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Oocyte triplet pairing for electrophysiological investigation of gap junctional coupling

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Abstract

Gap junctions formed by expressing connexin subunits in *Xenopus* oocytes provide a valuable tool for revealing the gating properties of intercellular gap junctions in electrically coupled cells. We describe a new method that consists of simultaneous triple recordings from 3 apposed oocytes expressing exogenous connexins. The advantages of this method is that in one single experiment, one oocyte serves as control while a pair of oocytes, which have been manipulated differently, may be tested for different gap junctional properties. Moreover, we can study simultaneously the gap junctional coupling of 3 different pairs of oocytes in the same preparation. If the experiment consists of testing the effect of a single drug, this approach will reduce the time required, as background coupling in control pairs of oocytes does not need to be measured separately as with the conventional 2 oocyte pairing. The triplet approach also increases confidence that any changes seen in junctional communication are due to the experimental treatment and not variation in the preparation of oocytes or execution of the experiment. In this study, we show the example of testing the gap junctional properties among three oocytes, two of which are expressing rat connexin36.

Keywords

carbenoxolone; junctional conductance; *Xenopus laevis*; synchrony; connexin36

1. Introduction

Connexin gap junction channels enable the intercellular, bidirectional transport of ions, metabolites, second messengers and other smaller molecules (reviewed by Meier and Dermietzel, 2006). Gap junctions formed by expressing connexin subunits in *Xenopus* oocytes provide a valuable tool for revealing the gating properties of intercellular gap junctions in electrically coupled cells (Ebihara, 1992). *Xenopus laevis* oocytes have been used to investigate junctional communication of a variety of ectopically expressed

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mammalian gap junction proteins (Dahl et al., 1987; Swenson et al., 1989; Ebihara, 1992). One problem has been that occasionally *Xenopus* oocytes can form endogenous Cx38 gap junctions (Ebihara et al., 1989; Gimlich et al., 1990). This problem is routinely prevented by the use of antisense Cx38 cRNA as in this present study. However, the effectiveness of this method has to be determined by measuring separate control pairs of oocytes to test for the lack endogenous coupling in the same experimental preparation. This adds a level of variation in that any differences seen between control vs. experimental pairs could be due to variations in preparation and/or execution of experimental treatments.

The discovery of mammalian connexin36 genes (Cx36), that have a preferential expression in neurons (Condorelli et al., 1998; Belluardo et al., 1999; Rash et al., 2000; Connors and Long, 2004), has considerably advanced our understanding of the prevalence and physiological importance of electrical neurotransmission. Cx36 is expressed strongly during development and although it is more weakly expressed in adults, it persists in specific neurons in the retina, hippocampus, neocortex, inferior olive, several brain-stem nuclei, and spinal cord, among others. Cx36 has been identified at ultrastructurally defined electrical synapses in many neuronal types that are believed to be electrically coupled (Fukuda et al., 2006; Christie et al., 2005; Rash et al., 2007; Hamzei-Sichani et al., 2007).

Several methods have been developed to measure gap junction coupling such dye transfer, scrape loading, gap-fluorescence recovery after photobleaching, the preloading assay, and local activation of a molecular fluorescent probe (LAMP), or by measuring electrical conductance and metabolic cooperation (reviewed by Abbaci et al., 2008). Unlike artificial dye transfer methods (Dakin et al., 2005), the dual intracellular recording technique still provides the most physiologically relevant and best temporal resolution for monitoring electrical coupling between connected pairs of cells (Spray et al., 1979). In this study, we have developed a triple oocyte pairing preparation to monitor simultaneously the electrical coupling between 3 putative gap junctional connections. This method offers the advantage of simultaneously measuring any endogenous, Cx38-dependent, background coupling in a control oocyte rather than having to separately measure control coupling in an separate pair of control oocytes. We used this technique to investigate how different pharmacological substances regulated junctional conductance. Here we report that the triple oocyte technique also enables detection of non-specific membrane changes. Because the control oocyte is measured simultaneously, we have increased confidence that any changes in coupling are due to the experimental treatment rather than variation in the preparation of oocytes or execution of the experiment.

