Micromolar Levels of Intracellular Calcium Reduce Gap Junctional Permeability in Lens Cultures

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Purpose. To investigate in bovine and embryonic chicken lens cultures the effects of elevated intracellular calcium on the permeability of gap junctions. To determine the changes in intracellular calcium using fura-2. To detect any changes in the phosphorylation of connexin43 after ionophore treatment.

Methods. Lucifer yellow was micro-injected into individual cells, and dye spread to neighboring cells was evaluated. Intracellular calcium levels were measured using the calcium indicator, fura-2. Cultures were also labeled with 32P-orthophosphate followed by immunoprecipitation with antibodies specific for the gap junction protein, connexin43.

Results. Bovine lens cultures incubated in the presence of either A23187 or ionomycin showed a reduction in intercellular dye transfer. The intracellular calcium concentrations in bovine cells were increased from a mean value of 0.11 ± 0.009 μM in the controls to a mean of 0.40 ± 0.073 μM with ionomycin treatment. Subsequent addition of EGTA to the medium decreased the intracellular calcium concentrations to a mean of 0.26 ± 0.113 μM and reversed the inhibition of dye spread found with ionomycin. With ionomycin in the medium, the phosphorylated form of connexin43 was not as prominent as in the controls. In contrast, these same treatments had no detectable effect on junctional permeability in the embryonic chicken lens cultures. Dye spread was equally extensive and rapid under control and ionophore conditions, even though fura studies showed an elevation in intracellular calcium levels.

Conclusions. In the bovine cultures, physiologically relevant changes in the levels of cytoplasmic calcium markedly reduced dye transfer. The increase in cytoplasmic calcium was correlated with a change in the phosphorylation level of connexin43. The regulation of junctional communication in the chick lens cultures appears to differ significantly from that in the bovine system. Invest Ophthalmol Vis Sci. 1994; 35:3332-3341.

Gap junctions are cell surface specializations which contain intercellular channels allowing direct exchange of ions and other small molecules between neighboring cells (for a review see refs. 1 and 2). Movement through these channels is passive and nonspecific. Functionally, gap junctions are thought to be involved in tissue homeostasis, regulation of cell growth, and embryonic development.

The permeability of gap junctions is determined by the number of channels between coupled cells and by the fraction of open channels, in addition to single channel properties. Factors such as cytoplasmic pH, intracellular calcium concentration and protein phosphorylation have been shown to regulate gap junctional permeability. Regulation may involve a direct action on the channel protein, altering its conformation, or a cascade of events involving second messengers and protein kinases.

In the lens, an extensive junctional network links epithelial cells, fiber cells, and possibly epithelial–fiber cell combinations, although there remain questions about the extent of the epithelial–fiber cell communication. The junctional network is thought to play a critical role given the avascular nature of the lens and the fact that certain metabolic functions, e.g. Na+, K+–activated ATPase activities, are concentrated in the lens epithelial cells. Junction-mediated cellular communication may also be required for normal lens development.
The importance of gap junctions in the lens suggests that there must be well-developed mechanisms for regulating permeability, especially in cases of injury to either epithelial or fiber cells. Although little is currently known about the control of junctional permeability in the lens, there is increasing evidence indicating a role for calcium. Calcium has been shown to modify transfer through cell-to-cell channels in other cell systems\(^9\) to affect junctional permeability in different lens preparations,\(^25\) and to affect lens junctional structure.\(^28\)

In the present study, we investigated the effect of elevated calcium levels on junctional permeability between cells in both bovine and embryonic chicken lens cultures. The bovine cultures were chosen because the biochemistry of the bovine lens has been evaluated extensively with respect to regulatory questions, including protein phosphorylation.\(^29\) The chicken system was selected because it mimics lens differentiation in vivo,\(^31\) providing a good model for studying junctional regulation during lens development. Calcium-sensitive dyes were used to monitor calcium concentration changes in individual lens cells, and these changes were correlated with gap junction permeability as measured by fluorescent dye transfer. Our results indicate that physiological changes in cytoplasmic calcium markedly reduce junctional permeability in the bovine cultures but not in the chicken cultures.

