue permeability, staurosporine was toxic to the lens epithelial cells after exposure times of 4 hr or more. Since significant changes in Cx49 were not consistently observed until after 4 hr of β-TPA treatment, staurosporine was used only to study those β-TPA-dependent changes in Cx43 which occurred prior to 4 hr of treatment.

### *β-TPA Treatment of Lens Epithelial Cultures Results in a Loss of Cx49 from Purified Membranes*

Cx49 in isolated lens membranes migrates with an apparent $M_r$ of 64–70 kDa, but in membranes isolated from lens

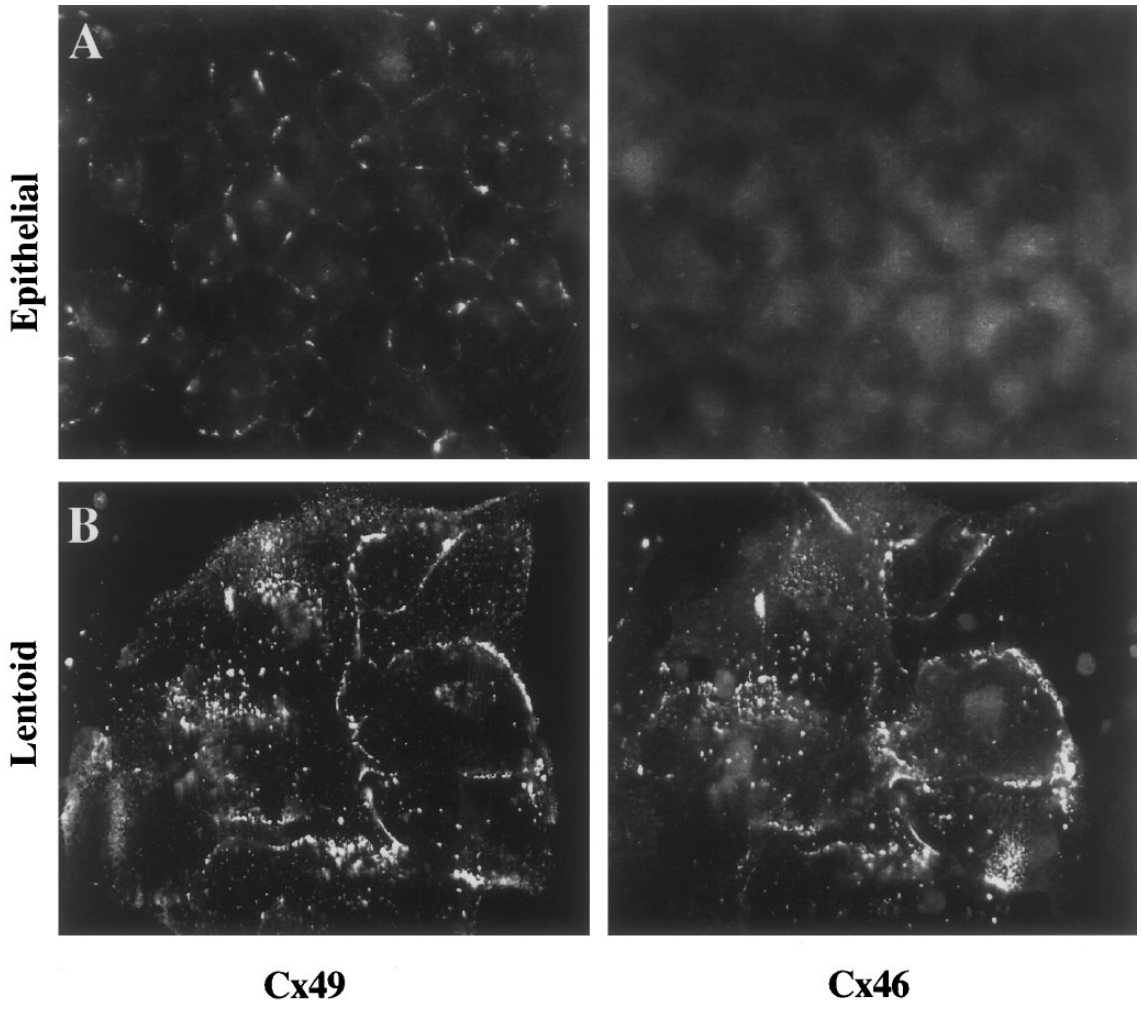


**FIG. 4.** Immunolocalization of Cx49 and Cx46 in untreated and $\beta$-TPA-treated ovine lens epithelial and lentoid cells, respectively. (A) 21-day-old lens epithelial cell cultures were labeled with both anti-Cx49 (1:100) detected with fluorescein-conjugated secondary antibody (1:200) and anti-Cx46 (1:100) detected with rhodamine-conjugated secondary antibody (1:500), as described under Materials and Methods. (B) A representative confocal image of a 20-day-old differentiated lens culture containing lentoids treated with $\beta$-TPA (50 ng/ml) for 24 hr and labeled with both anti-Cx49 (1:100) detected with fluorescein-conjugated secondary antibody (1:200) and anti-Cx46 (1:100) detected with rhodamine-conjugated secondary antibody (1:500).

cultures it migrates as two species of approximately 60 and 64 kDa on SDS–PAGE (TenBroek *et al.*, 1994). Following phosphatase treatment of membranes isolated from either the lens or lens cultures, a fraction of the slower form of Cx49 migrates faster, but the kinase responsible for the altered mobility is unknown (TenBroek *et al.*, 1994). To establish whether the loss of Cx49 from cell interfaces following 4–6 hr of $\beta$-TPA treatment (Fig. 3) was accompanied by changes in either the amount of Cx49 immunoreactivity or a shift in its mobility on SDS–PAGE, Western immunoblots of membranes derived from the 28-day-old $\beta$-TPA-treated lens epithelial cultures were labeled with antibody to Cx49. While there was no evidence of a decrease in Cx49 mobility following treatment of the lens cultures with $\beta$-TPA for 4–6 hr, the amount of Cx49 detected in the purified membranes decreased significantly (30–36% of controls) and was negligible by 24 hr of exposure to $\beta$-TPA (0–15% of controls) (Fig. 5B). Cx49 could again be detected 48 hr after the removal of $\beta$-TPA (Fig. 5B). The small amount of Cx49 detected 24 hr after the addition of $\beta$-TPA (Fig. 5B) may reflect the presence of lentoid cells, since they occasionally appear in differentiating epithelial cultures after 28 days. Thus, Cx49 was similarly examined in 14- to 21-day-old lens epithelial cultures, which are significantly less differentiated as measured by numbers of lentoids. Treatment of the younger cultures with $\beta$-TPA for 2 hr significantly depressed Cx49 levels detected by Western immunoblotting, and the protein was not detectable by 25 hr of $\beta$-TPA treatment. The Cx49 protein levels also failed to recover from $\beta$-TPA treatment as quickly as in 28-day-old cultures. Treatment with $\alpha$-TPA did not signifi-

cantly affect the level of Cx49 (Fig. 8). Thus, Cx49 is more sensitive to β-TPA in the younger, potentially less differentiated, lens epithelial cells.