2. Materials and Methods

2.1. Plasmid preparation

Connexin 36 was cloned from 7-day old Sprague-Dawley rat mesopontine tegmentum cDNA (Heister et al., 2007) using primers 5'-CACCATGGGGGAATGGACCATC (fwd) and 5'-CACATAGGCGGAGTCACTGGACTG (rev). The PCR product was cloned into pENTR (Invitrogen, CA) using Gateway technology. A *Xenopus* expression destination vector, pXen DEST CV5, was built from pXen1 (Macnicol 1994 gene) and pcDNA3.2/V5-DEST (Invitrogen, CA). GST was removed from pXen1 by digestion with Nco I/Xba I (essentially reverting it back to pSP64T, Kreig and Melton 1984) and the remaining plasmid blunt ended with Klenow. The C-terminal V5 tag and the Gateway recombination cassette from pcDNA3.2/V5-DEST, was amplified by PCR with 5' phosphorylated primers (5' GCTAGTTAAGCTATCAACAAGTTTGTAC (fwd), 5' CCGTTTAAACTCATTACTAACCGGTAC (rev)) using Pfu for blunt ended ligation into the digested pXen. pENTR Cx36 was recombined into pXen DEST CV5 to make pXen

Cx36-V5. All plasmids were sequenced to verify integrity. RNA was transcribed using SP6 (Promega) as previously described (Melton et al., 1984).

2.2. Oocyte preparation

All animal methods were approved by the UAMS IACUC, AUP# 2864. Oocyte preparation was optimized from several protocols (Charlesworth et al., 2006; Choe and Sackin, 1997; Ebihara, 1992). Adult female *Xenopus* frogs were anesthetized with 0.2% tricaine buffered with 0.3% sodium bicarbonate, and ovaries removed into oocyte isolation medium (96 mM NaCl, 2 mM KCl, 5 mM MgCl₂, 5 mM Hepes, pH 7.6). Oocytes were digested 2 × 30 min with 2 mg/ml collagenase type II (Sigma) and 2 mg/ml hyaluronidase type II (Sigma) in the above medium with gentle agitation. Remaining follicles were dislodged by agitation for a further 30 min in oocyte isolation medium supplemented with 0.6 mM CaCl₂. Oocytes were rinsed well with Ca²⁺ containing oocyte isolation followed by frog L-15 (described in Macacha and Haun, 2002). Oocytes were stored for several days in frog L-15 at 18°C with the medium changed at least daily. Two days before recording oocytes were injected (Nanoject Variable Microinjection Apparatus, model 3-000-203-XV, Drummond Scientific, Broomall, PA) with 23 nl total volume containing 50 ng each RNA as described in results. All oocytes were injected with 50 ng Cx38 antisense morpholino (ctttaacaactcccatcctgccatg) (Genetools, OR) or an antisense Cx38 oligonucleotide (15 ng/oocyte) to block endogenous expression of this connexin (Ebihara, 1996; Teubner et al., 2000). Control oocytes were incubated overnight with neutral red to distinguish them from experimental oocytes. The day before recording, oocytes were incubated in calcium-containing oocyte isolation medium supplemented with sucrose 460 mosmol/l to shrink the oocyte away from the vitelline membrane. After 10–15 min the vitelline membrane was manually peeled away using watchmakers forceps. Oocytes were placed in close apposition in agarose wells and incubated overnight at 18°C, in frog L-15.

2.3. Electrophysiological recordings

Oocytes were viewed with an upright microscope (Olympus BX51WI, Tokyo, Japan) placed on an X-Y translator (Somapatch-O-XY, Soma Scientific Instruments, Inc., Irvine, CA) installed on an anti-vibration table (30" × 48", TMC 63-543, Peabody, MA). The microscope was equipped with a 2X non-water immersed objective and a high-resolution 3-CCD color camera (Hitachi HV-D30, Imaging Products Group, Little River, SC). The S-video output of this camera was connected to the S-video input of a 17" LCD flat panel television (Samsung SyncMaster 710MP, resolution: 1280 × 1024 pixels) to monitor the impalement of the oocytes. High resolution photos were taken using an eight megapixel digital camera (Nikon, Coolpix 8700) connected to the microscope eyepiece via an adapter (MM99-5700, Martin Microscope Company, Easley, SC).

