Lens Connexin Channels Have Differential Permeability to the Second Messenger cAMP

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PURPOSE. Gap junction channels exhibit connexin specific biophysical properties, including the selective intercellular passage of larger solutes, such as second messengers. Here, we have examined the cyclic nucleotide permeability of the lens connexins, which could influence events like epithelial cell division and differentiation.

METHODS. We compared the cAMP permeability through channels composed of Cx43, Cx46, or Cx50 using simultaneous measurements of junctional conductance and intercellular transfer. For cAMP detection, the recipient cells were transfected with a CAMP sensor gene, the cyclic nucleotide-modulated channel from sea urchin sperm (SpIH). cAMP was introduced via patch pipette into the cell of the pair that did not express SpIH. SpIH-derived currents were recorded from the other cell of a pair that expressed SpIH. cAMP permeability was also directly visualized in transfected cells using a chemically modified fluorescent form of the molecule.

RESULTS. cAMP transfer was observed for homotypic Cx43 channels over a wide range of junctional conductance. Homotypic Cx46 channels also transferred cAMP, but permeability was reduced compared with Cx43. In contrast, homotypic Cx50 channels exhibited extremely low permeability to cAMP, when compared with either Cx43, or Cx46.

CONCLUSIONS. These data show that channels made from Cx43 and Cx46 result in the intercellular delivery of cAMP in sufficient quantity to activate cyclic nucleotide-modulated channels. The data also suggest that the greatly reduced cAMP permeability of Cx50 channels could play a role in the regulation of cell division in the lens.

Keywords: connexins, permeability, membrane channels, lens epithelium

Multicellular animals require communication between cells for the coordinated growth and development of tissues. This communication can derive from the binding of circulating growth factors to receptors that activate intracellular signal transduction cascades and generate second messengers, or by directly propagated signals between cells through the intercellular channels present in gap junctions. Both signal transduction pathways and gap junction channels are known to play important roles in the growth of the eye lens.1–8 However, studies have yet addressed whether differences in second messenger permeability through connexin channels made of Cx43, Cx46, and Cx50 contribute to the intercellular communication necessary for normal lens growth.

Gap junctions were initially assumed to be nonselective9,10; however, experiments carefully examining the movement of ions and dyes between coupled cells have revealed connexin-dependent differences in permeation.11–14 Innovative technical approaches have allowed this type of analysis to be extended to signaling molecules like IP3 and cAMP.15–19 Thus, different connexin channels are functionally distinct in terms of their conductance and permeability to small molecules.15,16,20–22 Genetic studies in mice have documented that such functional differences between gap junction channel types are important, because the loss of one connexin cannot be compensated for by replacement with other connexins.23–25

Characterization of mouse models with single or double knock-outs and knock-ins of Cx43, Cx46, and Cx50 have found distinct roles for each lens connexin in vivo.24,26–29 For example, a growth deficit in Cx50 knockout lenses is caused by a transient reduction in epithelial cell mitosis7 that occurs on postnatal days 2 to 3 (P2–P3). Cx50 provides the majority of coupling between epithelial cells at this age, and functional replacement of Cx50 by Cx46 maintains the same magnitude of coupling, but does not restore normal cell division and lens growth.24,30 Lens mitosis is stimulated by growth factors, which in turn activate intracellular signaling cascades.31,32 Thus, it is possible that Cx50 and Cx46 have different permeability to the second messengers arising from growth factor-mediated signal transduction, resulting in a difference of recruitment of neighboring cells into mitosis on P2 to P3.

The lens presents an ideal system to explore differential permeability of gap junctions to second messengers, as it only expresses three connexins in two cell types.1,33–35 Cx43 and Cx50 are present in epithelial cells, while Cx46 and Cx50 form the abundant gap junction channels between fibers.34,35 There is also an extensive literature on the developmental consequences of genetic manipulation of lens connexins in mice.33,34,37 That can be correlated with potential differences in second messenger permeation. Here, we have used patch clamp electrophysiologic and fluorescent imaging approaches to determine the permeation of cAMP through Cx43, Cx46, and Cx50 channels in vitro. We found that Cx43 and Cx46 were highly permeable to cAMP, whereas Cx50 channels exhibited extremely low permeability to this second messenger. Com-
bined with previously published work from Cx50-deficient mice.7,24,26,30 these data suggest that the reduced cAMP permeability of Cx50 could play a role in the regulation of cell division in the postnatal lens.

