Lens Connexin Channels Have Differential Permeability to the Second Messenger cAMP

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Submitted: April 9, 2019
Accepted: August 13, 2019

Citation: Valiunas V, Brink PR, White TW. Lens connexin channels have differential permeability to the second messenger cAMP. Invest Ophthal Vis Sci. 2019;60:3821–3829. https://doi.org/10.1167/iovs.19-27302

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 be directly propagated 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. However, no 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 nonselective; however, experiments carefully examining the movement of ions and dyes between coupled cells have revealed connexin-dependent differences in permeation. Innovative technical approaches have allowed this type of analysis to be extended to signaling molecules like IP3 and cAMP. Thus, different connexin channels are functionally distinct in terms of their conductance and permeability to small molecules. 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.

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. For example, a growth deficit in Cx50 knockout lenses is caused by a transient reduction in epithelial cell mitosis 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. Lens mitosis is stimulated by growth factors, which in turn activate intracellular signaling cascades. 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. Cx43 and Cx50 are present in epithelial cells, while Cx46 and Cx50 form the abundant gap junction channels between fibers. There is also an extensive literature on the developmental consequences of genetic manipulation of lens connexins in mice 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. 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 cells 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. 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. 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. 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. Recipient cells were transfected with the cyclic nucleotide-modulated channel from sea urchin (SpIH) 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). SpIH and RFP-transfected cells were co-cultured. Green eGFP and red RFP signals were visualized under a fluorescence microscope and pairs consisting of one SpIH 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 SpIH, and SpIH-derived currents were recorded from the cell expressing SpIH. Five hundred micromolar cAMP was dissolved in the pipette solution and allowed to flow into the cell by diffusion. An electrophysiologic assay for intercellular transfer of cAMP through gap junction channels was used to compare statistical differences between slopes obtained from linear regression (GraphPad Prism, San Diego, CA, USA). Analysis of covariance (ANCOVA) was used to compare statistical differences between slopes fitted from linear regression (GraphPad Prism, San Diego, CA, USA).

 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 channels. (Fig. 1) using the SpIH to detect cytoplasmic cAMP. In single cells, when no cAMP was delivered through the patch pipette, SpIH 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 Cx46, Cx50, and Cx43 (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 ΔI/Δt, 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. For the Cx43 cell pair shown in Figure 3A, ΔI/Δt 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 diffusion through the 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 measured was greater, it never reached saturation during the finite period that cells could be recorded from, so ΔI/Δt 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 pairs 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 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 ΔI/Δt, 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. For the Cx43 cell pair shown in Figure 3A, ΔI/Δt 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 diffusion through the 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 measured was greater, it never reached saturation during the finite period that cells could be recorded from, so ΔI/Δt 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 ± 35 seconds, compared with 211 ± 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 ± 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
Relative permeability (cAMP/K<sub>i</sub>) yielded a slope of 0.048/10<sup>6</sup>M.<sub>C0</sub> and Cx50 (open circles, n = 7), and Cx50 (open circles, n = 10). The solid lines correspond to first order regressions with the slopes given in the Table.

An average volume of 1.8 pL<sup>47</sup> so the total number of cAMP molecules that transfer to the recipient cell in 1 s for a Cx46 junctional conductance of 1 nS is (1.8 pL) (7.71 × 10<sup>10</sup> M/s/nS) (6.02 × 10<sup>15</sup>) = 8354 molecules/s/nS. Using the unitary conductance of Cx46 (146 pS), the number of cAMP molecules passing per channel per second can be calculated as 8354 molecules/s/nS (146 pS/channel × 1 nS) = 1220 molecules/channel/second. This cAMP permeability was 5-fold less than that calculated for Cx43 (6095 molecules/channel/second, Table).

For Cx50, cAMP permeability was estimated to be 35-fold less than that of Cx43, (176 molecules/channel/second, Table). However, it should be noted that in 5 of 10 experiments performed with Cx50 cell pairs there was no observed increase in SpIH (i.e., ΔI/Δt had a value of zero), which resulted in an R<sup>2</sup> value of 0.17 for the fit of the ΔI/Δt versus gap junctional conductance plot for Cx50 (P = 0.23). Despite the reduced level of certainty in our calculation of Cx50 permeability, these data clearly show that Cx43 and Cx46 channels have a much greater permeability to cAMP than Cx50.

