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Functional Effects of Casein Kinase I-Catalyzed Phosphorylation on Lens Cell-to-Cell Coupling

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Abstract. The functional consequence of the casein kinase I-catalyzed phosphorylation of the lens gap junctional protein connexin49 was investigated using a sheep primary lens cell culture system. To determine whether the phosphorylation of connexin49 catalyzed by endogenous casein kinase I results in an altered junctional communication between lens cells, the effect of the casein kinase I-specific inhibitor CKI-7 on Lucifer Yellow dye transfer between cells in the lens culture was examined. Dye transfer was analyzed in cultures of different ages because we have demonstrated previously that the expression of connexin49 increases as the cultures age while that of connexin43, which is likely not a substrate for casein kinase I, has been shown to decrease [Yang & Louis (1999) Invest. Ophthalmol. Vis. Sci. 41: 2568–2564]. In 9-day old lens cultures, in which gap junctions are composed primarily of connexin43, CKI-7 had little effect on the rate of dye transfer between lens cells. In contrast, treatment of 15-day and 28-day old cultures with CKI-7 resulted in a significant increase in the rate of dye transfer. Thus, the extent of this CKI-7-dependent increase in cell-to-cell communication was positively correlated with the level of expression of connexin49, the major casein kinase I substrate in lens plasma membranes. These results suggest that the casein kinase I-catalyzed phosphorylation of connexin49 decreases cell communication between connexin49-containing gap junctions in the lens.

Key words: Connexin — Lens — Gap junction — Phosphorylation — Regulation — Casein kinase I — Sheep

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Introduction

In multicellular organisms, intercellular communication mediated by gap junctions is one of the mechanisms by which cells communicate with each other to coordinate their activities. Gap junctions are clusters of transmembrane channels that connect the cytoplasmic compartments of neighboring cells and allow molecules smaller than approximately 1000 daltons to pass directly between cells (Bennett et al., 1991; Saez et al., 1993; Kumar & Gilula, 1996). Genes encoding the structural components of gap junction channels have been cloned and comprise a family of highly related proteins, called connexins (Bruzzone, White & Paul, 1996; Kumar & Gilula, 1996) with six connexins aligned to form a channel structure containing a central aqueous pore called a hemichannel or a connexon (Musil & Goodenough, 1993; Laird, 1996).

The ocular lens is one of the least complicated systems in which to study the function and regulation of gap junctions. The lens is an avascular tissue composed of only two cell types: a layer of epithelial cells on the anterior surface, and fiber cells that differentiate from the epithelial cells and comprise the bulk of the lens volume. These cells exchange ions, second messengers, metabolites, and nutrients through a network of gap junction channels that provide cytoplasmic connections between the fiber and epithelial cells of the lens (Rae et al., 1996). This network of gap junctions is believed to be critical for maintaining lens homeostasis and transparency (Goodenough, 1979; Goodenough, 1992).

Three connexins have been identified in mammalian and avian lenses. Connexin43 is exclusively present in the epithelial cells of the lens (Beyer et al., 1989; Musil, Beyer & Goodenough, 1990b), connexin50 has been identified in both epithelial and fiber cells (Kistler, Kirkland & Bullivant, 1985; White et al., 1992; Dahm et

al., 1999) while connexin46 is restricted to the fiber cells (Paul et al., 1991; Goodenough, 1992). The mechanisms responsible for regulating the gating and permeability properties of these connexins in the lens remain to be defined. However, all three connexins have been reported to be substrates for protein kinases (Voorter & Kistler, 1989; Musil et al., 1990a; 1990b; Musil & Goodenough, 1991; Jiang, Paul & Goodenough, 1993; Jiang et al., 1994; Warn-Cramer et al., 1998; Cheng & Louis, 1999). We have demonstrated recently that the phosphorylation of connexin49, the sheep homologue of mouse connexin50, is catalyzed by lens membrane-associated casein kinase I (Cheng & Louis, 1999). However, the functional effects of this phosphorylation remain to be defined.

A primary sheep lens culture system that mimics the early stages of lens fiber cell differentiation and possesses functional gap junctions was developed previously in this laboratory (TenBroek, Johnson & Louis, 1994; TenBroek, Louis & Johnson, 1997). We have shown that in the first 10 days in culture, connexin43 is the major connexin expressed (Yang & Louis, 2000). Subsequently, the connexin43 mRNA and protein levels decline while those of connexin49 increase (Yang & Louis, 2000). This "switch" in the expression of connexin43 and connexin49 appears to precede the expression of connexin44 which is not a substrate for casein kinase I (Arneson, Cheng & Louis, 1995) and remains at a relatively low level compared to that of connexin43 and connexin49 at all stages of these cultures (Yang & Louis, 2000). Therefore, in the first 10 days in culture, gap junctions are composed mainly of connexin43, while in 15-day or older cultures, the ratio of connexin49 protein/connexin43 protein significantly increases.

To determine the role of the casein kinase I-catalyzed phosphorylation of connexin49, we have examined the effect of CKI-7, a casein kinase I inhibitor (Chijiwa, Hagiwara & Hidaka, 1989), on junctional communication between cells in the sheep lens primary cell culture system. In this report we demonstrate that the membrane-associated casein kinase I activity is present in the lens cultures and that connexin43 is not a substrate for casein kinase I. Furthermore, we demonstrate that in connexin49-containing lens cultures, CKI-7 effected a significant increase in the rate of Lucifer Yellow dye transfer between lens cells. Our data thus suggest that the casein kinase I-catalyzed phosphorylation of connexin49 decreases cell communication between connexin49-containing gap junctions in the lens.

