Biophysical characterization of zebrafish connexin35 hemichannels

Valiunas, Virginijus, Rickie Mui, Elizabeth McLachlan, Gunnar Valdimarsson, Peter R. Brink, and Thomas W. White. Biophysical characterization of zebrafish connexin35 hemichannels. *Am J Physiol Cell Physiol* 287: C1596–C1604, 2004. First published July 28, 2004; doi:10.1152/ajpcell.00225.2004.—A subset of connexins can form unopposed hemichannels in expression systems, providing an opportunity for comparison of hemichannel gating properties with those of intact gap junction channels. Zebrafish connexin35 (Cx35) is a member of the Cx35/Cx36 subgroup of connexins highly expressed in the retina and brain. In the present study, we have shown that Cx35 expression in *Xenopus* oocytes and N2A cells produced large outward whole cell currents on cell depolarization. Using whole cell, cell-attached, and excised patch configurations, we obtained multichannel and single-channel current recordings attributable to the Cx35 hemichannels (Ihc) that were activated and increased by stepwise depolarization of membrane potential (Vm) and deactivated by hyperpolarization. The currents were not detected in untransfected N2A cells or in control oocytes injected with antisense Cx38. However, water-injected oocytes that were not treated with antisense showed activities attributable to Cx38 hemichannels that were easily distinguishable from Cx35 hemichannels by a significantly larger unitary conductance (γhc: 250–320 pS). The γhc of Cx35 hemichannels exhibited a pronounced Vm dependence; i.e., γhc increased/decreased with relative hyperpolarization/depolarization (γhc was 72 pS at Vm = −100 mV and 35 pS at Vm = 100 mV). Extrapolation to Vm = 0 mV predicted a γhc of 48 pS, suggesting a unitary conductance of intact Cx35 gap junction channels of −24 pS. Channel gating was also Vm dependent: open time declined with negative Vm and increased with positive Vm. The ability to break down the complex gating of intact intercellular channels into component hemichannels in vitro will help to evaluate putative physiological roles for hemichannels in vivo.

Connexin; gating; retina

**CONNEXINS (Cx)** are a family of proteins that were originally identified as one of the molecular components of vertebrate gap junction channels (4, 19, 34). Decades of ensuing research have since established that connexins provide a pathway for direct intercellular communication by connecting adjacent cells via gap junction channels and allowing the specific and selective exchange of small ions, nutrients, metabolites, and signaling molecules (15, 23, 41). A complete gap junction channel is made from two hemichannels contributed by each of the cells in communication. The hemichannels are hexamers of connexin proteins that are encoded by a multigene family containing at least 20 members (42, 57). Each connexin isofrom produces intercellular channels with unique properties of size selectivity, ion permeability, conductance, and gating (5, 23, 50).

In addition to forming gap junction channels between two communicating cells, many connexins can also form voltage-activated hemichannels within single plasma membranes (1, 2, 7, 14, 46, 49, 51, 55). Analysis of unopposed hemichannels in functional expression systems provides a unique opportunity to compare hemichannel properties with those of intact gap junction channels between coupled cells (47). A detailed knowledge of hemichannel conductance and gating is also a prerequisite for evaluating whether hemichannels might play any significant physiological roles in vivo (3, 11, 24, 52).

In situ hemichannel activity was first documented in horizontal cells isolated from fish retina (9, 28). To date, at least seven connexins have been identified in the retinas of several species of fish (8, 25, 29, 35, 36, 54, 59). Only two of these, Cx35 and Cx52.6, have been shown to be capable of forming active hemichannels in functional expression systems (32, 55, 56, 59), although neither has been characterized at the single channel level. This is potentially significant, because hemichannel activity has been implicated as an important component of feedback inhibition between cones and horizontal cells in the outer plexiform layer of the fish retina (20, 25, 40). It also has been suggested that hemichannels expressed in other retinal cell types may also affect synaptic transmission (59). In the perch and zebrafish retina, Cx35 has been immunolocalized to the inner and outer plexiform layers (29, 36), suggesting that it may be one candidate connexin to mediate hemichannel effects on synaptic transmission.