### *Protein Kinase C Is Detected in both the Epithelial and the Lentoid Cell*

To determine whether the observed loss of gap junction sensitivity to β-TPA in the lentoid was due to a loss of PKC during lens development, differentiating lens cultures were immunofluorescently labeled with antibodies specific for PKC-α, a β-TPA-sensitive isoform of PKC. Both epithelial and lentoid cells showed significant levels of PKC-α labeling (Fig. 9). Although PKC-α typically migrates from the cytosol to the membrane in the presence of β-TPA (50 ng/ml), an obvious translocation of PKC-α was not seen in the lens epithelial cell using immunolabeling and confocal microscopy. PKC-α was found in both cytosol and membranes of untreated lentoid cells. Following treatment with the tumor promoter, less PKC-α was detected in the cytoplasm of the lentoid but no change was detected in the membrane labeling. Membrane label was not detected using secondary antibody alone (not shown). The epithelial and lentoid cells could not be examined individually by protein analysis because it is not yet possible to physically separate the two cell types.

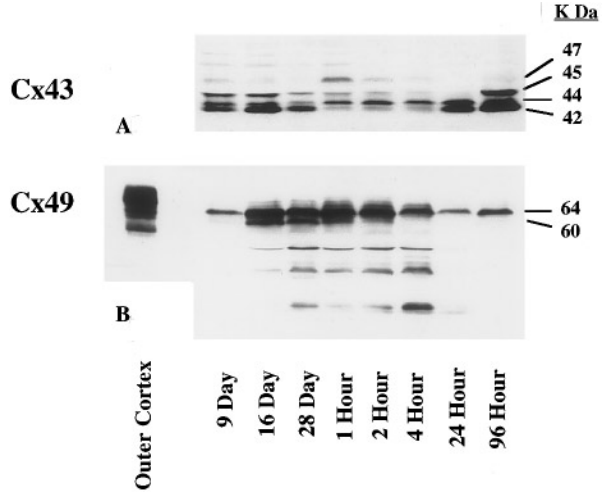


**FIG. 5.** Western immunoblots of Cx43 and Cx49 in ovine lens cell membranes. (A) Immunoblot showing Cx43 in membranes prepared from outer cortical lens fiber cells and from ovine lens epithelial cell cultures at 9, 16, and 28 days and Cx43 in membranes prepared from 28-day-old cultures following treatment with β-TPA (50 ng/ml) for 1, 2, 4, 24, or 96 hr (48 hr in β-TPA and 48 hr in recovery medium). The immunoblot was probed with anti-Cx43 (1:8000), followed by goat anti-rabbit peroxidase-labeled antibody (1:10,000), and the peroxidase was detected by ECL. (B) Immunoblot showing Cx49 in membranes prepared from outer cortical lens fiber cells and from ovine lens epithelial cell cultures after 9, 16, and 28 days in culture and Cx49 in membranes prepared from 28-day-old lens cultures following β-TPA treatment for 1, 2, 4, 24, or 96 hr (48 hr with β-TPA followed by 48 hr without the phorbol ester). The immunoblot was probed with anti-Cx49 (1:350), followed by goat anti-mouse peroxidase-labeled antibody (1:8000).

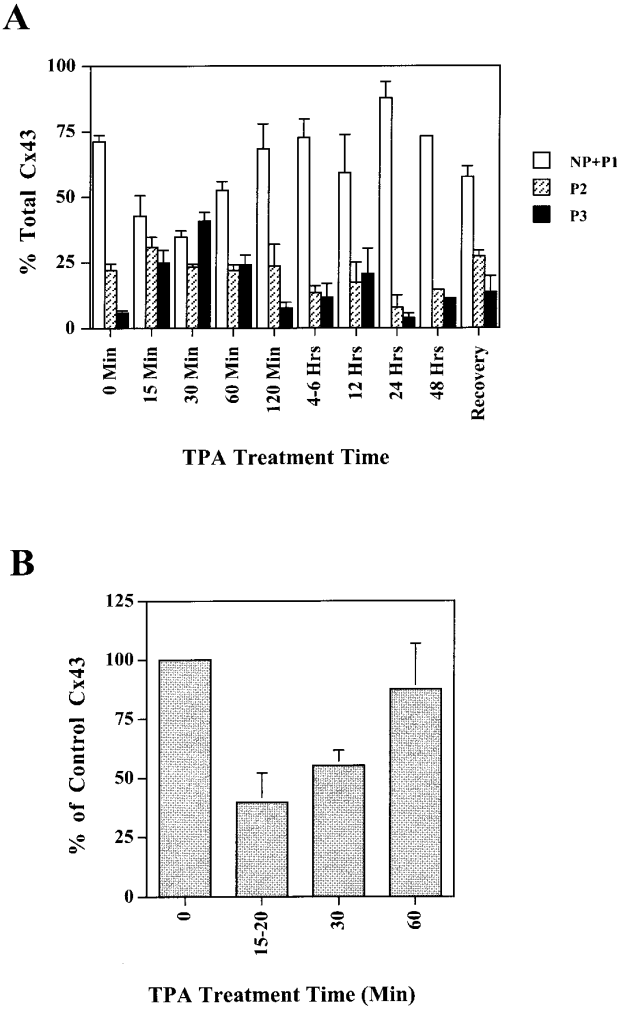


**FIG. 6.** (A) Relative levels of the different phosphorylated forms of Cx43 following treatment of lens cultures with β-TPA. The data represent the mean of 10 experiments in which Western immunoblotting of Cx43 was performed following treatment of 19- to 28-day-old lens epithelial cell cultures with 50 ng/ml β-TPA for the indicated time periods. The immunoblots were probed with anti-Cx43 (1:8000), followed by goat anti-rabbit peroxidase-labeled antibody (1:10,000), and the peroxidase was detected as described under Materials and Methods. The levels of the different phosphorylated forms of Cx43 were analyzed densitometrically and expressed relative to control values. Data are shown as the mean of these values ± SEM. (B) Cx43 levels in ovine lens culture membranes following β-TPA treatment for the indicated times. Values shown are adjusted for sample load and expressed as the percentage of the control levels of Cx43.

