Title:

Connexins are mechanosensitive

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ABSTRACT

Connexins form gap junction channels that provide a hydrophilic path between cell interiors. Some connexins, particularly the lens connexins, \textit{cx46 and cx50} and their orthologs, can form functional hemichannels in nonjunctional membranes. These hemichannels are a non-selective conduit to the extracellular medium and may jeopardize cell survival. The physiological function of hemichannels has remained elusive, but it has been postulated that hemichannels are involved in ATP-release caused by mechanical stimulation.

Here we show with single channel and whole cell electrophysiological studies that Cx46 hemichannels are mechanosensitive, like other families of ion channels and membrane bound enzymes. The hemichannel response to mechanical stress is bipolar. At negative potentials, stress opens the channel, and at positive potentials stress closes it. Physiologically, Cx46 hemichannels may assist accommodation of the ocular lens by providing a transient path for volume flow as the lens changes shape.

\textbf{Key words:} connexin, mechanical stress, lens
INTRODUCTION

Connexins, the subunits of gap junction channels, typically form closed hemichannels in nonjunctional cell membranes, and only after two hemichannels dock, will the channels open and create a hydrophilic pathway between cell interiors. Exceptions to this rule exist for two members of the connexin family of proteins, connexin46 and connexin50. Both are expressed in the lens where they can provide not only gap junction channels, but also nonjunctional conductances (12). When expressed exogenously, both of these connexins form open hemichannels (2, 31, 50). The properties of hemichannels are similar to the complete gap junction channels, both in terms of permeability and the gating effects of various agents, including pH. Open hemichannels, being nonselective, are generally deleterious to cell health. Oocytes expressing \textit{\textit{ex} 46 or \textit{ex} 50}\textit{Cx}46 or \textit{Cx}50 will die unless the hemichannels are kept shut by an elevated extracellular calcium concentration (11).

Other connexins, including \textit{\textit{ex} 43 and \textit{ex} 38}\textit{Cx}43 and \textit{Cx}38, have also been reported to form open hemichannels (10, 25). However, these were only observed under special experimental conditions, typically with unphysiological micromolar extracellular calcium. Recently, a series of papers have invoked connexins in the release of cellular ATP and NAD\textsuperscript{+} to the extracellular space through nonjunctional hemichannels (5, 8, 24, 41). These agents can act as extracellular second messengers (21). Calcium waves propagate through many cell networks (4, 7, 36, 37, 42), and connexins are known to play a crucial role in the intracellular propagation process (44). The intracellular mode utilizes gap junctions to pass InsP3 from cell to cell (6, 35). The extracellular mode involves
release of ATP and its binding to purinergic receptors (14, 37). Connexin hemichannels may be involved in this mode since they are permeant to ATP (24, 41).

Mechanical stress can initiate Ca\textsuperscript{2+} waves (40), and it is well documented that mechanical stress can result in ATP release (16, 47). The simplest scenario in which connexin hemichannels could initiate mechanosensitive calcium waves would be if they were mechanosensitive and permeant to ATP.

In the present study, we tested the sensitivity of hemichannels to mechanical stress. We chose Cx46 hemichannels because they are well characterized at both the macroscopic and single channel levels (11, 31, 32, 45). Cx46 is present in the ocular lens, and hemichannels might have a physiological role permitting rapid fluid equilibration under the mechanical stresses associated with accommodation.

**METHODS:**

**Preparation of oocytes**

Oocytes were prepared as described previously (9). *Xenopus laevis* oocytes were isolated by incubating small pieces of ovary in 2 mg ml\textsuperscript{-1} collagenase in calcium free oocyte Ringer’s solution (OR2) and stirring at 1 turn/second for three hours at room
temperature. After being thoroughly washed with regular OR2, oocytes devoid of follicle
cells and having an uniform pigmentation were selected and stored in OR2 at 18 °C.

**In vitro transcription of mRNAs**

Cx46 cloned into the expression vector pSP64T was obtained from Dr. D.L. Paul (31). mRNA was transcribed by Sp6 RNA Polymerase from 10 µg of *EcoRI*-linearized plasmid using the mMessage mMACHINE kit (Ambion). mRNA was quantified by absorbance (260nm), and the proportion of full-length transcripts was checked by agarose gel electrophoresis. 20 nl of mRNA (50 ng/µl) was injected into oocytes. The injected oocytes were then transferred into fresh OR2 medium with elevated Ca$^{2+}$ (5 mM) to keep the gap junction hemichannels closed and incubated at 18 °C for 18-24 hours. For electrophysiological recordings, oocytes were transferred to regular OR2.