Materials and Methods

Materials

Sheep lenses were obtained from John Morrell & Co. (Sioux Falls, SD); CKI-7 was purchased from Seikagaku America Inc. (Rockville, MD); unless indicated, other chemicals were purchased from Sigma Chemical Company (St. Louis, MO). All chemicals used were of reagent grade or the highest grade available.

Lens Cultures

Primary lens cell cultures were grown on 35 mm Falcon brand culture dishes that were coated with 2 ml of poly-DL-polyornithine (0.1 mg/ml) 0.15 M borate buffer (pH 8.6), for 2 hours at room temperature. Dishes were then rinsed three times with double distilled water and dried under ultraviolet light for 20–30 min. Eyes were removed from freshly slaughtered sheep and kept on ice for up to six hours before culturing. Lenses were then removed under sterile conditions as described previously (TenBroek et al., 1994) and placed in Hank's balanced salt solution without Ca^{2+} and Mg^{2+} (HBSS-CMF, pH 7.3). Approximately 20 nicks were made along the equator of each lens and the lenses were digested with trypsin (2.5 mg/ml) for 15 to 30 minutes at 37°C; cold HBSS-CMF was then added to slow the reaction. The solution was triturated 20–30 times to dislodge cells from the lenses, and cells were subsequently centrifuged (230 × g for 5 min) and resuspended in Medium 199 containing 10% fetal calf serum, penicillin (100 U/ml), and streptomycin (100 mg/ml). Two ml of this cell suspension was placed in one pre-coated 35-mm culture dish and incubated at 37°C in a humidified atmosphere with 5% CO_2 to allow cells to adhere. The medium was removed after 24 hours and centrifuged as before to pellet cells not adherent to the dishes. These cells were resuspended in fresh medium and reapplied to the culture dishes. Medium was replaced every other day throughout the period of cell culture.

Preparation of the Membrane KCl Extract, and Buffer-washed or Urea-washed Membranes from Primary Lens Cultures or Intact Lenses

Primary lens culture cells were scraped off the culture dishes using a rubber policeman and suspended in HBSS-CMF buffer containing 1 mM phenylmethylsulfonyl fluoride, leupeptin, aprotinin, and pepstatin A (at 1 mg/ml each). The suspension was centrifuged at 500 × g for 5 min, then the pelleted cells were homogenized in Tris buffer [(in mM) 25 Tris-HCl (pH 7.4), 1 phenylmethylsulfonyl fluoride, 2 benzamidine, 2 β-mercaptoethanol, 1 mg/ml each leupeptin, aprotinin, and pepstatin A] and centrifuged at 50,000 × g for 15 min. The membrane pellet was washed twice with Tris buffer, resuspended in the same buffer and stored at −80°C (buffer-washed membranes). Urea-washed membranes were prepared by subsequently extracting membranes twice with 8 M urea and centrifugation at 170,000 × g for 15 min. The membrane pellet was washed once with Tris buffer, centrifuged at 50,000 × g for 15 min, resuspended in Tris buffer, and stored frozen at −80°C. Alternatively, buffer-washed membranes were resuspended in Tris buffer containing 0.6 M KCl and centrifuged at 50,000 × g for 10 min. The supernatant fraction, which contained casein kinase I activity (Cheng & Louis, 1999), was collected, dialyzed against Tris buffer overnight, recentrifuged at 50,000 × g for 10 min, and the supernatant stored at −80°C. All procedures were performed on ice or at 4°C.

Preparation of urea-washed membranes and KCl extract from intact lenses was performed as described previously (Cheng & Louis, 1999).

Preparation of Sheep Cardiac Membranes

Five grams of sheep cardiac tissue was homogenized and suspended in Tris buffer containing (in mM) 25 Tris-HCl (pH 7.4), 1 phenylmethyl-

sulfonyl fluoride, 2 benzamidine, and 2 β-mercaptoethanol, and 1 mg/ml each leupeptin, aprotinin, and pepstatin A; these and all other steps were performed on ice or at 4°C. The suspension was centrifuged at 500 × g for 5 min to remove particulate material. The supernatant was subsequently homogenized with a Dounce tissue homogenizer and centrifuged at 50,000 × g for 15 min. The resulting pellets were subsequently washed twice with 8 M urea and centrifuged at 170,000 × g for 15 min. The membrane pellet was then washed once with Tris buffer, centrifuged at 50,000 × g for 15 min, resuspended in Tris buffer, and stored frozen at −80°C.

Phosphorylation of Lens Membranes or Casein

Buffer-washed or urea-washed membranes isolated from primary lens cultures were incubated at 37°C for 60 min in 10 mM $MgCl_2$, 25 μM [γ-^{32}P]ATP, 10 mM Tris-HCl buffer (pH 7.5). When indicated, 5 units of casein kinase I was also included. The reaction was stopped by centrifugation at 50,000 × g for 15 min at 4°C and resuspending the resulting pellet in SDS-PAGE sample buffer (Laemmli, 1970). The membranes were then electrophoretically fractionated on a 12% SDS-polyacrylamide gel, stained with Coomassie blue R-250, and dried under vacuum. Phosphorylated components were detected in autoradiograms by exposing the dried gel to Kodak Max X-ray film (Eastman Kodak Co., Rochester, NY).

Urea-washed membranes isolated from intact lenses were incubated at 37°C for 60 min in 10 mM $MgCl_2$, 25 μM [γ-^{32}P]ATP, 10 mM Tris-HCl buffer (pH 7.5), and the membrane KCl extract isolated from intact lenses or the primary lens cultures. The reaction was stopped and samples processed as described above. Phosphorylation of casein was performed as described previously (Cheng & Louis, 1999).