**MATERIALS AND METHODS**

**Cell Culture**

HeLa cells were plated on glass coverslips, grown to 50% confluence and transiently transfected with mouse Cx46 using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Rat Cx43 and human Cx50 stably transfected HeLa cells15,38 were also used.

**Electrophysiologic Measurements**

A dual voltage-clamp method and whole-cell/perforated patch recording were used to control the membrane potential of paired cells and to measure currents.15,39 Coverslips with adherent cells were transferred to a chamber on the stage of an inverted microscope equipped with fluorescence imaging. Cells were perfused at room temperature with bath solution containing (in mM) NaCl, 150; KCl, 10; CaCl2, 2; HEPES, 5 (pH 7.4); glucose, 5; CsCl, 2; and BaCl2. 2. Patch pipettes were filled with solution containing (in mM) K+ aspartate, 120; NaCl, 10; MgATP, 3; HEPES, 5 (pH 7.2); EGTA, 10. For perforated patch experiments, the pipette solution contained 30 to 50 μM β-escin.40 Pipettes were pulled from glass capillaries (Harvard Apparatus, Holliston, MA, USA) with a horizontal puller (DMZ-Universal, Zeitz-Instrumente, Martinsried, Germany). The resistance of the pipettes measured 2 to 5 MΩ.

**cAMP Flux Studies**

cAMP transfer through connexin channels was investigated using cell pairs.15,16 Recipient cells were transfected with the cyclic nucleotide-modulated channel from sea urchin (SpH)41,42 subcloned into pIRE2-EGFP (Takara Bio USA, Mountain View, CA, USA). Donor cells were transfected with red fluorescent protein (RFP; pIRE2-DsRed2; Takara Bio USA). SpH and RFP-transfected cells were co-cultured. Green eGFP and red RFP signals were visualized under a fluorescent microscope and pairs consisting of one SpH and one RFP-transfected cells were chosen for cAMP transfer measurements.

**CAMP was introduced via patch pipette into the cell of the pair that did not express SpH, and SpH-derived currents were recorded from the cell expressing SpH. Five hundred micromolar of cAMP (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in the pipette solution. To maintain a constant cytoplasmic concentration of CAMP during measurement of its permeation through connexin channels, the phosphodiesterase inhibitor IBMX (200 μM; Sigma-Aldrich) was added to the bath solution to prevent degradation of exogenously delivered CAMP. An adenylate cyclase inhibitor 2’,5’-dideoxyadenosine (5 μM; Calbiochem, Burlington, MA, USA) was added to the pipette and bath solutions to prevent endogenous CAMP synthesis. Quantitative CAMP flux (molecules/channel/s) and unitary channel permeability (P) were calculated as described.12,15,39,45

**Fluorescent e-CAMP Transfer**

CAMP permeability was visualized in transfected cells using a fluorescent form of the molecule, 1, N6-Ethenoadenosine-3’,5’-cyclic monophosphate (e-CAMP; Biolog Life Science Institute, Bremen, Germany). e-CAMP is an analogue of cyclic AMP in which the N1 and the N6 nitrogen atoms in the adenine nucleobase are connected by an etheno bridge to form a tricyclic ring system.44,45 Five millimolar e-CAMP was dissolved in the pipette solution. One cell in a pair, or cluster, was patched with a pipette containing e-CAMP for up to 10 minutes to allow fluorescent CAMP passage from source cells to the adjacent cells. e-CAMP was imaged at regular intervals using a digital CCD-camera (HRm Axiocam; Carl Zeiss, Thornwood, NY, USA). At the end of each cell pair experiment, the second cell was patched and the junctional current between the cells was measured.16 The outline of each cell, and an area of background, were manually drawn in the image using ImageJ software (http://image.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD, USA).16 Fluorescent intensities for recipient and source cells were corrected by subtracting the background intensity. Ratios of the corrected fluorescent intensities of the recipient to the source cell were calculated at the 5-minute time point.

**Statistical Analysis**

Student’s t-test or one-way ANOVA were used for two- or three-way comparisons of primary data, respectively (OriginLab, Northampton, MA, USA). Analysis of covariance (ANCOVA) was used to compare statistical differences between slopes fitted from linear regression (GraphPad Prism, San Diego, CA USA).