**Optical Analysis of cAMP Passage Through Cx43 and Cx50 Channels**

To confirm the significant difference in cAMP permeability between Cx43 and Cx50, 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.<sup>44,45</sup> We initially tested the ability of ε-cAMP to permeate through Cx43 gap junction channels by attaching a pipette filled with the dye to a single cell within a small group of HeLa cells expressing Cx43 (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 Cx43 channels. When the experiment was repeated using Cx50 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 Cx43 and Cx50 channels, we performed simultaneous measurements of gap junction conductance and ε-cAMP fluorescent flux in isolated cell pairs.<sup>15,16</sup> Data are shown for a cell pair expressing Cx43 (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 fluorescence 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 Cx43 expressing pair had high levels of ε-cAMP fluorescence after 5 minutes (Fig. 7B). In contrast, a Cx50 cell pair with a gap junctional conductance of 16 nS (Fig. 7C) showed little passage of ε-cAMP.

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**TABLE.** CAMP Permeability of Cx43, Cx46, and Cx50 Channels

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Cx43</th>
<th>Cx46</th>
<th>Cx50</th>
</tr>
</thead>
<tbody>
<tr>
<td>ΔI/Δt vs. G&lt;sub&gt;i&lt;/sub&gt; slope (s&lt;sup&gt;-1&lt;/sup&gt;/nS) ± SE</td>
<td>2.7 ± 0.3 × 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>3.7 ± 0.5 × 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>3.9 ± 5 × 10&lt;sup&gt;-5&lt;/sup&gt;</td>
</tr>
<tr>
<td>Unitary conductance (pS)</td>
<td>55</td>
<td>146</td>
<td>200</td>
</tr>
<tr>
<td>CAMP flux (molecules/channel/sec)</td>
<td>6095 (5880*)</td>
<td>1220</td>
<td>176</td>
</tr>
<tr>
<td>Relative permeability (cAMP/K&lt;sub&gt;i&lt;/sub&gt;)</td>
<td>0.18</td>
<td>0.014</td>
<td>0.0015</td>
</tr>
<tr>
<td>Single channel permeability (cm&lt;sup&gt;2&lt;/sup&gt;/s)</td>
<td>2.0 × 10&lt;sup&gt;-14&lt;/sup&gt;</td>
<td>4.1 × 10&lt;sup&gt;-15&lt;/sup&gt;</td>
<td>5.8 × 10&lt;sup&gt;-16&lt;/sup&gt;</td>
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</table>

* Previously calculated value for Cx43 from Kanaporis et al.<sup>15</sup>
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 e-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 e-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 e-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 reduced by temperature with average Q10 values of approximately 1.1 to 1.3. However, it should be noted that the Q10 value for an aqueous solution of KCl is 1.2 to 1.3, 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 solvent. Connexin Q10 values are not consistent with structural changes within the channel, but rather strongly suggest that the channel is dominated by solvent and not by interactions of solute with the channel wall. 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. In contrast, deletion of either Cx43 or Cx46 did not reduce mitosis or produce growth defects. 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 junctional conductance between cells. The intracellular concentration of cAMP oscillates during the cell cycle and significantly contributes to the regulation of the Go/G1 transition. 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. During the preceding embryonic stage of lens growth, the diameter grows at a steady linear rate and the volume increases as a smooth exponential. In contrast, early postnatal growth of the lens is oscillatory in a manner repressed in organ culture by the pulsatile administration of growth factors, and that corresponds with 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. This correlation was further strengthened by the observations that postnatal epithelial cell proliferation was significantly reduced following the lens specific knockout of the p110α catalytic subunit of phosphoinositide 3-kinase, and that the magnitude of gap junctional conductance mediated by Cx50 channels was specifically upregulated by p110α. 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.

Acknowledgments

Supported by National Institutes of Health (Bethesda, MD, USA) Grants EY013163 and EY026911 (TWW), and GM088181 (VV).

Disclosure: V. Valiunas, None; P.R. Brink, None; T.W. White, None

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