Phosphorylation of Cardiac Membranes

Sheep cardiac membranes were incubated at 37°C for 60 min in 10 mM $MgCl_2$, 25 μM [γ-^{32}P]ATP, 10 mM Tris-HCl buffer (pH 7.5) and 5 units of purified casein kinase I. The reaction was stopped by centrifugation at 45,000 × g for 15 min at 4°C and resuspending the resulting membrane pellet in SDS-PAGE sample buffer. The membranes were then electrophoretically fractionated on a 12% SDS-polyacrylamide gel, stained with Coomassie blue R-250, and dried under vacuum. Phosphorylated components were detected in autoradiograms by exposing the dried gel to Kodak Max X-ray film.

Western Blotting of Lens Membrane or Cardiac Membrane Proteins

Lens or cardiac membrane proteins were electrophoretically fractionated on 12% polyacrylamide gels and transferred overnight to nitrocellulose membranes (Schleicher and Schuell, Keene, NH) at 300 mA in a Transfer-Blot electrophoretic transfer cell (Bio-Rad, Hercules, CA). The nitrocellulose membranes were then incubated in 3% nonfat dry milk/PBS buffer (pH 7.4)/0.2% Tween-20 (blocking solution) for one hour, hybridized at room temperature for 1–2 hour with connexin43 antibody (Chemicon, Temecula, CA), diluted in blocking solution, and washed extensively with PBS buffer (pH 7.4)/0.2% Tween-20 at least 3 times. The nitrocellulose membranes were subsequently incubated at room temperature for one hour with horseradish peroxidase-coupled anti-mouse IgG + IgM antibody (KPL, Gaithersburg, MD) diluted in blocking solution, and washed extensively with PBS buffer (pH 7.4) containing 0.2% Tween-20. Immunoreactive bands were detected following incubation of the nitrocellulose membranes with ECL Western blotting detection reagent (Amersham Life Science Inc., Arlington Heights, IL) for 20 sec to 1 min followed by exposure to Kodak Max X-ray film.

Lucifer Yellow Microinjection of the Lens Cells

Cells in the lens culture were microinjected with a 4% (wt/vol) Lucifer Yellow CH solution by overcompensation of the negative capacitance circuit in the amplifier until the impaled cell was brightly fluorescent. 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. After each injection, the image of dye transfer between the injected and neighboring cells was captured and recorded at the indicated time by a computer connected to the camera with the software package Image-1/Fluorescence (Universal Imaging Corp., PA). Subsequently, from the recorded images, cells with brightly fluorescent cytoplasm and nucleus were counted as the number of neighboring cells having received dye from the injected cell as described previously (TenBroek et al., 1994).

Statistics

Data were analyzed by analysis of variance (ANOVA) using the software SuperANOVA (version 1.1, Abacus Concepts Inc.). The two-tailed probability (P) values were considered significant when <0.05.

Results

Characterization of the Membrane-associated Casein Kinase I Activity in the Primary Lens Culture System

It has been demonstrated previously that phosphorylation of connexin49 in lens membranes is catalyzed by membrane-associated casein kinase I which can be extracted from lens membranes by 0.6 M KCl (Cheng & Louis, 1999). To confirm that membrane-associated casein kinase I is also present in cells of the primary ovine lens cell culture, membranes were isolated from 9-, 15-, and 28-day lens cultures, respectively, and extracted with 0.6 M KCl. The resulting KCl extract from each of these cultures was then incubated with [γ-^{32}P]ATP and urea-washed membranes isolated from whole sheep lenses [from which most peripheral proteins including any endogenous casein kinase I activity had been removed (Arneson et al., 1995)]. The membranes were then precipitated by centrifugation and proteins fractionated by SDS-PAGE. The resulting autoradiogram demonstrated that all these different KCl extracts were able to catalyze the phosphorylation of connexin49 in the urea-washed lens membranes (Fig. 1, lanes *2–4*). As a positive control, phosphorylation of connexin49 catalyzed by the KCl extract of membranes isolated from intact lenses (Fig. 1, lane *1*) was also shown. Incubation of the urea-washed lens membranes with [γ-^{32}P]ATP alone did not result in the phosphorylation of any lens membrane protein (Fig.

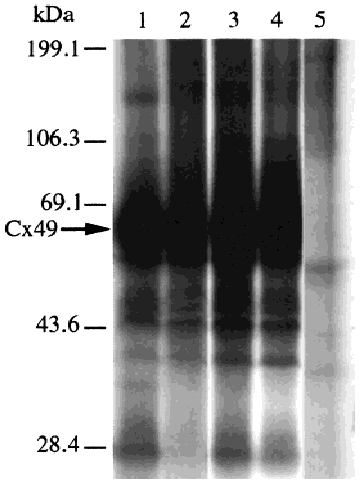

Fig. 1. Phosphorylation of urea-washed lens membranes by the KCl extract isolated from sheep primary lens cell culture membranes. Urea-washed, sheep outer cortical lens membranes (30 μg) were incubated with [γ-^{32}P]ATP and the KCl extract of membranes isolated from either sheep lenses (lane *1*), 9-day lens cell cultures (lane *2*), 15-day lens cell cultures (lane *3*), 28-day lens cell cultures (lane *4*), or buffer alone (lane *5*). Samples were then processed for electrophoresis and autoradiography as described in Materials and Methods. The arrow indicates the mobility of connexin49.

