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Calmodulin and protein kinase C regulate gap junctional coupling in lens epithelial cells

Monica M. Lurtz and Charles F. Louis
Department of Biology, Georgia State University, Atlanta, Georgia 30303

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Lurtz, Monica M., and Charles F. Louis. Calmodulin and protein kinase C regulate gap junctional coupling in lens epithelial cells. *Am J Physiol Cell Physiol* 285: C1475–C1482, 2003. First published August 13, 2003; 10.1152/ajpcell.00361.2002.—The mechanisms regulating the permeability of lens epithelial cell gap junctions in response to calcium ionophore or ATP agonist-mediated increases in cytosolic Ca^{2+} (Ca_i^{2+}) have been investigated using inhibitors of calmodulin (CaM) and PKC. Cell-to-cell transfer of the fluorescent dye AlexaFluor594 decreased after the rapid and sustained increase in Ca_i^{2+} (to micromolar concentrations) observed after the addition of ionophore plus Ca^{2+} but was prevented by pretreatment with inhibitors of CaM but not PKC. In contrast, the delayed, transient decrease in cell-to-cell coupling observed after the addition of ATP that we have reported previously (Churchill G, Lurtz MM, and Louis CF. *Am J Physiol Cell Physiol* 281: C972–C981, 2001) could be prevented by either the direct or indirect inhibition of PKC but not by inhibition of CaM. Surprisingly, there was no change in the relative proportion of the different phosphorylated forms of lens connexin43 after this ATP-dependent transient decrease in cell-to-cell coupling. Although BAPTA-loaded cells did not display the ATP-dependent transient increase in Ca_i^{2+}, the delayed, transient decrease in cell-to-cell dye transfer was still observed, indicating it was Ca_i^{2+} independent. Thus CaM-mediated inhibition of lens gap junctions is associated with sustained, micromolar Ca_i^{2+} concentrations, whereas PKC-mediated inhibition of lens gap junctions is associated with agonist activation of second messenger pathways that are independent of changes in Ca_i^{2+}.

calcium; connexin43; lens gap junctions

GAP JUNCTIONS ARE INTERCELLULAR channels that allow direct communication of the cytoplasms of neighboring cells. These channels are comprised of six connexin subunits in each membrane forming a connexon, with two opposing connexons forming a gap junction. Although gap junctions are relatively nonspecific ion channels with pores large enough to allow passage of molecules up to 1,000 Da, some selectivity is seen depending upon the connexin (34). At least 20 human connexins have been identified, with most exhibiting a tissue-specific expression pattern (44). Thus the mammalian lens expresses three distinct connexin (Cx) proteins, Cx43, Cx46, and Cx50, in both the human and mouse, whereas in sheep the homologs are Cx43, Cx44, and Cx49, respectively.

The mammalian lens is an avascular organ composed of two cell types, epithelial and fiber cells. Cx43 is the major connexin protein expressed in epithelial cells, whereas Cx44 and Cx49 are expressed in fiber cells. Epithelial cells, which form a single-cell layer on the anterior surface of the lens, migrate to the equatorial region of the lens as they mature where they begin differentiating into fiber cells. The developing fiber cells elongate and lose most of their organelles, affording lens transparency. As a result, metabolism in the lens fiber cells is anaerobic, requiring the movement of glucose into and the products of metabolism out of these cells. The mammalian lens utilizes a microcirculatory system, with inward flow at the anterior and posterior poles and outward flow at the equatorial poles (11). Aquaporins are proposed to mediate the inward circulation and gap junctions to mediate the outward circulation (13, 17, 47). Failure of lens cells to maintain homeostatic microcirculation can result in the development of regions of opacity, termed cataracts (3, 11, 33). Increased cytosolic calcium concentration (Ca_i^{2+}) in lens cells has been correlated with lens cataract development (9, 20, 28, 45) and changes in gap junction permeability (8, 10, 14). Furthermore, the ubiquitous calcium-binding protein calmodulin (CaM) has been proposed to physically interact with gap junctions (18, 40, 41) and has been proposed to modulate fiber cell coupling in intact rat lenses (14).

The use of connexin-knockout mice has implicated gap junctions in cataract formation. For example, knockout of either Cx46 or Cx50 in the mouse resulted in cataract formation in the lens nuclear region (16, 43). Interestingly, knockout of the mouse Cx50 gene and simultaneous knockin of the Cx46 gene in the Cx50 locus results in cataract-free, although smaller than normal-sized, lenses (42), indicating Cx50 is essential for normal lens development. Although Cx43-knockout mice die shortly after birth, their lenses exhibit a phenotype characteristic of early stage cataractogenesis (15).

In lens epithelial cells, Ca_i^{2+} can be modulated by extracellular purinergic (P_{2U}) receptor agonists (7, 8, 30), and P_{2U} receptors have been identified pharmacologically in cultures of sheep lens epithelial cells (6). These receptors are coupled to the activation of PLC

Address for reprint requests and other correspondence: M. M. Lurtz, Dept. of Biology, Georgia State Univ., MSC 8L0389, 33 Gilmer St SE Unit 8, Atlanta, GA 30303-3088 (E-mail: bioml l@langate.gsu.edu).

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that in turn produces inositol-1,4,5-trisphosphate (IP_3) that effects the release of Ca^{2+} from the endoplasmic reticulum and diacylglycerol, a potent activator of PKC. Cx43 is the predominant connexin expressed in sheep lens cultures, but as the cultures age, Cx43 expression decreases and Cx49 expression increases (46). Cx43 contains several amino acid substrates for PKC in its carboxyl region (21, 24), and activation of PKC by the phorbol ester PMA in sheep lens cultures has been shown to result in the phosphorylation of this connexin with a corresponding reduction in cell-to-cell dye coupling (38).