**RESULTS**

**Measurement of CAMP Permeability**

We developed a method for the quantitative detection of CAMP transfer through connexin channels15,16 (Fig. 1) using the SpH41 to detect cytoplasmic CAMP. In single cells, when no CAMP was delivered through the patch pipette, SpH channels passed a low level of current (Fig. 1A, black line). When 50 μM CAMP was introduced into the cytoplasm through the patch
Comparison of Intercellular cAMP Transfer by Different Lens Connexins

Quantitation of differences in cAMP permeability between connexins was achieved by monitoring the time course of the increase in the SpIH dependent tail current relative to the junctional conductance between cells. The change in SpIH tail current was first plotted as a function of time for individual cell pairs expressing Cx43, Cx50, and Cx46 (Fig. 3A). For a Cx46 cell pair (open triangles), the tail current rose steadily to a new saturated value of approximately 200 pA within approximately 150 seconds following cAMP delivery to the source cell. In contrast, a Cx50 cell pair (open circles) showed a much slower rate of increase in SpIH tail current that did not reach saturation within 300 seconds. In a Cx43 cell pair (filled circles), the tail current rose rapidly to a new saturated value of approximately 1450 pA in approximately 20 seconds. For cell pairs expressing Cx43 and Cx46, primary data like these allowed calculation of the parameter \( \Delta I/\Delta t \), which is the net change in the SpIH current normalized to its maximum value, divided by the net time required to attain the tail current increase as we have previously shown.\(^{15}\) For the Cx43 cell pair shown in Figure 3A, \( \Delta I/\Delta t \) was obtained by fitting the linear part of the current-time relationship (Fig. 3B, filled circles), and represents the SpIH current increase due to cAMP accumulation in the recipient cells, after permeation through connexin channels from the source cell. Data for the Cx46 cell pair (Fig. 3B, open triangles) required more time to reach saturation, resulting in a reduced slope compared with Cx43. In the 50% of Cx50 cell pairs where the SpIH current measurably increased, it never reached saturation during the finite period that cells could be recorded from, so \( \Delta I/\Delta t \) had to be estimated from linear fits of the entire data sets that showed consistent SpIH current increase over time (Fig. 3C, open circles).

Data showing the increase in SpIH current following delivery of cAMP to the neighboring cell were collected from 7 to 10 individual cell pairs expressing either Cx43, Cx46, or Cx50. These aggregate data from all of the experiments were plotted in Figure 4. For Cx43 (\( n = 9 \)) and Cx46 (\( n = 7 \)), the SpIH tail current showed a 3.6 ± 0.6-fold and 2.6 ± 0.8-fold increase, respectively. In contrast, only half of the Cx50 cell pair

with a junctional conductance of 31 nS, the magnitude of the SpIH current in the recipient cell increased following introduction of cAMP into the source cell (Fig. 2A), indicating the cell-to-cell passage of cAMP through Cx46 channels. In a Cx50 cell pair with a gap junctional conductance of 16 nS, the magnitude of the SpIH current did not change at all during the 5-minute period following introduction of cAMP into the donor cell (Fig. 2B), suggesting little, or no intercellular passage of cAMP through Cx50 channels. Expanded records of the SpIH current at the time when the whole-cell patch was first opened (Fig. 2C, black line), and 280 seconds later (red line) illustrated the different responses of the lens connexins. The cells transfected with Cx46 gap junction channels had an approximately 4-fold increase in the magnitude of the SpIH current. This showed that CAMP was being delivered from the source cell and could permeate through Cx46 channels. In the Cx50 expressing cell pair, there was no increase in the magnitude of the SpIH current. This suggested that very little cAMP was being delivered from the source cell, and that CAMP diffusion through Cx50 gap junction channels was negligible. For comparison, an expanded record from a cell pair with Cx43 channels showed that CAMP diffusion increased the magnitude of the SpIH current approximately 5-fold, as previously reported.\(^{15}\) Thus, channels made of Cx43, Cx46, and Cx50 exhibited qualitatively different cAMP permeability.