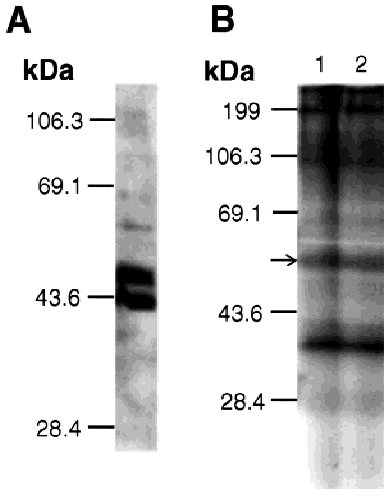

Fig. 2. Phosphorylation and Western blot analysis of membranes isolated from the sheep primary lens cell cultures. (*A*) Urea-washed membranes (5μg) isolated from lens cultures were analyzed by Western blotting using a connexin43-specific antibody as described in Materials and Methods. (*B*) Buffer-washed membranes (60 μg) isolated from lens cultures were incubated at 37°C for 60 min in 10 mM $MgCl_2$, 25 μM [γ-^{32}P]ATP, 10 mM Tris-HCl buffer (pH 7.5) in lane *1;* casein kinase I (5 units) was included in lane *2*. Samples were then processed for electrophoresis and autoradiography as described in Materials and Methods. The arrow indicates the mobility of connexin49.

1, lane *5*). In addition, each of these KCl extracts was able to catalyze the phosphorylation of casein (*data not shown*). Together, these data demonstrated that, at all three stages tested (9-day, 15-day, and 28-day), membrane-associated casein kinase I activity is present in cells of this lens culture system.

Does Casein Kinase I Catalyze the Phosphorylation of Connexin43?

We have shown previously that in contrast to connexin49, connexin44 is not a substrate for the lens membrane-associated casein kinase I activity (Arneson et al., 1995; Cheng & Louis, 1999). Although connexin43 is also present in the ovine lens cultures, there is no prior report indicating that casein kinase I catalyzes the phosphorylation of this connexin in mammalian lenses.

To determine whether connexin43 is a substrate for casein kinase I, membranes were isolated from 9-day lens cultures and the presence of connexin43 in these membranes was confirmed by Western blot analysis using a connexin43-specific antibody (Fig. 2*A*). Subsequently, buffer-washed membranes isolated from the 9-day cultures, which should retain most peripheral membrane proteins including the endogenous casein kinase I activity (Arneson et al., 1995), were incubated with [γ-^{32}P]ATP with or without exogenously added casein kinase I. The membranes were then precipitated by centrifugation and proteins analyzed by SDS-PAGE and autoradiography. As shown in Fig. 2*B*, incubation of the buffer-washed membranes with [γ-^{32}P]ATP alone (Fig. 2*B*, lane *1*) or with [γ-^{32}P]ATP and exogenously added casein kinase I (Fig. 2*B*, lane *2*) showed identical results. Namely, several proteins, including a 60–65 kDa component corresponding to the M_r of connexin49 in these lens cultures (Yang & Louis, 2000), were phosphorylated; notably, there was no 42–47 kDa phosphorylated component corresponding to the M_r of connexin43 (Fig. 2*A*). These data indicate that neither the endogenous membrane-associated casein kinase I nor the exogenously added casein kinase I catalyzes the phosphorylation of connexin43.

To further confirm that casein kinase I does not catalyze the phosphorylation of connexin43, sheep cardiac muscle membranes were isolated because connexin43 is abundantly expressed in this tissue (Manjunath, Goings & Page, 1985). The presence and mobility of connexin43 in the sheep cardiac membranes were first confirmed by Western blot analysis using a connexin43-specific antibody (Fig. 3*A*, lane *1*). As a negative control, membranes isolated from the outer cortical fiber cells of ovine lenses were also examined and, as reported previously, showed no detectable connexin43 immunoreactivity (Fig. 3*A*, lane *2*). To determine whether casein kinase I catalyzes the phosphorylation of connexin43 in cardiac membranes, urea-washed cardiac membranes were incubated with purified casein kinase I and [γ-^{32}P]ATP, and the membrane proteins were then analyzed by SDS-

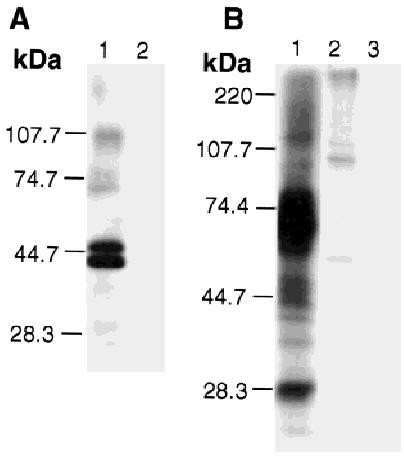

Fig. 3. Phosphorylation and Western blot analysis of membranes isolated from sheep cardiac muscle. (*A*) Urea-washed sheep cardiac membranes (5 μg) (lane *1*) and urea-washed sheep outer cortical lens membranes (5 μg) (lane *2*) were subjected to Western blot analysis using a connexin43-specific antibody. (*B*) Urea-washed sheep outer cortical lens membranes (30 μg) (lane *1*), or urea-washed sheep cardiac membranes (75 μg) (lanes *2* and *3*), were incubated at 37°C for 60 min in 10 mM $MgCl_2$, 25 μM [γ-^{32}P]ATP, 10 mM Tris-HCl buffer (pH 7.5) in the presence of casein kinase I (5 units). The cardiac membranes in lane *2* were pretreated with alkaline phosphatase prior to this subsequent incubation with the phosphorylation mixture. Membranes were then analyzed by SDS-PAGE and autoradiography.

