Video Article

Isolation and Kv Channel Recordings in Murine Atrial and Ventricular Cardiomyocytes

Clemens Köhncke[1,2], Ulrike Lisewski[1], Leonhard Schleußner[1], Carolin Gaertner[1], Saskia Reichert[1], Torsten K. Roepke[1,3]

[1]Experimental and Clinical Research Center (ECRC), Charité Medical Faculty and Max-Delbrück Center for Molecular Medicine (MDC)

[2]Medical Department, Division of Cardiology, Campus Virchow-Klinikum, Charité - Universitätsmedizin Berlin

[3]Medical Department, Division of Cardiology and Angiology, Campus Mitte, Charité - Universitätsmedizin Berlin

Correspondence to: Torsten K. Roepke at Torsten-Kai.Roepke@charite.de

URL: http://www.jove.com/video/50145
DOI: doi:10.3791/50145

Keywords: Physiology, Issue 73, Medicine, Cellular Biology, Molecular Biology, Genetics, Biomedical Engineering, Anatomy, Cardiology, Cardiac Output, Low, Cardiomyopathies, Heart Failure, Arrhythmias, Cardiac, Ventricular Dysfunction, Cardiomyocytes, Kv channel, cardiac arrythmia, electrophysiology, patch clamp, mouse, animal model

Date Published: 3/12/2013

Citation: Köhncke, C., Lisewski, U., Schleußner, L., Gaertner, C., Reichert, S., Roepke, T.K. Isolation and Kv Channel Recordings in Murine Atrial and Ventricular Cardiomyocytes. *J. Vis. Exp.* (73), e50145, doi:10.3791/50145 (2013).

Abstract

KCNE genes encode for a small family of Kv channel ancillary subunits that form heteromeric complexes with Kv channel alpha subunits to modify their functional properties. Mutations in KCNE genes have been found in patients with cardiac arrhythmias such as the long QT syndrome and/or atrial fibrillation. However, the precise molecular pathophysiology that leads to these diseases remains elusive. In previous studies the electrophysiological properties of the disease causing mutations in these genes have mostly been studied in heterologous expression systems and we cannot be sure if the reported effects can directly be translated into native cardiomyocytes. In our laboratory we therefore use a different approach. We directly study the effects of KCNE gene deletion in isolated cardiomyocytes from knockout mice by cellular electrophysiology - a unique technique that we describe in this issue of the *Journal of Visualized Experiments*. The hearts from genetically engineered KCNE mice are rapidly excised and mounted onto a Langendorff apparatus by aortic cannulation. Free Ca^{2+} in the myocardium is bound by EGTA, and dissociation of cardiac myocytes is then achieved by retrograde perfusion of the coronary arteries with a specialized low Ca^{2+} buffer containing collagenase. Atria, free right ventricular wall and the left ventricle can then be separated by microsurgical techniques. Calcium is then slowly added back to isolated cardiomyocytes in a multiple step comprising washing procedure. Atrial and ventricular cardiomyocytes of healthy appearance with no spontaneous contractions are then immediately subjected to electrophysiological analyses by patch clamp technique or other biochemical analyses within the first 6 hours following isolation.

Video Link

The video component of this article can be found at http://www.jove.com/video/50145/

Protocol

1. Animal Anesthesia and Organ Harvesting

1. Anaesthetize the mouse by intraperitoneal (i.p.) injection of Ketamine (200 mg/kg BW) and Xylazine (20 mg/kg BW).
2. To anticoagulate inject 250 IU Heparin i.p. to avoid blood clotting and thrombus formation.
3. Wait until deep narcosis is reached, which is characterized by areflexia. To check for areflexia, test corneal reflex by gently touching the cornea or test flight reflex by tail pinching.
4. Transfer the mouse onto operating table and fix it in supine position.
5. Incise the skin and the abdominal wall below the xiphoid and perform clamshell thoracotomy: Extend the cut to both sides along the costal arch and subsequently cut ribs in the medial axillary line, deflect the rib cage upwards.
6. Open pericardium, locate great vessels. Gently press heart caudal to better display the aorta. Clamp the aorta using forceps.
7. Place the heart in concavity of a pair of scissors and dissect all connecting vessels with one single cut. Make sure to preserve a large enough part of the ascending aorta for Langendorff cannulation.
8. Transfer excised heart immediately to a Petri dish filled with ice cold and pre-oxygenized solution 1 (for solutions, see **Table 1**).