**Solutions:**

OR2 solution in mM: 82.5 NaCl, 2.5 KCl, 1.0 MgCl$_2$, 1.0 CaCl$_2$, 1.0 Na$_2$HPO$_4$, 5.0 HEPES, antibiotics (Penicillin, 10,000 units/ml; Streptomycin, 10 mg/ml), pH7.5).

Hypotonic solution: 2.5 KCl, 1.0 MgCl$_2$, 1.0 CaCl$_2$, 1.0 Na$_2$HPO$_4$, 5.0 HEPES, pH7.5).

Hypertonic solution: 82.5 NaCl, 2.5 KCl, 1.0 MgCl$_2$, 1.0 CaCl$_2$, 1.0 Na$_2$HPO$_4$, 5.0 HEPES, pH7.5), 100 sucrose.
Voltage clamp

Whole cell voltage clamp recording was performed with two intracellular electrodes as described (9).

Patch clamp technique

Single ex46Cx46 hemichannels were studied by the patch-clamp technique (19) using a WPC-100 amplifier (E.S.F. Electronic, Goettingen, Germany). Currents were filtered at 5 kHz, digitized using a VR-10B digital data recorder, and stored on video tape. The recordings were transferred to a Power Macintosh (Apple) computer using an ITC-18 Computer Interface (Instrutech Corporation) and analyzed. Acquisition and analysis were done with the Acquire and TAC programs (both from Bruxton Corporation).

The vitelline membrane was removed and the oocyte washed once before it was transferred to a new dish containing potassium gluconate solution (KGlu solution: 140 mM KGlu, 10 mM KCl, 5.0 mM TES, pH 7.5). Patch pipettes were made from borosilicate glass tubing (1.5-0.86 mm, #GC150F-15, Warner Instrument Corporation), drawn using a Flaming-Brown Micropipette Puller (Model P-97, Sutter Instrument Company), and polished with a microforge (Narishige Scientific Instruments) to an inside diameter of 0.5-1μm with a resistance of 10-20 MΩ in KGlu solution. The pipette and bath usually contained the KGlu solution.

Negative pressure was applied to the membrane patch pneumatically through the pipette holder. The pressure, measured with a water manometer, was established first in a
reservoir with a syringe. Step changes of pressure were then applied to the patch by connecting the pipette to the reservoir or atmospheric pressure with a valve.

**Statistics**

Channel kinetics were analyzed only for patches containing single channels. Student’s paired t-tests were used to quantitate the effects of stress on single channels and whole oocyte conductance.

**RESULTS**

**Effect of mechanical stress on single ex46Cx46 hemichannels**

To test whether ex46Cx46 hemichannels are sensitive to mechanical stress, we used single channel patch clamp. Cell-attached or excised membrane patches with single ex46Cx46 hemichannels were stressed by applying suction to the pipette(18). Cx46 hemichannels were easily identified by their channel properties: a unit conductance of ~300pS, poor selectivity between cations and anions, and a complex voltage-dependent gating (32, 33, 45). At positive potentials, the channels dwelled mainly in a
subconductance state. At potentials negative to –30 mV, the channels closed over a time scale of seconds, due to activation of a “loop gate” (31, 32, 45).

Figure 1 shows the activity of a Cx46 hemichannel at –50 mV after a jump back from a positive holding potential. The frequency of channel openings declined over the first 20s, but application of negative pressure reactivated the channel with a latency of about 1s. Activity was maintained as long as the pressure was applied. With the release of pressure, the channels closed after passing through substates. Figure 2 shows a multichannel record with two negative pressure jumps that induced intense channel activity. The release of suction ended activity abruptly, but that was followed by a slow (~ 1s) return to steady state activity. Such rebound behavior has been reported for mechanosensitive potassium channels (probably TREK-1) in rat heart (30), and snail heart (29). The rebound was postulated to represent retensioning of the cytoskeleton upon release of stress, and was highly variable. Since the stimulus rise time was <100ms, the latency was not instrument based.