Full evaluation of the potential roles for zebrafish Cx35 in hemichannel-mediated inhibition in vivo first requires a complete documentation of hemichannel conductance and gating properties in vitro. Toward this goal, we have shown that *Xenopus* oocytes injected with Cx35 cRNA and N2A cells transfected with Cx35 cDNA develop large, outward whole cell currents on cell depolarization. Using cell-attached and excised patch configurations, we further demonstrated that the hemichannel conductance (γhc) and gating of Cx35 hemichannels exhibited a pronounced dependence on membrane voltage (Vm). γhc increased with relative hyperpolarization and decreased on depolarization, whereas channels gated closed with negative Vm and opened with positive Vm. The ability to document the conductance and gating of Cx35 hemichannels will help to clarify whether their functional properties are consistent with current models of hemichannel feedback inhibition in the retina.
MATERIALS AND METHODS

Expression constructs. For expression studies in Xenopus oocytes, the Cx35 coding sequence was subcloned into pCS2+ (48) as previously described (29). This construct was linearized with NotI, gel purified, and used as template (1 μg of DNA) to produce capped cRNAs using the mMessage mMachine kit (Ambion). For expression studies in N2A cells, the Cx35 coding region was released from pCS2+ with EcoRI (New England Biolabs, Beverly, MA) and subcloned into the EcoRI site of the bicistronic vector pIRE2-EGFP (BD Biosciences/Clontech, Palo Alto, CA). This plasmid contains the internal ribosome entry site (IRES) of the encephalomyocarditis virus between the multiple cloning site and the coding sequence of the enhanced green fluorescent protein (EGFP). This permits both the gene of interest and the enhanced form of the Aequorea victoria GFP gene to be translated from a single bicistronic mRNA, allowing an efficient selection of transfected cells for functional studies.

Oocyte preparation. Stage V and VI oocytes were isolated from Xenopus laevis (Nasco, Fort Atkinson, WI), defolliculated by collagenase digestion, and cultured in modified Barth’s (MB) medium. Cells were injected with a total volume of 40 nl of either an antisense oligonucleotide (3 ng/cell), to suppress endogenous Xenopus Cx38, or a mixture of antisense plus Cx35 cRNA (40 ng/cell), using a Nanjet II Auto/Oocyte injector (Drummond, Broomall, PA). After overnight incubation, oocytes were immersed for a few minutes in hypertonic solution to strip the vitelline envelope, transferred to petri dishes containing MB medium, and manually paired with the vegetal poles apposed for gap junction channel analysis. For hemichannel analysis, single oocytes were devitellinized and processed as described below. Electrophysiological recordings were made 48 h after cRNA injection.

Whole cell electrical recordings in Xenopus oocytes. The functional properties of hemichannels and gap junction channels were assessed by dual voltage clamp (43). Current and voltage electrodes (1.2-mm diameter; Glass Company of America, Millville, NJ) were pulled to a resistance of 1–2 MΩ with a horizontal puller (Narishige, Tokyo, Japan) and filled with 3 M KCl, 10 mM EGTA, and 10 mM HEPES, pH 7.4. Voltage clamping of oocyte pairs was performed using two GeneClamp 500 amplifiers (Axon Instruments, Foster City, CA) controlled by a PC-compatible computer through a Digidata 1320A interface (Axon Instruments). pCLAMP 8.0 software (Axon Instruments) was used to program stimulus and data collection paradigms. Current outputs were filtered at 50 Hz, and the sampling interval was 10 ms. To determine voltage-gating properties of gap junction channels, transjunctional potentials (Vj) of opposite polarity were generated by hyperpolarizing or depolarizing one cell for 3 s in 20-mV steps (over a range of ±120 mV) while the second cell was clamped at −40 mV. Currents were measured at the end of the voltage pulse, at which time they approached steady state (Ijss), and the macroscopic conductance (gmm) was calculated by dividing Ijss by Vj. gmm was then normalized to the values determined at ±20 mV and plotted against Vj. To characterize hemichannels, single oocytes were assessed with a two-electrode voltage-clamp procedure (14). Cells were initially clamped at −40 mV. Depolarizing voltage steps (−20 to +80 mV at 20-mV intervals) were imposed for 5 s, and whole cell currents were recorded. To test the calcium dependence of Cx35 hemichannel currents, MB medium supplemented with 2 mM CaCl2 was used. Membrane current values were measured at the end of the pulse and plotted against the clamped membrane potential (Vm).