**MATERIALS AND METHODS**

**Culturing**

The method for culturing the bovine lens cells was essentially as described in a previous report.\(^32\) Calf eyes were removed shortly after death. The eyes were maintained in cold phosphate-buffered saline (PBS) containing penicillin and streptomycin until the lenses could be removed, approximately 1.5 hours after slaughter. The lenses with capsules attached were isolated from surrounding tissues, placed in tubes containing TD buffer (140 mM NaCl, 5 mM KCl, 0.7 mM Na\(_2\)HPO\(_4\), 5 mM D-glucose, 25 mM Tris-HCl, pH 7.4), and the cells dissociated in 0.015% trypsin (Gibco, Grand Island, NY) at 37°C for 30 minutes on a shaker at 100 to 150 rpm. At the end of this incubation, cells were pooled and pelleted (15 minutes, 1000 rpm). The pellets were resuspended in Media 199 (Gibco) containing 10% fetal bovine serum (HyClone, Logan, UT) and antibiotic-antimycotic agents (Sigma, St. Louis, MO). The cell suspension was filtered through three layers of sterile lens paper (Curtin Matheson Scientific, Wood Dale, IL) to remove any cell clumps and capsule material. The filtrate was plated at 0.5 to 1.0 \(\times\) \(10^6\) cells per 35 mm tissue culture dish. The plates were precoated with a 0.6 mg/ml solution of collagen (Celtrix Laboratories, Palo Alto, CA), applied as a thin layer and dried under ultraviolet light. The cultures were maintained at 37°C in a 5% CO\(_2\) incubator. Lenses from 10-day-old chicken embryos were cultured as previously reported.\(^31\) The animal research adhered to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research.

**Experimental Conditions and Microinjections**

Intercellular dye transfer was determined by iontophoresing a fluorescent tracer into a single cell within a confluent area and monitoring dye movement into surrounding cells. Dye transfer was recorded under each of three different conditions: in a control solution (PBS with the calcium concentration equal to 0.9 mM, pH 7.4, room temperature); after subsequent addition of a calcium ionophore solution (incubated for at least 15 minutes); and after removal of the ionophore and addition of 2 mM EGTA. The ionophores A23187 and ionomycin were dissolved in dimethyl sulfoxide (DMSO) and used at a final concentration of 0.5 mM or 0.075 mM, respectively. The control and EGTA solutions contained an equivalent concentration of DMSO.

For each condition, 6 to 10 cells per dish were injected. The cells were viewed on a Zeiss (Thornwood, NY) IM35 inverted microscope using a 32X objective. Electrodes for dye injection were prepared from 1.0 mm OD Prism capillary tubes (Dagan, Minneapolis, MN), using a Flaming-Brown Micropipette Puller (Sutter Instrument, San Rafael, CA). The tips of the electrodes were filled by capillary action with a 1% solution of lucifer yellow (Sigma, St. Louis, MO) in water. Single cells were impaled and iontophoretically injected with the dye using a 2 nA hyperpolarizing current for 10 seconds (A310 Accupulsor, World Precision Instruments, Sarasota, FL). The cells were viewed for 3 to 5 minutes on a videomonitor using a silicon-intensified target camera (SIT, Dage-MTI, Michigan City, IN). The process was recorded on videotape for later analysis. Fluorescent and phase-contrast photographs of the cells were taken approximately 1 minute after impalement with 35-mm Tri-X film (Eastman Kodak, Rochester, NY). From the photographs, the number of cells containing dye were counted.

**Fura-2**

Bovine lens cells cultured on glass coverslips were loaded with 2 mM fura-2 AM, the cell permeant acetoxymethyl ester of fura-2, for 30 minutes at room temperature. After rinsing with buffered saline, the cells were placed on the stage of a Zeiss IM35 for imaging. Fluorescent images of the cells were obtained using a 50W mercury lamp, filter wheel (Microvideo Instruments, Avon, MA), and SIT camera (DAGE-MTI).
The fluorescent intensities resulting from excitation at 340 nm and 380 nm were digitized from a point over each cell. Fifty readings at each wavelength were obtained and averaged for each cell. These average pixel values were used to calculate the free calcium concentration at that point. The average intracellular free calcium concentration reported for each condition was obtained from the indicated number of cells from three experiments with two separate preparations.