Cx46 Channels Have Greater cAMP Permeability Than Cx50

Data obtained using the cAMP permeation assay in cell pairs expressing either Cx46 or Cx50 gap junction channels are shown in Figure 2. The source cell pipette contained 500 \( \mu \)M cAMP, and was opened in whole-cell mode at the time indicated by a black asterisk. In a Cx46 expressing cell pair pipette, SpIH bound cAMP, producing an increased channel open probability and membrane current flow (Fig. 1A, red line). Detection of gap-junction-mediated movement of cAMP between cells was accomplished by co-culturing cell pairs where the recipient cell (cell 1) expressed the connexin to be tested and SpIH. The source cell (cell 2) expressed only the connexin which is the net change in the SpIH current normalized to its maximum value, divided by the net time required to attain the tail current increase as we have previously shown.\(^{15}\) For the Cx43 cell pair shown in Figure 3A, \( \Delta I/\Delta t \) was obtained by fitting the linear part of the current-time relationship (Fig. 3B, filled circles), and represents the SpIH current increase due to cAMP accumulation in the recipient cells, after permeation through connexin channels from the source cell. Data for the Cx46 cell pair (Fig. 3B, open triangles) required more time to reach saturation, resulting in a reduced slope compared with Cx43. In the 50% of Cx50 cell pairs where the SpIH current measurably increased, it never reached saturation during the finite period that cells could be recorded from, so \( \Delta I/\Delta t \) had to be estimated from linear fits of the entire data sets that showed consistent SpIH current increase over time (Fig. 3C, open circles).

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pairs $(n = 10)$ showed a SpIH current increase, resulting in a mean $1.1 \pm 0.2$-fold change (Fig. 4A, $P < 0.05$, one-way ANOVA). For Cx43, the mean time to SpIH tail current saturation was $52 \pm 35$ seconds, compared with $211 \pm 45$ seconds for Cx46. In all of the Cx50 expressing cell pairs, the SpIH tail current failed to reach saturation during the limited time frame that the cell pair could be stably recorded by dual whole-cell patch clamp (between 5 and 10 minutes). This limited us to plotting the elapsed time without achieving SpIH saturation for Cx50, which underestimates the actual saturation time, and had a mean value of $365 \pm 105$ seconds. Despite this underestimation, Cx50 showed a significantly slower response than either Cx43 or Cx46 (Fig. 4B, $P < 0.05$, one-way ANOVA). In contrast to the changes in SpIH tail current, the mean values of gap junctional conductance between the cell pairs for Cx43 (13 ± 6 nS), Cx46 (11 ± 10 nS), and Cx50
cAMP flux can be calculated as follows: \( \frac{0.00037 \text{ s}}{C_0} \left( \frac{0.048}{10^7} \right) \), and \( C_x50 \) (conductance for \( C_x43 \) (dose-response curve yielded a slope of \( \frac{0.048}{\text{concentration}} \)), and \( N_A \) is Avogadro’s number. HeLa cells have cAMP molecules that transfer from the source cell to the recipient cell in 1s for a \( C_x46 \) junctional conductance of 1nS is \( (1.8 \text{ pL}) (7.71 \times 10^{-9} \text{ M/s/nS}) (6.02 \times 10^{23}) = 8354 \) molecules/s/nS. Using the unitary conductance of \( C_x46 \) (146 pS), the number of cAMP molecules passing per channel per second can be calculated as \( (8554 \text{ molecules/s/nS}) (146 \text{ pS/channel/}\text{1nS}) = 1220 \) molecules/channel/second. This cAMP permeability was 5-fold less than that calculated for \( C_x43 \) (6095 molecules/channel/second, Table).

For \( C_x50 \), cAMP permeability was estimated to be 35-fold less than that of \( C_x43 \), (176 molecules/channel/second, Table). However, it should be noted that in 5 of 10 experiments performed with \( C_x50 \) cell pairs there was no observed increase in SpIH (i.e., \( \Delta I/\Delta t \) had a value of zero), which resulted in an \( R^2 \) value of 0.17 for the fit of the \( \Delta I/\Delta t \) versus gap junctional conductance plot for \( C_x50 \) (\( P = 0.23 \)). Despite the reduced level of certainty in our calculation of \( C_x50 \) permeability, these data clearly show that \( C_x43 \) and \( C_x46 \) channels have a much greater permeability to cAMP than \( C_x50 \).