2. Preparation of Heart and Langendorff Perfusion

1. Cannulate the aorta with a 1.8F steel cannula. Make sure to avoid air embolism.
2. Fixate aorta on the cannula with a surgical suture and flush coronaries with 1 ml of solution 1.
3. Connect cannula with a Langendorff apparatus.

4. Make sure time from thoracotomy to Langendorff cannulation does not exceed 120 sec to avoid extended ischemia/reperfusion injury to the myocardium.
5. Perfuse heart with 10 ml of Ca^{2+} free solution 2 (4 ml/min).
6. Perfuse heart with collagenase solution 3 for 8 min (4 ml/min).

3. Microsurgical Dissociation of Cardiac Chambers

1. Transfer heart into a pre-warmed 100-mm Petri dish containing low Ca^{2+} solution 4.
2. Carefully remove aortic and other non-cardiac tissue with scissors and discard it.
3. Separate atria and ventricles and continue with each chamber separately. Keep cells immersed in solution 4. Use small volumes (less than 5 ml).

4. Further Dissociation of Cardiomyocytes

1. Atrial cardiomyocytes:
    1. To individualize atrial cardiomyocytes transfer the atria into a separate pre-warmed 100-mm culture dish and dissociate the tissue through gently pulling it apart with fine forceps. Ensure an almost complete dissociation of the tissue.
    2. Use a 1 ml pipette with an enlarged fire-polished plastic pipette tip to suspend the cells in 1 ml of solution 5 for 5 min.
    3. Separate the cells from debris by using a cell filter (200 µm mesh size).
    4. Add 5 ml solution 5 to the cell suspension and centrifuge for 2 min at 16 x g at room temperature.
    5. The following steps are operated under a cell culture hood. Discard the supernatant and re-suspended the pellet in 5 ml of solution 6.
    6. After sedimentation by gravity for 10 min in a 15 ml tube, centrifuge for 1 min at 16 x g at room temperature. Remove the supernatant. Re-suspend the cells depending on their quantity in 1-5 ml of solution 6.

2. Ventricular cardiomyocytes:
    1. Dissect the left ventricular region of interest with fine forceps in 5 ml of solution 4.
    2. Suspend cells by gently pipetting until most of the cells are separated. Transfer the cell solution after filtering (200 µm mesh size) into a 50 ml tube, add a volume of 25 ml.
    3. Centrifuge for 2 min at 16 x g at room temperature.
    4. The following steps are operated under a cell culture hood. Remove supernatant, re-suspend the pellet in 25 ml of solution 6 and allow sedimentation of the cells for 10 min.
    5. Count the cells and remove the supernatant, add 25 - 50 ml solution 6 on the cells.

5. Preparation of Cardiomyocytes for Cellular Electrophysiology, Biochemical or IF Studies

1. For electrophysiology studies, keep the myocytes in solution 6 in a 50 ml tube and inhibit sedimentation.
2. For biochemical studies, *e.g.* calcium imaging, plate myocytes on laminin coated cell culture dish (final concentration 20 µg/ml laminin in PBS).
3. For immunofluorescence staining prepare a cell culture dish plate with glass cover slips and coated with laminin solution (final concentration 50 µg/ml laminin in PBS).
    1. Remove the solution before plating myocytes. Plate the cells and control the cell density by using a microscope.
    2. Let the myocytes adhere to cover slip for 1 hr at 37 °C in 2 % CO_2, remove the solution and start immediately with a standard staining procedure protocol.
    3. Incubate with fixative, *e.g.* 4% PFA in PBS (pH 7.5) for 10 min at room temperature and follow with three PBS washing steps for 5 min each.
    4. To permeabilize the cells and to inhibit unspecific antibody binding incubate the myocytes with 10 % serum, 0.3% Triton, 0.2% BSA in PBS for 30 min at room temperature.
    5. Incubate with the primary antibody for 1 hr at 37 °C and wash as described before.
    6. Incubate with the secondary antibody for 1 hr at room temperature. To counterstain the nuclei and α-actinin use DAPI and fluorochrome conjugated phalloidin (Alexa Fluor 488, Invitrogen).
    7. After washing, transfer the glass cover slips carefully on silane treated microscope slides and embed cells in fluorescence mounting medium.