Digitized intensities were obtained with a video digitizer on board an Apple II microcomputer (Apple, Cupertino, CA), and ratios were calculated after background subtraction, using the formula described by Gynkiewicz. To calculate calcium concentrations in the cells, the ratio of the values at 340 and 380 nm was determined under zero calcium conditions (Rmin). This was done in Ca++-free PBS after microinjecting EGTA into the cell (current injection with a microelectrode loaded with 2 mM EGTA). The Rmin value was 0.83. Under saturated calcium conditions (Rmax), the ratio was calculated from cells placed in Ca++-free PBS and injected with CaCl2 (current injection with 10 mM CaCl2 in the microelectrode). Rmax was 4.37. F0 and Fs were digitized fluorescence values obtained with 380 nm excitation from cells injected with ETA or calcium, respectively. The F0/Fs ratio was 5.86. The Kd value of 190 nM was an estimate from standard curves generated with calcium buffers as previously described.

**32P-Orthophosphate Labeling and Immunoprecipitation**

Bovine cultures were rinsed three times and starved in serum-free, phosphate-deficient Dulbecco's modified essential medium (Sigma) for 20 minutes. 32P-orthophosphate (8500 to 9120 Ci/mmol) (DuPont, NEN, Boston, MA) was added at 0.375 mCi/0.5 ml medium to each 35 mm dish for 4 hours at 37°C in a 5% CO2 environment. Cultures were washed three times with cold Dulbecco's phosphate-buffered saline (ICN Flow) and solubilized with cold lysis buffer (0.025 M Tris-HCl, 0.1 M NaCl, 10 mM EDTA, 50 mM NaF, 500 mM Na3VO4, 0.5% Triton X-100, 0.8% SDS, 0.02% NaN3, 1 mM phenylmethylsulfonyl fluoride, 1 mg/ml pepstatin, 1 mg/ml leupeptin, pH 8.0). DNA was sheared by drawing the samples through a 25-gauge needle.

Rabbit antibodies prepared to peptides derived from the N-terminal 20 residues and the C-terminal 23 residues of connexin43 were used in combination for immunoprecipitation. The immunoprecipitation protocols for antibody incubation, protein A addition, and washes were from Laird et al. Washed immunobeads were solubilized in sample buffer (2% SDS, 5% β-mercaptoethanol, 10% glycerol, 0.005% bromphenol blue, 62.5 mM Tris-HCL, pH 6.8) and loaded on 12% acrylamide gels following the method of Laemmli. Bio-Rad low range molecular weight SDS-PAGE standards were also run. After drying the gels under vacuum, direct autoradiography was performed using Kodak XAR film.

**RESULTS**

**Morphology of Cultured Bovine Lens Cells and Embryonic Chicken Lens Cells**

Bovine cultures consisted primarily of large, flat, irregularly shaped cells, similar to previous reports. Small areas of the cultures were organized into a polygonal, pavement-like pattern similar to the chicken lens cultures. Mounds of cells formed, but further work must be done to determine if they are "lentoidal" in nature.

Chicken lens cultures initially consisted of single layers of flat epithelial cells that acquired a polygonal shape. Subsequently, linearly aligned elongated cells became apparent. These cells may correspond to the cells in the equatorial region of the intact lens. Near these elongated cells, complex multicellular, multilayered structures, termed lentoids, developed and displayed characteristics similar to those of lens fiber cells in vivo.

**Effect of Elevated Intracellular Calcium on Junctional Permeability**

The effects of increased intracellular calcium levels on junctional permeability in bovine and chicken lens cultures were studied using the calcium ionophores A23187 and ionomycin. In untreated bovine cultures, the flat, irregularly shaped cells and the polygonal, pavement-like cells demonstrated rapid dye transfer to several neighboring cells after impalement. In all three stages of cells in untreated chicken cultures (epithelial cells, elongated cells, and lentoids), dye transferred rapidly and extensively from the injected cell to multiple tiers of surrounding cells. Similar results were seen with the DMSO controls in both bovine and chicken cultures (Tables 1 to 3, Figs. 1 and 2). Because the chicken lens cells were much smaller than the bovine lens cells, a larger number of cell bodies became apparent after dye injection.