### Optical Analysis of cAMP Passage Through \( C_x43 \) and \( C_x50 \) Channels

To confirm the significant difference in cAMP permeability between \( C_x43 \) and \( C_x50 \), we directly visualized cAMP permeability through lens connexin channels using a fluorescent form of the molecule (ε-cAMP). ε-cAMP has a long fluorescent lifetime, and retains the physiologic activity of native cAMP.\(^{45} \) We initially tested the ability of ε-cAMP to permeate through \( C_x43 \) gap junction channels by attaching a pipette filled with the dye to a single cell within a small group of HeLa cells expressing \( C_x43 \) (Fig. 6A, \( n = 6 \)). After opening the patch in whole-cell mode, dye transfer to half of the neighboring cells was detected within 5 minutes (Fig. 6B), and virtually all of the cells within 10 minutes (Fig. 6C), confirming that like native cAMP, ε-cAMP easily passed through \( C_x43 \) channels. When the experiment was repeated using \( C_x50 \) transfected HeLa cells (Fig. 6D, \( n = 7 \)), very little transfer of ε-cAMP to neighboring cells was observed after either 5 (Fig. 6E) or 10 (Fig. 6F) minutes following the opening of the patch pipette.

To quantitatively compare the transfer of fluorescent cAMP through \( C_x43 \) and \( C_x50 \) channels, we performed simultaneous measurements of gap junction conductance and ε-cAMP fluorescent flux in isolated cell pairs.\(^{15,16} \) Data are shown for a cell pair expressing \( C_x43 \) (Fig. 7A), where one patch pipette filled with ε-cAMP was attached to a single cell within the pair. The patch was opened in whole-cell mode, and ε-cAMP fluorescence was recorded for 5 minutes. At the end of fluorescent recording, the second cell was patched, and the magnitude of gap junctional conductance between the cells was determined to be 12 nS. Both cells in the \( C_x43 \) expressing pair had high levels of ε-cAMP fluorescence after 5 minutes (Fig. 7B). In contrast, a \( C_x50 \) cell pair with a gap junctional conductance of 16 nS (Fig. 7C) showed little passage of ε-cAMP.

### Table. CAMP Permeability of \( C_x43 \), \( C_x46 \), and \( C_x50 \) Channels

| Parameter                              | \( C_x43 \)                              | \( C_x46 \)                              | \( C_x50 \)                              |
|----------------------------------------|------------------------------------------|------------------------------------------|------------------------------------------|
| \( \Delta I/\Delta t \) vs. \( G_s \) slope (s\(^{-1}\)/nS) ± SE | 2.7 ± 0.3 \times 10^{-3}  | 3.7 ± 0.5 \times 10^{-4} | 3.9 ± 5 \times 10^{-5} |
| Unitary conductance (pS)               | 55                                      | 146                                      | 200                                      |
| cAMP flux (molecules/channel/sec)       | 6095 (5880\(^*\))                       | 1220                                     | 176                                      |
| Relative permeability (cAMP/K\(^+\))   | 0.18                                     | 0.014                                    | 0.0015                                   |
| Single channel permeability (cm\(^2\)/s) | 2.0 \times 10^{-14}                      | 4.1 \times 10^{-15}                      | 5.8 \times 10^{-16}                      |

\(^*\) Previously calculated value for \( C_x43 \) from Kanaporis et al.\(^{15} \)
to the second cell of the pair 5 minutes after the opening of the patch pipette (Fig. 7D). The mean (±SD) junctional conductance for cell pairs tested were 11 ± 1.4 and 17 ± 1.0 nS for Cx43 and Cx50, respectively (Fig. 7E, n = 3, P > 0.05, Student’s t-test). The extent of cAMP transfer was determined by calculating the ratio of fluorescent intensity in the recipient cell over that in the donor cell after 5 minutes. These ratios were 0.92 ± 0.18 for Cx43, and 0.09 ± 0.07 for Cx50 (Fig. 7F, P < 0.05, Student’s t-test). Consistent with our results for native cAMP above, we found that cAMP permeated through Cx43 channels much more readily than through Cx50 channels.