We have demonstrated previously that in sheep lens cultures, there is a sustained elevation of Ca_i^{2+} after the addition of ionophore plus elevated extracellular Ca^{2+} concentration and that this results in a sustained inhibition of gap junction coupling (8). In contrast, purinergic receptor activation results in a transient elevation in Ca_i^{2+} and a delayed, transient inhibition of gap junction coupling (8). In this study, we have used pharmacological approaches to demonstrate that different mechanisms are responsible for these different responses of gap junctions to elevated Ca_i^{2+}.

MATERIALS AND METHODS

Materials. Sheep eyes were obtained from Iowa Lamb (Hawarden, IA). Characterized fetal calf serum was purchased from Hyclone (Logan, UT). Fura 2-AM, BAPTA-AM, and Alexa Fluor 594 (AF594) were from Molecular Probes (Eugene, OR). Monoclonal anti-mouse Cx43 primary antibody (MAB3068, IgG1), goat anti-mouse IgG, and horseradish peroxidase (HRP)-conjugated secondary antibodies were purchased from Chemicon International (Temecula, CA). Bisindolylmaleimide I (BIM-1) was obtained from Calbiochem (San Diego, CA). Medium 199, Hanks' balanced salt solution without divalent cations ($HBSS^{--}$) or with divalent cations ($HBSS^{++}$), PMA, staurosporine, U73122, U73343, ionomycin, calmidazolium (CDZ), *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7), ATP, and all other chemicals were purchased from Sigma Chemical (St. Louis, MO).

Cell culture. Primary cultures of sheep lens epithelial cells were prepared as described previously (37). Briefly, eyeballs removed from freshly slaughtered lambs were packaged on ice and the lenses were removed 4–6 h later. Lenses were nicked around the equator and anterior surface before a 30-min incubation at 37°C with 2.5 mg/ml trypsin, after which the reaction was quenched by the addition of 40 ml of ice-cold $HBSS^{--}$, and the lenses were triturated 20 times to dissociate the cells. After centrifugation (230 *g* for 5 min at 4°C), the dissociated differentiating epithelial cells were resuspended in Medium 199 (with 10 M HEPES and 4.2 mM $NaHCO_3$, pH 7.2) supplemented with 10% vol/vol FBS, 100 U/ml penicillin, and 0.1 mg/ml streptomycin. Cells were plated at a density of 1×10^5 cells/ml (2 ml/35-mm dish) on sterile, poly-*d*, L-ornithine-coated glass coverslips in a humidified 37°C incubator with 5% CO_2. Cells grown in culture for 8–28 days were used in the experiments described here. Only confluent cells displaying the cobblestone appearance typical of epithelial cells were used in these studies. Lentoids, characterized by multiple layers of transparent cells with poorly defined cell borders, were not used in these studies.

Calcium imaging. Cells were loaded with the Ca^{2+} indicator fura 2-AM (1 μM) by incubating the cells in the dark for 30 min at ambient temperature. The buffer containing fura 2-AM was removed, and the cells were rinsed with fresh $HBSS^{++}$ buffer and then incubated an additional 30 min in 2 ml $HBSS^{++}$ buffer (with 10 mM HEPES, 5 mM $NaHCO_3$, pH 7.2) to allow for complete hydrolysis of the intracellularly loaded fura 2-AM. Each coverslip of cells was used to form the bottom of a 2-ml microincubation chamber for imaging (Harvard Apparatus, Holliston, MA) that was performed on a Nikon TE300 (Nikon, Melville, NY) inverted microscope fitted with a 75-watt xenon short arc lamp and a Hamamatsu charge-coupled device digital camera (Hamamatsu, Bridgewater, NJ) and supported on a vibration isolation table (Technical Manufacturing, Peabody, MA). Images were collected using MetaFluor software (Universal Imaging, v. 3.5, Downington, PA). A Metaltek filter wheel (Metaltek Instruments, Raleigh, NC) housing the necessary excitation filters for fura 2 and the appropriate Nikon filter blocks for fura 2 emission and AF594 optics were moved to position as needed during an experiment. Ca_i^{2+} was measured ratiometrically ($\lambda_{340}/\lambda_{380}$) with fura 2 throughout the experiment in the injected cell and the cells adjacent to the injected cell. Ca^{2+} concentrations were determined as described previously (8).

Assessment of gap junctional coupling using AF594 dye transfer. A 1 mM solution of AF594 was iontophoretically injected into cells with a Duo 773 electrometer (World Precision Instruments, Sarasota, FL), using an A310 Accupulser (World Precision Instruments) over the course of 1 min with a pulse protocol of 5 ms every 100 ms for 3 s total. Images of cells were recorded 5 min after injection of AF594 dye, and the number of cells receiving dye was counted. A control AF594 injection was performed on each coverslip of cells to establish that the cells were communicating. At the end of each experiment, the coverslip with cells was placed in a 35-mm petri dish, sealed, flash frozen in liquid nitrogen, and stored at −80°C for subsequent protein analysis.

Microinjection. Micropipettes were pulled from borosilicate glass capillaries with 1 mm outside diameter, 0.75 mm inner diameter, and an internal 100 micron microfilament (Dagan, Minneapolis, MN) on a P-97 Flaming/Brown micropipette puller (Sutter Instruments, Novato, CA). Micropipettes backfilled with a 1 mM solution of AF594 were mounted on a half cell with a chlorodized silver wire and used with a resistance between ~60–200 MΩ.