In the bovine cultures, there was a dramatic decrease in dye transfer after the addition of either ionophore (Fig. 3). The incidence and extent of dye transfer were reduced more by ionomycin when compared to A23187 treatment (see Table 1). Little (1 to 2 cells) or no transfer of dye was observed in 88% of the injections after exposure to ionomycin, and extensive
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TABLE 1. Incidence and Extent of Dye Transfer in Bovine Lens Cells in Response to Calcium Ionophores and Calcium-Free Conditions

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Cells Containing Dye*</th>
<th>% Reduced Transfer (3-4 cells)</th>
<th>Total Number of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>0% 7% 93% 5 or More</td>
<td>94% 6%</td>
<td>83</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>88%† 12% 0%†</td>
<td>94% 6%</td>
<td>16</td>
</tr>
<tr>
<td>A23187</td>
<td>65%† 28% 12%†</td>
<td>100% 0%</td>
<td>17</td>
</tr>
<tr>
<td>EGTA</td>
<td>0% 17% 88% 30%</td>
<td>95% 7%</td>
<td>14</td>
</tr>
</tbody>
</table>

* Photographs were taken approximately 1 minute after impalement. Dye transfer was evaluated during the actual experiment and by examination of the photographic records.
† Data were analyzed by a chi-square test for a 3 X 4 contingency table; significant compared to DMSO control, P < 0.0001.

TABLE 2. Incidence and Extent of Dye Transfer in Chicken Epithelial Lens Cells in Response to Calcium Ionophores

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Number of Cells Containing Dye*</th>
<th>Total Number of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>0% 17% 91%</td>
<td>14</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>8% 92% 12%</td>
<td>12</td>
</tr>
<tr>
<td>A23187</td>
<td>3% 97% 91%</td>
<td>12</td>
</tr>
</tbody>
</table>

* Photographs were taken approximately 1 minute after impalement. Dye transfer was evaluated during the actual experiment and by examination of the photographic records.

TABLE 3. Incidence and Extent of Dye Transfer in Chicken Elongated Cells and Lentoids in Response to Calcium Ionophores

<table>
<thead>
<tr>
<th>Treatment</th>
<th>% Transfer</th>
<th>Total Number of Injections</th>
</tr>
</thead>
<tbody>
<tr>
<td>Elongated</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>94%</td>
<td>16</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>94%</td>
<td>16</td>
</tr>
<tr>
<td>Lentoids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>100%</td>
<td>7</td>
</tr>
<tr>
<td>Ionomycin</td>
<td>95%</td>
<td>14</td>
</tr>
</tbody>
</table>

dye transfer was not observed. In the presence of A23187, 65% of the injections resulted in little or no transfer, whereas 12% of the injections showed transfer as extensive as controls.

In an attempt to reverse the reduction in channel permeability in the bovine cells, the ionophore medium was replaced with medium containing 2 mM EGTA. Extensive transfer was observed in 83% of the injections, indicating that the permeability changes were reversible upon calcium chelation (Table 1).

In addition to a semiquantitative analysis of the transfers based on photographic records, a precise quantitative analysis of dye intensities over time was performed to monitor dye transfer in the bovine lens cultures. A digital representation of fluorescence intensity was obtained for the injected cell and an adjacent cell, using a slit camera and an A/D converter on an Apple II microcomputer. The intensity was digitized every 3 seconds for 3 minutes, beginning with the initial filling of the cell with lucifer yellow (Fig. 4). Under control conditions (0.05% DMSO, Fig. 4A), the fluorescence intensities in the injected cell and the recipient cell were equivalent within 2 minutes. In the cells pretreated with 5 μM ionomycin (Fig. 4B), the injected cell maintained a constant, high level of fluorescence intensity, whereas the neighboring cells remained at background levels.

Neither A23187 or ionomycin affected junctional permeability in the three chicken lens cell types (Tables 2 and 3, Figs. 5 and 6). Dye spread was as extensive and rapid as in the DMSO controls, even after the A23187 concentration was increased to 15 mM or the calcium concentration in the media was elevated to 4.0 mM. In addition, chicken lens cells were preloaded with calcium by iontophoresing with 10 mM CaCl2 in the microelectrode. Lucifer yellow was then injected into a neighboring cell. In all cases, the dye transferred to the preloaded cells. These results suggest that under a wide range of intracellular and extracellular calcium concentrations, there was no effect on junctional permeability between chicken lens cells.

Analysis of Intracellular Concentrations of Free Calcium Using Fura-2

The dye injection studies strongly suggested that increased intracellular calcium concentrations affected junctional permeability in bovine lens cells but not in chicken lens cells. To verify that the ionophore treatment did indeed increase the cytoplasmic concentrations of free calcium in the two culture systems, the fluorescent calcium indicator fura-2 was used.

