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Mefloquine Effects on the Lens Suggest Cooperative Gating of Gap Junction Channels

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Abstract. Mefloquine (MFQ) selectively blocks exogenously expressed gap junction channels composed of C×50 but not C×46. The purpose of the current study was to evaluate MFQ effects on wild-type (WT) mouse lenses that express both C×50 and C×46 in their outer shell of differentiating fibers (DFs). Lenses in which C×46 was knocked into both C×50 alleles (KI) were used as controls; MFQ had no effect on coupling in these lenses. When WT lenses were exposed to MFQ, the DF coupling conductance decreased significantly, suggesting that C×50 contributes about 57% of the coupling conductance in DF and C×46 contributes 43%. Remarkably, in the presence of MFQ, the 43% of the channels that remained open did not gate closed in response to a reduction in pH, whereas in the absence of MFQ, the same pH change caused all the DF channels to gate closed. Since MFQ is a selective blocker of C×50 channels, it appears that C×46 channels lack pH-mediated gating in the absence of functional C×50 channels but are pH-sensitive in the presence of C×50 channels. These results suggest the two types of channels interact and gate cooperatively.

Key words: C×46 — C×50 — Pharmacology — Coupling conductance — C×46 knockin

Introduction

The mouse lens is an approximately spherical organ with several distinct gap junction domains. A single epithelial layer covers the anterior surface, where C×43 and C×50 are expressed. The interior of the lens comprises fiber cells that can be divided into two zones: the outer shell (the outer 15% of the lens radius) contains differentiating fibers (DFs), where C×43 is degraded and C×50 and C×46 are expressed; the inner core, (about 85% of the radius) contains mature fibers (MFs), which have no organelles and no new protein expression (reviewed in Mathias, Rae & Baldo, 1997). Studies of genetically modified mouse lenses that lack either C×46 or C×50 suggest that both connexins contribute significantly to the coupling conductance in DF; however, the MFs appear to be coupled exclusively by gap junction channels composed of C×46 (Gong et al., 1998; Baldo et al., 2001; Martinez-Wittinhan et al., 2004). These domains of gap junction coupling and connexin expression are shown in Figure 1.

The antimalarial drug mefloquine (MFQ) has been suggested to "be distributed to the lens and enhance age-related cataractogenesis" (Motten et al., 1999). In studies using exogenous expression of several connexins, Cruikshank et al. (2004) reported that MFQ selectively blocked gap junction channels made from C×50 but that, at a concentration of 10 μM, it had no measurable effect on channels made from C×46 and, at 30 μM, it caused a very small inhibition of C × 46 channels. Interestingly, C×46 and C×50 are the two connexin proteins that make the gap junction channels in the DFs of wild-type (WT) lenses. Prior to the current study, there were no data on the effect of MFQ on gap junction coupling in the lens; however, the cataractogenic effects of MFQ might occur via effects on lens gap junctions. One purpose of the current report was to determine whether MFQ selectively blocks C×50 channels in the DF of WT lenses.

Most connexins generate gap junctions whose conductance is decreased with a reduction in intracellular pH (Spray, Harris & Bennett, 1981), and the inhibitory pH is near the normal physiological value (Spray et al., 1982; Peracchia & Bernardini, 1984). The mechanisms for pH gating could be direct

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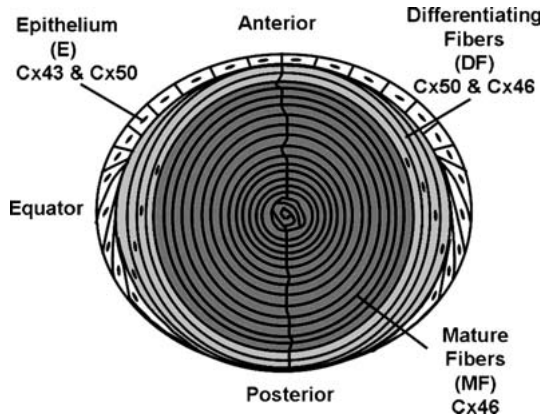

Fig. 1. A sketch of the lens' structure and the connexins that form functional gap junction channels in each domain.

protonation of connexins or indirect actions by intermediates (Ek et al., 1994; Yamaguchi, Huang & Ma, 1995; Ek-Vitorin et al.,1996; Morley, Taffet & Delmar, 1996; Peracchia & Wang, 1997; Trexler et al., 1999). Gap junction channels in DFs of the lens are sensitive to intracellular pH, but in the core MF region they are not pH-sensitive. The pH insensitivity in MFs is thought to be due to posttranslational modification of gap junction channels at the DF-to-MF transition. At this transition, C×46 and C×50 have their C termini cleaved (Lin et al., 1997) and C×50 channels may be rendered nonfunctional (Mathias et al., 1997; Gong et al., 1998). In pH-sensitivity studies of WT or C×46 knockout (KO) lenses, the DF gap junctions show pH gating. However, Baldo et al. (2001) found the DF gap junctions of C×50 KOs, whose channels are composed of C×46, to have significantly reduced pH gating. Lenses from C×50 KO mice are not very healthy, and the possibility exists that the change in pH gating was a secondary effect. In the current study, we used acute exposure to MFQ to evaluate the lack of functioning C×50 channels on the pH sensitivity of gap junction channels in the DFs of otherwise healthy WT lenses.

