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Whole-cell and Perforated Patch-clamp Recordings from Acutely-isolated Murine Sino-atrial Node Cells

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[Abstract] Cardiac pacemaker cells of the sino-atrial node are responsible for the initiation of the heart beat and express an array of ion channels. The patch-clamp technique is the gold standard method for investigating the function of ion channels expressed in electrically active cells. Conventional whole-cell and perforated patch-clamp techniques can be used to investigate ionic currents in the voltage-clamp mode and changes in membrane potential (*e.g.*, action potential) in the current-clamp mode. Here, we provide details of protocols used to measure spontaneous and triggered action potentials and whole-cell funny current If (HCN4) in single cardiomyocytes isolated from the mouse sino-atrial node (SAN).

Keywords: Cardiomyocytes, Sinoatrial node, Cardiac pacemaker, Funny current, Action potential, K_{ATP} current

[Background] The patch-clamp technique allows for the real-time measurement of ionic currents and membrane potential from electrically active cells (Hamill *et al.*, 1981). Electrically active SAN cells are also known as pacemaker cells as they initiate the beating of the heart. The automaticity of the SAN is in part due to the slow diastolic depolarization of the membrane initiated by the opening of If (HCN4) allowing the membrane potential to reach threshold and leading to firing of the SAN action potential. The If current (also known as the pacemaker current) is a non-selective sodium/potassium current activated at hyperpolarized membrane potentials and by intracellular cyclic adenosine monophosphate. Whole-cell voltage-clamp and current-clamp recordings allow for understanding of the role of various ionic currents underlying the SAN action potential in more details (Aziz *et al.*, 2018; Nobles *et al.*, 2018). Patch-clamp of isolated SAN cells can be challenging, here we provide detailed protocols for the measurement of ionic currents and action potentials using conventional and perforated whole-cell patch-clamp techniques.

Materials and Reagents

1. 13 mm glass coverslips (VWR, catalog number: 631-0148)
2. Thin filamented borosilicate glass capillaries (Harvard Apparatus, catalog number: 30-0066)
3. Thin borosilicate glass capillaries (Harvard Apparatus, catalog number: 30-0094)

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4. 12-well cell culture plates, CellStar (Greiner Bio-One, catalog number: 665 180)
5. Microloader pipette tips (Eppendorf, Thermo Fisher Scientific, catalog number: 10289651)
6. Adult mice, 6-12 weeks, *e.g.*, C57/Bl6 (The Jackson Laboratory, catalog number: 000664)
7. Laminin (Sigma-Aldrich, catalog number: L2020)
8. Deionized, filtered water (dH_2O) (Milli-Q, Merck Millipore, catalog number: ZRXQ003WW)
9. Sodium Chloride (NaCl) (Sigma-Aldrich, catalog number: S7653)
10. Potassium Chloride (KCl) (Sigma-Aldrich, catalog number: P9333)
11. Potassium Hydroxide pellets (KOH) (Sigma-Aldrich, catalog number: P6310)
12. Sodium Hydroxide pellets (NaOH) (Thermo Fisher Scientific, catalog number: S/4920/53)
13. HEPES (Sigma-Aldrich, catalog number: H3375)
14. D-Glucose (Sigma-Aldrich, catalog number: G5767)
15. L-aspartic acid ($C_4H_6KNO_4$) (Sigma-Aldrich, catalog number: A6558)
16. Potassium D-Gluconate ($C_6H_{11}KO_7$) (Sigma-Aldrich, catalog number: G4500)
17. Adenosine 5′-triphosphate (ATP) disodium salt (Sigma-Aldrich, catalog number: A7669)
18. Adenosine 5′-triphosphate (ATP) magnesium salt (Sigma-Aldrich, catalog number: A9187)
19. Ethylene glycol-bis (2-aminoethylether)-N,N,N’,N’-tetraacetic acid (EGTA) (Sigma-Aldrich, catalog number: E3889)
20. Phosphocreatine di(tris) salt (Sigma-Aldrich, catalog number: P1937)
21. Guanosine 5′-triphosphate (GTP) Sodium salt hydrate (Sigma-Aldrich, catalog number: G8877)
22. Amphotericin B (Sigma-Aldrich, catalog number: A4888)
23. DMSO (Sigma-Aldrich, catalog number: D4540)
24. Potassium D-Gluconate ($C_6H_{11}KO_7$) (Sigma-Aldrich, catalog number: G4500)
25. Calcium Chloride ($CaCl_2$) 1 M solution (Fluka, catalog number: 21114)
26. Magnesium Chloride ($MgCl_2$) 1 M solution (Fluka, catalog number: 63026)
27. Tyrode Extracellular solution (see Recipes)
28. Perforated Patch-Clamp intracellular solution (see Recipes)
29. Whole-Cell Current-Clamp solution (see Recipes)
30. Amphotericin stock solution (see Recipes)
31. Laminin-coated coverslips (see Recipes)