Figure 3 shows single channel activity at positive potentials. The channel was first identified at negative potential on the basis of its typical large conductance before switching to positive potentials where the channel exhibited the characteristic two conductance levels, typical for positive potentials. Since the channel is an inward rectifier, the full conductance is smaller at positive potentials. At positive potentials, the channel dwelled mainly in a subconductance state. Application of negative pressure reversibly inhibited channel activity in a classic stretch-inactivated mode of gating, with a
latency on the order of 1s. Thus, \textit{ex46Cx46} hemichannels are mechanosensitive in a paradoxical manner, incorporating features of stretch-activated channels (18) and stretch-inactivated channels (29). Stretch can activate or inhibit Cx46 channels dependent on membrane voltage. This is similar to the behavior of \textit{Shaker} channels expressed in oocytes (17); stretch opens closed channels and closes open channels.

Activation of \textit{ex46Cx46} hemichannels by mechanical stress at negative potentials was consistently observed only in cell-attached patches. In excised patches at negative potentials, we rarely saw activation, but inactivation usually persisted after excision at positive potentials. Apparently a cytoplasmic factor is required for full mechanosensitivity. The voltage dependence of gating in eukaryotic mechanosensitive channels is known to depend on the status of the cytoskeleton (20, 43).

An obvious question is whether we are observing endogenous SACs or connexins. The result is quite clear: they are two different channels (Figure 4). SACs are abundant in all oocytes, whereas the large conductance (280 pS) channel can only be observed in CX46 injected oocytes (32, 45). This channel is directly gated by pH (32, 46). The strongest argument that this channel has to be ascribed to Cx46 is the observation that channel properties change with mutating the CX46 sequence (22, 23, 32). The conductance and the selectivity are different between Cx46 (45) and endogenous SACs (20, 52). The voltage dependence of mechanosensitivity also changes. The voltage dependence of mechanosensitivity, the conductance and the selectivity are different between in cx46 and
endogenous SACs differs (20, 52). The endogenous channels are activated by mechanical stress at all potentials, and only inactivation is voltage dependent (Figure 5).

**Effect of osmotic stress in intact cells**

To test whether the mechanosensitivity of $\text{ex46Cx46}$ hemichannels is detectable in whole cell recordings, we subjected $\text{ex46Cx46}$ oocytes to osmotic stress. Cx46 activation is fatal to oocytes. The stochastic opening of a few channels causes depolarization, which in turn leads to the opening of more channels. Since $\text{ex46Cx46}$ hemichannels are closed at potentials more negative than $-30$ mV, and the resting membrane potential is negative to $-30$mV, the “fatal” activation of a large number of channels is a slow process, and cells die over several hours. To extend the lifetime of $\text{ex46Cx46}$ expressing oocytes, we incubated them in Ringer’s solution with high calcium (5 mM) to keep the hemichannels closed (11). For acute experiments the calcium concentration was lowered to its physiological value of 1 mM.

When the membrane potential of oocytes expressing $\text{ex46Cx46}$ was clamped at $-50$ mV the hemichannels were closed and the membrane conductance was approximately 1$\mu$S, a value similar to that of uninjected oocytes (11, 31, 32). Hypotonic solutions caused the oocytes to swell, and increased the membrane conductance (Figure 5-6). Upon switching back to normal Ringer’s solution, the conductance transiently increased, and then returned to resting values within about ten minutes. The transient increase in conductance is due to the reintroduction of the higher conductivity normal Ringers. Currents in
hypotonic medium are carried mainly by cytoplasmic anions, such as chloride, and the remaining extracellular salts (10 mM). Thus, the “instantaneous” current obtained just after restoration of normal Ringer’s solution represents the osmotically-activated change in conductance.

In accordance with the single channel data shown above, depolarized potentials reversed the response to hypotonic stress, reducing the membrane current (Figure 6-7). This reduction in current could be caused by a reduction in the single channel conductance (because of dilution of the permeant ionic species), or by stretch-inactivated gating (15, 29, 34).