Materials and Methods

Chemicals and supplies were obtained from Sigma (St. Louis, MO) unless otherwise noted. MFQ was generously provided by the Drug Synthesis and Chemistry Branch, Developmental Therapeutics Program, National Cancer Institute (Bethesda, MD).

Solution

Tyrode solution contained 137.7 mM NaCl, 5.4 mM KCl, 0.3 mM NaOH, 1 mM $MgCl_2$, 10 mM glucose and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, pH 7.4) with NaOH.

Lens Extraction

The creation of C×46 knockin (KI) mice is described by White (2002). WT or KI mice were killed by peritoneal injection of pentobarbital (100 mg/kg weight) in accordance with the SUNY at Stony Brook, Division of Laboratory Animal Research, guidelines; lenses were dissected from the eye and placed in a sylgard-lined chamber (Dow Corning, Midland, MI) in normal Tyrode solution at 36–37°C (Gong et al., 1998; Baldo & Mathias, 1992).

Impedance Studies

Impedance studies were performed with two microelectrodes. The microelectrodes were pulled to have a sturdy tip geometry that would penetrate the lens capsule without breaking. Their resistance was 1–3 MΩ when filled with 3 M KCl. Each was shielded with silver paint, and the paint was insulated from the bath by a coat of sylgard. One intracellular microelectrode was used to inject a wide-band current into a central fiber cell. The second intracellular microelectrode recorded the induced voltage at a distance, r (cm), from the lens' center. The current and voltage signals were sent to a Fast Fourier Analyzer (Hewlett Packard, Everett WA) for analysis and to compute the impedance in real time. In order to test the sensitivity of coupling to acidification, a measurement of the high-frequency series resistance (R_S) in either DFs or MFs was made while superfusing the lens with Tyrode solution that had been bubbled with 100% CO_2. Control studies were conducted in Tyrode solution equilibrated with air. At high frequencies, the membrane conductance was effectively short-circuited by the membrane capacitance; thus, nonspecific effects of MFQ on channels other than gap junctions did not affect these data.

The model of lens gap junctional coupling has been described (Mathias, Riquelme & Rae, 1991; Mathias, Rae & Eisenberg, 1981). The relationship between the high-frequency series resistance, R_S (Ω), and the underlying coupling conductances, G_{DF} and G_{MF} (S/cm^2), is given by

$$\begin{aligned} R_S &= \frac{1}{4\pi w G_{DF}}\left(\frac{1}{r}-\frac{1}{a}\right) && r \geq b = 0.85a \\ R_S &= \frac{1}{4\pi w G_{DF}}\left(\frac{1}{b}-\frac{1}{a}\right)+\frac{1}{4\pi w G_{MF}}\left(\frac{1}{r}-\frac{1}{b}\right) && r \leq b = 0.85a \end{aligned} \quad (1)$$

G_{DF} and G_{MF} are the coupling conductances per unit area of cell-to-cell contact and w (cm) is the width of a fiber cell (w ≈ 3 μm). The voltage recording microelectrode is at a distance, r (cm), from the center of a lens of radius a (cm). The DF-to-MF transition occurs at $r = b$ (cm) from the center. As can be deduced from the equation, R_S is the resistance of all the gap junctions between the point of recording and the surface of the lens. We measure R_S and the location of the recording in DF, then used equation 1 to determine G_{DF}. The microelectrode was then advanced into MFs and the recordings were repeated to determine G_{MF}.

Electrophysiological Data Analysis

Data analysis was done with Sigma Plot and Sigma Stat Software, version 4.0, and Microsoft (Redmond, WA) Excel on a Windows–98 PC.

Results

To facilitate comparison of data from different groups of lenses, all coupling conductances were normalized to the value in DF of WT lenses. However, G_{DF} is generally quite close to 1 S/cm^2 of cell-

to-cell contact, so the normalized values are close to their dimensional values.