Equipment

1. Digidata 1440A data acquisition system (Molecular Devices)
2. Axopatch 200B patch-clamp amplifier (Molecular Devices)
3. Patch-clamp recording chamber (Warner Instruments, RC-26 Chamber)
4. Inverted Microscope Nikon TE200 Eclipse (Nikon)
5. Micromanipulator (Patch Star) (Scientifica)
6. Pipette Puller PP-830 (Narashige)
7. Microforge MF-830 (Narashige)

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Software

1. pCLAMP 10 data acquisition and analysis software (Molecular Devices)
2. Origin 6.0 analysis software (OriginLab Corporation)
3. GraphPad Prism v6

Procedure

A. Pacemaker cardiomyocytes isolated from sinoatrial node (as described in Aziz *et al.*, 2020) are plated on to laminin-coated coverslips (Recipe 5) at least 1 h prior to recording.
Note: In our experience freshly isolated SAN cells can be used for patch-clamp for up to 4-5 h after isolation before they become difficult to patch-clamp.

B. Whole-cell patch-clamp/Perforated whole-cell patch clamp
   1. Pull pipettes from appropriate glass capillaries (with filament for whole-cell and without filament for perforated patch-clamp) with a 2-step pipette puller and polish them with the microforge, their resistance should be around 4 to 5 MΩ when filled with pipette solution for the whole-cell configuration (pipette resistance is measured using the patch-clamp amplifier and the pCLAMP recording software when the pipette tip is placed in the bath solution). For perforated patch-clamp, pipette resistance is around 1.3-1.6 MΩ.
   Note: The heat settings for the pipette puller need to be optimized for the required resistance. This will be different for individual machines, however as an example, we set our pipette puller (PP-830, Narashige) to 62 °C (1st pull) and 48 °C (2nd pull) for whole-cell patch pipettes (with filament) and to 6 °C (1st pull) and 43 °C (2nd pull) for perforated-patch pipettes (without filament). The second pull is important for the shape and size of the pipette.
   2. Take a coverslip from the plate using curved forceps and place it, cell side up, into the patch-clamp chamber. Perfuse the cells continuously with extracellular solution (most patch-clamp rigs have gravity-driven perfusion systems on them to allow continuous perfusion of solutions during experiments).
   3. Fill the pipette with the relevant intracellular solution (~half-full using a 1 ml syringe attached to a microloader pipette tip) and remove any air bubbles by gently flicking the pipette before placing it on the pipette holder. For perforated patch-clamp, first fill the tip of the pipette with amphotericin-free intracellular solution by carefully dipping the tip in this solution for a few seconds, then back fill the pipette with stock solution containing amphotericin (Recipe 2).
   Notes:
      a. *It is very important to remove any air bubbles so as not to compromise whole-cell access resistance and current/membrane potential measurements.*
      b. *It is advantageous to use non-filamented glass capillaries for perforated patch because it allows one to fill the end of the tip of the pipette with amphotericin-free solution, this helps*

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with seal formation by delaying perforation. The time for 'dipping' the tip (to fill the tip with amphotericin-free solution) is important as it can influence the time taken to perforate the cell–this time must be optimized. In general, we 'dip' the tip for 2 s initially and see how that affects seal formation and perforation. If seal formation is difficult or the cell starts to perforate before seal formation then a longer 'dipping' time is required.

4. Lower the pipette into the bath solution using the micromanipulator. After compensating for pipette offset (using the patch-clamp amplifier), under the microscope, approach and gently touch the chosen cell with the pipette to form a high resistance Giga-Ohm (GΩ) seal using negative pressure (suction).
5. For perforated patch, wait until the access resistance (Ra) reaches at least 20 MΩ (this depends on the efficacy of the amphotericin). For the whole-cell configuration apply more negative pressure to achieve whole-cell access. Ra should be below 10 MΩ. In both configurations, compensate for whole-cell capacitance and series resistance (70-80%) using the patch-clamp amplifier (this has in-built circuitry to compensate for both parameters). Note the whole-cell capacitance (Cm) for each cell.
6. For acquisition, set the filter at 2 kHz and sampling rate at 10 kHz.