To distinguish between these possibilities, we modified the protocol so that normal Ringer’s solution was hypertonic relative to pre-equilibrated oocytes. The oocytes were shrunk by equilibration for 30 minutes in hypertonic Ringer’s solution (normal Ringers supplemented with 100 mM sucrose). When the shrunken oocytes were exposed to normal Ringer’s solution, they now swelled but the membrane conductance still fell at depolarized potentials (Figure 7-8). Thus, even at normal ionic strength, depolarization inhibits the activation of swelling-induced currents, and the inhibition is most likely a function of the probability of being open, rather than changes in unitary conductance. The apparent crossover potential for these two competing trends of open probability is ~ –30 mV (data not shown).

The swelling response depended on expression of Cx46, since there was no response in uninjected oocytes or in oocytes expressing Cx43 (Figure 8-9). This agrees with earlier observations that hypoosmotic stress fails to activate oocyte currents
Furthermore, depolarizing the membrane from –50 mV to –20 mV did not induce a current in control oocytes, contrary to \textbf{ex46Cx46} expressing oocytes. (We avoided positive potentials and excessive hypotonicity to avoid activation of other currents (52), that might obscure the Cx46-mediated currents). None of the osmotic stresses induced a membrane current in control oocytes so that the conductance induced by hypotonic stress in \textbf{ex46Cx46} oocytes at –50 mV is caused by \textbf{ex46Cx46} hemichannel activation.

**DISCUSSION**

This is the first demonstration that connexins can be mechanosensitive. The response is connexin-specific, however. Neither \textbf{ex43} nor \textbf{ex38Cx43} or \textbf{Cx38} responded to hypo-osmotic stress. Oocytes exogenously expressing \textbf{ex43Cx43} do not form open hemichannels, while \textbf{ex38,Cx38}, which is endogenous to oocytes, only forms open hemichannels in the absence of extracellular calcium (10).

Since \textbf{ex46Cx46} is expressed at high levels exclusively in the lens, the mechanosensitivity of \textbf{ex46Cx46} hemichannels may play a physiological role. Under resting conditions, the membrane potential keeps the channels closed. During visual accommodation, the mechanical stress imposed on the cells to change the shape of the lens could open the hemichannels and allow a more rapid volume flow to relieve stress.
This process would be self-limiting, since depolarization rapidly closes the channels, preserving cell viability. Such a scenario is consistent with the findings that in normal resting lens fiber cells, there is no significant contribution of \textit{cx46} hemichannels to membrane currents (27). However, such currents become visible under certain experimental conditions, such as low calcium (12).

Although oocytes endogenously express mechanosensitive cation channels (20, 28, 48), they cannot be reliably activated by volume changes. This inability to evoke whole cell currents corresponding to the single channel currents, has been ascribed to several potential causes: a large ‘membrane reserve’ (53), limited water permeability (39), or to the presence of a stiff cytoskeleton that is disrupted during patch formation. The bipolar effect of potential on the kinetics of mechanosensitive ion channels is reminiscent of Shaker K\textsuperscript{+} channels. In that case it was postulated that the intermediate states of the channel, those between fully closed and fully open, are physically larger, and hence occupancy is favored by mechanical stress(17).

Nearly all cells release ATP with mechanical stress (13, 16, 38, 49, 51), and connexins have been suggested to mediate at least part of the process. Stress-induced ATP fluxes have been measured in normal oocytes, and the activation of endogenous mechanosensitive channels was invoked as a possible sensory mechanism (3). The present study establishes that, in principle, connexins can be mechanosensitive and can mediate both sensing and ATP release. Cx46 hemichannels are sufficiently large to permit the flux of ATP. For example, when \textit{cx46} hemichannels are active,
extracellular application of cAMP results in a CFTR-mediated chloride current that depends upon elevation of intracellular cAMP (33).

It has been suggested in the literature that connexins, such as Cx43, could form open hemichannels that mediate the physiological stress-induced release of ATP from cells (24, 25, 41). However, the Cx43 hemichannel conductance only occurs in unphysiological, low extracellular calcium. Complicating the simple view of the hemichannel as an ATP transporter/transducer, is evidence that ATP release may be vesicular (1, 26). Also, in astrocytes from Cx43 knock-out mice, intercellular calcium waves propagate in these cells mainly through an extracellular pathway (42) with the same velocity as in wild-type astrocytes (37).