One of the curves in Figure 2 illustrates the effect of superperfusing a WT lens with solution bubbled with 100% CO_2. After a 30-min exposure to CO_2, WT gap junction coupling conductance in the DF dropped to 10% of its initial value. This is a typical result that has been reported a number of times in lenses from different species (reviewed in Mathias et al., 1997). In all lenses, there is an extracellular path for current flow, and this path parallels the intracellular (cell-to-cell via gap junction) path. Curve fitting impedance data from WT lenses in physiological conditions (Baldo et al., 2001) suggest the extracellular path has a conductance (G_e) that is about 10% of that of the intracellular path. Thus, for the normalized data in Figure 2, the residual WT conductance after 30-min exposure to 100% CO_2 is thought to represent the extracellular conductance and the cell-to-cell coupling conductance becomes zero. If one subsequently removes the CO_2, the coupling conductance will eventually recover but the time to recover is significantly longer than the time to produce the uncoupling (Mathias et al., 1991). This asymmetry in time course probably reflects the high membrane permeability for CO_2, which allows it to easily enter cells of the lens, where it is converted into H^+ and HCO_3^-, which are relatively membrane-impermeant and thus difficult to transport out of the lens. Figure 2 also compares the effect of CO_2 on DF coupling conductance in WT and C×50 KO lenses. KO of C×50 channels caused the conductance to be reduced to less than half that of WT (0.46 in this example), but the remaining C×46 channels lost most of their pH sensitivity. In a 30-min exposure to CO_2, the conductance of WT fell to 0.1, which represents closure of essentially 100% of both C×50 and C×46 gap junction channels. In contrast, in C×50 KO lenses, after 30-min exposure to CO_2, the fractional conductance went from 0.46 to 0.30, which represents closure of about 56% of the C×46 channels. This surprising result (first presented in Baldo et al., 2001) suggested that C×46 channels in DFs might require association with C×50 channels for normal pH-sensitive gating. However, the lack of C×50 over the life span of these mice has produced lenses that are significantly less robust than WT (low resting voltage, low input resistance, cataract, undersized and a tendency to run down), so it was possible the reduced C×46 channel gating was an indirect effect. For example, a change in the phosphorylation state of C×46 could change its affinity for protons. This is a hypothetical example, but it illustrates one of many indirect effects that might lead to altered pH sensitivity.

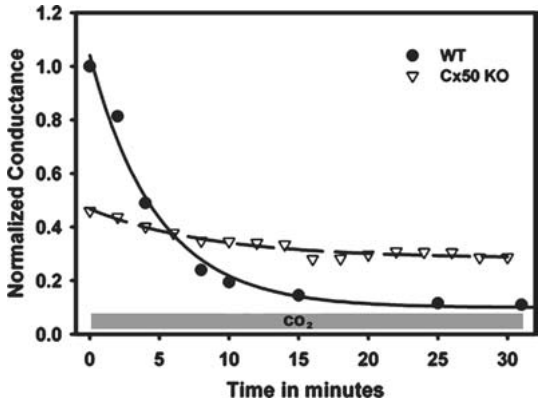

Fig. 2. pH gating. When WT lenses were exposed to 100% CO_2, coupling conductance dropped to near 10%. Since about 10% of the DF conductance is due to the extracellular path, which parallels the intracellular path (cell-to-cell via gap junctions), all of the DF gap junction channels appear to close. In contrast, the pH sensitivity of DF gap junction channels in C×50 KO lenses is significantly reduced. In a 30-min exposure to 100% CO_2, about 90% of WT channels close whereas only about 35% of the C×46 channels in the C×50 KO lens close, suggesting loss of C×50 channels has greatly reduced the pH sensitivity of DF gap junctions.

Effects of MFQ on Gap Junctional Coupling in WT lenses

Figure 3 illustrates the main finding of the current study. An experimental WT lens was exposed to 100 μM MFQ, whereas the control WT lens was exposed to dimethyl sulfoxide (DMSO, the vehicle used to get MFQ into the solution). About 1 h later, each lens was superperfused with 100% CO_2 solution. In the initial 50 min prior to CO_2 exposure, MFQ caused the DF coupling conductance to decrease ~50%, while DMSO caused no change in the control lens. At the end of this period, both lenses were normally transparent. Remarkably, when the MFQ-treated lens was superperfused with 100% CO_2 solution, the coupling conductance did not change, whereas when the DMSO lens was similarly treated, the coupling conductance went to near zero. The conductance of the DMSO lens dropped well below that of the MFQ-treated lens; hence, CO_2 induces closure of a group of channels that remain open in the MFQ-treated lens. Since MFQ has been shown to selectively block C×50 channels, the pH-insensitive channels are thought to be made from C×46; yet these same channels were pH-sensitive when C×50 channels were able to respond to the pH change. This is similar to what was observed in Figure 2; however, the effect of MFQ on pH sensitivity was much more dramatic and occurred in a relatively short time period. Moreover, both lenses in the experiment shown in Figure 3 were quite healthy in all regards except for the reduced DF coupling conductance in the MFQ-treated lens.