Cells and culture conditions. Experiments were carried out on N2A cells transiently transfected (Lipofectin; InVitrogen Life Technologies) with pCS2+ for zebrafish Cx35. N2A cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum, 100 μg/ml streptomycin, and 100 U/ml penicillin. The cells were cultured at 37°C in a CO2 incubator (5% CO2, 95% ambient air). To perform experiments, the cells were harvested and seeded onto sterile glass coverslips placed in multiwell culture dishes (−104 cells/cm2). Electrophysiological experiments were carried out on cells cultured for 1–3 days after transfection. Transfected cells were selected for electrophysiological analysis by GFP expression.

Solutions and pipettes. During patch-clamp experiments, oocytes were superfused with bath solution containing (in mM) 120 potassium aspartate, 10 NaCl, 2 CaCl2, 5 HEPES (pH 7.4), and 5 glucose, with 2 mM CsCl, BaCl2, and tetraethylammonium chloride included. For the Ca2+-free (0 Ca2+) bath solution, CaCl2 was omitted. The patch pipettes were filled with solution containing (in mM) 120 potassium aspartate, 10 NaCl, 3 MgATP, 5 HEPES (pH 7.2), and 10 EGTA (pCa ~8), filtered through 0.22-μm pores. For whole cell recording from N2A cells, potassium aspartate in the superfusate was replaced with an equal molar concentration of NaCl.

Electrical measurements. Glass coverslips with adherent cells were transferred to an experimental chamber perfused with bath solution at room temperature (~22°C). The chamber was mounted on the stage of an inverted microscope (Olympus IMT2). Patch pipettes were pulled from glass capillaries (code 7052; A-M Systems) with a horizontal puller (Sutter Instruments). When filled, the resistance of the pipettes measured ~1–2 MΩ. Experiments were carried out on single cells using the whole cell voltage-clamp technique. A selected cell was attached to a patch pipette connected to a micromanipulator (WR-88; Narishige Scientific Instrument) and an amplifier (Axopatch 200). This method permitted control of the membrane potential, Vm, and allowed measurement of the associated membrane current, Im.

Macroscopic hemichannel currents in Xenopus oocytes. Recordings from Xenopus oocytes injected with Cx35 cRNA showed slowly activating outward currents. These currents were absent in antisense-injected control oocytes not expressing Cx35, indicating that they were generated by Cx35 hemichannels. Figure 1A illustrates the voltage protocol showing that Vm was held at −40 mV and that periodic depolarizing voltage pulses of 5-s duration were then delivered to alter Vm to 80 mV in increments of 20 mV. Figure 1B shows control currents from an oocyte injected with antisense oligonucleotides, whereas Fig. 1C demonstrates a typical record from an oocyte that had been injected with Cx35 cRNA. The family of Im traces elicited by depolarizing (outward current) pulses is typical of other documented hemichannels like Cx46 (14). Im increased proportionally with Vm and showed a voltage- and time-dependent activation. As shown in Fig. 1D, addition of 2 mM Ca2+ to the external bath reduced the magnitude of Im in...
Cx35-expressing oocytes and shifted the voltage of activation to more positive potentials as previously documented for other connexin hemichannels (13). When external Ca$^{2+}$ is not elevated, the majority of membrane current in Cx35 cRNA-injected oocytes arose from currents through Cx35 hemichannels, $I_{hc}$ (Fig. 1E).

Multichannel recordings of $I_{hc}$ from Xenopus oocytes. Macroscopic records of $I_{hc}$ in Xenopus oocytes injected with Cx35 cRNA using standard voltage clamp showed $I_{hc}$ signals that were composites of hundreds to thousands of hemichannels. To analyze single-channel properties of Cx35 hemichannels in oocytes, we utilized cell-attached or cell-detached (inside-out) patch-clamp modes. Figure 2A shows multichannel currents through hemichannels recorded in the cell-attached configuration, in which the cell was superfused with Ca$^{2+}$-free solution. The currents shown were elicited by depolarization to 90 mV. Depolarization gave rise to a time-dependent increase in $I_{hc}$ exhibiting discrete current steps, indicating the sequential opening and closing of hemichannels (channel opening: upward deflections). At least 9–10 hemichannels were activated during the voltage step. Figure 2B shows currents elicited by biphasic ±50-mV pulses (upper trace). Hyperpolarization led to a closure of hemichannels and occasional channel flickering with short open time. Voltage reversal from hyperpolarization to depolarization led to an increase in $I_{hc}$ induced by opening of hemichannels. This record illustrates that channel open time increased and more hemichannels activated with positive potentials for Cx35.