C. Whole-cell funny current (If) measurements using the perforated patch-clamp technique
1. To record whole-cell If currents from SAN cells, the amplifier should be set to voltage-clamp mode (this is done via a switch on the amplifier interface).
2. The following solutions allow for detection and measurement of the If current: Perforated-Patch intracellular solution and Tyrode extracellular solution (Recipe 1).
3. Acquire If current-voltage relationship using the following step protocol (see Figure 1): Hold the cell at a holding potential of -40 mV. From -40 mV step to -130 mV for 1,300 ms and then to +40 mV for 400 ms (this step is to investigate inactivation properties) and then back to -40 mV for 350 ms. This is repeated a further 9 times with an incremental change of 10 mV for each step *e.g.*, -130, -120, -110 *etc.*

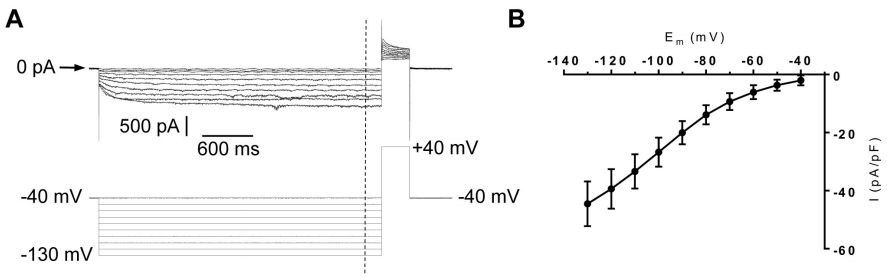

Figure 1. Whole-cell perforated-patch voltage-clamp recording of the If current from acutely isolated murine SAN cells. A. Representative whole-cell If current-voltage trace (top trace) and voltage-clamp protocol (bottom trace) used to acquire it. The dotted line denotes the point where the data was taken from to plot the steady-state current-voltage relationship in B. B. Steady-state current-voltage relationship for mouse If current in SAN cells. Data are shown as

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mean ± SEM, n=14 cells/6 mice.

D. Action Potential measurements using the perforated whole-cell patch-clamp (current-clamp mode, see Figure 2)

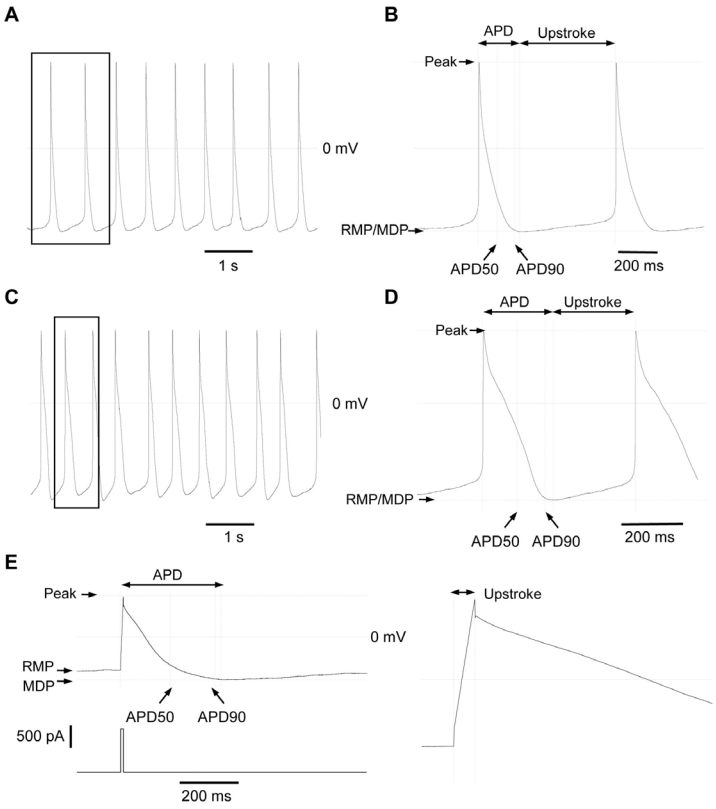

Figure 2. Current-clamp recordings measuring action potentials from acutely isolated murine SAN cells. A. Representative 6 s spontaneous action potential train from a single SAN cell using the perforated-patch configuration. B. Magnified from A, showing the various parameters that can be acquired from an action potential recording such as the resting membrane potential (RMP), maximum diastolic potential (MDP), peak potential, time to peak and action potential duration (APD), action potential duration at 50% and 90% amplitude (APD50 and APD90, respectively). C. Representative 6 s spontaneous action potential train from a single SAN cell using conventional whole-cell patch-clamp. D. Magnified from C, showing the various parameters as in B. E. Representative action potential trace using a 5 ms, 1,000 pA trigger (lower trace) showing the various parameters as in B and D. The left-hand trace shows a much quicker upstroke compared to spontaneous action potentials.