PAGE. The resulting autoradiogram is shown in Figure 3B. Whether or not the cardiac membranes were pretreated with alkaline phosphatase (Fig. 3*B*, lane *2:* alkaline phosphatase-pretreated cardiac membranes; lane *3:* untreated cardiac membranes), no protein in these membranes in the molecular weight range of ovine cardiac connexin43 (42–44 kDa, as shown in Fig. 3*A*, lane *1*) appeared to be significantly phosphorylated by the exogenously added casein kinase I. As a positive control for casein kinase I activity, the same amount of casein kinase I was incubated with urea-washed lens membranes, which resulted in the phosphorylation of connexin49 (Fig. 3*B*, lane *1*). Together, these data indicate that connexin43 is not a substrate for casein kinase I.

Functional Effect of CKI-7 on Gap Junctional Communication in Primary Lens Cultures of Different Ages

It has been shown previously that the amount of connexin43 protein did not change significantly in the first 10 days of the primary ovine lens culture, but declined to approximately 45–60% in 15- and 20-day old cultures (Yang & Louis, 2000). In contrast, the amount of connexin49 protein increased more than 20-fold from 5 to 20 days in culture (Yang & Louis, 2000). Thus, the ratio of connexin49 protein/connexin43 protein increases as the culture ages. In this lens cell culture system, cells reach confluence approximately five days after plating when they start to form an extensive network of communicating intercellular gap junction channels. Therefore, the ratio of connexin49 protein/connexin43 protein in intercellular channels should be lowest in the 9-day old cultures but should then increase significantly in the older cultures. Hence, to elucidate the functional consequence of the casein kinase I-catalyzed phosphorylation of connexin49, 9-, 15-, and 28-day old lens cell cultures were used to examine the effect of the casein kinase I-specific inhibitor CKI-7 (Chijiwa et al., 1989) on the junctional communication between the lens cells.

Cells in the primary lens culture were microinjected with the fluorescent dye Lucifer Yellow either prior to, or subsequent to the addition of CKI-7 diluted in serum-free medium 199 to give a final concentration of 400 μM CKI-7, 0.4% DMSO. Gap junctional communication in the presence or absence of CKI-7 was then quantitated by counting the number of adjacent cells receiving Lucifer Yellow dye from the microinjected cell within a defined period of time. We have shown previously that under the conditions used here, 400 μM CKI-7 inhibits the casein kinase I-catalyzed phosphorylation of connexin49 by more than 80% while having little effect on the activity of casein kinase II (for which, as we have shown previously, connexin49 is not a substrate) (Cheng & Louis, 1999). Similar concentrations of CKI-7 have been used in several previous in vitro and in vivo studies of casein kinase I action [e.g., Manno et al., (1995); Krantz et al., (1997); Bioukar et al., (1999)].

The time course of Lucifer Yellow transfer between lens cells prior to and subsequent to the addition of CKI-7 was first characterized in 9- and 28-day cultures that were derived from the same set of lenses. The cultures were first incubated for 2.5 hr with medium 199 containing 0.4% DMSO, microinjected with Lucifer Yellow, and the time course of dye transfer recorded by quantitating the number of neighboring cells receiving Lucifer Yellow from the microinjected cells at various times after each microinjection. This was taken as the control rate of dye transfer before CKI-7 treatment. The medium was then changed to medium 199 containing 400 μM CKI-7, 0.4% DMSO and the cultures were incubated for a further 2.5 hr, and assays for the time course of Lucifer Yellow transfer were repeated. The results are reported in Fig. 4. For the 28-day culture, addition of CKI-7 resulted in an increased rate of dye transfer when compared to the control rate (Fig. 4*A*), while cells in the 9-day culture showed no difference in the rate of dye transfer after addition of CKI-7 (Fig. 4*B*). If DMSO alone was added instead of CKI-7 after 2.5 hr, the rate of dye transfer was unaffected, irrespective of the age of the culture (*data not shown*). An example of Lucifer Yellow dye transfer between cells in a 28-day cell

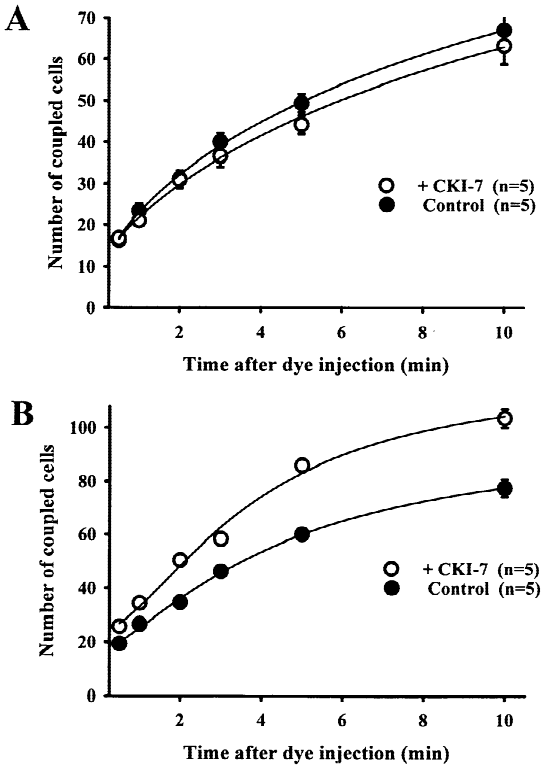

Fig. 4. Effect of CKI-7 on the time course of dye transfer between cells in the primary lens cell cultures. 9-day (*A*) and 28-day (*B*) primary lens cultures that were derived from the same set of lenses were incubated at 37°C in medium 199 containing 0.4% DMSO for 2.5 hr before Lucifer Yellow was injected into one cell and dye transfer between cells in this lens culture was determined (control) as described in Materials and Methods. Each lens culture was subsequently incubated at 37°C in medium 199 containing 0.4% DMSO and 400 μM CKI-7 for an additional 2.5 hr, and the time course of Lucifer Yellow transfer was measured (*+CKI-7*). The time course of dye transfer is expressed as the number of cells receiving Lucifer Yellow that were adjacent to the microinjected cells. Values represent means ± SEM. (The error bar for most data points fell within the diameter of the symbol); 5 microinjections were performed at each time point in one plate each of either a 9-day or a 28-day lens culture.