### *Cx49, but Not Cx43, Transcript Levels Are Altered by β-TPA Treatment*

The reduced levels of Cx49 protein detected following TPA treatment of the lens cultures could be the result of a

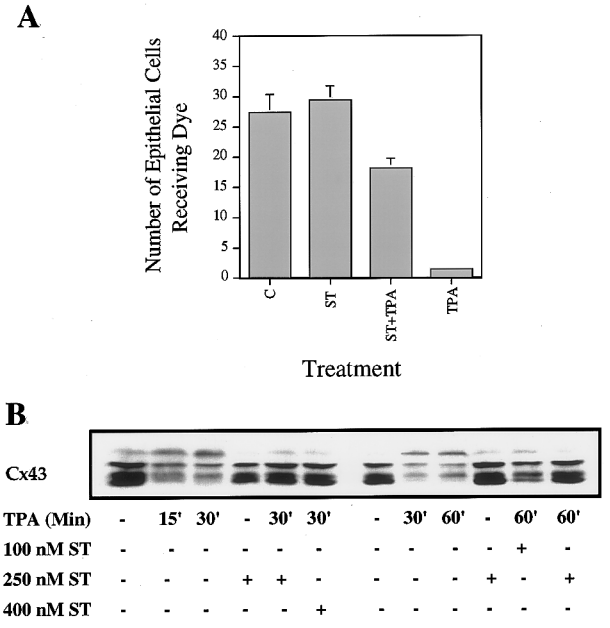


**FIG. 7.** (A) The effect of stauroporine on the $\beta$-TPA-mediated inhibition of dye transfer in ovine lens epithelial cell cultures. 20- to 24-day-old cultures were examined for their ability to communicate by microinjection of 4% Lucifer Yellow prior to and during preincubation with 250 n*M* staurosporine (as described under Materials and Methods). After 30 min of preincubation in staurosporine, $\beta$-TPA (50 ng/ml) was added to the same cultures and, after 1–1.5 hr, cell communication was determined using Lucifer Yellow dye transfer. The mean number of cells (±SEM) receiving dye 3 min after microinjection of Lucifer Yellow is reported for untreated controls (C), cells treated with staurosporine (ST), cells treated with both staurosporine and $\beta$-TPA (ST + TPA), and cells treated with only $\beta$-TPA (TPA). The data from the first three columns represent four separate experiments using two sets of age-matched lens cell cultures. Data reported in the TPA column represent three separate experiments in age-matched cultures. (B) Cx43 in $\beta$-TPA-treated ovine lens culture membranes incubated with or without staurosporine. Western immunoblot of membranes isolated from 21-day-old cultures treated for the indicated times with 50 ng/ml $\beta$-TPA with or without the indicated concentrations of staurosporine (ST). The cultures were pretreated with staurosporine for 30 min and further incubated for the indicated time under "TPA" when both staurosporine and $\beta$-TPA (50 ng/ml) were present.

decrease in transcription. Treatment of cells with phorbol esters has been shown to alter connexin mRNA concentrations in some cell types (Brissette *et al.*, 1991), but not in others (Asamoto *et al.*, 1991; Oh *et al.*, 1991). As an initial step in determining whether there was a significant change in either Cx43 or Cx49 mRNA synthesis following $\beta$-TPA treatment, 18-day-old ovine lens cultures were treated with $\beta$-TPA for either 0–48 or 48 hr and then allowed to recover for 48 hr in medium lacking $\beta$-TPA. RNA was then analyzed by Northern blotting using constituitively produced $\beta$-actin mRNA to normalize Cx43 and Cx49 mRNA levels.

A 3.0-kb mRNA species, which is similar to the size of the message detected in other Cx43-expressing cells (Musil *et al.*, 1990a), was detected in Northern blots of total lens culture RNA using a rat Cx43 cDNA probe. A small but significant increase in steady-state Cx43 mRNA was observed within 2–6 hr of exposure of lens cultures to $\beta$-TPA ($0.001 < P < 0.01$; $n = 4$). Cx43 mRNA remained slightly elevated through 24 hr and then decreased significantly (6 hr is greater than 48 hr, $0.01 < P < 0.02$; $n = 4$) before returning to control levels following removal of $\beta$-TPA (48 hr is less than 96 hr, $0.02 < P < 0.05$; $n = 4$) (Figs. 10A and 10B).

Cx49 mRNA levels were similarly examined. The Cx49 cDNA hybridized to a 6.7-kb mRNA in Northern blots of total RNA isolated from lens cultures. Smaller Cx49-related mRNA species were also routinely detected in spite of the stringent washing conditions (see Materials and Methods) and the apparently nondegraded Cx43 and $\beta$-actin mRNAs. Similar to the disappearance of Cx49 protein following $\beta$-TPA treatment, $\beta$-TPA treatment for 6 hr resulted in a significant decrease in Cx49 mRNA ($P < 0.05$). Message levels were not measurable following 12 hr of exposure to $\beta$-TPA, but Cx49 mRNA could again be detected 48 hr after removal of the $\beta$-TPA (Figs. 10A and 10B).

To confirm the immunofluorescent antibody labeling studies which showed that Cx46 was present only at interfaces between enlarged epithelial cells and lentoids (Fig. 3A), a rat Cx46 cDNA was used to reprobe Northern blots used in the above experiments and also Northern blots of RNA isolated from highly differentiated cultures. This Cx46 probe did not hybridize to Northern blots of control or $\beta$-TPA-treated lens epithelial culture RNA under conditions of low stringency, although it did detect a 2.6-kb message in RNA isolated from more differentiated cultures containing lentoids (data not shown). Thus, the lower kilobase transcripts detected with the Cx49 probe (Fig. 10A) do not appear to represent cross-reactivity with Cx46 mRNA.

## DISCUSSION

The differentiating ovine lens culture system allows for an *in vitro* examination of cell-to-cell communication in the

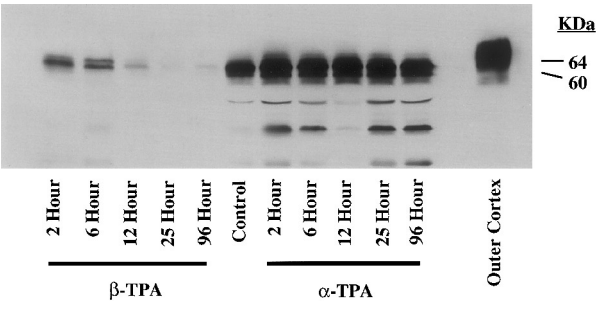


**FIG. 8.** Western immunoblot analysis of the effect of $\beta$-TPA on Cx49 in 14-day-old ovine lens epithelial cultures. Immunoblot of membranes isolated from 14-day-old cultures treated for the indicated times with either 50 ng/ml $\alpha$-TPA or 50 ng/ml $\beta$-TPA. The immunoblot was probed with anti-Cx49 (1:350), followed by goat anti-mouse peroxidase-labeled antibody (1:8000).