Membrane isolation. Cells from previously frozen coverslips were harvested by adding 500 μl of ice-cold *buffer 1* (in mM: 25 Tris base, 100 NaCl, 10 EDTA, 50 NaF, 0.5 Na_3VO_4, pH 8.0) with freshly added protease inhibitors (2 mM PMSF, 1 mg/l each: aprotinin, leupeptin, and pepstatin) to each frozen petri dish, physically removing the cells from the coverslip with a cell scraper (Sarstedt, Newton, NC) and transferring the suspended cells to a thick-walled 1.5-ml microfuge tube (Beckman/Coulter, Fullerton, CA). Coverslips were washed with an additional 400 μl of ice-cold *buffer 1* supplemented with protease inhibitors, and the final volume in each sample was brought to 1 ml. Samples were vortexed, and the cells were sedimented at 1,260 *g* at 4°C in a Beckman TLA100 centrifuge for 5 min. The supernatant was removed, and the sedimented cells were resuspended in 100 μl of ice-cold buffer 1 with protease inhibitors with homogenization (30 times with a 1-ml glass-on-glass Potter-Elvehjem tissue homogenizer; Bellco Glass, Vineland, NJ). *Buffer 1* with protease inhibitors was added to a final volume of 1 ml, capped, vortexed, and incubated on ice for 10 min. The lysed cells were centrifuged at 100,000 *g* at 4°C for 30 min in a Beckman TLA 100 centrifuge. The supernatant was removed and discarded, and the sedimented membranes were resuspended in 65 μl of ice-cold *buffer 2* (10 mM HEPES, pH 7.2) by drawing the membranes through a 50-μl Hamilton

GASTIGHT 1700 syringe with cemented needle (Sigma Aldrich, St. Louis, MO) until the membrane suspension was homogenous (~30 times). The protein concentration of each membrane preparation was determined using the micro bicinchoninic acid (BCA) assay using bovine serum albumin standards (35). Membranes were either used immediately or flash-frozen in liquid nitrogen and stored at −80°C.

Dephosphorylation of proteins. Dephosphorylation of lens membrane proteins was accomplished by incubation of the membranes with calf intestinal alkaline phosphatase as previously described (2). Briefly, 15 μg of protein (30 μl total volume) were incubated with 0.1 U/μl alkaline phosphatase in 0.1 M Tris (pH 7.5) supplemented with 0.1 M $MgCl_2$ and protease inhibitors (1 mM PMSF; 1 mg/l each: aprotinin, leupeptin, and pepstatin) for 1 h at 37°C. Immediately after the incubation period, sample buffer was added to each reaction mixture, heated at 65°C for 4 min, cooled on ice, and electrophoretically separated.

Western immunoblotting. After electrophoretic analysis of proteins on 10% PAGE gels with a 5% stacking gel in 1% SDS (23), gels were equilibrated with transfer buffer for 15 min before electrophoretic transfer to PVDF membranes (Schleicher and Schuell, Keene, NH). Electrophoresis was conducted overnight on a Hoefer Scientific (San Francisco, CA) SE 600 series vertical slab gel unit at 6–8 mA, and protein transfer was achieved using the Bio-Rad (Richmond, CA) Trans-Blot cell per manufacturer's instructions. The nonspecific antibody binding sites on the PVDF membranes were blocked in PBS-Tween (137 mM NaCl, 2.7 mM KCl, 1.8 mM Na_2HPO_4, and 0.1% vol/vol Tween-20, pH 7.2), containing 3% wt/vol dried milk for 3 h at ambient temperature, or overnight at 4°C, with gentle agitation. After being blocked of nonspecific antibody binding sites, membranes were washed three times for 10 min each in fresh PBS-Tween without dried milk before overnight incubation (4°C, gentle agitation) with mouse-anti Cx43 antibody (1:1,000 dilution in PBS-Tween with 3% dried milk). The primary antibody solution was removed, and membranes were washed three times for 15 min in fresh PBS-Tween with 3% dried milk before the addition of secondary antibody (1:2,000 dilution of goat-anti mouse IgG conjugated to HRP in PBS-Tween with 3% dried milk) for 2–3 h at ambient temperature, or overnight at 4°C, with gentle agitation. Secondary antibody-containing solution was removed, membranes were washed as described above, and immunoreactive components were detected on Kodak BIOMAX MS film after membranes were incubated for 1 min with ECL detection reagent (Amersham Pharmacia Biotech, Piscataway, NJ). Immunoblots were stored in PBS-Tween without milk at 4°C, and all experiments were repeated a minimum of three times.

Data handling and statistical analysis of the data. Injection data were pooled, averaged, and expressed as means ± SE. The Ca_i^{2+} for each field of cells was determined by measuring the average value for the injected cell, and for each of the cells immediately adjacent to the injected cell, and then calculating the mean for the group. Values for identical treatments were pooled, averaged, and expressed as means ± SE. Statistical differences were tested for multiple treatments against the appropriate controls using one-way ANOVA, and differences between two samples were conducted using a *t*-test. Differences from control value were considered significant when $P < 0.01$.

RESULTS

We have shown previously that when AF594 dye was injected into a single cell in a confluent monolayer of sheep lens cells with resting Ca_i^{2+} (50–100 nM), the dye spreads to the adjacent cells (8). It was also demonstrated that experimentally elevating Ca_i^{2+} to ~1 μM in the presence of ionomycin (2 μM) and elevated extracellular Ca^{2+} (Ca_o^{2+}) (11.8 mM) significantly reduced cell-to-cell transfer of AF594. In Fig. 1, *B* and *D*, respectively, AF594 transferred to 15 cells when resting Ca_i^{2+} was 110 ± 9 nM, but only to 5 cells when the average Ca_i^{2+} was raised to 1,200 ± 140 nM with ionomycin and elevated Ca_o^{2+}, consistent with the previously reported data. Summary data for resting and elevated Ca_i^{2+} conditions are shown in Fig. 2 and Table 1.