Similar hemichannel voltage dependence was observed in current records obtained in the cell-detached patch mode. Excised inside-out patches obtained from oocytes usually contained several functioning hemichannels (Fig. 3A). During membrane hyperpolarization to −50 mV (upper trace), $I_{hc}$ revealed discrete steps indicating the opening and closing of hemichannels (channel opening: downward deflections). The vertical curve at right represents an all-points histogram of the current trace and indicates the involvement of at least two hemichannels during hyperpolarizing voltage. The distances between the peaks were of comparable amplitudes, 2.8 and 3.0 pA, and corresponded to conductance steps of ~56–60 pS. The following depolarization led to the sequential opening of hemichannels, resulting in the activity of at least seven to nine hemichannels by the end of the depolarizing pulse. The current histogram revealed current transitions of 2.1–2.3 pA, which translates to 42–46 pS. Thus gating of Cx35 hemichannels exhibited a dependence on membrane voltage, gating closed with negative $V_m$, and gating open with positive $V_m$.

Voltage dependence of single hemichannel conductance. $g_{hc}$ was determined using voltage pulses of different amplitude and of either polarity in the cell-detached patch configuration. The data were sampled, averaged, and plotted versus $V_m$. Figure 3B shows the voltage dependence of $g_{hc}$.
Polarizing voltage tends to reduce the time in the open state, gating of Cx35 hemichannels to the closed state. Thus hyperpolarization and decreased with depolarization. The extrapolated single channel conductance at \( V_m = 0 \) was 48 pS, consistent with the low value of \( \gamma_j \) reported for the mouse ortholog of Cx35 (44, 45). Therefore, unitary conductance of Cx35 hemichannels exhibited a pronounced rectification of unitary conductance as a function of \( V_m \), a common feature of hemichannels that has been reported for other connexins analyzed (39, 46, 49, 51).

Dependence of single channel activity on membrane potential. Records obtained from Cx35-expressing oocytes in the cell-detached mode with a few single operational hemichannels were also used to study channel kinetics. The analysis of longer records (\( \approx 10 \)-s duration) allowed us to demonstrate that the gating was voltage dependent at the single channel level. Figure 4 shows the operation of two to three Cx35 hemichannels in a cell-detached patch during different \( V_m \) steps. At a \( V_m \) of \(-30 \) mV, the channels were frequently open, with a few transitions to the closed state (upward current deflections). Increasing \( V_m \) to \(-50 \) and \(-70 \) mV resulted in an increased gating of Cx35 hemichannels to the closed state. Thus hyperpolarizing voltage tends to reduce the time in the open state, consistent with the macroscopic records, in which ensembles of channels behave as outward rectifiers.

Exogenous versus endogenous hemichannels in oocytes. Endogenous membrane currents obtained from oocytes that were not treated with antisense Cx38 oligonucleotides and were injected with H\(_2\)O instead of Cx35 cRNA showed different properties compared with those expressing Cx35. Figure 5A illustrates \( I_m \) records in cell-attached mode from oocytes injected with antisense oligonucleotides to Xenopus Cx35. Currents elicited by \( \pm 50 \)-mV pulses showed no discrete gating steps, in sharp contrast to oocytes injected with Cx35 cRNA (see Fig. 2), and represented the leak current of the patch, which was not subtracted from any of the records. However, oocytes not treated with Xenopus Cx35 antisense oligonucleotides and injected with water displayed hemichannel activity that was likely attributable to endogenous Cx38 (10). Figure 5B shows a multichannel recording of Cx35 hemichannels in the cell-attached configuration. Again, the vertical curve at right is an all-points histogram. The unitary conductance of the operational channels was voltage dependent and yielded values of \( \approx 320 \) and 250 pS for \( V_m \) values of \(-30 \) and \(30 \) mV, respectively. Thus endogenous oocyte Cx38 hemichannels were efficiently suppressed by antisense oligonucleotide treatment.