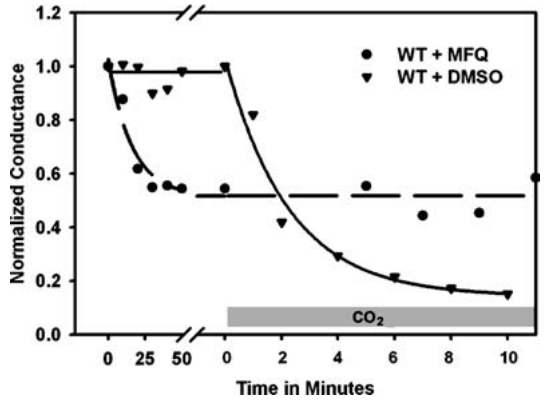

Fig. 3. Time course of MFQ blockade and its effect on pH sensitivity. A WT lens was exposed to 100 μM MFQ for 60 min. A control lens was exposed to DMSO (the vehicle used to get MFQ into solution) for the same period. The DF coupling conductance in the MFQ-treated lens dropped to 50%, whereas the control lens conductance was constant. After the initial measurement of DF coupling conductance, each lens was superfused with solution that had been bubbled with 100% CO_2. Coupling conductance in the control lens showed a typical response in which about 90% of its gap junction channels closed in a period of 10 min. In contrast, gap junction channels in the MFQ-treated lens became pH-insensitive. Since MFQ selectively blocks C×50 channels, this result suggests that the remaining C×46 channels require the presence of C×50 channels for pH sensitivity (i.e., there is cooperative gating).

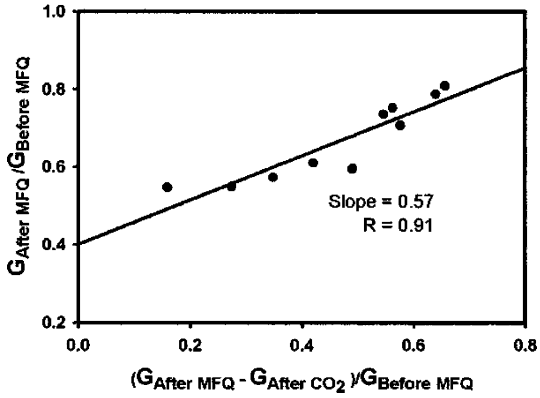

Fig. 4. Fractional DF coupling conductance after 1 h block with MFQ as a function of the fractional change in conductance after treatment with CO_2. The bathing solution contained 10 μM MFQ, but the concentration reaching the DF gap junctions appears to be less, possibly due to the propensity of MFQ to come out of solution. This graph shows that the MFQ effect on conductance is correlated with its effect on pH sensitivity.

In the absence of MFQ, the effect of 100% CO_2 was always complete closure of all gap junction channels in all WT lenses from all species studied. The lens in Figure 3 was one of three MFQ-treated lenses that showed complete loss of pH sensitivity. These data support the hypothesis that C×50 and C×46 channels respond cooperatively to changes in pH and that in WT lenses it is the presence of C×50 channels that confers pH sensitivity to C×46 channels.

MFQ did not always completely eliminate pH gating as shown in Figure 3; in some experiments, blockade was significantly less than half of the initial conductance and pH gating was also partially present. We can think of several possibilities for this variability. First, the fractional conductance due to C×50 (G_{50}) could be variable. However, total DF coupling conductance in WT lenses ($G_{50} + G_{46}$) was quite consistent, as was the residual DF conductance in either C×50 (G_{46}) or C×46 (G_{50}) KO lenses, suggesting that the stoichiometry of expression was consistent. Hence, variations in the amount of G_{50} seemed unlikely but possible. Second, the concentration of MFQ used could be near its dissociation constant for C×50 channels, and small variations in either the concentration of MFQ or the dissociation constant could have caused the variability in blockade. We therefore tried using higher concentrations of MFQ, but the variability persisted, suggesting this was not the cause. MFQ is a fairly hydrophobic molecule; hence, it is difficult to get into solution, is prone to come out of solution and binds nonspecifically to membranes. A third possible cause of the variability is that varying degrees of blockade represented varying concentrations of MFQ in the extracellular spaces of DFs, whereas the stoichiometry of C×50 expression relative to that of C×46 was constant. The data shown in Figure 4 support this hypothesis.

If the concentration of MFQ in the lens was a variable, then one would expect variable effects on pH sensitivity as well as on conductance. In WT lenses, pH-dependent closure of all C×50 channels results in closure of all C×46 channels, whereas when MFQ is maximally effective, as in Figure 3, there is no pH-mediated closure of either C×50 or C×46 channels. To extend this idea, one would expect that if 50% of G_{50} is blocked by MFQ, then 50% of G_{46} will be pH-insensitive and 50% will be pH-sensitive. To extend this hypothesis to our data, Figure 4 graphs the fractional change in conductance after CO_2 as a function of the fractional conductance after block with 10 μM MFQ. The data from 10 lenses do indeed fall on a straight line (correlation coefficient $R = 0.91$), indicating the two effects were correlated.