CaM inhibitors prevent the ionomycin-dependent decrease in cell-to-cell coupling. CaM regulates the functional activity of many proteins, and recent evidence suggests that CaM modulates Cx32 function (29). To determine the possible role of CaM in the ionomycin plus elevated Ca_o^{2+}-dependent closure of sheep lens gap junctions, lens cell cultures were pretreated with the CaM inhibitor CDZ (10 μM) for 20 min before the addition of ionomycin for 5 min and the subsequent elevation of Ca_o^{2+}. In the presence of CDZ, increasing Ca_i^{2+} to 1.41 ± 0.13 μM did not result in a decrease in cell-to-cell dye transfer (Fig. 1*F*; summary data in Fig.

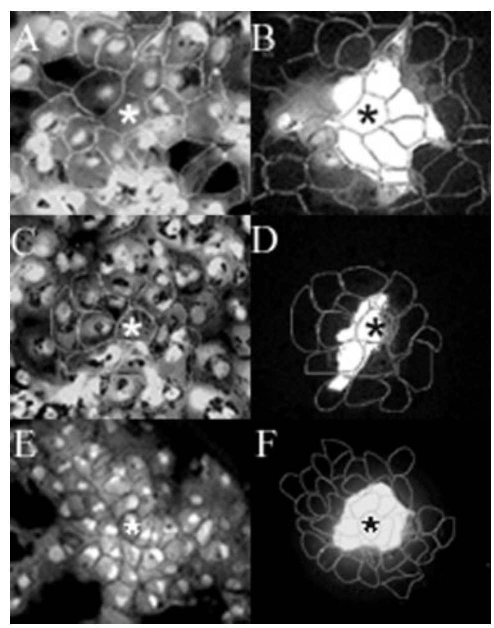

Fig. 1. The effect of a sustained increase in intracellular Ca^{2+} (Ca_i^{2+}) on cell-to-cell dye transfer between lens cells. Confluent monolayers of lens cells were loaded with the Ca^{2+} indicator fura 2. *A*, *C*, and *E*: λ_{380} images of fura 2 loaded lens epithelial cells. *B*, *D*, and *F*: Alexa Fluor 594 (AF594) cell-to-cell dye transfer. *A* and *B*: in the absence of ionomycin plus elevated (11.8 mM) extracellular calcium (Ca_o^{2+}), cells exhibited a resting Ca_i^{2+} of 110 ± 9 nM. Injection of AF594 resulted in dye transfer to 15 cells. *C* and *D*: addition of ionomycin plus 11.8 mM Ca_o^{2+} resulted in a sustained elevation of Ca_i^{2+} to 1,200 ± 140 nM. AF594 dye now only transferred to 5 cells. *E* and *F*: cells pretreated with the calmodulin inhibitor (CDZ, 10 μM) exhibited elevated Ca_i^{2+} (1,430 ± 350 nM) after the addition of ionomycin and 21.8 mM Ca_o^{2+}, and AF594 dye transferred to 19 cells. In each panel, an asterisk denotes the cell in which AF594 dye was microinjected.

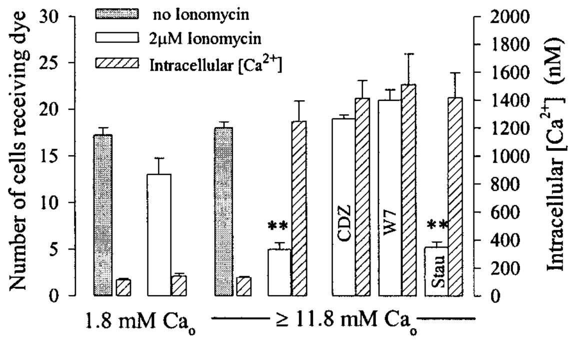

Fig. 2. Effect of calmodulin inhibitors on cell-to-cell dye transfer between lens cells after the addition of ionomycin plus elevated Ca_o^{2+}. In the absence of ionomycin (solid bars), AF594 transferred to 17.2 ± 0.8 cells (n = 21) in 1.8 mM Ca_o^{2+} and 18.0 ± 0.6 cells (n = 6) in 11.8 mM Ca_o^{2+}; $[Ca_i^{2+}]$ in 11.8 mM Ca_o^{2+} (hatched bars) was 112 ± 9 and 131 ± 9 nM, respectively. In the presence of 2 μM ionomycin (open bars), dye transferred to 13.0 ± 1.7 cells (n = 3) in 1.8 mM Ca_o^{2+} and 5.0 ± 0.7 cells (n = 9) in 11.8 mM Ca_o^{2+}; Ca_i^{2+} was 139 ± 20 and 1,250 ± 150 nM, respectively. Pretreatment with either 10 μM CDZ or 100 μM *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide (W7) for 30 min before addition of ionomycin plus 21.8 mM Ca_o^{2+} resulted in cell-to-cell dye transfer to 18.8 ± 0.5 cells (n = 6; $[Ca_i^{2+}]$ = 1,410 ± 130 nM) and 21.0 ± 1.1 cells (n = 4; $[Ca_i^{2+}]$ = 1,510 ± 220 nM), respectively. Thirty minutes pretreatment with 10 nM staurosporine (stau) before addition of ionomycin plus 11.8 mM Ca_o^{2+} resulted in dye transfer to 5.2 ± 0.6 cells (n = 5; $[Ca_i^{2+}]$ = 1,420 ± 180 nM). A significant change in dye transfer (P < 0.01) is denoted by a double asterisk.