Single hemichannel currents derived from Cx35 also had a smaller unitary conductance than endogenous Xenopus Cx38 hemichannels. Figure 5C shows recordings of Cx35 hemichannels in the cell-detached mode from oocytes. A voltage-ramp protocol (\( \pm 100 \) mV/2 s) evoked rectifying currents with a voltage-dependent unitary channel conductance, i.e., 75 pS at \( V_m = -95 \) mV, and channels remained open at depolarizing voltages. Currents evoked by voltage ramp (\( \pm 100 \) mV/2 s) from water-injected oocytes (Fig. 5D) also exhibited rectification and yielded unitary channel conductance of 300 pS at \( V_m = -100 \) mV and of 250 pS at \( V_m = 100 \) mV, attributable to Xenopus Cx38 hemichannels (10). Therefore, endogenous Cx38 hemichannels were easily distinguished from the exogenous Cx35 hemichannels by their significantly larger unitary conductance.

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Fig. 3. Unitary conductance of Cx35 hemichannels is voltage dependent. A: a hemichannel current recorded in the cell-detached patch clamp mode from an oocyte injected with Cx35 cRNA revealed hemichannel activity (\( I_{hc} \)) in response to the application of voltage steps to \( \pm 50 \) mV from a holding potential of 0 mV (\( I = 0 \)). B: a plot illustrating the voltage dependence of single hemichannel conductance (\( \gamma_m \)). Values were obtained from 7 oocytes in the cell-detached patch-clamp mode and then averaged and plotted versus \( V_m \). The continuous line corresponds to the best fit of data to a second-order exponential and predicts that \( \gamma_m = 48 \) pS at \( V_m = 0 \) mV. Data represent means \( \pm \) SD.

Fig. 4. Dependence of single Cx35 hemichannel activity on membrane potential. Representative traces of hemichannel activity (\( I_{hc} \)) during extended \( V_m \) steps of \(-30 \), \(-50 \), and \(-70 \) mV are shown. At higher hyperpolarizing voltages, Cx35 hemichannels gated more frequently to the closed state. Currents were recorded in cell-detached patch-clamp configuration.
Cx35-transfected cells, the associated membrane current were recorded in untransfected control N2A cells (Fig. 6). Voltage pulses of 5-s duration were then delivered to alter I_Cx38 hemichannels. Dashed line indicates zero current level (n = 3). No discrete channel events were observed. B: single hemichannel currents (cell-attached patch) recorded from water-injected oocytes without antisense oligonucleotides (n = 3) revealed activity attributable to Xenopus Cx38 hemichannels. Dashed line indicates zero current level (I = 0); dotted lines represent discrete current steps indicative of opening and closing of hemichannels. The current histograms yield unitary conductances of ~320 or 250 pS for V_m of -30 or +30 mV, respectively. Xenopus Cx38 and zebrafish Cx35 both display distinctive voltage-dependent unitary conductance. C: recording from an oocyte injected with Cx35 cRNA. A voltage-ramp protocol (±100 mV/2 s) evoked rectifying currents with voltage-dependent unitary channel conductance. D: recording from a water-injected oocyte without antisense oligonucleotides. Xenopus Cx38 hemichannel currents evoked by a voltage ramp (±100 mV/2 s) also exhibited rectification.

**Hemichannel activity in N2A cells transfected with Cx35.** To compare the effects of expression system on Cx35 hemichannels and intact gap junction channels, we transfected N2A cells with Cx35 cDNA. Figure 6A illustrates the voltage protocol used to activate whole cell channel hemichannel currents in transfected cells. A family of I_m traces was elicited by depolarizing (outward currents) and hyperpolarizing pulses (inward currents). The records were obtained from N2A cells superfused with Ca^{2+}-free solution. After whole cell recording conditions were established, the V_m was clamped to 0 mV and the bipolar voltage pulses of 5-s duration were then delivered to alter V_m in 20-mV steps from ±10 to ±90 mV. No significant currents were recorded in untransfected control N2A cells (Fig. 6B). In Cx35-transfected cells, the associated membrane current (I_m) increased with depolarization (V_m) and showed a voltage- and time-dependent activation, whereas hyperpolarization induced inward currents that deactivated with time (Fig. 6C). In Cx35-expressing N2A cells, a plot of I_m vs. V_m (Fig. 6D) demonstrated the same hemichannel current-voltage relationship as recorded in oocytes. Thus Cx35-expressing N2A cells developed currents that activated slowly, with depolarizing voltage steps that were similar to those recorded from Xenopus oocytes (see Fig. 1), and the functional behavior of Cx35 hemichannels was not influenced by the type of cell in which they were expressed.