Three fine motorized micromanipulators, 4 axes each, (MX7500, Siskiyou Corp., Grants Pass, OR) controlled by remote devices were installed around a custom made plexiglass recording chamber fixed with a rod to the table and mounted above the microscope condenser. The motorized micromanipulators hold 3 headstages of 2 dual channel patch clamp amplifiers (Multiclamp 700B, Molecular Devices, Sunnyvale, CA). Headstage sensitivity was adjusted using the Multiclamp 700B Commander interface to 50 MΩ (optimized for a maximum current of 200 nA).

Electrodes were pulled from borosilicate glass capillaries with an inner filament (1.5 mm outer diameter, 0.84 mm inner diameter, WPI, Sarasota, FL) on a pipette puller (P-97, Sutter Instrument Company, Novato, CA). Electrodes were filled with a solution containing 3 M KCl, 10 mM Hepes (pH 7.4) and have a resistance ranging between 0.9 and 1.1 MΩ. All gap junction channels undergo regulation by pHi. Unlike other connexin channels which

close by acidification, Cx36 channels undergo unique regulation by pHi since their activity is inhibited by alkalosis rather than acidosis (González-Nieto et al., 2008; but see Teubner et al., 2000). Therefore, a pH buffer (HEPES 10 mM) was included in the intracellular solution and the pH was adjusted to 7.4. Oocytes were superfused with a Marc's Modified Ringers (MMR, Ubbels et al., 1983) containing in mM: NaCl 100, KCl 2, MgSO₄ 1, CaCl₂ 2, HEPES 5 and pH was adjusted to 7.4. Healthy oocytes had a membrane potential lower than -60 mV. Drugs and solutions of different ionic content were applied to the slice by switching the perfusion with a three-way valve system.

Analog signals were low-pass filtered at 2 kHz, and digitized at 5 kHz using a Digidata-1440A interface and pClamp10.2 software (Molecular Devices, Sunnyvale, CA). In triplet recordings, we acquired simultaneously 6 signals representing the current and voltage of each of the 3 oocytes. Series resistance compensation was performed after oocyte impalement using the bridge balance in current clamp mode by neutralizing the fast component in the voltage response to a -10 nA pulse of 1 sec duration (for discussion of possible errors resulting from series resistance, see Wilders and Jongsma, 1992).

To measure cell responses to current pulses of incremental amplitude, we designed a current clamp protocol in pClamp software, in which we acquired 11 sweeps each of 15 sec duration and we inject current pulses of 1 sec duration at time 0.2, 5.2 and 10.2 sec respectively in cell#1, cell#2 and cell#3. The current pulses have an initial amplitude of -40 nA in the first sweep and were incremented by 10 nA in each subsequent sweep. Voltage coupling ratio was defined as ratio of voltage change in cell #2 over voltage change in cell #1 in response to injection of a -20 nA pulse current of one sec duration in cell#1. The purpose of current clamp experiments (Fig. 1) was to estimate the voltage coupling ratio, which was determined as the ratio of voltage changes in both cells at the end of 1 sec current pulse injected in one cell. Even though the membrane time constants exceeded 1 sec, both the injected cell and the coupled cell exhibited similar membrane time constants. Therefore, the measurement of the voltage coupling ratio was not affected by the time chosen to determine voltage changes in each cell even though steady state has not been fully reached by the end of 1 sec. In contrast, in voltage clamp experiments (Fig. 2), steady state current responses were always reached by the end of 1.5 sec voltage steps. In voltage clamp mode, oocytes were held at a voltage identical to their resting membrane potential. The junctional conductance from cell#1 to cell#2 was estimated by measuring the steady-state current change in cell#2 in response to injection of a -50 mV step (duration 1.5 s) into cell #1, and then dividing the current produced (nA) by 50 mV. For a more accurate measurement of junctional conductance and to eliminate potential errors due to electrode access resistance and space clamp issues, please refer to previous studies (Van Rijen et al., 1998; Veenstra 2001).