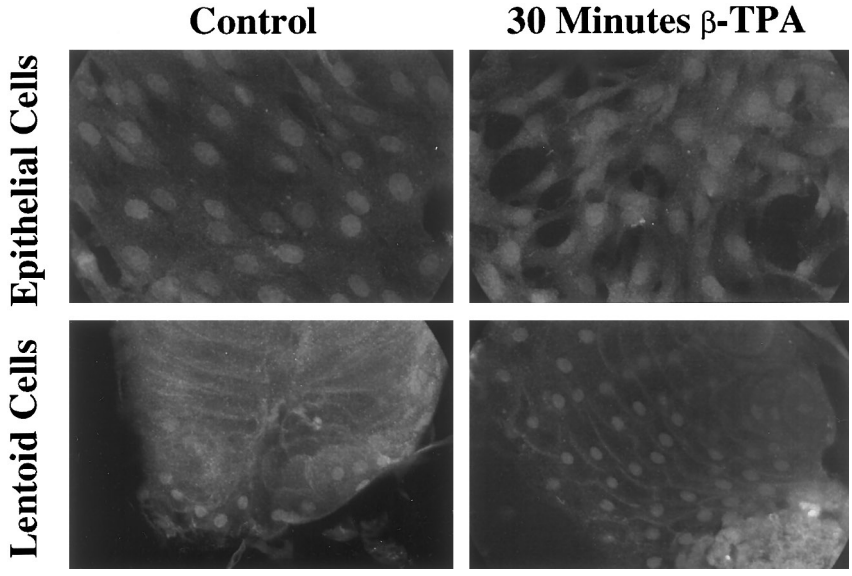


**FIG. 9.** Immunolocalization of PKC-$\alpha$ in untreated and $\beta$-TPA-treated ovine lens epithelial and lentoid cells. Representative confocal images of epithelial and lentoid cells in control or $\beta$-TPA-treated (50 ng/ml; 30 min) differentiating cultures labeled with anti-PKC-$\alpha$ (1:500) detected with rhodamine-conjugated secondary antibody (1:500) as described under Materials and Methods.

developing lens, a tissue which possesses distinct cell types and at least three different connexin proteins (Kistler and Bullivant, 1989; Musil *et al.*, 1990a; Paul *et al.*, 1991; Ten-Broek *et al.*, 1994). By characterizing the effects of $\beta$-TPA on the ovine lens culture system, we have shown that different connexins within the same cell exhibit differential responses to the same protein kinase activator and that the response of a specific connexin to protein kinase activation can differ depending on the stage of lens cell development. Furthermore, these studies indicate that a change in the complement of connexins expressed by the cell during differentiation can significantly alter the regulatory characteristics of cell-to-cell communication mediated by gap junctions.

### *β-TPA Treatment Has Different Effects on the Cx43 and Cx49 Gap Junctions within Lens Cultures*

An important finding of this investigation is that Cx43 and Cx49 respond differentially to a $\beta$-TPA-induced increase in PKC activity in the lens epithelial cell. While $\beta$-TPA initially inhibits intercellular dye transfer mediated by both Cx43 and Cx49, the subsequent effects of $\beta$-TPA on the two connexins are quite different. Both Cx43 and Cx49 disappear from epithelial cell interfaces in response to the activation of PKC, but the loss of Cx43 from interfaces appears to be more rapid. In spite of its disappearance from the surface, a significant level of Cx43 is maintained in the total membrane fraction. In contrast, the loss of immunofluorescently labeled Cx49 from the cell surface correlates with its loss from membrane fractions and is coincident with a decrease in the level of Cx49 mRNA. Cx43 mRNA levels instead show a slight but significant increase which is not coincident with either an increase in intercellular communication or in intracellular Cx43 protein. These contrasting effects indicate that the controls governing the synthesis and turnover of Cx43 and Cx49 fundamentally differ.

The transient increase in Cx43 mRNA levels and the significant decrease in Cx49 mRNA levels following $\beta$-TPA treatment suggest that the synthesis of these two connexins could be regulated by PKC during lens development *in vivo*. Indeed, the 5′-untranslated region of mouse Cx43 contains an AP1 element (Sullivan *et al.*, 1993), to which the protein complex fox-jun binds (Vyas, 1990, No. 524). A recent study indicates that this site controls the induction of Cx43 transcription following PKC activation in uterine smooth muscle (Jiang *et al.*, 1996). Further sequence analysis of the ovine Cx49 gene will indicate whether it too includes any of the known $\beta$-TPA-responsive elements. Differential regulation of connexin synthesis and trafficking to the intercellular interfaces by this tumor promoter has also been demonstrated in other systems (Berthoud *et al.*, 1993; Brissette *et al.*, 1991; Laing *et al.*, 1994). For example, in C9 cells, derived from rat liver epithelium, both Cx26 and Cx43 are lost from cell interfaces following $\beta$-TPA treatment, but following the removal of $\beta$-TPA, Cx43 reappears at interfaces significantly earlier than does Cx26 (Berthoud *et al.*, 1993).

Although Cx43 and Cx49 colocalize to the same gap junction plaques in ovine lens cultures, Cx49 is detected at cell interfaces for up to 5 hr after the $\beta$-TPA-induced loss of Cx43 (Fig. 2 and data not shown). It is unlikely that such an extreme difference can be accounted for by differences in antibody affinity alone. This result suggests that the majority of the lens epithelial connexin channels are homotypic (i.e., Cx49/Cx49 and Cx43/Cx43) rather than hetero-

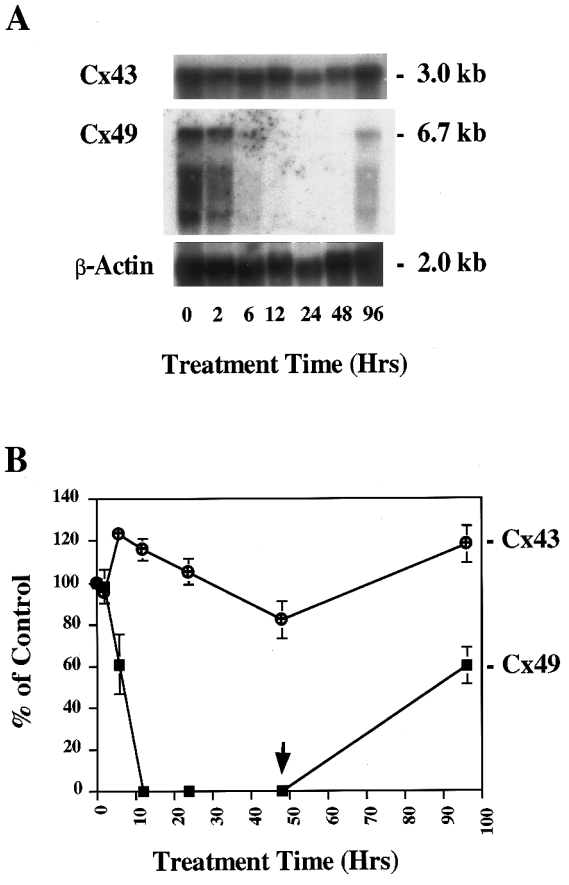


**FIG. 10.** Northern blot analysis of the effect of $\beta$-TPA on Cx43 and Cx49 mRNA in ovine lens epithelial cell cultures. (A) 19-day-old lens epithelial cultures were treated with $\beta$-TPA for 0, 2, 6, 12, 24, 48, or 48 hr, with 48 hr of recovery prior to RNA isolation and Northern blotting (see Materials and Methods). (B) A compilation of four experiments identical to that shown in A, where Cx43 and Cx49 message levels were first calculated as the percentage of control and then normalized relative to the densitometric intensity of the $\beta$-actin message. Data are expressed as the mean ± SEM.

typic (i.e., Cx43/Cx49) and is in agreement with studies which have shown that mouse Cx50 and rat Cx43 expressed in oocytes are unable to form heterotypic channels (White *et al.*, 1995a). Scanning transmission electron microscopy and mass analysis of rodent gap junctions provides evidence consistent with a model in which homomeric channels of two different connexins may segregate into distinct domains within a single gap junction (Sosinsky, 1995). Such a model helps to explain the results of our study in which Cx43 and Cx49 are differentially downregulated in spite of their colocalization.