2 and Table 1); comparable results were obtained using the CaM inhibitor W7 (1.51 ± 0.22 μM; Fig. 2 and Table 1). To determine the possible role of PKC in this Ca^{2+}-dependent inhibition of gap junctional coupling, cells were pretreated for 20 or 30 min with of the PKC inhibitor staurosporine (10 nM). As depicted in Fig. 2, PKC inhibition failed to prevent the ionomycin plus elevated Ca_o^{2+}-dependent decrease in cell-to-cell dye transfer (Table 1).

Addition of extracellular ATP results in a delayed, transient decrease in cell-to-cell dye coupling. In contrast to the sustained elevation in Ca_i^{2+} mediated by ionomycin plus elevated Ca_o^{2+}, addition of ATP (100 μM) to the extracellular medium resulted in a transient increase in Ca_i^{2+} in the lens cell cultures (8, 45). We have shown previously that after the addition of ATP, there is a time-dependent reduction in cell-to-cell coupling (after ~4 min), with a return to the original levels of cell-to-cell coupling within 20 min after ATP addition (8). In Fig. 3, *A*, *C*, and *E*, fura 2 images of lens cell cultures (λ_{ex}380 nm) define the cell boundaries for determination of AF594 cell-to-cell dye transfer (Fig. 3, *B*, *D*, and *E*). In the absence of ATP (Fig. 3*B*), 15 cells received dye; 5 min after ATP addition to the extracellular medium, the number of cells receiving dye was reduced to three cells (Fig. 3*D*). Thirty minutes after ATP was added, cell coupling assessed by AF594 dye transfer had returned to pre-ATP addition levels (Fig. 3*E*, 16 cells).

CDZ does not prevent the ATP-dependent, delayed, transient reduction in cell-to-cell coupling. To determine whether the ATP-dependent decrease in cell-to-cell coupling involved CaM, cells were pretreated with the CaM inhibitor CDZ (10 μM) for 20 min before injection of AF594 into a single cell in the lens culture. As shown in Table 2, the presence of CDZ itself did not affect lens cell-to-cell coupling as assessed by the number of cells to which AF594 dye transferred. CDZ pretreatment also failed to prevent the ATP-dependent transient decrease in lens cell-to-cell coupling.

Inhibition of PKC prevents the ATP-dependent transient reduction in cell-to-cell coupling. Purinergic receptors are coupled to pathways that activate PKC,

Table 1. *Effect of ionomycin and inhibitors of CaM or PKC on cell-to-cell dye coupling in lens epithelial cultures*

Treatment	Number of Cells Receiving Dye	n
1.8 mM Ca_o^{2+}	17.2 ± 0.8	21
Ionomycin + 1.8 mM Ca_o^{2+}	13.0 ± 1.7	3
Ionomycin + 11.8 mM Ca_o^{2+}	5.0 ± 0.8*	5
CDZ + 1.8 mM Ca_o^{2+}	18.4 ± 1.1	23
CDZ + 11.8 mM Ca_o^{2+}	18.4 ± 1.3	19
CDZ + ionomycin + 21.8 mM Ca_o^{2+}	18.8 ± 0.5	6
W7 + 1.8 mM Ca_o^{2+}	19.1 ± 0.5	14
W7 + ionomycin + 1.8 mM Ca_o^{2+}	19.3 ± 1.0	4
W7 + ionomycin + 21.8 mM Ca_o^{2+}	21.0 ± 1.1	4
Stau + ionomycin + 11.8 mM Ca_o^{2+}	5.2 ± 0.6*	5

Values are stated as means ± SE; n = number of observations. Stau, staurosporine; CDZ, calmidazolium; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide; Ca_0^{2+}, extracellular Ca^{2+}. *Value significantly smaller than control values (P < 0.01).

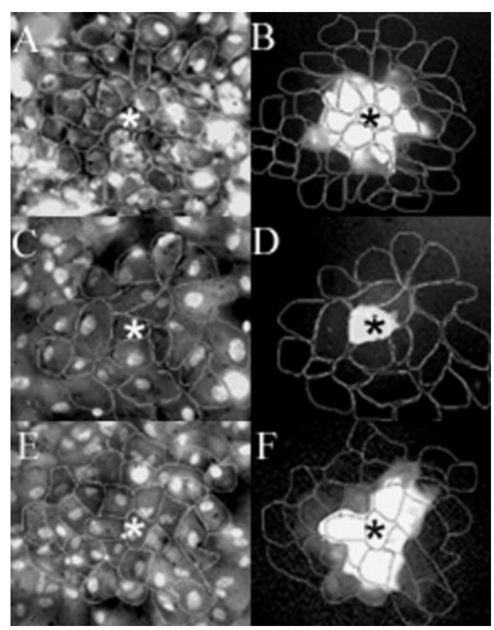

Fig. 3. Effect of extracellular ATP on cell-to-cell dye transfer between lens cells. Lens cell cultures were loaded with the calcium indicator fura 2. *A*, *C*, and *E*: cells prior to measurement of cell-to-cell coupling with AF594 dye in *B*, *D*, and *F*. *A* and *B*: in the absence of ATP, AF594 transferred to 15 cells. *C* and *D*: 5 min after the addition of 100 μM, ATP AF594 transferred to 3 cells. *E* and *F*: AF594 transferred to 16 cells 31 min after the addition of 100 μM ATP. In each panel, an asterisk denotes the cell into which AF594 dye was microinjected.