This observation had some interesting implications. Assume P_M is the fraction of C×50 channels blocked by MFQ and the effective dissociation constant of [MFQ] for C×50 channels is given by K_M.

$$P_M = \frac{[MFQ]}{[MFQ + K_M]} \quad (2)$$

Assuming that MFQ has either come out of solution or is binding nonspecifically to membranes, [MFQ] is an unknown variable and hence P_M is also a variable; however, it is experimentally defined by the fractional blockade. The total conductance, G_{DF}, is given by

$$G_{DF} = G_{46} + G_{50} + G_e \quad (3)$$

G_e is the conductance due to the extracellular space, which is about 10% of the total conductance. After lenses have been exposed to MFQ, the measured conductance is labeled G_M; after exposure to CO_2, the measured conductance is labeled G_{CO2}. Thus, by definition,

$$\frac{G_M}{G_{DF}} = \text{Fractional conductance after blockade with MFQ} \quad (4)$$

$$\frac{G_{CO_2}}{G_{DF}} = \text{Fractional conductance after } CO_2 \quad (5)$$

The fractional conductance after blockage with MFQ is dependent on C×50 and can be expressed as

$$\frac{G_M}{G_{DF}} = \frac{G_e + G_{46} + G_{50}\,(1 - P_M)}{G_{DF}} = 1 - P_M \frac{G_{50}}{G_{DF}} \quad (6)$$

Our hypothesis is that C×46 gating is dependent on the availability of unblocked C×50 channels; therefore, we assume that

$$\frac{G_{CO_2}}{G_{DF}} = \frac{G_e + P_M G_{46}}{G_{DF}} = \frac{1 - (G_{46} + G_{50})}{G_{DF}} + P_M \frac{G_{46}}{G_{DF}} \quad (7)$$

Figure 4 graphs G_M/G_{DF} *vs.* $(G_M - G_{CO_2})/G_{DF}$. The above analysis suggests

$$\frac{G_M - G_{CO_2}}{G_{DF}} = (1 - P_M)\frac{(G_{46} + G_{50})}{G_{DF}} \quad (8)$$

Based on Equations 6 and 8, Figure 4 represents

$$1 - P_M \frac{G_{50}}{G_{DF}} \textit{ vs. } (1 - P_M)\frac{(G_{46} + G_{50})}{G_{DF}} \quad (9)$$

The implicit variable is P_M; hence, the slope of the data in Figure 4 is given by

$$\text{slope} = \frac{G_{50}}{G_{46} + G_{50}} \quad (10)$$

Therefore, the linear regression provides an estimate of the fraction of the total conductance contributed by C×50 channels.

MFQ Effects on C×46 KI Lenses

As a control, we looked at the effect of MFQ on the KI lenses, which express no C×50 and should therefore not be affected by MFQ. We chose the KI lenses over the C×50 KO lenses because the KO lenses were unhealthy and had a mild cataract and KI of C×46 into the C×50 gene locus produces a robust lens with no opacity (Martinez-Wittinghan et al., 2004; White, 2002). As can be seen in Figure 5A, MFQ had no effect on the DF coupling conductance in C×46 KI lenses.

As a second control, we examined the effect of MFQ on pH sensitivity in the KI lenses. As reported (Martinez-Wittinghan et al., 2004), the pH sensitivity of C×46 channels in the lens appears to be determined by the locus of gene expression. The C×46 KI lenses regain much of the pH sensitivity that was lost in the C×50 KO lenses. We do not understand this rather odd result (see Martinez-Wittinghan et al., 2004 for some speculations); however, the KO lenses provided a useful control to see if any of the MFQ effects on pH sensitivity occur via effects on C×46. Figure 5B shows the DF conductance after a 10-min exposure to 100% CO_2. In either the absence or presence of MFQ, the conductance fell to about 37% of its initial value. Thus, MFQ does not appear to affect pH gating through any interaction with C×46.

Fractions of DF Coupling Conductance Due to C×50 and C×46

The slope of the linear regression in Figure 4 gives $G_{50}/(G_{46} + G_{50}) = 0.57$, hence by subtraction $G_{46}/(G_{46} + G_{50}) = 0.43$. Assuming the value of G_e is the same in MFQ-treated and untreated lenses, this method of estimating the fractional conductances eliminates the effect of G_e and directly determines the fractional coupling conductance due to each connexin. These fractions are in reasonable agreement with the residual DF conductance of $G_e + G_{46} = 0.44$ in the C×50 KO lenses (Baldo et al., 2001) or $G_e + G_{50} = 0.32$ in the C×46 KO lenses (Gong et al., 1998). However, these fractions are both less than 1, suggesting that KO of one connexin had indirect effects on the remaining conductance, which was due to channels made from the other connexin. In either KO, the lenses were less robust than normal (as previously described in the introductory comments to Results), and it would not be surprising if there were indirect effects on the residual coupling conductance. Lastly, our previous data (Gong et al., 1998; Baldo et al., 2001; Martinez-Wittinhan et al., 2004) suggested functional C×50 channels are lost at the DF-to-MF transition. The loss of G_{50} results in a reduction of normalized coupling conductance from $G_{DF} = 1$ to an average $G_{MF} = 0.44 \pm 0.06$ (Gong et al., 1998; Baldo et al., 2001; Martinez-Wittinhan