6. Cellular Electrophysiology

1. Perform Whole-cell patch-clamp recordings on freshly isolated atrial and ventricular cardiomyocytes at room temperature.
2. Transfer healthy appearing cardiomyocytes into perfusion chamber filled with a defined volume of extracellular bath solution. 117 mM NaCl, 4 mM KCl, 1 mM KH_2PO_4, 4 mM $NaHCO_3$, 1.7 mM $MgCl_2$, 3 mM $CoCl_2$, 10 mM HEPES, 10 mM glucose, and 0.02 mM Tetrodotoxin (TTX), (pH 7.4). Use NaOH for pH value adjustment.
3. Use proper patch clamp equipment (*i.e.* IX71 inverted microscope, a Multiclamp 700B Amplifier, a Digidata 1440A acquisition system and PC with pClamp10.3 software (Molecular Devices)).
4. Use patch pipettes with resistances of 3-5 MΩ when filled with intracellular solution containing 130 mM KCl, 2 mM $MgCl_2$, 11 mM HEPES, 11 mM EGTA, 5 mM Na_2ATP, 0.4 mM Na_2GTP, 5 mM Na_2CP and 4.9 mM $CaCl_2$ (pH 7.2). Use KOH for pH value adjustment.
5. Evoke outward K+ currents during 4.5-sec voltage steps to test potentials between -60 and +50 mV in 10-mV increments from a holding potential of -70 mV after a 20-msec prepulse to -40 mV.

6. Make sure leak currents are always <100 pA.
7. For dissection of different K+ currents use specific inhibitors such as 4-aminopyridine (4-AP; ICN Biomedicals, Irvine, CA, USA), Heteropodatoxin 2 (HpTx2; Alomone) or Tetraethylammonium (TEA; Sigma). Stock solutions should be prepared in extracellular bath solution, and applied directly to the closest possible vicinity of the cell via a microtip after "baseline" recordings. Equilibration should be allowed for 2-3 min before "drug" recordings.
8. For analysis normalize current amplitudes in individual cells to cell size (whole-cell membrane capacitance). Analyze data offline by using pClamp10.3 software (Molecular Devices) or comparable software.

Representative Results

Isolation of adult murine cardiomyocytes from genetically engineered mice to study the function of specific genes of interest *in vitro* has become a powerful tool to further understand cardiac pathophysiology. This method is currently used by only a small but increasing number of basic science laboratories worldwide. However, isolation of adult ventricular murine cardiomyocytes can be tricky and needs to be done thoroughly and repetitively by experienced hands. **Figure 1** shows freshly isolated exemplar atrial and ventricular cardiomyocytes. For ventricular cardiomyocytes we recommend to only use rod- shaped, striated ventricular myocytes of healthy appearance with no spontaneous contractions. Compared with ventricular myocytes, atrial myocytes are shorter and thinner. The characteristic striation and rod-shape of ventricular cardiomyocytes is missing in adult atrial murine cardiomyocytes. The yield for ventricular cardiomyocytes isolated from an intact mouse heart is $5x10^5$ - $1x10^6$. For atrial cardiomyocytes it is significantly less. We generally expect to isolate about 5 - 25,000 atrial cells from one mouse heart. Once isolated, cardiomyocytes can be subjected to different *in vitro* techniques including electrophysiological recordings (**Figure 1**). We recommend to use isolated cardiomyocytes within the first 6 hr after isolation. **Figure 1** shows exemplar Kv channel outward recordings (on the right) of atrial and ventricular cardiomyocytes evoked by different depolarization steps by whole-cell patch clamp technique. The characteristic shape of murine adult ventricular and atrial Kv channel repolarizing currents can be seen over a defined time course (here 4 sec, **Figure 1**). Please note that the current amplitude is significantly lower in murine adult atrial cardiomyocytes compared to ventricular cardiomycytes.

Solution	Contents (in mM, if not specified differently)
1	117 NaCl, 4 KCl, 10 HEPES, 1 KH_2PO_4, 4 $NaHCO_3$, 1.7 $MgCl_2$, 10 Glucose
2	117 NaCl, 4 KCl, 10 HEPES, 1 KH_2PO_4, 4 $NaHCO_3$, 1.7 $MgCl_2$, 10 Glucose + 229.5 µM EGTA
3	117 NaCl, 4 KCl, 10 HEPES, 1 KH_2PO_4, 4 $NaHCO_3$, 1.7 $MgCl_2$, 10 Glucose, $CaCl_2$ 0.1 + 0.8 g/L Collagenase Type 2 (300 U/L)*
4	117 NaCl, 4 KCl, 10 HEPES, 1 KH_2PO_4, 4 $NaHCO_3$, 1.7 $MgCl_2$, 10 Glucose, $CaCl_2$ 0.2 + 0.8 g/L Collagenase Type 2 (300 U/l)* + BSA (1 g/L)
5	117 NaCl, 4 KCl, 10 HEPES, 1 KH_2PO_4, 4 $NaHCO_3$, 1.7 $MgCl_2$, 10 Glucose, $CaCl_2$ 0.5 + BSA (1 g/L)
6	117 NaCl, 4 KCl, 10 HEPES, 1 KH_2PO_4, 4 $NaHCO_3$, 1.7 $MgCl_2$, 10 Glucose, $CaCl_2$ 1.0 + BSA (1 g/L)

Table 1. Isolation solutions. Equilibrated for 10 min with Carbogen (95% O_2, 5%CO_2), at 37 °C pH=7.4 (NaOH). * Activity may depend on batch number, so previous testing of collagenase activity is recommended.