Lens cultures were loaded with fura-2 and treated under conditions identical to those used for the lucifer yellow microinjection experiments (Tables 1 to 3). In the presence of DMSO, the approximate calcium concentration measured in the bovine cells was 0.11 ± 0.009 μM (Table 4). After incubation with 5 μM ionomycin, the intracellular concentration of free calcium rose to 0.40 ± 0.073 μM within 10 minutes (Table 4). Additional calcium was then added to the medium to saturate the system and to demonstrate the effectiveness of the fura measurements in the presence of the ionophore. Raising the extracellular calcium concentration from 0.9 mM to 12 mM caused the intracellu-
FIGURE 1. Fluorescent (A) and phase-contrast (B) photographs of bovine lens cultures treated with 0.05% DMSO. Here and in subsequent figures, the images were recorded 1 minute after injection with 1% lucifer yellow. Note that dye spread to a number of cells, with the various nuclei staining more intensely than the cytoplasm. The cells overlap so it is difficult to distinguish cell boundaries. The change in fluorescent intensity was recorded and is shown in Figure 4A. Bar = 25 μm.

FIGURE 2. Embryonic chicken lens cultures (epithelial and elongated cells) treated with 0.05% DMSO. Note that dye transferred to a number of the chicken lens cells, which are much smaller than the bovine cells. Bar = 25 μm.

lar calcium concentration to increase to greater than 1.0 μM, indicating that the system was not saturated during the dye transfer experiments. Subsequent addition of EGTA to the medium, which had been shown to reverse the inhibition of dye transfer, decreased the intracellular calcium concentrations to 0.26 ± 0.113 μM (Table 4).

The results with fura-2 in the chicken cultures showed that the calcium concentrations in the presence of ionophore also increased; however, the levels were more variable from cell to cell than in the bovine cultures. The highest increase occurred in the elongated lens cells.

Immunoprecipitation of Gap Junction Proteins From Lens Cultures

These studies focused on connexin43 because it is the only gap junction protein known to link cells throughout the lens epithelium. Western immunoblot experiments, with two antibodies directed against either the N-terminal or the C-terminal of connexin43, previously revealed the presence of connexin43 in the cultures of bovine lens cells. Results after electrophoresis of the immunoprecipitated 32P-orthophosphate labeled proteins from bovine lens cultures with connexin43 antibodies are shown in Figure 7. With preimmune antibodies, no bands were apparent in the Mr = 43,000 to 47,000 range (lane 1). With untreated control cultures, connexin43 appears as a major phosphorylated band at Mr = 44,000 (lane 2). Addition of ionomycin for 1 hour reduced the labeling intensity, indicating a decreased level of phosphorylation (lane 3).
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FIGURE 3. Bovine lens cultures treated with 5 μM ionomycin. Dye is retained in what appears to be a single cell, with overlapping processes from other cells modifying the fluorescence image. The fluorescence intensity was monitored with time and is shown in Figure 4B. Fluorescence microscopy (A), a combination of fluorescence and phase microscopy (B). Bar = 25 μm.

DISCUSSION

Previous studies with bovine lens cultures and chicken lens cultures, along with the present findings, have shown that cells in both systems are coupled by gap junctions. This was determined by the intracellular microinjection and subsequent intercellular transfer of the fluorescent tracer, lucifer yellow. It has also been shown that the dye transfer is sensitive to octanol in both the bovine cultures and the chicken cultures, reinforcing the idea that this communication is due to gap junctions linking the cells.

We have studied the effects of intracellular calcium levels on junctional permeability in bovine and embryonic chicken lens cultures to analyze the regulatory properties of gap junctions between lens cells. An increase in intracellular free calcium altered junctional permeability in the bovine cultures, reducing or eliminating the transfer of dye. The effect of calcium on junctional permeability was reversible because the addition of extracellular EGTA quickly restored lucifer yellow transfer.

The junctional permeability of the chicken lens cells was not detectably altered with increased intracellular calcium.
FIGURE 5. Embryonic chicken epithelial lens cells treated with 7.5 μM A23187. Note the extensive dye transfer in the presence of ionophore, unlike that observed with bovine lens cells. Bar = 25 μm.

cellular calcium because dye transfer to neighboring cells appeared to be as rapid and extensive as under control conditions. As with the bovine cultures, the dye transfer has been shown to be sensitive to treatment with octanol.43 Because the transfer of fluorescent dyes serves as a way to evaluate gap junction communication in these cells, these results suggest differences in the regulation of gap junction permeability.