**DISCUSSION**

We used two approaches to compare cAMP permeability through gap junction channels formed from different lens connexins, a quantitative electrophysiologic assay relying on activation of the SpIH channels, and direct microscopic observation of the cell-to-cell passage of cAMP, a fluorescent form of the molecule. Using both approaches, Cx50 showed greatly reduced cAMP permeability compared with Cx43. Cx46 also had much greater cAMP permeability than Cx50, although not as high as that of Cx43. The calculated cAMP flux values for single gap junction channels were 6095,
1220, and 176 molecules/s for Cx43, Cx46, and Cx50, respectively (Table).

We performed our cAMP permeability experiments at room temperature (~25°C), and the conductance of connexin channels is moderately influenced by temperature with average Q10 values of approximately 1.1 to 1.3.49–53 However, it should be noted that the Q10 value for an aqueous solution of KCl is 1.2 to 1.3,54 which is the same magnitude as the values reported for connexin channels. This suggests that the modest temperature effects observed for connexin channel conductance are largely due to changes in solute mobility within the channel. In the context of our work, the profound differences in cAMP permeability that we documented between Cx43, Cx46, and Cx50 channels are unlikely to vary significantly between 25°C and 37°C.

Knockout of Cx50 from the mouse lens resulted in decreased epithelial mitosis in the first postnatal week, and a significant reduction of lens growth.7,26,55 In contrast, deletion of either Cx43 or Cx46 did not reduce mitosis or produce growth defects.7,28 suggesting that a specific activity of Cx50 was required for normal postnatal epithelial mitosis and lens growth to occur. Cx50 provides most of the gap junctional coupling in the lens epithelia in the early postnatal period, and genetic replacement of Cx50 by Cx46 did not restore normal cell division, despite resulting in the same magnitude of functional conductance between cells.24,50 The intracellular concentration of cAMP oscillates during the cell cycle and significantly contributes to the regulation of the G0/G1 transition.46,57 If differences in intrinsic cAMP permeability through connexin channels were important for normal epithelial cell division to proceed, then one could predict that neither Cx46 or Cx43 could replace the function of Cx50, as they both exhibit much greater permeability to cAMP.

Lens growth changes in the early postnatal period, where we observed a significant decrease in epithelial cell division in Cx50-deficient lenses.7,50 During the preceding embryonic stage of lens growth, the diameter grows at a steady linear rate and the volume increases as a smooth exponential.58,59 In contrast, early postnatal growth of the lens is oscillatory in a manner reproduced in organ culture by the pulsatile administration of growth factors, and that corresponds with the timing of the epithelial cell cycle.60,61 Our data showed that Cx50 channels were much less permeable to cAMP than either Cx43 or Cx46. The elevation of cAMP levels directly affected epithelial cell proliferation in the lens epithelium.62–64 and if Cx50 were the dominant connexin expressed when cells were actively dividing, its low intrinsic permeability would allow cells to autonomously regulate their cytoplasmic cAMP levels independently of their neighboring cells. In contrast, the high levels of cell-to-cell transfer of cAMP through Cx43 or Cx46 channels following the deletion, or genetic replacement of Cx50, could disrupt the epithelial cells ability to independently regulate their cytoplasmic cAMP due to flux between neighboring cells, and contribute to the altered cell proliferation seen in the mouse models.

Previous studies in mice have found a correlation between the functional activity of Cx50 and maximal postnatal proliferation in epithelial cells. On postnatal days 2 to 3, 20% or more of all lens epithelial cells were labeled following a 1-hour exposure to BrdU, and Cx50 provided 60% or more of the total magnitude of epithelial coupling. In older lenses, epithelial proliferation was reduced to 5% or less of total cells, and the contribution of Cx50 to gap junctional coupling was reduced to 25% or less by postnatal day 28, with the majority of coupling at this age provided by Cx43.50 This correlation was further strengthened by the observations that postnatal epithelial cell proliferation was significantly reduced following the lens specific knockout of the p110ζ catalytic subunit of phosphoinositide 3-kinase, and that the magnitude of gap junctional conductance mediated by Cx50 channels was specifically upregulated by p110ζ.65 Together, these studies have pinpointed a critical role for Cx50 channels in lens epithelial cell proliferation. Our current work has now identified profound differences in the permeability of the second messenger cAMP through lens connexin channels as one possible explanation for why Cx50 channels were required for the proper regulation of postnatal lens mitosis.

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