### *β-TPA-Induced Inhibition of Gap Junction Communication between Epithelial Cells Correlates with the Phosphorylation of Cx43*

A significant decrease in cell-to-cell dye transfer is observed within 15 min of addition of $\beta$-TPA to lens epithelial cultures, although the amounts of immunofluorescently detected Cx43 and Cx49 at cell interfaces appear to be unchanged. Thus, it is likely that $\beta$-TPA treatment leads to a reduction of both Cx43 and Cx49 gap junction permeability. The concomitant change in the level of phosphorylation of Cx43 indicates that the diminished permeability of Cx43 gap junctions may be a result of an increased phosphorylation of this protein and consequent gating of the Cx43 channel. The stimulation of PKC by $\beta$-TPA has been well characterized (Kikkawa *et al.*, 1989), and studies in other systems have also suggested that the phosphorylation of Cx43 is responsible for a decreased communication (Berthoud *et al.*, 1992; Brissette *et al.*, 1991) and a decrease in Cx43 channel conductivity (Kwak *et al.*, 1995a,b) following treatment with $\beta$-TPA. In fact, purified Cx43 has recently been shown to be directly phosphorylated on ser368 by PKC *in vitro* (Lampe *et al.*, submitted for publication). Both the epithelial and lentoid cells express the most prevalent of the TPA-sensitive PKC isoforms, PKC-$\alpha$. The inhibition of $\beta$-TPA effects on Cx43 by preincubation with staurosporine further indicates that phosphorylation is a prerequisite for the $\beta$-TPA-induced inhibition of dye transfer and the loss of Cx43 from the cell surface. More than one protein kinase may be responsible for the different effects of $\beta$-TPA on lens gap junctions, as the lowest concentration of staurosporine tested inhibited the $\beta$-TPA-mediated loss of Cx43 from epithelial cell interfaces but neither completely prevented the shift in Cx43 mobility (increase in phosphorylation) nor the inhibition of dye transfer.

Whereas an increase in the degree of phosphorylation of Cx43 correlates with the inhibition of gap junction communication between lens epithelial cell (e.g., 15 min), it is not clear whether the inhibition of communication by $\beta$-TPA is also associated with an increase in the phosphorylation of Cx49, although Cx49 gap junction permeability to Lucifer Yellow is clearly diminished. These Cx49-containing gap junctions are likely functional, as Cx50-transfected HeLa cells support significant levels of Lucifer Yellow dye transfer, and the amino acid sequence of Cx49 shares an 87% identity with mouse Cx50 (Yang and Louis, 1996). Cx49 in cortical lens membranes is not phosphorylated *in vitro* by PKC, but instead can be phosphorylated by a previously unidentified lens protein kinase (Arneson *et al.*, 1995). It is possible that the permeability of Cx49-containing gap junctions is thus inhibited by another kinase which is activated following $\beta$-TPA treatment. However, phosphorylation of Cx49 may not be required for inhibition of gap junction communication by PKC, as Lucifer Yellow dye transfer was inhibited and Cx26 channels were less frequently observed in Cx26-transfected SKHep cells treated with $\beta$-TPA, despite the fact that Cx26 does not appear to be a phosphoprotein (Kwak *et al.*, 1995a). Determining the molecular basis for the inhibition of Cx49 channel permeability by $\beta$-TPA will require further studies, since the only antibody with high affinity for the Cx49 expressed in ovine lens cultures is the IgM monoclonal antibody 6-4-B2-C6. Although a number of protocols were tested, including a procedure utilizing the mannan-binding protein (Nevens *et al.*, 1992)

(Pierce Biochemicals), Cx49 could not be cleanly immunoprecipitated from either $^{35}$S- or $^{32}$P-labeled ovine lens cultures using this IgM antibody (data not shown). Dye transfer was not inhibited between the Cx50-transfected HeLa cells by β-TPA treatment, so Cx50 phosphorylation was not examined.

### *Differentiation Affects the Regulation of Lens Gap Junctions*

The β-TPA-dependent inhibition of cell-to-cell communication between the epithelial cells but not the lentoid cells in lens cultures demonstrates that lens cell differentiation results in fundamental changes which affect cell-to-cell communication. Certain studies indicate that the response of epithelial and fiber cell gap junctions to agents affecting junctional communication may differ. For example, the inhibition of intercellular communication between lentoid cells requires a higher concentration of octanol than that required to uncouple epithelial cells in both embryonic chick and ovine lens cultures (unpublished data; (TenBroek *et al.*, 1994)). Our study shows that differences in the regulation of communication between epithelial and fiber cells can result from not only the changing expression of connexins during differentiation, but also from the differential regulation of the same connexin in the two cell types.

The replacement of Cx43 by Cx46 in the differentiating lens cultures could change the sensitivity of gap junctions to β-TPA. Evidence for functional differences between these two connexins is provided by experiments in which the expression in oocytes of Cx46, but not Cx43, result in osmotic lysis (Paul *et al.*, 1991). However, the coincident expression of Cx49, which is expressed by the epithelial cells after 5 days and also by the more differentiated lentoid cells appearing after 4–5 weeks in culture, complicates the interpretation of this study. Cx49 is significantly affected by β-TPA in the epithelial cell, but gap junction permeability and the localization and the synthesis of Cx49 are not inhibited by β-TPA in the lentoid cell. Gap junctions have been shown to be regulated differently by the same protein kinase in unrelated cell types (e.g., cAMP-dependent protein kinase (Bennett *et al.*, 1991)), but this report is the first describing a significant change in the regulation of a specific connexin during the differentiation of a single cell type. That dye transfer between Cx50-HeLa cells is insensitive to β-TPA but sensitive between the lens epithelial cells expressing Cx49 (Cx50 homologue) underscores the fact that this connexin can be regulated differently in unrelated cell types.

The insensitivity of gap junctions in the lentoid to β-TPA may mirror the situation *in vivo*. Fiber cell gap junctions in the intact lens are insensitive to certain uncoupling agents which typically effect the uncoupling of epithelial cell gap junctions (Miller and Goodenough, 1986; Rae *et al.*, 1982; Schuetze and Goodenough, 1982). The degree of sensitivity appears to be dependent upon the stage of differentiation, as embryonic chick lens fiber cells are uncoupled by elevated $CO_2$ up to stage 14, after which time the same cells are insensitive to such treatment (Schuetze and Goodenough, 1982). It has been proposed that the avascularity of the lens makes it dependent on gap junction-mediated metabolic cooperation between cells to maintain homeostasis (Piatigorsky, 1981) and that the closure of a significant number of connexin channels between fiber cells could disrupt the existing ion balance and result in cataract formation (Kistler and Bullivant, 1989). Thus, to prevent cataract, the fiber cell gap junction may be refractory to agents which inhibit gap junction communication in the less differentiated cells of the epithelial layer.