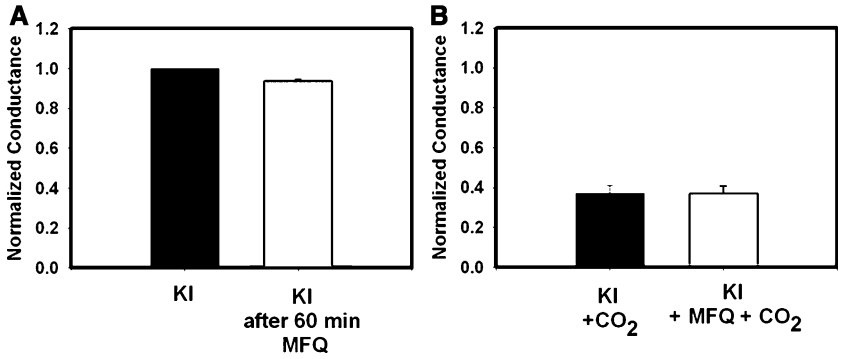

Fig. 5. MFQ effects on KI lenses. The normalized coupling conductance of KI lenses is compared after treatment with MFQ. (*A*) Coupling. KI lenses did not have a significant change in coupling conductance after treatment with 10 μM MFQ for 60 min. Normalized coupling after MFQ was 0.93 ± 0.01 (n = 9). (*B*) Gating. This panel compares the pH gating of KI lenses treated with MFQ for 60 min before the pH trial (KI + MFQ + CO_2) *vs.* nontreated KI lenses (KI + CO_2). There was no change in pH gating when lenses were treated with MFQ. KI + MFQ + CO_2 coupling conductance was 0.37 ± 0.04 (n = 10) *vs.* KI + CO_2 coupling conductance of 0.37 ± 0.04 (n = 9). All data are presented as mean ± SEM. Thus, in the absence of C×50 channels, MFQ had no effect on DF coupling conductance or the pH sensitivity of DF gap junction channels.

et al., 2004), which again includes G_e; hence, these data suggest $G_{50}/(G_{46} + G_{50}) = 0.62 \pm 0.06$ and $G_{46}/(G_{46} + G_{50}) = 0.38 \pm 0.06$ (mean ± standard error of the mean [SEM] for three groups). These two WT lens estimates of the fractional conductance due to C×50 and C×46 are illustrated in Figure 6.

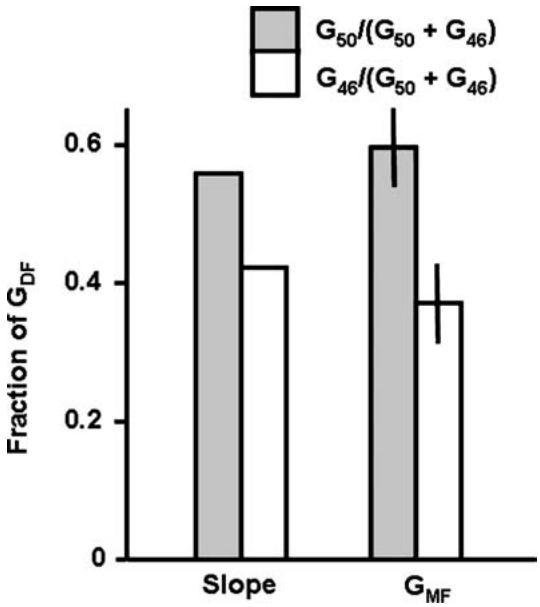

Fig. 6. Fraction of DF coupling conductance contributed by C×50 or C×46. Two different methods of determining these fractions were employed. The first is based on the slope of the linear regression shown in Figure 5. The second is an average based on the values of G_{MF} reported by Gong et al. (1998), Baldo et al. (2001) and Martinez-Wittinhan et al. (2004). The rationale for these different approaches is described in the text.

Discussion

The most remarkable finding in this study was that, in WT lenses, C×50 is required in some way in order for all channels in the DF to be pH-sensitive. Jiang & Goodenough (1996) found evidence that some lens gap junction channels are heteromers formed from a mix of C×46 and C×50; however, their data did not address the relative number of such channels. A seemingly simple explanation for pH sensitivity would be that all channels in the DF are heteromers, containing at least one pH-sensitive C×50 subunit and that one C×50 subunit is sufficient to confer pH sensitivity on the channel. However, there are problems with this hypothesis. First, C×50 is initially expressed in the epithelium, where there is no C×46 expression, so these channels are homomers containing only C×50. For them to form heteromers in the DF, the initial channels would have to be disassembled and reassembled with a mixture of C×46 and C×50 subunits. Second, at the DF-to-MF transition, functional C×50 channels appear to be lost (Gong et al., 1998; Baldo et al., 2001; Martinez-Wittinhan et al., 2004), leaving MF channels composed of only C×46. If the DF channels were all heteromers of C×50 and C×46, they would have to be disassembled at the DF-to-MF transition and reassembled as homomeric C×46 channels in the MF. Lastly, the data presented here provide the most compelling evidence against the heteromer hypothesis. Assume all DF channels contain one or more C×50 subunits but MFQ blocks half the channels, possibly because some minimum number of C×50 subunits is required for MFQ binding. The remaining unblocked channels still have the C×50 subunits that caused them to be pH-sensitive before MFQ, so they should still be pH-sensitive after MFQ; but they are not.