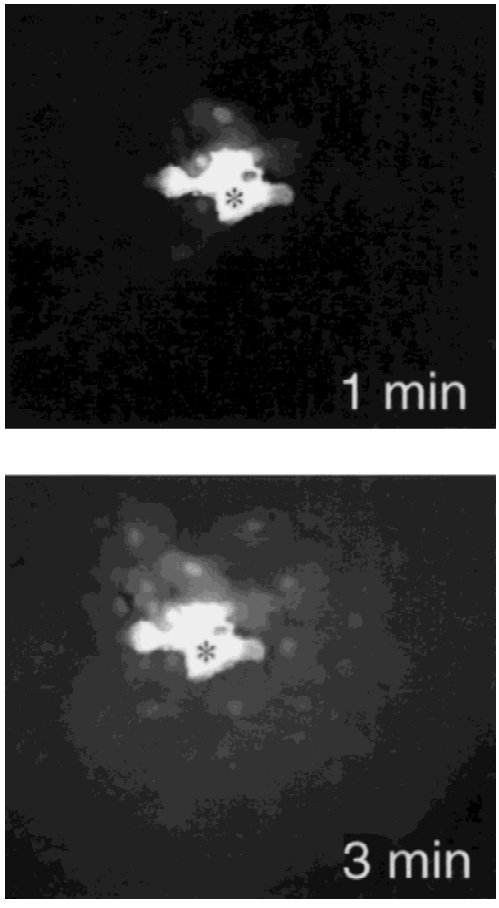

Fig. 5. An example of Lucifer Yellow dye transfer between cells in a 28-day sheep primary lens cell culture. The recorded images of Lucifer Yellow dye transfer 1 and 3 min following the microinjection of a cell in a 0.4% DMSO-treated 28-day primary lens culture, are shown. Asterisk indicates the microinjected cell.

culture 1 and 3 min after the microinjection of Lucifer Yellow is shown in Fig. 5.

The experiment described in Fig. 4 was further repeated in three different sets of lens cell cultures where Lucifer Yellow dye transfer was quantitated before and after CKI-7 treatment of 9-, 15-, and 28-day cells in 3 sets of cultures derived from different sets of lenses. Lens cultures were first incubated for 2.5 hr with 0.4% DMSO, and the number of adjacent cells receiving dye was counted 2 min after each microinjection to determine the control value. Medium was replaced and the cultures were then incubated with 400 μM CKI-7/0.4% DMSO for another 2.5 hr, and dye transfer assays were repeated. Results are reported in the Table. One plate of 9- or 28-day cells from each of the three sets of lens cultures was analyzed in this experiment, while the 15-day cells were from two of the three sets of lens cultures. The data consistently showed that addition of CKI-7 did not result in any significant changes in the rate of dye transfer between cells in the 9-day old lens cultures. However, in the 15- and 28-day old cultures, in which the level of expression of connexin49 has been shown to be significantly increased (Yang & Louis, 2000), treatment with CKI-7 significantly increased the rate of dye transfer between these cells. This rate increased approximately 29% and 49-68% in the 15-day and the 28-day old cultures, respectively, after CKI-7 treatment. That is, the CKI-7-dependent increment in the rate of dye transfer was greater in 28-day cultures indicating a positive correlation between the action of CKI-7 and the level of expression of connexin49 in these cultures.

The effect of CKI-7 on dye transfer in 28-day old lens cultures can be observed by 30 min after the addition of inhibitor to the lens culture. As shown in Fig. 6, when dye transfer in a 28-day old culture was analyzed

Table. Effect of CKI-7 on gap junctional communication as measured by dye transfer in primary lens cultures

Days of culture	Number of cells receiving dye[a]		+CKI-7/control (%)
	Control	+CKI-7	
9	36.8 ± 1.9 (22)[b]	36.1 ± 1.9 (22)	98
9	45.6 ± 2.1 (23)	50.6 ± 2.7 (15)	111
9	29.2 ± 1.1 (20)	34.1 ± 1.2 (22)	117
15	35.2 ± 1.3 (31)	45.5 ± 2.2 (29)*	129
15	37.3 ± 1.5 (28)	48.0 ± 2.1 (28)*	129
28	32.0 ± 1.3 (30)	53.7 ± 2.0 (23)*	168
28	38.1 ± 1.0 (24)	60.3 ± 1.4 (21)*	158
28	33.3 ± 0.6 (24)	49.6 ± 1.0 (21)*	149

[a] Cells were first treated with 0.4% DMSO for 2.5 hr (control), then with CKI-7 + 0.4% DMSO for another 2.5 hr (+CKI-7). After each treatment, gap junctional communication was determined by measuring the number of cells receiving Lucifer Yellow dye as described in Materials and Methods. The number of cells receiving dye from the microinjected cells was measured 2 min after each microinjection.

[b] Values are reported as the mean ± SEM. The number of cells microinjected with Lucifer Yellow in each treatment is indicated in parentheses. Three different sets of lens cultures were used to derive the 9-day and 28-day data and two different sets of lens cultures were used to derive the 15-day data.