To determine whether the whole cell current-voltage relationships shown in Figs. 1 and 6 were similar to the recordings from excised patches of Cx35 hemichannels shown in Fig. 3, we quantified the single channel behavior in excised patches by measuring the mean current during the last 0.5 s of the voltage pulse and plotting it as a function of V_m. Comparison of the current-voltage relationship for macroscopic whole cell Cx35 hemichannel currents with microscopic I_m recorded in cell-detached patches is shown in Fig. 7. Plots were normalized to the current value at the highest voltage tested. Typically, the magnitude of hemichannel currents increased six- to sevenfold over a 60-mV depolarization from the voltage at which hemichannel activity was first evident in the whole cell records. Thus the Cx35 single hemichannel behavior of smaller unitary conductance, longer open time, and more active chan-
nels with increasing depolarization correlated well with the whole cell current data.

Analysis of Cx35 hemichannel activation kinetics. The voltage-gating characteristics of Cx35 hemichannels were further explored by analyzing the kinetics of hemichannel opening for values of $V_m \geq 40$ mV, i.e., sufficient to induce significant outward currents. Figure 8A shows representative results obtained for fitting the first 1 s of hemichannel activation in oocytes injected with Cx35 cRNA (top) and N2A cells transfected with Cx35 (bottom). In all cases, the time-dependent increase in $I_{hc}$ was well fit by a single exponential function. As shown in Fig. 8B, there are no significant differences between the mean values of $\tau$, which were essentially constant with increasing driving force in both cell systems and varied between 0.4 and 0.6 s. Cx35 gap junction channels previously showed current decay with $\tau$ values of 0.4–0.5 s, and $\tau$ also failed to increase with higher driving voltage (56). Thus voltage-independent $\tau$ values appear to be a characteristic property of the Cx35/Cx36 connexin subgroup and present in both gap junction channels and hemichannels.

Gating properties of Cx35 gap junction channels. To compare the macroscopic properties of Cx35 gap junction channels in different expression systems, we used paired *Xenopus* oocytes injected with Cx35 cRNA and N2A cell pairs transfected with Cx35 cDNA. Figure 9A shows representative gap junction currents recorded from oocytes (top) and N2A cells (bottom), respectively. Bipolar pulses were applied to step $V_j$ in 20-mV increments and generate a family of currents from oocyte cell pairs and N2A cell pairs, as shown. The junctional current $I_j$ increased proportionally with $V_j$ and showed a voltage- and time-dependent inactivation. The amplitudes of $I_j$ were determined at the beginning (instantaneous; $I_{j,\text{inst}}$) and end of each pulse (steady state; $I_{j,\text{ss}}$) to estimate the conductances $g_{j,\text{inst}}$ and $g_{j,\text{ss}}$, respectively. The values of $g_{j,\text{ss}}$ were then normalized with respect to $g_{j,\text{inst}}$ and plotted versus $V_j$. As shown in Fig. 9B, this resulted in a symmetrical relationship for both types of cell pairs. The continuous and dashed lines represent best fits to a Boltzmann equation (43) whose parameters are given in Table 1. These findings are consistent with previous reports on Cx35 gap junction channels in paired oocytes (29, 36, 56) and show that the functional behavior of Cx35 gap junction channels was not modified by the cellular environment in which they were expressed.