The protein kinase C activator, 12-O-tetradecanoylphorbol-13-acetate (TPA), has also shown differential effects on junctional permeability in these two lens culture systems. Although TPA dramatically reduced permeability in the bovine cultures,32 there were no apparent effects on dye transfer in the chicken cultures.43 The regulation of junctional communication in the two systems differs significantly.

In the mammalian lens, calcium does play a role in regulating gap junctional communication. This calcium-dependent gating of junctional channels could involve a variety of different mechanisms because calcium is known to be required in numerous regulatory pathways. For example, calcium could act by binding directly to junctional proteins44 or indirectly via calmodulin.45 Alternatively, it could be activating a protein kinase,32 protein phosphatase,46 or adenylate cyclase.47 Calcium-activated proteolysis is unlikely to be involved because we have found the calcium effect to be reversible. Our results suggest that changes in cytoplasmic calcium affect phosphorylation/dephosphorylation.

FIGURE 6. Embryonic chicken elongated lens cells treated with 5 μM ionomycin. Note the extensive dye transfer in the presence of ionophore, unlike that observed with bovine lens cells. Bar = 25 μm.

TABLE 4. Cytoplasmic Calcium Levels in Cultured Bovine Cells as Measured With Fura-2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Mean Free Calcium</th>
<th>SEM</th>
<th>n</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>DMSO</td>
<td>0.106 mM</td>
<td>.009</td>
<td>51</td>
<td></td>
</tr>
<tr>
<td>Ionomycin*</td>
<td>0.398 mM</td>
<td>.073</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>Calcium added</td>
<td>1.112 mM</td>
<td>.216</td>
<td>59</td>
<td></td>
</tr>
<tr>
<td>EGTA</td>
<td>0.256 mM</td>
<td>.113</td>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>

* P = 0.0002 (compared to DMSO).
FIGURE 7. SDS-PAGE of immunoprecipitated 32P-orthophosphate labeled bovine lens cultures with connexin43 antibodies, specific for both the N-terminus and the C-terminus. Lane 1, untreated cultures incubated with preimmune serum; lane 2, untreated cultures incubated with immune sera; lane 3, cultures treated with ionomycin (5 pM) 1 hour before the end of labeling and then incubated with immune sera.

ylation of the connexin protein. Previously, connexin43 was the only gap junction protein identified in the lens epithelium of various species17,18 and connexin43 had been detected in bovine lens cultures.32 Ionophore treatment, which elevated intracellular calcium concentrations and reduced junctional permeability in the bovine cultures, was correlated with a reduction in the phosphorylation of connexin43. The Mr = 44,000 band probably corresponds to a phosphorylated form of connexin43 described by Musil et al.48 No higher molecular weight bands were detected. Under increased calcium conditions, a kinase other than protein kinase C is likely involved because an activator of C kinase enhances connexin43 phosphorylation in bovine lens cultures, in conjunction with a decrease in junctional permeability.85 Future studies will need to address a variety of questions, including the nature of the protein kinase involved in connexin43 phosphorylation.

The change in the level of connexin43 phosphorylation in the presence of ionomycin represents an exciting starting point for exploring the molecular mechanisms of junctional regulation in response to calcium in the bovine cultures. It is particularly intriguing that such physiological increases in cytoplasmic calcium led to significant reductions in junctional permeability in the bovine cultures. This may be important in understanding various aspects of lens physiology. For example, the literature contains numerous reports that calcium concentrations become elevated during aging and cataract formation in human lenses.49-55 The calcium levels described in these reports substantially exceeded the concentrations we have shown to affect gating of junctional channels. Highly localized calcium increases have also been reported in discrete opacities in a diabetic model for cataractogenesis.50 Thus, reductions in junctional permeability, which would be extensive at the 1 μM calcium level, could precede opacification in the development of cataracts.

Key Words
calcium, gap junctions, lens, phosphorylation, dye injection

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References
10. Spray DC, White RL, Campos de Carvalho A, Harris


42. Reynhout JK, Klukas KA, Lampe PD, Johnson RG. Inhibition of gap junction communication in cultured...
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