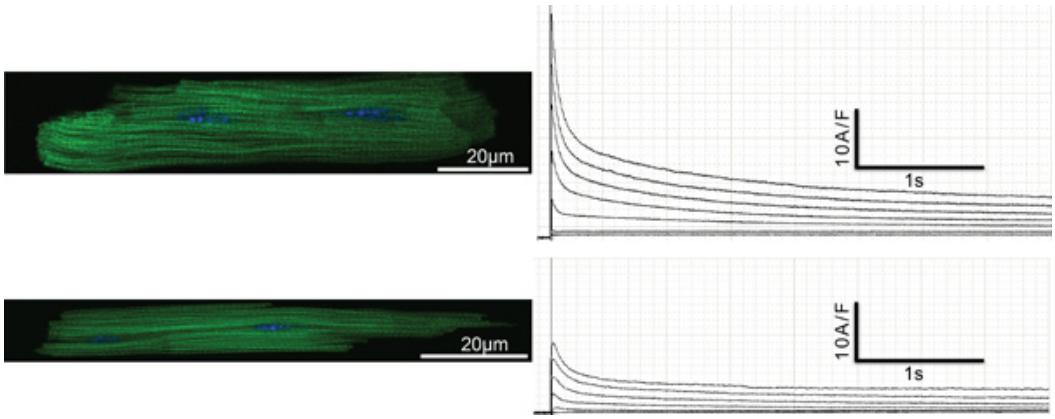

Figure 1. Upper Left: Exemplary ventricular cardiomyocyte stained with DAPI (nuclei, blue) and Alexa Flour 488 Phalloidin (α-actinin, green). **Upper Right:** Exemplary traces of whole cell patch clamp recording. **Lower Left:** Exemplary atrial cardiomyocyte stained with DAPI (nuclei, blue) and fluorochrome conjugated Phalloidin (α-actinin, green). **Lower right:** Exemplary traces of whole cell patch clamp recording.

Discussion

With the growing development of genetically engineered mouse strains to study cardiac function and cardiac pathology related to gene deletion there is also an increasing interest in specialized methods to study effects of the specific gene deletion *in vitro*. In our laboratory we study the roles of a family of Kv channel ancillary subunits on cardiac repolarization. The *KCNE* genes comprise a family of 5 genes (*KCNE1-5*) that play

important roles in human ventricular and atrial repolarization [11, 15]. *KCNEs* are single transmembrane domain Kv channel ancillary subunits that cannot pass any potassium currents on their own, but they can form complexes with Kv channel alpha subunits and modulate their functional properties such as gating, conductance, pharmacology and trafficking within the cell significantly. Mutations and common polymorphisms in *KCNE2* for example are associated with inherited and acquired forms of the Long QT Syndrome (LQTS) [1, 16].

Pioneer work in isolation and electrophysiological characterization of different Kv channel alpha subunits and their contribution to rat but also murine ventricular and atrial repolarization has been done by the group of Dr. Nerbonne at the Washington University of St. Louis in the 1990s [3, 5, 7]. However, significantly less is known about the contribution of Kv channel ancillary subunits to atrial and ventricular repolarization within the rodent myocardium. *KCNEs* have mainly been studied in heterologous expression systems such as CHO cells and *Xenopus laevis* oocytes. Using these methods *KCNE* subunits have been shown to be highly promiscuous in forming heteromeric Kv channel complexes identifying a range of different Kv channel alpha subunits as potential partners for *KCNEs*[10]. However, we cannot be sure whether these specific heteromeric Kv channel complexes also occur in native cardiomyocytes or if the observed heteromeric *KCNE* Kv channel alpha subunit partnerships are heterologous expression artifacts [1]. We therefore adopted the isolation technique and Kv channel recording protocols from the Nerbonne laboratory and modified them as indicated in the protocol section of this article to dissect the different Kv channels in murine repolarization, which are controlled by *KCNE* genes. Using this approach we recently found that the Kv channel ancillary subunit *KCNE2* controls two distinct Kv currents in the murine ventricular myocardium ($I_{Kslow,1}$ and $I_{to,f}$). By application of specific inhibitors for different potassium channels we were able to show that deletion of the murine *KCNE2* gene causes a significant reduction in both *Kv1.5* and *Kv4.2* currents [14]. Regulation of *Kv1.5* by *KCNE2* was not previously known, whereas regulation of *Kv4.2* by *KCNE2* has already been demonstrated *in vitro* by heterologous expression studies [20].