Differences between the regulation of epithelial and lentoid gap junction communication could be explained by changes in PKC isoform expression. PKC isoforms differ in their substrate specificity, cofactor requirements, and localization (Saxon *et al.*, 1994), and the particular PKC isoforms expressed in certain tissues have been shown to vary with the level of differentiation (Leli *et al.*, 1993; Saxon *et al.*, 1994). We have detected significant expression of PKC-α, one of the most prevalant of the β-TPA-sensitive PKC isoforms, in both epithelial and lentoid cells. In the lentoid cell, PKC-α localized to both the cytosol and the plasma membrane before treatment with β-TPA, but to only the plasma membrane after treatment. Since the activation of PKC-α has been shown to result in its relocalization from cytosolic to membrane fractions (Ito *et al.*, 1988), lentoid PKC may already be significantly activated prior to β-TPA treatment. Prior modification of lentoid Cx46 or Cx49 (e.g., by phosphorylation) could render these connexins inaccessible to further phosphorylation.

It is unlikely that changes in the regulation of gap junction channel conductance are due to the formation of heterotypic Cx46/Cx49 channels (White *et al.*, 1995b), because Cx49 is expressed and present at interfaces between communication-competent epithelial cells 2–3 weeks before the appearance of Cx46. Nevertheless, these two connexins colocalize in the lentoid (TenBroek *et al.*, 1994) and also in the fiber cell (Paul *et al.*, 1991) membrane, so the coincident expression of Cx46 in the lentoid cell could have an effect on Cx49 channel function. Alternatively, since Cx49 also colocalizes with Cx43 in the cultured epithelial cells, the downregulation of Cx43 which is associated with lens cell differentiation (Musil *et al.*, 1990a; TenBroek *et al.*, 1994) could result in a change in Cx49 function.

Numerous cytoskeletal and morphological changes are associated with the differentiation of epithelial cells to fiber cells *in vivo* (Piatigorsky, 1981), some of which may occur in the differentiating lens cultures. The β-TPA-dependent disappearance of Cx49 from the lens epithelial cell surface is temporally correlated with a change in these cells to a fibroblast-like morphology with few cell contacts. However, β-TPA fails to alter the morphology of, or to induce a loss of cell contacts between, lentoid cells, and the localization of Cx49 and Cx46 in the lentoid cell plasma membrane are unaffected even by prolonged β-TPA treatment. Furthermore, dye transfer is not affected between Cx50-HeLa cells until after 24 hr of β-TPA treatment, at which time an obvious loss of cell contact is seen (not shown). β-TPA-induced shape changes have been noted in many cell types

(Ben-ze'ev, 1987; Schliwa *et al.*, 1984) and result from a rearrangement of cytoskeletal (Schliwa *et al.*, 1984) and adhesion (Ben-ze'ev, 1987) elements. Cytoskeletal components and actin-like filaments have also been found to be associated with gap junction structures (Larsen *et al.*, 1979; Lo *et al.*, 1994), and an interdependence between adhesion systems and gap junctional communication has been implicated in several studies (Meyer *et al.*, 1992; Musil *et al.*, 1990b). Differences between the cytoskeletal and adhesion components in epithelial and lentoid cells or the regulation of these components by protein kinases may explain why lentoid cells do not change in shape or why lentoid gap junctions are not internalized following treatment of lens cultures with $\beta$-TPA.

This investigation of the effect of $\beta$-TPA on the connexins of lens epithelial and lentoid cells in culture describes a potentially important change in gap junction regulation during differentiation in the lens. The differential effects of $\beta$-TPA on the gap junctions of the epithelial and the lentoid cells may be attributable to cell-specific expression of different complements of connexins as well as to other physiological and protein differences between the two cell types. Further studies examining the protein kinase signaling pathways in the undifferentiated epithelial cell and in the differentiating fiber cell should help us to better understand why gap junction sensitivity to PKC changes during the development of the mammalian lens.

## ACKNOWLEDGMENTS

The authors thank Dr. Todd Starich for his helpful advice and both Ding I. Yang and Grant Churchill for the many hours they spent on the road to obtain the fresh lenses used in these studies. This research was supported by National Institutes of Health Grants GM-46277 and EY-05684, by National Eye Institute Training Grant EY-07133-01, and by National Cancer Institute Training Grant CA-09607.

## REFERENCES

Arneson, M. L., Cheng, H., and Louis, C. (1995). Characterization of the ovine-lens plasma membrane protein-kinase substrates. *Eur. J. Biochem.* **234,** 670–679.

Asamoto, M., Oyamada, M., El Aoumari, A., Gros, D., and Yamasaki, H. (1991). Molecular mechanisms of TPA-mediated inhibition of gap-junctional intercellular communication: Evidence for action on the assembly or function but not the expression of connexin 43 in rat liver epithelial cells. *Mol. Carcinog.* **4,** 322–327.

Ben-ze'ev, A. (1987). The role of changes in cell shape and contacts in the regulation of cytoskeleton expression during differentiation. *J. Cell Sci. Suppl.* **8,** 293–312.

Bennett, M. V. L., Barrio, L. C., Bargiello, T. A., Spray, D. C., Hertzberg, E., and Saez, J. C. (1991). Gap junctions: New tools, new answers, new questions. *Neuron* **6,** 305–320.

Berthoud, V. M., Cook, A. J., and Beyer, E. C. (1994). Characterization of the gap junction protein connexin56 in the chicken lens by immunofluorescence and immunoblotting. *Invest. Ophthalmol. Visual Sci.* **35,** 4109–4117.

Berthoud, V. M., Ledbetter, M. L. S., Hertzberg, E. L., and Saez, J. C. (1992). Connexin43 in MDCK cells: Regulation by a tumor-promoting phorbol ester and calcium. *Eur. J. Cell Biol.* **57,** 40–50.

Berthoud, V. M., Rook, M. B., Traub, O., Hertzberg, E. L., and Saez, J. C. (1993). On the mechanisms of cell uncoupling induced by a tumor promoter phorbol ester in clone 9 cells, a rat liver epithelial cell line. *Eur. J. Cell Biol.* **62,** 384–396.

Beyer, E. C. (1993). Gap junctions. *Int. Rev. Cytol.* **137C,** 1–37.

Brewitt, B., and Clark, J. I. (1988). Growth and transparency in the lens, an epithelial tissue, stimulated by pulses of PDGF. *Science* **242,** 777–779.