* Significantly different from controls ($P < 0.01$).

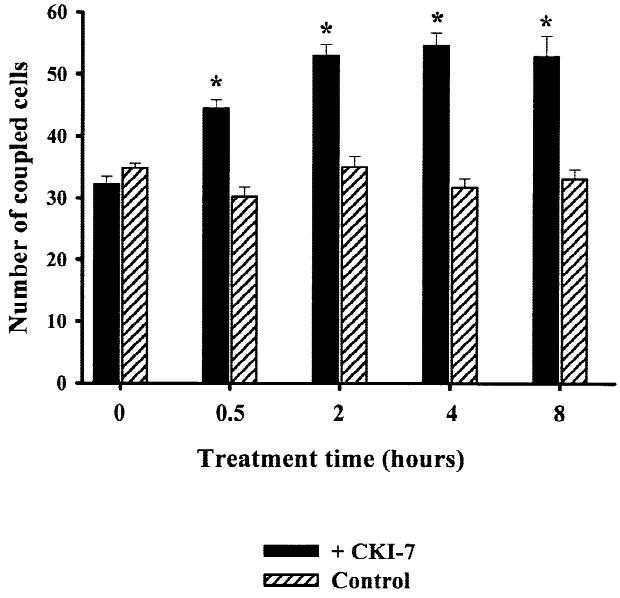

Fig. 6. Lucifer Yellow dye transfer following different times of incubation of the sheep lens cell cultures with CKI-7. Lucifer Yellow transfer between cells in a 28-day primary lens cell culture was analyzed following treatment with M199 medium containing 0.4% DMSO and 400 μM CKI-7 (*+CKI-7*). As a control, another 28-day primary lens culture was also analyzed for Lucifer Yellow transfer following treatment with M199 medium containing 0.4% DMSO alone (*Control*). The extent of dye transfer is expressed as the number of cells adjacent to the microinjected cells that received Lucifer Yellow dye 2 min after each microinjection. 12 to 15 microinjections were performed and assayed at each time point; values represent means ± SEM. Asterisk indicates the data statistically different ($P < 0.01$) from the value of zero hr incubation.

following incubation of the culture with 400 μM CKI-7 for various time periods, an increased rate of dye transfer can be detected within 30 min of the addition of CKI-7, and this effect reached a maximum by 2 hr and was maintained for at least up to 8 hr. As a control, dye transfer in another 28-day old culture incubated with 0.4% DMSO alone for the same period of time was also analyzed and the rate of dye transfer was shown to be unaffected by this reagent.

Discussion

This study has characterized the effect of the casein kinase I-specific inhibitor, CKI-7, on gap junctional communication in a sheep primary lens cell culture system. We have demonstrated the presence of membrane-associated casein kinase I in these cells, and that connexin43 is likely not a substrate of casein kinase I. In the presence of CKI-7, Lucifer Yellow dye transfer between cells in older lens cultures that contain higher levels of connexin49 (Yang & Louis, 2000) is increased, suggesting that the casein kinase I-catalyzed phosphorylation of connexin49 decreases cell communication between connexin49-containing gap junctions in the lens.

Multiple connexins (connexin43, connexin44, and connexin49) are expressed in this sheep lens culture system, so the observed changes in gap junctional communication after CKI-7 treatment could be attributed to altered levels of phosphorylation of one or more of these three connexins. However, evidence presented here indicates that connexin43 is likely not a substrate for casein kinase I. Furthermore, CKI-7 had no obvious effect on the rate of dye transfer between cells in 9-day old cultures in which connexin43 is the major connexin protein (Yang & Louis, 2000) even though membrane-associated casein kinase I activity is present at all three stages of the lens cultures examined here, including the 9-day cultures (Fig. 1). Thus, the CKI-7-induced increase in the rate of dye transfer in the older (15- and 28-day) cultures was unlikely due to changes in the activity of connexin43-containing junctional channels.

It has been shown previously that connexin44 is not a substrate of casein kinase I (Arneson et al., 1995), and that there is no obvious temporal change in connexin44 expression in this culture system (Yang & Louis, 2000). Thus, the ability of CKI-7 to increase the rate of dye transfer only in the older cultures cannot be attributed to changes in connexin44 expression level or activity in these cultures. Although these results could be explained by the expression of a nonconnexin casein kinase I substrate that only regulates the junctions in older cultures,

this seems less likely as no phosphoproteins have been identified that are associated with, and directly regulate, connexin44-containing gap junction channels. Therefore, it appears more likely that the action of CKI-7 on gap junctions in the lens cultures results from its ability to inhibit the phosphorylation of connexin49. Furthermore, that CKI-7 did not affect the rate of cell-to-cell transfer of Lucifer Yellow in the 9-day lens cultures suggests that this chemical does not have nonspecific effects on gap junction channels. Rather, these data suggest that the CKI-7-induced increase in the rate of dye transfer between cells in the older lens cultures was due to changes in the connexin composition of the gap junction channels in these older cultures. Taken together, these results suggest that CKI-7 increases cell-to-cell communication in the older lens cultures by specifically altering the phosphorylation state of connexin49 and thus increasing cell communication of connexin49-containing gap junctions.