Membrane potential cross-correlograms were constructed from 100 sec simultaneous recording samples from each pair of cells. Membrane potential traces were offset by their mean and the traces were then reduced by group averaging to one point per 5 ms. Cross-correlograms of the conditioned traces were constructed and normalized by the square root of the product of the autocorrelation values of the 2 traces at zero lag time. The coefficients of correlation (theoretical maximum = 1) measures the degree to which changes in membrane potential of each cell were correlated. The coefficients of correlation of the membrane potential were considered significant if they were at least 3 times higher than the standard deviation (SD) of the those of the shuffled membrane potentials (confidence limit >99.97%).

Data, expressed as mean $\pm$ SEM, were statistically analyzed using paired t-test (Origin 7.0 software, Microcal Software Inc., Northampton, MA) unless otherwise stated.

3. Results

This study reports a modified version of using *Xenopus* oocytes to measure junctional coupling. We made recordings from 25 triplets of oocytes, one of the oocytes in each triplet served as a control and was distinguished by a labeling it with a red dye (or by positioning the pigmented side upward) while the 2 others oocytes of the triplet expressed connexin36 (Fig. 1). In 2 of 25 triplets, the gap junctional conductance between the oocytes expressing Cx36 was below 10 nS suggesting inadequate connexin36 expression and these triplets were not considered for further analysis. The average junctional conductance of the remaining triplets was 123 ± 11 nS (range 18–341 nS, $n = 46$ junctions from 23 triplets).

Voltage clamp experiments indicate that the gap junctions were symmetric in that application of voltage steps of opposite polarity in the stimulated cells produced in the coupled cells currents of similar amplitude but opposite polarity (Fig. 2). In 22 of the 23 oocyte triplets there was a significant voltage coupling ratio between oocytes expressing Cx36 ($54 \pm 3\%$, $n = 46$ junctions, range 22–101%). In the remaining triplet the Cx36-expressing oocytes had a voltage coupling ratio of 5% and 8%. In contrast, the voltage coupling ratio between the control oocytes and Cx36-expressing oocytes was near 0 ($n = 46$ putative junctions). This result indicates that the rate of effectiveness of our technique is very high, that functional gap junctions were formed only between oocytes that express connexin36 and that under our incubation conditions no gap junctions were formed from endogenous connexin 38. In addition, the absence of voltage coupling in the control oocyte indicates that no cytoplasmic bridges were formed among paired oocytes.

The voltage coupling ratio between oocytes injected with constitutively active Cx36 ($84 \pm 5\%$) was significantly higher than that between oocytes injected wild type Cx36 ($53 \pm 8\%$) ($n = 8$ junctions from 4 triplets, $P = 0.005$, unpaired t-test).

Oocytes expressing Cx36 had a more depolarized resting membrane potential (-67 ± 0.8 mV) compared to control oocytes (-74 ± 9 mV, $n = 24$ comparisons from 12 triplets, $P < 0.0001$, paired t-test). Moreover, the resting membrane potentials of the Cx36-expressing oocytes in each triplet were very similar and significantly correlated using linear fit ($R = 0.82$; $P = 0.001$). This result suggests that oocytes coupled by gap junctions tend to adjust their resting membrane potential to the same level.

We measured the input resistance of oocytes in 12 triplets; in each triplet, 2 of the 3 oocytes expressed Cx36. The input resistance represents the sum of the non-junctional membrane resistance and the junctional resistance and it was calculated in current clamp mode after performing bridge balance to cancel series resistance, which includes pipette resistance and cytoplasmic access resistance. The input resistance of oocytes expressing Cx36 was significantly lower (0.75 ± 0.04 M Ω) than that of control oocytes (1.05 ± 0.05 M Ω , $n = 24$ pairs, $P < 0.00001$, paired t-test). This result suggests that gap junctions significantly lower the input resistance of oocytes. Although one study has reported that Cx36 failed to form hemichannels (Al-Ubaidi et al., 2000), recent findings indicate that neurons may release ATP by way of Cx36 hemichannels (Schock et al., 2008). Therefore, we cannot exclude the possibility of formation of hemichannels by Cx36, which may contribute to the decrease in input resistance.

