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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 are that in one single experiment, 1 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 3 oocytes, 2 of which are expressing rat 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 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.,

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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

Connexin36 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 $MgCl_2$, 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 $CaCl_2$. Oocytes were rinsed well with Ca^{2+} containing oocyte isolation followed by frog L-15 (described in Machaca 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 viteline 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 in. × 48 in., TMC 63-543, Peabody, MA). The microscope was equipped with a 2× 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 in. 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, $MgSO_4$ 1, $CaCl_2$ 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 s 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 s duration and we inject current pulses of 1 s duration at time 0.2, 5.2 and 10.2 s