The above-described three lines of evidence against the heteromer hypothesis suggest that the

majority of lens fiber cell gap junction channels are homomers, composed of either C×50 or C×46. If so, there must be cooperative interactions between homomeric C×46 and C×50 channels such that pH-mediated closure of C×50 channels induces closure of C×46 channels. A working hypothesis is shown in Figure 7. The suggestion is that, in the lens, normally expressed C×46 channels are pH-insensitive but each functional C×50 channel that is gated closed due to a reduction in pH induces closure of a neighboring C×46 channel(s). Thus, in the C×50 KO lens or when MFQ is present or in the MF, the C×46 channels are not pH-sensitive; these are the experimental observations behind Figure 7.

An interesting unanswered question concerns the nature of the cooperative interaction between the channels. In gap junction plaques, channels pack rather closely, so it is possible that there is a direct physical interaction between channels, similar to that shown in Figure 7. A direct physical interaction is difficult to study until two-dimensional crystals of gap junction channels are available for structural studies like those recently reported for the fiber cell water channel AQP0 (Gonen et al., 2004, 2005). Immunostaining suggests that channels made from C×46 and C×50 coexist in most plaques; however, there are also regions within plaques where immunostaining shows only one connexin or the other (e.g., Fig. 3 of Gong et al., 1998). Thus, the interaction may be a nucleation phenomenon in which closure of channels in one region of a plaque propagates to all regions of the plaque. There may also be a linking protein that couples channel gating within a plaque. Immunoprecipitation studies of C×46 and C×50 show that each associates with the tight junction protein ZO-1 (Nielsen et al., 2003). A lens-specific ZO-1 KO would provide a useful test of this possibility; however, to our knowledge, such a mouse is not available at this time. We are left with an intriguing observation that may take a significant amount of time to understand at a more mechanistic level.

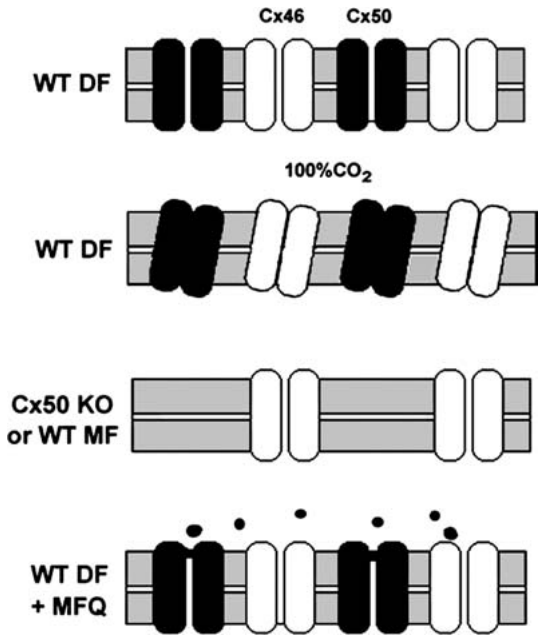

Fig. 7. Sketch of a possible model to explain the results presented here and in our previous studies of connexin KO lenses.

Cooperative Gating of Gap Junction Channels

Although the data presented here are probably the most dramatic demonstration of cooperative gating of gap junction channels, there are previous suggestions in the literature that cooperative gating exists. Manivannin, Mathias & Gudowska-Nowak (1996) derived a Markov model of cooperative gating. The physical observation that motivated this analysis was from dual whole-cell patch-clamp records containing just a few channels; often, two or more channels would either open or close simultaneously. This is a highly improbable event, and to see it happen rather frequently suggested cooperative gating between neighboring channels. Valiunas and Weingart (2001) suggested cooperative voltage-dependent gating between C×30 channels. Their conclusion was based on comparison of gating of single channels and multichannels. Although these were solid indications of cooperativity between neighboring gap junction channels, the effects were subtle and did not seem to have great physiological significance. In the WT lens, pH-dependent gating of DF gap junction channels is also unlikely to be of direct significance. However, the fact that gating is possible in DFs but lost in MFs suggests there could be a regulatory role for gating of DF channels in response to some physiological signals.

pH Sensitivity of C×46 Channels

Exogenous expression of C×46 has invariably produced gap junction channels that are pH-sensitive (Eckert, 2002; White et al., 1994; Stergiopoulos et al., 1999). Moreover, even in the lens, when C×46 was synthesized on the C×50 gene locus, the resulting channels appeared to be pH-sensitive (Martinez-Wittinghan et al., 2004). The puzzling observation is, therefore, when C×46 is synthesized on the C×46 gene locus the resulting channels appear to be pH-insensitive, at least in the absence of functional C×50 channels. The primary sequence of the protein is the same in all of these situations; hence, the difference must reside in posttranslational modifications. In the lens, when C×46 is synthesized on the C×50 gene locus, the protein is initially made in the epithelium, whereas it is normally synthesized only in the DF. These two physically different locations contain very

different cytoplasmic and membrane proteins (reviewed in Mathias et al., 1997), and it is likely they also contain different regulatory proteins. These regulatory proteins, through phosphorylation, glycation or other modifications, could eliminate the H^+ binding site that is otherwise present. This is our working hypothesis, and in the future we plan to look for gene locus-specific differences in posttranslational modifications of the two fiber cell connexins.