In 3 of the 23 oocyte triplets, one of the oocytes expressing Cx36 exhibited spontaneous membrane potential or current fluctuations (duration 2–5 sec) that were transferred with attenuation to the other Cx36-expressing oocyte but not to the control oocyte (Fig. 3). The coefficient of cross-correlation of the spontaneous membrane potentials in these 3 pairs of connected oocytes was $C = 0.96 \pm 0.1$, which indicates a high degree of synchronous activity ($C = 1$ being the theoretical maximum). In the remaining connected oocytes, there

were infrequent similar slow changes in membrane potentials with no detectable fluctuations. Although the source of the fluctuations could not be determined, this result shows that membrane fluctuations generated in one oocyte are transferred reliably to the other electrically coupled oocyte.

The gap junction blocker carbenoxolone (500 μ M) decreased the junction conductance ($n = 3$) by about 60%. The effect of carbenoxolone was slow, reaching its maximum inhibition within 25 to 35 min and it was slowly reversible. This inhibitory effect was associated in some cases with Cx36-independent effects such as an increase in membrane conductance and a slowly developing inward current (Fig. 4, control oocyte).

4. Discussion

The oocyte pairing system has the advantage of testing drug on isolated expressed channels without the concomitant unspecific effects on neuronal network. We can study simultaneously the gap junctional coupling of 3 different pairs of oocytes. If the experiment consists of testing the effect of a single drug, this approach will reduce the time required, as background coupling in control pairs of oocytes does not need to be measured separately as with the conventional 2 oocyte pairing.

The most prominent features of the mouse Cx36 channel, functionally expressed in *Xenopus* oocytes, are its low voltage sensitivity, small single channel conductance and inability to form hemichannels and heterotypic gap junction channels with many other connexins (Srinivas et al., 1999; Al-Ubaidi et al., 2000; Teubner et al., 2000). The electrical coupling properties of Cx36 gap junctions can better be studied than the dye coupling properties because Cx36 channels seems to be only permeable to neurobiotin (MW 287) but not to fluorescent dyes such as Lucifer Yellow and DAPI (Teubner et al., 2000; Al-Ubaidi et al., 2000).

Similar to previous studies, our results indicate that the current-voltage relationship of the connexins 36 gap junction is linear suggesting no voltage-dependency. Our results demonstrate that the intrinsic fluctuations generated in some oocytes are reliably transferred to other coupled oocytes. The mechanism of generating oscillations in *Xenopus* oocytes is unclear but it involves at least 2 second messengers: IP3 and intracellular calcium. The spontaneous and evoked oscillations in oocytes were found to be mediated by chloride currents and may be enhanced by mechanical stimulation and activation of different types of membrane receptors (Oron et al., 1985; Parekh et al., 1993; Hülsmann et al., 1998).

The oocyte expression system provides an excellent preparation to test the direct blockade properties of gap junction blockers since there are few receptors expressed on the membrane. So far, carbenoxolone is still the most commonly used gap junction blocker, since it is believed to be devoid of major side effects, although the literature is controversial about the specificity of carbenoxolone. Carbenoxolone is a water-soluble hemisuccinate derivative of 18 β -glycyrrhetic acid which is known to suppress the gap-junctional channels of electrically coupled cells in a wide variety of tissues (Davidson et al., 1986; Davidson and Baumgarten, 1988). In agreement with previous studies, we found that carbenoxolone attenuate gap junctional conductance (Fig. 4), and the effects of carbenoxolone were slow and very weakly reversible (Rekling et al., 2000; Schoppa and Westbrook, 2002; Hayar et al., 2005; Placantonakis et al., 2006).

Although there are some gap junction blockers that are relatively more potent, such as mefloquine (Cruikshank et al., 2004), many recent studies have recognized that there is no specific pharmacological antagonist of gap junctions (Rozental et al., 2001) and that carbenoxolone is not an exception (Rekling et al., 2000; Rouach et al., 2003; Vessey et al.,