Studies of lens connexin43-, connexin46- (rodent homologue of sheep connexin44), or connexin50- (rodent homologue of sheep connexin49) knockout mice have all been reported (Gao & Spray, 1998; Gong et al., 1997; White, Goodenough & Paul, 1998). The phenotypes of the lenses in these different lines of mice differed, indicating that these three connexins may have different functions in the intact lens. The connexin46- and connexin50-knockout mice developed cataracts (Gong et al., 1997; White et al., 1998), while the connexin 43-knockout mice exhibited signs of early stages of cataract formation (Gao & Spray, 1998). Whereas deletion of either the connexin43 or connexin46 gene had no obvious effects on lens formation or lens fiber cell differentiation, deletion of connexin50 resulted in mice that exhibited a reduced rate of growth of the lens and the eye. Therefore, among the lens connexins, connexin50 appears to play a critical role in the early stages of lens development and fiber cell differentiation (White et al., 1998).

In spite of the temporal changes in the levels of expression of connexin43 and connexin49 in this lens culture system, the basal level of dye transfer between these cells (as represented by the control values of the rate of dye transfer in the Table) did not change significantly as the cultures aged. Yang and Louis (2000) have shown that compared to 5-day old cultures, the level of connexin43 protein is reduced approximately 50% by 20 days in culture. In contrast, connexin49 protein level is increased more than 20-fold over this same time period. Thus, it might be expected that the gap junction-mediated cell-to-cell communication in the older cultures would differ from that in the younger cultures. However, this was not the case, indicating that the activity of connexins is tightly regulated so that the cells can maintain a constant level of gap junction-mediated cell-to-cell communication as the cultures differentiate. Recognizing the distinct gating and permeability properties of the three different connexins in the lens (White et al., 1994; Ebihara, Berthoud & Beyer, 1995; Moreno et al., 1995; Srinivas et al., 1999), the potential existence of both heterotypic (White et al., 1994) and heteromeric (Ebihara et al., 1999; Jiang & Goodenough, 1996) gap junction channels between the three lens connexins, and the turnover of the connexin proteins, multiple mechanisms are likely required to maintain a constant level of cell-to-cell communication in such a differentiating lens culture system. Concentrations of CKI-7 that inhibit casein kinase I effect an increase in Lucifer Yellow dye transfer in the older lens cultures suggesting that casein kinase I, which catalyzes the phosphorylation of connexin49, inhibits gap junctional communication in the connexin49-containing lens cell cultures. This could play an important role in regulating cell-to-cell communication as the lens cultures age. Thus, under basal conditions there would be a pool of connexin49 in the lens cells that is inhibited by casein kinase I-catalyzed phosphorylation; on the addition of CKI-7, the activity of casein kinase I is inhibited so that an endogenous protein phosphatase activity could now catalyze the dephosphorylation of this pool of connexin49 resulting in an increased level of cell-to-cell communication.

Although the role of casein kinase I in regulating cellular functions in any tissue remains poorly understood, the involvement of casein kinase I in two cellular processes has been described. In the nucleus of the cell, casein kinase I has been proposed to regulate DNA metabolism (Cegielska & Virshup, 1993; Hoekstra et al., 1991), whereas cytoplasmic casein kinase I has been proposed to play a role in regulating membrane structure and cell morphogenesis (Lu, Soong & Tao, 1985; Eder, Soong & Tao, 1986; Robinson et al., 1993; Manno et al., 1995). Membrane-associated casein kinase I has been shown to decrease the affinity between cytoskeletal elements or reduce membrane mechanical stability (Lu et al., 1985; Eder et al., 1986; Manno et al., 1995). The importance of the cytoskeleton in remodeling and maintaining cell integrity during lens development has been demonstrated (for review, *see* Wride, 1996), as has association of cytoskeletal components with gap junctions (Larsen et al., 1979; Lo, Mills & Kuck, 1994; Toyofuku et al., 1998). Hence, disassembly of the cytoskeleton and inhibition of gap junctional communication by casein kinase I-catalyzed phosphorylation of cytoskeletal proteins and connexin49, respectively, may play an important role in lens fiber cell differentiation. Alternatively, as an integral membrane protein, connexin49 may make a significant contribution to the lens membrane/cytoskeletal superstructure. Hence, connexin49 may be regulated together with cytoskeletal proteins by casein kinase I in order to make adjustments for this structure during fiber cell elongation and growth.

Other factors that have been shown to be fundamental for lens development and have also been shown to be substrates for casein kinase I, include the insulin receptor (Rapuano & Rosen, 1991) and the p75 tumor necrosis factor (TNF) receptor (Beyaert et al., 1995). It has been shown that lens epithelial cells express insulin receptors, and changes in the binding affinity of insulin with its receptors was correlated with changes in the differentiation state of lens epithelial cells (Bassas et al., 1987). However, the functional consequence of casein kinase I-catalyzed phosphorylation of insulin receptors has not been determined. The casein kinase I-catalyzed phosphorylation of the p75 TNF receptor in lymphoid-origin cells has been shown to inhibit apoptosis mediated by this receptor (Beyaert et al., 1995), whereas TNF has been proposed to play a role in the degradation of lens fiber cell DNA and nuclei (a process similar to apoptosis) that occurs during lens fiber cell differentiation (Wride, 1996). Thus, it is possible that casein kinase I regulates a number of biochemical steps critical for lens fiber cell development and differentiation, and that regulation of gap junctional communication is one of the links in this complex process.

In conclusion, this study provides evidence that casein kinase I may regulate cell-to-cell communication between connexin49-containing gap junctions in vivo. Furthermore, these data suggest that casein kinase I catalyzes the phosphorylation of connexin49 but not the phosphorylation of connexin43 or connexin44, indicating differential mechanisms for the regulation of these three connexins in the mammalian lens. Of the three lens connexins, connexin49 appears to be critically important for lens fiber cell differentiation and lens development, indicating that the possible regulation of connexin49 by casein kinase I may play an important role in these processes.

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