Atrial fibrillation (AF) is the most frequent sustained arrhythmia in clinical practice and is associated with significant morbidity and mortality [4]. Mutations in all different KCNEs have recently been associated with AF in humans. Recently, two non-synonymous mutations were found in *KCNE1* in patients with AF that were not present in the control group (Olesen *et al.*, 2012). Mechanistically, heterologous expression studies identified a gain-of-function effect in *KCNQ1* currents as the likely underlying mechanism. Furthermore, recently it was shown that *KCNE1*$^{-/-}$ mice suffer from spontaneous episodes of paroxysmal AF [17]. In a study evaluating 28 unrelated Chinese families with lone AF, Yang *et al.* identified a mutation in *KCNE2*, which resulted in an arginine-to-cysteine substitution (R27C). Functional analysis of the mutant channel in heterologous expression studies revealed a gain-of-function effect on *KCNQ1* channels (I_{Ks}) [18]. However, *KCNE2* can also form complexes with a range of other Kv channel α-subunits including *Kv1.5*, which has also been implicated in AF. We have recently shown that *KCNE2* can modulate *Kv1.5* currents in the murine ventricle leading to a prolongation of the ventricular APD in mice [15]. Therefore, disruption of atrial *Kv1.5* currents by *KCNE2* dysfunction could also represent the underlying arrhythmogenic mechanism for AF in our *KCNE2*$^{-/-}$ mouse model and/or in patients harboring mutation in *KCNE2* suffering from AF. Mutations in *KCNE3* were also recently identified in a patient with familial AF [8]. Electrophysiological recordings revealed an increased activity of *Kv4.3/KCNE3* and *Kv11.1/KCNE3* generated currents by the mutation, thereby conferring susceptibility of mutation carriers to faster cardiac action potential repolarization and thus vulnerability to re-entrant wavelets in the atria. The *KCNE3* V17M missense mutation however had no effect *KCNE3/KCNQ1* currents despite the fact that *KCNE3* forms functional complexes with *KCNQ1* in the myocardium. Furthermore, *KCNE3* was recently shown to be up regulated in a human population with valvular AF supporting the hypothesis that *KCNE3* plays a role not only in familial AF but also valvular AF [6]. Recently, a single nucleotide polymorphism (G/T) was identified in *KCNE4* resulting in a glutamic acid (Glu, E)/aspartic acid (Asp, D) substitution at position 145 of the *KCNE4* peptide [19]. Subsequent functional analysis of this polymorphism revealed that the *KCNE4* polymorphism exerts the effect of "gain of function" on the *KCNQ1* channels [9]. Again, these studies were undertaken in heterologous expression systems and experimental evidence from native cardiomyocytes is still lacking. More recently, an isolated non-familial case of AF with a missense (L65F) mutation has been identified in a Danish cohort of 158 patients with AF [12]. Furthermore, a polymorphism in *KCNE5* (C97T) was identified within the same study population, which was associated with a higher risk for developing AF [13]. Interaction of both mutant β-subunits (*KCNE2* and *KCNE5*) with the *KCNQ1* channel produced a gain-of-function effect with increased IKs currents. The *KCNQ1* α-subunit of the IKs channel can associate with any one of the five accessory β-subunits (*KCNE1-5*). Therefore *KCNQ1* seems to be a likely candidate α-subunit partner when searching for a molecular substrate in the pathogenesis of *KCNE*-associated AF. However, given the pronounced promiscuity of *KCNE* subunits other molecular targets are possible and we therefore intend to specifically investigate these possible KCNE/Kv alpha-subunit interactions. Studies in our laboratory are therefore also currently aimed to elucidate the mechanisms behind KCNE-associated AF utilizing the technique we describe in this article - isolation of murine atrial cardiomyocytes and subsequent *in vitro* electrophysiological recordings with application of specific inhibitors to different Kv channel alpha subunits and biochemical analyses of native cardiomyocytes from *KCNEx* knockout mice.

Disclosures

We have nothing to disclose.

Acknowledgements

This work was funded by grants from Deutsche Forschungsgemeinschaft (DFG), Fritz-Thyssen-Stiftung and Charité/MDC.

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