2004). In addition to its blockade properties of gap junctions, carbenoxolone was reported to inhibit synaptic transmission by blocking calcium channels (Vessey et al., 2004). Moreover, the input resistance and neuronal excitability of preBötC neurons and cultured hippocampal and cortical neurons were significantly reduced by carbenoxolone (100 μ M; Reklings et al., 2000; Rouach et al., 2003). Carbenoxolone, like other glycyrrhizin derivatives, has also been shown to inhibit the Na⁺-K⁺-ATPase (Zhou et al., 1996). This could explain why in addition to its blockade of the gap junction coupling between oocytes, we found that carbenoxolone induced an inward current and an increase in membrane input conductance even in control oocytes that did not express connexin 36. Unfortunately, other gap junction blockers are not any better than carbenoxolone in terms of specificity, and the direct pharmacological evidence of gap junction blockade will still have to await the development of more specific gap junction blockers that do not interfere with membrane channels, receptors and transporters. The triplet oocyte recording system described here should help these efforts as the third control oocyte is there specifically as a simultaneous internal control to assess Cx36-independent actions of pharmacological substances. In addition, it is possible using oocyte triplet pairing to compare the gating properties of 2 junctions to another control junction in the same preparation. If the experiment consists of testing the effect of a substance that modulates gap junctional conductance, our method will increase productivity 3 times compared to conventional 2 oocytes pairing, because the effect of this substance is tested simultaneously on 3 gap junctions. Moreover, in one single experiment, one pair of oocytes could serve as control while a third oocyte, which has been manipulated differently, will be tested for different gap junctional properties with the other 2 oocytes.

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Figure 1.

Simultaneous recordings from an oocyte triplet. (A) Left panel: Photo of the impalement of the 3 oocytes which were placed in an agar well in a recording chamber superfused with an MMR solution and viewed with a 2X objective. Right panel: Photo of the oocyte triplet taken via the ocular piece. Note that the control oocyte is labeled by a red dye in order to be able to distinguish it from the 2 other oocytes that express connexin 36. (B) Representative simultaneous intracellular triple recordings in current clamp mode showing the voltage responses to current pulses of 1 sec duration and incremental amplitude (from -40 nA to 40 nA, using 10 nA increment) injected at different time intervals in each oocyte. Note that injection of current in the transfected oocytes (red and green traces) produced voltage responses in the same cells as well as the other transfected oocytes. In contrast, injecting current pulses in the control oocyte (blue traces) produced no effect in any of the 2 oocytes.

Figure 2.

Current-voltage relationship in electrically coupled and uncoupled oocytes. (A) In voltage clamp mode, oocytes were held at a voltage identical to their resting membrane potential. Voltage steps of 1.5 sec duration of incremental amplitude (from -60 mV to 60 mV, increment of 10 mV) were applied at different intervals. Note that application of voltage steps in the Cx36-expressing oocytes (red and green traces) produced current responses in the same cells as well as the other Cx36-expressing oocyte. In contrast, application of voltage steps in the control oocyte (blue traces) produced no effect in any of the other 2 oocytes. (B) Current-voltage relationships show that the control cell had higher input resistance than the transfected cell. (C) The slopes of the junctional current-voltage traces of

the transfected cells were similar suggesting identical junctional conductance. The linearity of these traces indicates that junctional conductance was voltage-independent.

Figure 3.

Synchronous membrane potential fluctuations in oocytes expressing Cx36. (A) A typical example of current clamp recordings from an oocyte triplet in which one of the Cx36-expressing oocytes (red trace) exhibited membrane potential oscillations that were transferred with attenuation the other Cx36-expressing oocyte (green trace) but not to the control oocyte (blue trace). (B) Crosscorrelograms of the membrane potential waveforms indicate that the oscillations in the cells expressing Cx36 were perfectly synchronous (black traces) with a coefficient of correlation approaching 1 and a peak lag time of about 100 ms (see arrow in right panel with expanded X axis) indicating that the oscillations in cell#1 lead those in cell#3. In contrast, the crosscorrelograms of membrane potential traces between the Cx36-expressing cells and the control cell show no significant correlation (gray traces).

Figure 4.

Effects of carbenoxolone on membrane properties and junctional conductance of an oocyte triplet. Carbenoxolone induced an inward current (top panel) and an increase in membrane input conductance (middle panel) in all 3 pairs of oocytes which include 2 oocytes that expressed connexin 36 (red and blue traces) and one oocyte which served as a control (blue traces). Carbenoxolone reduced the junctional conductance between the 2 Cx36-expressing oocytes.