**DISCUSSION**

Hemichannel activity has been recorded from oocytes injected with cRNA for a number of connexins (1, 2, 12, 14, 32, 46, 55). In addition, hemichannel activity also has been documented for several connexins in transfected cell systems (7, 49, 51). In this study, we have shown that injection of zebrafish Cx35 cRNA into oocytes results in hemichannel activity and, furthermore, that transfection of N2A cells with Cx35 cDNA produces currents similar to those observed in the oocyte assay. Each experimental cell system was able to replicate the macroscopic data of the other, fortifying the notion that the properties revealed were those of Cx35 and were not due to an endogenous connexin in oocytes or N2A cells.

We also have characterized single and multichannel activity using cell-attached and -detached patch-clamp methods and have found that Cx35 behaved similarly to the other documented hemichannels (46, 49, 51). Cx35 hemichannels are
Cx35 HEMICHANNEL PROPERTIES

Zebrafish Cx35 is highly expressed in the brain and retina, and structural analysis of both the gene and the protein clearly demonstrate that Cx35 belongs to the Cx35/36 subgroup of orthologous connexins (29). Immunohistochemical analysis has previously localized zebrafish Cx35 in the retina in both the inner plexiform layer, a synaptic layer for ganglion cells and interneurons such as bipolar cells and amacrine cells, and the outer plexiform layer, where dendrites of horizontal cells and bipolar cells synapse with photoreceptors (29). This pattern is consistent with many other studies showing Cx35/36 labeling in the retinas of other species (16, 17, 21, 22, 30, 31, 36, 45, 58). Within the fish brain, Cx35 has been localized in the mixed chemical and electrical synapses of goldfish Mauthner neurons (37, 38). To date, all of these immunolocalizations are consistent with the formation of patent Cx35 gap junction channels that function as classic electrotonic synapses (60), although asymmetric Cx35/36 labeling has been reported in the cone pedicle (27), which could correspond to the presence of either heterotypic gap junction channels or hemichannels.

Recently, hemichannel activity was proposed as a common mechanism mediating feedback inhibition between cones and horizontal cells in the outer plexiform layer of the vertebrate retina (25, 25, 40). Although Cx35 was not specifically implicated in this instance, its abundance in the retina makes it worthwhile to consider whether its functional properties, or the properties of other well-characterized hemichannels, are consistent with the model of feedback inhibition proposed (20). At issue is whether or not hemichannels have physiological properties consistent with their proposed function in synaptic transmission. On the basis of the present work and previous characterization of other connexin hemichannels, we can first define the minimal necessary conditions for hemichannel function in vivo.

Assuming hemichannels are stable structures within the membrane in vivo, the first important parameter limiting their function then appears to be the concentration of extracellular Ca\textsuperscript{2+}. Previous studies have established that hemichannels are gated closed in the presence of millimolar Ca\textsuperscript{2+} levels presumably via interaction with the alleged “loop gate” (14, 18, 46, 49). Under what conditions would the interstitial fluid surrounding a cell be depleted of Ca\textsuperscript{2+}? One possibility is a restricted space that is small in volume, where the membrane surfaces facing the space would have the capability to remove Ca\textsuperscript{2+}. At least two of these criteria could be met in the case of cone pedicles and horizontal cells in the retina (25). They appear to interact in a restricted space with a small volume, although there is no direct experimental evidence to confirm voltage gated and behave similarly to outward rectifiers as has proven to be the case for all the other hemichannels analyzed at the single channel level. This voltage-gating behavior is consistent with both fast voltage-dependent gating and the slower purported “loop gating” (6, 39, 46), with the latter being sensitive to extracellular Ca\textsuperscript{2+}. In addition, Cx35 is able to form typical gap junction channels in both oocyte pairs and N2A cell pairs, as shown in Fig. 8. The macroscopic data obtained from oocyte cell pairs confirm those previously published (29). In combination with the data obtained from N2A cell pairs in this study, we have demonstrated that Cx35 gap junction channel voltage-dependent properties are not altered by the choice of cell type used as an expression system.