Brissette, J. L., Kumar, N. M., Gilula, N. B., and Dotto, G. P. (1991). The tumor promoter 12-O-Tetradecanoylphorbol-13-acetate and the ras oncogene modulate expression and phosphorylation of gap junction proteins. *Mol. Cell. Biol.* **11,** 5364–5371.

Caldes, T., Alemany, J., Robcis, H. L., and de Pablo, F. (1991). Expression of insulin-like growth factor I in developing lens is compartmentalized. *J. Biol. Chem.* **266,** 20786–20790.

Chanson, M., Bruzzone, R., Spray, D. C., Regazzi, R., and Meda, P. (1988). Cell uncoupling and protein kinase C: Correlation in a cell line but not in a differentiated tissue. *Am. J. Physiol.* **255,** C699–C704.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyananiate-phenol-chloroform extraction. *Anal. Biochem.* **161,** 156–159.

Dermietzel, R., Traub, O., Hwang, T. K., Beyer, E. C., Bennett, M. V. L., and Spray, D. C. (1989). Differential expression of three gap junction proteins in developing and mature brain tissues. *PNAS USA* **86,** 10148–10152.

Elfgang, C., Eckert, R., Lichtenberg-Frate, H., Butterweck, A., Traub, O., Klein, R. A., Hulser, D. F., and Willecke, K. (1995). Specific permeability and selective formation of gap junction channels in connexin-transfected HeLa cells. *J. Cell Biol.* **129,** 805–817.

Fromaget, C., Aoumari, A. E., Dupont, E., Briand, J. P., and Gros, D. (1990). Changes in the expression of connexin43, a cardiac gap junctional protein, during mouse heart development. *J. Mol. Cell. Cardiol.* **22,** 1245–1258.

Gupta, V. K., Berthoud, V. M., Atal, N., Jarillow, J. A., Barrio, L. C., and Beyer, E. C. (1994). Bovine connexin44, a lens gap junction protein: Molecular cloning, immunologic characterization, and functional expression. *Invest. Opthalmol. Visual Sci.* **35,** 3747–3758.

Ito, T., Tanaka, T., Yoshida, T., Onoda, K., Ohta, H., Hagiwara, M., Itoh, Y., Ogura, M., Saito, H., and Hidaka, H. (1988). Immunocytochemical evidence for the translocation of protein kinase C in human megakaryoblastic leukemic cells: Synergistic effects of $Ca^{2+}$ and activators of protein kinase C on the plasma membrane association. *J. Cell Biol.* **107,** 929–937.

Jiang, G. E., Fishman, G. I., Garfield, R. E., and Andersen, J. (1996). Activation of protein kinase C in human uterine smooth muscle induces connexin-43 gene transcription through an AP-1 site in the promoter sequence. *J. Biol. Chem.* **271,** 23667–23674.

Jiang, J. X., Paul, D. L., and Goodenough, D. A. (1993). Posttranslational phosphorylation of lens fiber Connexin46: A slow occurence. *Invest. Ophthalmol. Visual Sci.* **34,** 3558–3565.

Kadle, R., Zhang, J. T., and Nicholson, B. J. (1991). Tissue-specific distribution of differentially phosphorylated forms of Cx43. *Mol. Cell. Biol.* **11,** 363–369.

Kikkawa, U., Kishimoto, A., and Nishizuka, Y. (1989). The protein kinase C family: Heterogeneity and its implications. *Annu. Rev. Biochem.* **58,** 31–44.

Kistler, J., and Bullivant, S. (1989). Structural and molecular biology of the eye lens membranes. *In* ''Critical Reviews in Biochemistry and Molecular Biology'' (D. Goodenough, Ed.), Vol. 24(2), pp. 151–181.

Kistler, J., Kirkland, B., Gilbert, K., and Bullivant, S. (1986). Aging of lens fibers: Mapping membrane proteins with monoclonal antibodies. *Invest. Ophthalmol. Visual Sci.* **27,** 772–780.

Kumar, N. B. (1991). Gap junctions: A multigene family. *Semin. Cell Biol.* **3,** 3–16.

Kwak, B. R., Hermans, M. M. P., De Jonge, H. R., Lohmann, S. M., Jongsma, H. J., and Chanson, M. (1995a). Differential regulation of distinct types of gap junction channels by similar phosphorylating conditions. *Mol. Biol. Cell.* **6,** 1707–1719.

Kwak, B. R., Vanveen, T. A. B., Analbers, L. J. S., and Jongsma, H. J. (1995b). TPA increases conductance but decreases permeability in neonatal rat cardiomyocyte gap junction channels. *Exp. Cell Res.* **220,** 456–463.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227,** 680–685.

Laing, J. G., Westphale, E. M., Engelmann, G. L., and Beyer, E. C. (1994). Characterization of the gap junction protein, connexin45. *J. Membr. Biol.* **139,** 31–40.

Lampe, P. D. (1994). Analyzing phorbol ester effects on gap junctional communication: A dramatic inhibition of assembly. *J. Cell Biol.* **127,** 1895–1905.

Larsen, W. J., Tung, H. N., Murray, S. A., and Swensen, C. A. (1979). Evidence for the participation of actin microfilaments and bristle coats in the internalization of gap junction membrane. *J. Cell Biol.* **83,** 576–587.

Lau, A. F., Kanemitsu, M. Y., Kurata, W. E., Danesh, S., and Boynton, A. L. (1992). Epidermal growth factor disrupts gap-junctional communication and induces phosphorylation of connexin43 on serine. *Mol. Biol. Cell* **3,** 865–874.

Lavenius, E., Parrow, V., Nanberg, E., and Pahlman, S. (1994). Basic FGF and IGF-1 promote differentiation of human SH-SY5Y neuroblastoma cells in culture. *Growth Factors* **10,** 29–39.

Leli, U., Shea, T. B., Cataldo, A., Hauser, G., Grynspan, F., Beermann, M. L., Liepklans, V. A., Nixon, R. A., and Parker, P. J. (1993). Differential expression and subcellular localization of protein kinase C alpha, beta, gamma, delta, and epsilon isoforms in SH-SY5Y neuroblastoma cells: Modifications during differentiation. *J. Neurochem.* **60,** 289–298.

Lo, W. K., Mills, A., and Kuck, J. F. (1994). Actin filament bundles are associated with fiber gap junctions in the primate lens. *Exp. Eye Res.* **58,** 189–196.

Louis, C. F., Hur, K. C., Glavan, A. C., TenBroek, E. M., Jarvis, L. J., Eccleston, E. D., and Howard, J. B. (1989). Identification of an 18,000-dalton protein in mammalian lens fiber cell membranes. *J. Biol. Chem.* **264,** 19967–19973.

Meyer, R. A., Laird, D. W., Revel, J. P., and Johnson, R. G. (1992). Inhibition of gap junction and adherens junction assembly by connexin and A-CAM antibodies. *J. Cell Biol.* **119,** 179–189.

Miller, T. M., and Goodenough, D. A. (1986). Evidence for two physiologically distinct gap junctions expressed by the chick lens epithelial cell. *J. Cell Biol.* **102,** 194–199.