Table 2. *Effect of ATP in the presence and absence of inhibitors on dye transfer in lens epithelial cell cultures*

Treatment	Number of Cells Receiving Dye	*n*
Control	17.6 ± 0.5	8
ATP	5.5 ± 0.2*	6
U73122	23.7 ± 0.8	7
U73122 + ATP	23.4 ± 0.5	5
U73343	17.5 ± 0.3	4
U73343 + ATP	7.0 ± 0.6	3
Stau	19.6 ± 0.8	8
Stau + ATP	20.2 ± 0.6	5
BAPTA	22.2 ± 1.0	5
BIM-1*	20.3 ± 0.6	6
BIM-1 + ATP	21.0 ± 0.7	5
BAPTA + ATP	6.4 ± 0.9*	5
CDZ	18.5 ± 1.6	14
CDZ + ATP	4.8 ± 1.4*	5

Values are stated as means ± SE; *n* = number of observations. BIM-1, bisindolylmaleimide I. *Value significantly smaller than control values ($P < 0.01$).

and purinergic receptors have been identified pharmacologically in lens cells (6). It has also been shown that direct activation of PKC by phorbol ester results in a decreased lens cell-to-cell dye coupling in both chicken lens cultures (4) and sheep lens cultures (38). We therefore tested whether activation of PKC was a necessary step in the decreased lens cell-to-cell coupling after the addition of ATP to the extracellular medium. In the absence of ATP, lens cells pretreated with the PKC inhibitor staurosporine (10 nM) for 20 min transferred AF594 dye to 19.6 ± 0.8 cells (Fig. 4*C* and Table 2). After the addition of extracellular ATP to these staurosporine-treated cells, the number of cells to which AF594 transferred was now unchanged after 5 or 10 min (Fig. 4*C* and Table 2). Pretreatment of lens cell cultures with the PKC inhibitor BIM-1 (100 nM, 30 min; Table 2) also prevented the ATP-mediated transient decrease in cell-to-cell dye transfer.

Inhibition of PLC prevents the ATP-dependent, transient reduction in cell-to-cell coupling. Activation of PLC results in the production of both IP_3 and diacylglycerol, which in turn activates PKC and so provides one possible mechanism for the ATP-dependent decrease in lens cell-to-cell dye transfer. To determine whether activation of PLC was a necessary step in the ATP-dependent transient decrease in cell-to-cell coupling, cells were pretreated with the PLC inhibitor U73122 (2 μM) for 20 min before AF594 injection. U73122 addition to the medium did result in a significant increase in the basal level of cell-to-cell dye transfer. However, this was now unaltered at any time after the further addition of ATP to the extracellular medium (Fig. 4*B* and Table 2). U73343, the inactive analog of U73122, did not prevent the ATP-mediated delayed and transient decrease in cell-to-cell dye coupling (Table 2).

A transient increase in Ca_i^{2+} is not required for the ATP-dependent, transient decrease in cell-to-cell coupling. Our data in Table 2 indicate that CaM does not mediate the ATP-dependent, transient decrease in lens cell-to-cell coupling. It was therefore of interest to determine whether this transient decrease in cell-to-cell coupling depended on the ATP-dependent transient increase in Ca_i^{2+} (Fig. 5). To answer this, cells were first loaded with fura 2-AM and subsequently loaded with the nonfluorescent calcium chelator BAPTA-AM. These cells exhibited a basal Ca_i^{2+} that was within the normal range of resting concentrations we have reported previously in primary cultures of sheep lens cells (compare Fig. 5, *timepoint a*, and Ref. 8). However, as expected, the BAPTA-loaded cells exhibited no apparent increase in Ca_i^{2+}, either transient or sustained, after the addition of ATP to the extracellular medium (Fig. 5, *timepoints b* and *c*). Although loading with BAPTA prevented the ATP-dependent, transient increase in Ca_i^{2+}, cell-to-cell coupling was significantly reduced 5 min after the addition of ATP to the extracellular medium (Fig. 4*D* and Table 2).

Purinergic receptor activation of PKC does not alter the electrophoretic mobility of the different phosphorylated forms of Cx43. PKC has been shown to catalyze the phosphorylation of Cx43 in its carboxyl region (24). Indeed, in lens cell cultures, activation of PKC by the phorbol ester PMA results in the phosphorylation of Ser368 in the COOH terminus of Cx43 (25) and induces a shift in electrophoretic mobility detectable by Western blot analysis (24, 38). Because our data indi-