Table 1. Comparison of the Boltzmann parameters for C × 35 gap junctional channels between the Xenopus oocyte and N2A cell expression systems

<table>
<thead>
<tr>
<th>Cell</th>
<th>(V_f)</th>
<th>(V_{0.5}), mV</th>
<th>(G_{\text{max}})</th>
<th>(A)</th>
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<tbody>
<tr>
<td>Oocyte</td>
<td>+</td>
<td>87</td>
<td>0.29</td>
<td>0.05</td>
</tr>
<tr>
<td>Oocyte</td>
<td>-</td>
<td>-81</td>
<td>0.28</td>
<td>0.05</td>
</tr>
<tr>
<td>N2A</td>
<td>+</td>
<td>81</td>
<td>0.27</td>
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</tr>
<tr>
<td>N2A</td>
<td>-</td>
<td>-72</td>
<td>0.26</td>
<td>0.05</td>
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</tbody>
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Gap junctional conductance between pairs of Xenopus oocytes or N2A cells was measured by dual voltage clamp in response to increasing transjunctional potentials (\(V_j\)) of opposite polarity. Normalized gap junctional conductance at steady state (\(g_{\text{max}}\)) was plotted as a function of \(V_j\) with \(G_{\text{max}}\), set as unity. Data were fit to a Boltzmann equation of the form \(g_{\text{max}} = \left(1 + \exp\left\{A(V_j - V_{0.5})\right\}\right) G_{\text{max}}\), where \(G_{\text{max}}\) is the minimum conductance value as estimated from the Boltzmann fit and \(V_{0.5}\) is the voltage at which half-maximal decrease of \(g_{\text{max}}\) is measured. The cooperativity constant (\(A\)), reflecting the voltage sensitivity of the channel, reflects the equivalent number of electron charges moving through the transjunctional field. The plus and minus signs for \(V_j\) refer to the polarity of the transjunctional potential.

Fig. 9. Gating of Cx35 gap junction channels in Xenopus oocytes and N2A cells. A: responses of gap junction currents (\(I_j\)) to transjunctional voltages (\(V_j\)) obtained from Xenopus oocytes injected with Cx35 (top) or N2A cells transfected with Cx35 (bottom). \(I_j\) decayed similarly as a function of \(V_j\) and time in both cell systems. B: dependence of normalized gap junction conductance at steady state (\(g_{\text{max}}\)) on \(V_j\). Data from Xenopus oocytes (○, \(n = 7\)) and N2A-transfected cells (●, \(n = 6\)) were plotted as a function of \(V_j\) and fit to a Boltzmann equation whose parameters are given in Table 1. Data represent means ± SE.
these assumptions. The third requirement, significant reduction of extracellular Ca$^{2+}$, has yet to be shown.

A second critical parameter for the activation of hemichannels is a depolarization of membrane voltage. All hemichannels characterized to date, including zebrafish Cx35, activate upon depolarization and gate closed upon hyperpolarization (7, 14, 46, 49, 51, 55). In the retinal feedback inhibition model (20, 25, 40), hemichannel current flow is assumed to increase as the horizontal cells hyperpolarize. This is counterintuitive to every documented example of hemichannel voltage dependence. Thus two important criteria for hemichannel activation, removal of extracellular calcium and cell depolarization, are inconsistent with their playing a role in feedback inhibition between cones and horizontal cells as previously proposed.

Pharmacological evidence for a hemichannel role in inhibition was provided by the use of the gap junction blocker carbenoxolone, which blocked feedback inhibition, and this effect was attributed to the specific loss of hemichannel activity (25). This view was recently challenged by a study showing that application of carbenoxolone at the same concentrations (100 μM) to patch-clamped, isolated cone photoreceptors directly reduced the cone Ca$^{2+}$-current (53). Thus carbenoxolone directly inhibited cone Ca$^{2+}$ channels and synaptic transfer independently of horizontal cell current, and this alone could explain the reduction in signaling previously attributed to inhibition of horizontal cell hemichannels.

The demonstration that some connexins can form functional hemichannels in vitro is currently not sufficient to allow a mechanistic understanding of how they impact cellular function, especially in the absence of supporting data showing that specific ionic requirements and other special conditions necessary for hemichannel gating have been met in the target tissue. To date, there is no direct in vivo or in situ demonstration of hemichannel activity that unequivocally demonstrates involvement in any form of extracellular mediated communication, even if elusive, hypothesis (3, 11, 20, 24, 52). Future hypotheses of hemichannel relevance to organ physiology must first be based on a thorough understanding of specific hemichannel functional properties of the connexin(s) involved. For Cx35, the present study provides an initial foundation for future investigation of this growing area of research.

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