Whether other connexins, or gap junction channels, are mechanosensitive is not yet known. Although Cx43 hemichannel activity has only been found under unphysiological bath conditions, it is possible that second messengers may modify the gating of the channel to function in vivo.
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**ACKNOWLEDGEMENTS:**

This work was supported by the National Institute of Health grant GM48610.
FIGURE LEGENDS

Figure 1. Stretch-activation of cx46 hemichannels.

Single channel currents were recorded from a membrane patch of an oocyte exogenously expressing cx46. The membrane potential was held at –50 mV. Negative pressure (~ 40 mbar) was applied by suction to the patch pipette during the time indicated by the line.

Open probability was determined for five membrane patches from different oocytes. Means ± SE are plotted; statistical significance of differences between the activity before and during application of stretch uses a paired t-test as indicated: *, p<0.05.

Figure 2. Multichannel data at –50 mV from a patch of an oocyte expressing connexin 46.

Mechanical stress (indicated by the bars) reversibly increased channel activity.

Figure 3. Mechanical stress shuts the cx46 channel at positive potentials.

Single channel currents were recorded from a membrane patch excised from an oocyte exogenously expressing cx46. The membrane potential was held at +50 mV, where the channel dwells primarily in a subconductance state. Negative pressure (~ 40 mbar) was applied to the patch pipette during the time indicated by the line. The channel was closed reversibly by mechanical stress. In this particular recording channel inhibition was observed with some latency. Open probability was determined from four different membrane patches using channels in the fully open states and the subconductance states.
Means ± SE are plotted, n is given above the bars; the statistical significance of differences between before and during application of stretch based on paired t-tests is indicated: *, p<0.05, **, p<0.01.

Figure 4. The oocyte’s endogenous stretch activated channel (SAC) has different unit conductance and different kinetics than Cx46. The channels were held at –50 mV in excised inside-out membrane patches. Two SACs with ~ 50 pS unit conductance were active. A single ~270 pS channel was observed in a patch excised from a Cx46 expressing oocyte.

Figure 5. Response of a SAC to membrane stress. Recording conditions were the same as in Figure 1.

Figure 6. Macroscopic membrane conductance induced by osmotic stress. Cx46 expressing oocytes were held at –50 mV and exposed to hypotonic solution (OR2 sans NaCl) as indicated. Hyperpolarizing 5 mV voltage pulses (top trace) at a rate of 12/minute were applied and induced the currents shown in the bottom trace. Cx46 hemichannels are closed at this potential. Osmotic stress resulted in the development of a large membrane conductance. A quantitative analysis was performed at the times indicated by the roman numerals. During exposure to hypotonic solutions, the ionic strength is reduced and leads to attenuated currents. Thus, the conductance increase upon washout, like a typical “tail current”, more likely reflects the elevated probability of channels being open as a result of osmotic stimulation.
(Means ± SE are plotted, n is given above the bars; statistical significance of difference between pre and post application of osmotic stress based on paired t-tests is indicated: *, p<0.05, **, p<0.01).

Figure 6.7. Depolarization reverses the response to osmotic stress.
When held at –20 mV ex46Cx46 hemichannels in oocytes are responsible for a large membrane conductance. Application of osmotic stress at this potential reversibly diminished the conductance. Subsequent application of CO2 also reduced conductance, consistent with its origin in ex46Cx46 hemichannels.

Figure 7.8. Reduction of membrane conductance by osmotic stress at –20 mV is not caused by the reduction of ionic strength.
Oocytes were equilibrated for 30 minutes in hypertonic solution (OR2 plus 100mM sucrose). Exposure to regular OR2 reversibly reduced the conductance. (Means ± SE are plotted, n is given above the bars; statistical significance of difference between pre and post application of osmotic stress based on paired t-tests is indicated: *, p<0.05).

Figure 8.9. Osmotic stress does not affect the membrane conductance of uninjected oocytes or in oocytes expressing ex43-Cx43. a) Oocytes were held at –50 mV and hypotonic solution was applied at the time indicated. Conductance was determined at the time points indicated by the roman numerals and plotted. b) Oocytes were preincubated
for 30 minutes in hypertonic solution held at –50mV and then stepped to –20 mV.

Regular OR2 solution was applied as indicated. None of the interventions resulted in a significant change of membrane conductance.
Figure 1
Figure 3
Figure 5
Figure 7
Figure 8
Figure 9