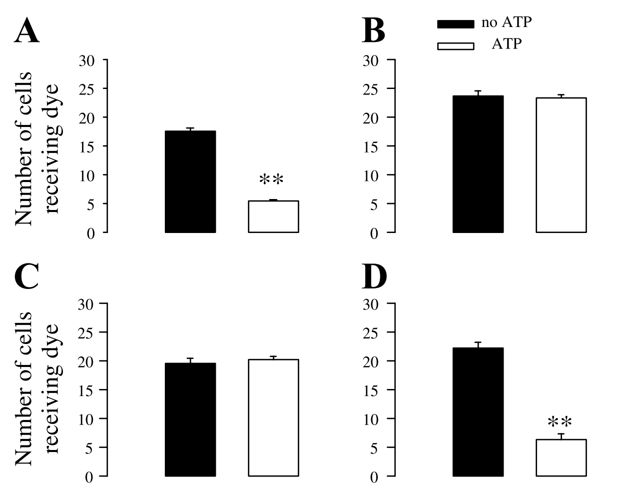

Fig. 4. The effect of U73122, staurosporine, and BAPTA on cell-to-cell dye transfer between lens cells after the addition of extracellular ATP. Lens cell cultures were loaded with the Ca^{2+} indicator fura 2, and cell-to-cell coupling was assessed by AF594 dye transfer. A transient increase in Ca_i^{2+} was mediated by the addition of 100 μM ATP. *A*: in the absence of ATP, AF594 transferred to 18.3 ± 0.8 cells (n = 17). Five to ten minutes after the addition of 100 μM ATP, AF594 transferred to 5.0 ± 0.7 cells (n = 5). *B*: in cells pretreated for 20 min with 2 μM U73122, AF594 transferred to 23.7 ± 0.8 cells (n = 7), and 5–10 min after addition of 100 μM ATP, AF594 transferred to 23.4 ± 0.5 cells (n = 5). *C*: in cells pretreated for 20 min with staurosporine (10 nM), AF594 transferred to 19.6 ± 0.8 cells (n = 8), and 5–10 min after addition of 100 μM ATP, AF594 transferred to 20.2 ± 0.6 cells (n = 5). *D*: in cells loaded with BAPTA, AF594 transferred initially to 22.2 ± 1.0 cells, and to 6.4 ± 0.7 cells (n = 5) 5–10 min after the addition of 100 μM ATP. A significant change in dye transfer ($P < 0.01$) is denoted by a double asterisk.

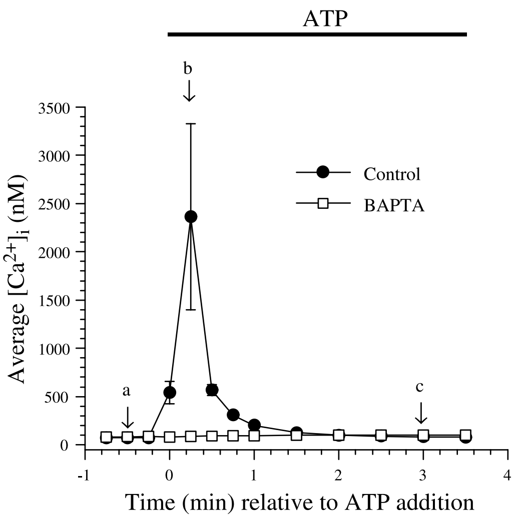

Fig. 5. Effect of BAPTA on the ATP-dependent Ca_i^{2+} transient in lens cells. The Ca_i^{2+} of sheep lens cells was measured as a function of time relative to extracellular addition of ATP. Arrows a, b, and c indicate the timepoints 30 s before ATP addition and at 5 s or 3 min after ATP addition, respectively. Cells exhibited a transient increase in Ca_i^{2+} after addition of 100 μM ATP (●; a: 71 ± 2 nM, b: 2,360 ± 960 nM, and c: 82 ± 2 nM; n = 15 cells), whereas cells preloaded with BAPTA exhibited no significant change in Ca_i^{2+} after the addition of extracellular ATP (□; a: 82 ± 11 nM, b: 87 ± 14 nM, and c: 99 ± 25 nM; n = 16 cells).

cate PKC activation is required for the delayed, transient decrease in cell-to-cell dye transfer observed after the addition of extracellular ATP, it was important to determine whether the decrease in cell coupling was associated with a change in the electrophoretic mobility of the different phosphorylated forms of Cx43. To address this question, membrane preparations from individual plates of lens cells used in experiments either in the absence of ATP, or exposed to 100 μM ATP for 10 min, were analyzed for changes in Cx43 mobility by Western blot analysis. Alkaline phosphatase dephosphorylation of the lens proteins was used as a control to confirm that the mobility shift in Cx43 was phosphorylation dependent as previously demonstrated (25, 38). Western blot analysis of lens membranes indicated that Cx43 migration results in two immunoreactive bands [41 kDa (P0) and 43 kDa (P1); see Fig. 6, *lane 1*]. Incubation of the lens cultures with ATP for 10 min did not result in any changes in the relative mobility of these two immunoreactive forms of Cx43 (Fig. 6, *lane 2*). The relative proportion of each immunoreactive band was calculated for each lane (*lane 1*: 54% ± 1.5% P0, 46% ± 1.5% P1; *lane 2*: 54% ± 2.5% P0, 46% ± 2.5% P1, n = 3) and indicates that there is no change in the relative proportion of these different Cx43 forms after purinergic receptor activation of PKC. Alkaline phosphatase treatment of the lens membranes (Fig. 6, *lanes 3* and *4*) demonstrates that the P1 form of Cx43 was indeed due to phosphorylation this protein.

DISCUSSION

The data presented here indicate two different mechanisms by which lens gap junctions are regulated in primary cultures of lens epithelial cells. A reduction in cell-to-cell dye transfer after a sustained increase in Ca_i^{2+} was dependent on CaM, whereas a delayed and transient reduction in dye transfer after purinergic receptor activation required PKC activation. This provides the first report demonstrating that whereas lens gap junctions can be regulated by elevated Ca_i^{2+}, lens cell gap junctions respond differently to Ca^{2+} signaling pathways depending on the mechanism by which Ca_i^{2+} is elevated in these cells.

A sustained elevation in Ca_i^{2+} in primary lens cultures mimicking the precataract state was accomplished by the addition of ionomycin and an increased Ca_o^{2+}; addition of ionomycin alone did not affect cell-to-cell dye transfer. The effect of ionomycin addition alone has been shown to release Ca^{2+} from the endoplasmic reticulum, followed by capacitative Ca^{2+} influx and a return of Ca_i^{2+} to resting levels (8), consistent with the effects of ionomycin on human umbilical vein endothelial cells (27). Only by increasing the Ca_o^{2+} to ≥11.8 mM in the presence of ionomycin did Ca_i^{2+} increase to micromolar concentrations, resulting in the partial inhibition of gap junctions. Pretreatment of the lens cell cultures with CaM inhibitors does not prevent the increase in Ca_i^{2+} but does prevent the partial inhibition of the gap junctions, indicating this inhibition is CaM dependent.