Contributions of C×50 and C×46 to Gap Junction Coupling in the Lens

Based on MFQ blockade, our data suggest C×50 channels contribute about 57% of the DF coupling conductance and C×46 channels contribute 43%. Previous data from C×50 KO or C×46 KO lenses (Gong et al., 1998; Baldo et al., 2001; Martinez-Wittinhan et al., 2004) indicated that both contribute significantly to the coupling conductance of the DF, but the actual fraction was difficult to determine because of the poor conditions of these lenses. In the current study, the lenses were healthy, so we think these estimates of the fractional conductances are the most reliable. Moreover, they are consistent with the data from connexin KO lenses.

The KO studies also suggested that C×50 channels did not contribute to the coupling conductance in the MF, and since zero is an unequivocal number, this result is probably quantitatively as well as qualitatively correct. We initially thought that MFQ would provide a good tool to detect C×50 channels in the MF. However, our protocol measures MF coupling conductance indirectly as the series of DF and MF coupling resistances, then by subtraction obtains the MF coupling resistance (see Methods). Unfortunately, the variability of MFQ blockade in connection with our protocol to determine G_{MF} made it difficult to reliably detect whether there was any small effect of MFQ on G_{MF}. There was no indication of any effect, but given the uncertainty, we abandoned this line of experimentation. Nevertheless, the MFQ-determined fractional contribution of C×46 to G_{DF} fits very well with the value of G_{MF}; hence, the data are compatible with the suggestion in the KO studies that G_{MF} is due only to C×46 channels.

The single-channel conductance of C×50 channels (about 220 pS) is significantly larger than that of C×46 channels (about 140 pS) when they have been studied in exogenous expression systems (Hopperstad, Srinivas & Spray, 2000; Srinivas et al., 1999). The difference in single-channel conductances may be the reason for the difference in contributions to the macroscopic G_{DF}. Moreover, the C×46 KI lenses had a reduced G_{DF} in comparison to WT, and this reduction could be quantitatively explained if the C×50 gene locus expressed the same number of open C×46 channels as C×50 channels; however, the smaller single-channel conductance of the C×46 channels resulted in the reduced G_{DF} (Martinez-Wittinghan, 2004). Baldo et al. (2001) reported $G_{DF} = 1.05$ S/cm^2 of cell-to-cell contact, but this included the conductance of the extracellular spaces, G_e. Thus, $G_{50} + G_{46} \approx 1.0$ S/cm^2 and, based on the MFQ data, $G_{50} \approx 0.57$ S/cm^2 and $G_{46} \approx 0.43$ S/cm^2. Dividing these macroscopic conductances by their respective single-channel conductances gives the density of open channels for each type of connexin: $N_{50} = 2.6 \times 10^9$ cm^{-2}, $N_{46} = 3.0 \times 10^9$ cm^{-2} open channels per area of cell-to-cell contact. Not only are the densities of open channels similar but there have been reports in the literature that C×46 and C×50 proteins are expressed in about a 1:1 ratio (Kistler, Christie & Bullivant, 1988; Konig & Zampighi, 1995). A 1:1 ratio of open channels with a 1:1 ratio of expressed channels implies that either type of channel has the same open probability. Taken altogether, the data suggest that the C×46 or C×50 gene locus of expression produces about the same number of channels with about the same open probability at normal pH.

Summary

We have presented fairly direct evidence that pH sensitivity of lens DF gap junction channels depends on cooperative gating between pH-sensitive C×50 channels and pH-insensitive C×46 channels. The nature of this cooperative bond is not known; it could be as simple as close proximity or somewhat more complex, involving a bridging protein that couples neighboring channels or a very complex cascade of events. Further work is needed to determine the nature of this cooperative bond. The idea of cooperative pH gating in the DF but loss of functional C×50 channels at the DF-to-MF transition appears to explain both the reduction in coupling conductance of the MF relative to the DF and the lack of pH sensitivity for MF gap junction (C×46) channels. However, we know far too little about the events that occur at the DF-to-MF transition, and this may be too simple an explanation. Lastly, the MFQ data in connection with other data on C×46/C×50 expression and C×46 or C×50 single-channel conductances fit together in a rather simple scheme. The implications are, in the lens, either the number of channels expressed or the open probability of the channels is similar regardless of the locus of gene expression or the primary sequence of the connexin. However, the pH sensitivity of C×46 channels does appear to depend on the locus of gene expression. The mechanism by which the gene locus affects the functional properties (pH sensitivity) of C×46 channels remains to be explained.

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