Murray, W. A., and Gainer, H. S. C. (1989). Regulation of gap junctional communication by protein kinases. *In* ''Cell Interactions and Gap Junctions'' (N. Sperelakis and W. C. Cole, Eds.), Vol. I, pp. 97–106. CRC Press, Boca Raton, FL.

Musil, L. S., Beyer, E. C., and Goodenough, D. A. (1990a). Expression of the gap junction protein connexin43 in embryonic chick lens: Molecular cloning, ultrastructural localization, and post-translational phosphorylation. *J. Membr. Biol.* **116,** 163–175.

Musil, L. S., Cunningham, B. A., Edelman, G. M., and Goodenough, D. A. (1990b). Differential phosphorylation of the gap junction protein connexin43 in junctional communication-competent and -deficient cell lines. *J. Cell Biol.* **111,** 2077–2088.

Musil, L. S., and Goodenough, D. A. (1991). Biochemical analysis of connexin43 intracellular transport, phosphorylation, and assembly into gap junctional plaques. *J. Cell Biol.* **115,** 1357–1374.

Musil, L. S., and Goodenough, D. A. (1993). Multisubunit assembly of an integral plasma membrane channel protein, gap junction connexin43, occurs after exit from the ER. *Cell* **74,** 1065–1077.

Nevens, J. R., Mallia, K. A., Wendt, M. W., and Smith, P. K. (1992). Affinity chromatographic purification of immunoglobulin M antibodies utilizing immobilized mannan binding protein. *J. Chromatogr.* **597,** 247–256.

Oh, S. Y., Grupen, C. G., and Murray, A. W. (1991). Phorbol ester induces phosphorylation and down-regulation of connexin43 in WB cells. *Biochim. Biophys. Acta* **1094,** 243–245.

Paul, D. L., Ebihara, L. J., Takemoto, L. J., Swenson, K. I., and Goodenough, D. A. (1991). Connexin46, a novel lens gap junction protein, induces voltage-gated currents in nonjunctional plasma membrane of *Xenopus* oocytes. *J. Cell. Biol.* **115,** 1077–1089.

Pelletier, D. B., and Boynton, A. L. (1994). Dissociation of PDGF receptor tyrosine kinase activity from PDGF-mediated inhibition of gap junctional communication. *J. Cell Physiol.* **158,** 427–434.

Pepper, M. S., and Meda, P. (1992). Basic fibroblast growth factor increases junctional communication and connexin 43 expression in microvascular endothelial cells. *J. Cell. Physiol.* **153,** 196–205.

Piatigorsky, J. (1981). Lens differentiation in vertebrates: A review of cellular and molecular features. *Differentiation* **19,** 134–153.

Presta, M., Maier, J. A. M., and Ragnotti, G. (1989). The mitogenic signalling pathway but not the plasminogen activator-inducing pathway of basic fibroblast growth factor is mediated through protein kinase C in fetal bovine aortic endothelial cells. *J. Cell. Biol.* **109,** 1877–1884.

Rae, J. L., Thomson, R. D., and Eisenberg, R. S. (1982). The effect of 2-4 dinitrophenol on cell to cell communication in the frog lens. *Exp. Eye Res.* **35,** 597–609.

Reynhout, J. K., Lampe, P. D., and Johnson, R. G. (1992). An activator of protein kinase C inhibits gap junction communication between cultured bovine lens cells. *Exp. Cell. Res.* **1948,** 337–342.

Richardson, N. A., Chamberlain, C. G., and McAvoy, J. W. (1993). IGF-1 enhancement of FGF-induced lens fiber differentiation in rats of different ages. *Invest. Ophthalmol. Visual Sci.* **34,** 3303–3312.

Saxon, M. L., Zhao, X., and Black, J. D. (1994). Activation of protein kinase C isozymes is associated with post-mitotic events in intestinal epithelial cells *in situ*. *J. Cell Biol.* **126,** 747–763.

Schliwa, M., Nakamura, T., Proter, K. R., and Euteneuer, U. (1984). A tumor promoter induces rapid and coordinated reorganization of actin and vinculin in cultured cells. *J. Cell Biol.* **99,** 1045–1059.

Schuetze, S. M., and Goodenough, D. A. (1982). Dye transfer between cells of the embryonic chick lens becomes less sensitive to $CO_2$-treatment with development. *J. Cell Biol.* **92,** 694–705.

Sosinsky, G. (1995). Mixing of connexins in gap junction membrane channels. *Proc. Natl. Acad. Sci. USA* **92,** 9210–9214.

Stauffer, K. A. (1995). The gap junction proteins beta 1-connexin (connexin-32) and beta 2-connexin (connexin-26) can form heteromeric hemichannels. *J. Biol. Chem.* **270,** 6768–6772.

Sullivan, R., Ruangvoravat, C., Joo, D., Morgan, J., Wang, B. L., Wang, X. K., and Lo, C. W. (1993). Structure, sequence, and expression of the mouse Cx43 gene encoding connexin 43. *Gene* **130,** 191–199.

TenBroek, E., Arneson, M., Jarvis, L., and Louis, C. (1992). The distribution of the fiber cell intrinsic membrane proteins MP20 and connexin46 in the bovine lens. *J. Cell. Sci.* **103,** 245–257.

TenBroek, E. M., Louis, C. F., and Johnson, R. J. (1994). Cell-to-cell communication in a differentiating ovine lens culture system. *Invest. Ophthalmol. Visual Sci.* **35,** 215–228.

Towbin, H., Staehelin, T., and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.

Traub, O., Look, J., Dermietzel, R., Brummer, F., Hulser, D., and Willecke, K. (1989). Comparative characterization of the 21 kD and 26 kD gap junction proteins in murine liver and cultured hepatocytes. *J. Cell Biol.* **108,** 1039–1051.

White, T. W., Bruzzone, R., Goodenough, D. A., and Paul, D. L. (1992). Mouse Cx50, a functional member of the connexin family of gap junction proteins, is the lens fiber protein MP70. *Mol. Biol. Cell* **3,** 711–720.

White, T. W., Bruzzone, R., Goodenough, D. A., and Paul, D. L. (1995a). Chimeric connexins reveal the molecular basis for novel properties of lens intercellular channels reconstituted in paired *Xenopus* oocytes. *In* ''Intercellular Communication Through Gap Junctions'' (Y. Kanno, K. Kataoka, Y. Shiba, Y. Shibata, and T. Shimazu, Eds.), Vol. 4, pp. 387–390. Elsevier, Amsterdam.

White, T. W., Paul, D. L., Goodenough, D. A., and Bruzzone, R. (1995b). Functional analysis of selective interactions among rodent connexins. *Mol. Biol. Cell* **6,** 459–470.

Yang, D., and Louis, C. F. (1996). Molecular cloning of sheep connexin49 and its identity with MP70. *Curr. Eye Res.* **15,** 307–314.

Received for publication April 21, 1997
Accepted July 31, 1997