Although our data are consistent with CaM interacting with lens gap junctions, there are no apparent CaM-binding IQ domains in these connexins. However, Török et al. (39) have shown that a synthetic peptide corresponding to the 16 NH_2-terminal amino acids of Cx43 binds to CaM, suggesting CaM may regulate Cx43-containing lens gap junctions.

It is well documented that a sustained elevation in lens Ca_i^{2+} is an early event in the development of many types of cataract (9, 19, 26; for review, see Ref. 12). However, the mechanisms responsible for this elevated Ca_i^{2+} are poorly understood. Because gap junction coupling between cells allows for the cell-to-cell passage of many different molecules, including signaling molecules, inhibiting communication of damaged or apoptotic cells with healthy cells would be expected to limit further cell damage in a tissue. Thus it is likely that CaM-dependent closure of gap junctions when there is a sustained elevation of Ca_i^{2+}, as is thought to occur in cataract, may provide a protective mechanism for the lens after injury.

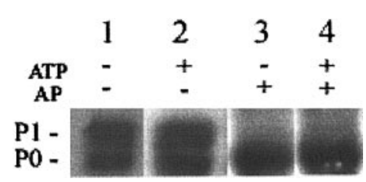

Fig. 6. Western blot analysis of connexin43 (Cx43) in lens cell membranes. The electrophoretically fractionated proteins of membranes isolated from lens cell cultures were immunoblotted with α-Cx43 antibody. *Lane 1*: no treatment; *lane 2*: treated with 0.1 mM ATP (10 min); *lane 3*: incubated with alkaline phosphatase (AP) only; *lane 4:* ATP-treated (0.1 mM, 10 min) and then incubated with alkaline phosphatase.

In contrast to the relatively rapid decrease in cell-to-cell coupling after an ionomycin-dependent sustained elevation in Ca_i^{2+}, the decrease in cell-to-cell coupling in response to extracellular application of ATP required several minutes. The decrease in cell coupling occurred within 5 min of ATP addition, and communication returned to pre-ATP addition levels 20 min post-ATP addition. Previous pharmacological evaluation of these sheep lens cultures indicated the presence of purinergic receptors (6); therefore, it was suspected that ATP activated a purinergic receptor-signaling pathway resulting in the activation of PLC generating IP_3 and diacylglycerol. Although both IP_3 and diacylglycerol can activate PKC, diacylglycerol activation of PKC is Ca^{2+} independent (1). If PKC activation via purinergic receptor stimulation is a necessary step in this mechanism, it follows that inhibition of an intermediate step in this pathway would prevent downstream signaling and subsequent inhibition of gap junctions. Indeed, inhibition of PLC or PKC before ATP addition did prevent the ATP-dependent, transient decrease in cell coupling, thus supporting this hypothesis. Because a number of steps in this pathway are Ca^{2+} dependent, it was important to determine whether the ATP-dependent transient decrease in cell coupling depended on the ATP-dependent, transient increase in Ca_i^{2+}. When cells were loaded with the cell-permeant form of the Ca^{2+} chelator BAPTA, the ATP-dependent transient increase in Ca_i^{2+} was inhibited but not the transient decrease in cell coupling. We conclude that direct activation of PKC activation by diacylglycerol effects the delayed and transient reduction in cell-to-cell dye transfer observed in these lens epithelial cell cultures.

Thus the agonist-dependent, transient decrease in cell-to-cell coupling is not mediated by the same mechanism as that observed after a sustained elevation in Ca_i^{2+} with ionomycin and increased Ca_o^{2+}.

We have shown previously that of the three lens connexins, Cx43 is the major PKC substrate in the lens epithelial cell culture and that activation of PKC by PMA in sheep lens cell cultures results in the phosphorylation of this connexin that is correlated with an electrophoretic mobility shift and a decrease in cell coupling (38). Thus it was important to determine whether ATP-dependent purinergic receptor activation promoted changes in the level of phosphorylation of this connexin indicated by an electrophoretic mobility shift. Western blot analysis of lens epithelial cell cultures indicated no obvious change in the level of Cx43 phosphorylation as assessed by a mobility shift indicating it is unlikely the substrate for PKC in this situation. There are several possible explanations. One is that although amino acid substrates for PMA-activated PKC have been identified in Cx43 (25), other Cx43 residues may also be phosphorylated by PKC after purinergic receptor activation, and phosphorylation of these residues does not necessarily result in a mobility shift in Cx43 in SDS-PAGE gels (36). Alternatively, it is possible that purinergic receptor activation of PKC might result in the phosphorylation of Cx44 (3) or Cx49 that is also present in these lens cultures (38). It is always possible that although a small change in the level of Cx43 phosphorylation might not be detected as a mobility shift in SDS-PAGE, it may be sufficient to significantly decrease cell coupling. Finally, it is possible that PKC catalyzes the phosphorylation of a nonconnexin substrate that in turn activates a pathway that leads to gap junction closure. There is considerable evidence for such pathways in the regulation of gap junctions in other tissues (22, 31, 32).

In conclusion, we have identified two mechanisms modulating gap junctional coupling in sheep lens cells. CaM-dependent inhibition of lens gap junctional coupling is evident with sustained, micromolar levels of Ca_i^{2+}, whereas agonist-induced PKC activation results in a transient decrease in lens gap junctional coupling. The mechanism by which PKC is activated and the molecular events following its activation that lead to gap junction closure in the lens are the subject of ongoing studies in this laboratory.

DISCLOSURES

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