1. To measure spontaneous action potentials from SAN cells use the following solutions: Perforated-Patch intracellular solution (Recipe 3) and Tyrode extracellular solution (Recipe 1).
2. Once an adequate whole-cell access resistance is reached and whole-cell parameters compensated, switch to current-clamp mode. The y-axis label on pCLAMP should change to

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mV.

3. Acquire spontaneous action potentials using a gap-free recording protocol (no trigger is applied-the gap-free protocol is a continuous recording without any user-defined parameters other than the length of recording).
4. If spontaneous action potentials are not firing, it is possible to initiate action potential firing using a trigger–a short 5 ms current pulse (between 300 and 900 pA is usually optimal). The frequency of the trigger can be set as desired. For example, 1 Hz (1 trigger/second) or 3 Hz (3 triggers/second).

Note: Where a cell lacks spontaneous activity, action potentials can be triggered by injecting a current pulse. The size of the pulse can vary from cell to cell and must be optimized to trigger a full action potential.

E. Action Potential measurements using conventional whole-cell patch-clamp (current-clamp mode–set using the amplifier interface) (see Figure 2)
   1. Spontaneous and triggered action potentials can be measured in the conventional whole-cell patch-clamp configuration. To record action potentials or changes in membrane potential in this configuration use the following solutions: Whole-Cell Current-Clamp intracellular solution (Recipe 4) and Tyrode extracellular solution (Recipe 1).
   2. After 'going' whole-cell and compensating for whole-cell capacitance switch to current-clamp mode.
   3. Record spontaneous or triggered action potentials (see Procedure D).

   Note: The whole-cell current-clamp intracellular solution has a 'low' EGTA concentration (less buffering of Ca^{2+})–in our hands, this is conducive for better action potential recordings over a longer period of time compared to intracellular solutions with higher EGTA.

Data analysis

1. Data from voltage- and current-clamp recordings can be initially analyzed using Clampfit (part of the pCLAMP software package). Further analysis can be carried out using MS Excel, Microcal Origin and GraphPad Prism.
2. For voltage-clamp recordings (see Nobles *et al*., 2018), plotting the voltage at each step (x) vs current amplitude (y) gives a current (I)-voltage (V) relationship (see Figure 1). The current amplitude can be normalized to cell size by using the Cm. From the I-V relationship is it possible to assess changes in the activation profile and voltage-dependence when the cell is challenged pharmacologically for example. Ideally, cells isolated from at least 5 mice should be subjected to patch-clamp recordings. More information can be ascertained from the raw current traces. For example, time course and rate of activation and inactivation.
3. Action potential measurements from current-clamp recordings can initially be analyzed using the event detection function on Clampfit (analysis module from the pCLAMP software), this

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measures beating frequency, action potential amplitude, inter-event intervals and variability. This can also be done manually on Clampfit for quality control. In addition, parameters such as resting membrane potential (RMP), maximum diastolic potential (MDP), peak potential, time to peak and action potential duration (APD), action potential duration at 50% and 90% amplitude (APD50 and APD90, respectively). Additional AP durations, for example at 20%, 30% etc. amplitudes can also be calculated. Upstroke velocity can be calculated from action potential amplitude and time-to-peak (action potential amplitude (mV)/time-to-peak (ms)). See Aziz *et al.*, 2018 for details.

Recipes

Note: The solutions are filtered through a 0.22 micron filter (Millipore) and either used immediately on the day or frozen in aliquots.

1. Tyrode Extracellular solution
   140 mM NaCl
   5.4 mM KCl
   2.0 mM $CaCl_2$
   1.0 mM $MgCl_2$
   5 mM HEPES
   5.5 mM D-Glucose
   Adjust the pH to 7.4 with NaOH
2. Amphotericin stock solution (60 µg/µl)
   3 mg Amphotericin B
   50 µl DMSO
   Vortex thoroughly (may need sonication if dissolving problematic). This solution can be aliquoted and stored at -20 °C for up to 2 weeks
   Working concentration is 240 µg/ml (4 µl of stock in 1 ml of intracellular solution)
3. Perforated Patch-Clamp Intracellular solution
   130 mM L-Aspartic acid
   10 mM NaCl
   2.0 mM ATP-Na salt
   0.1 mM GTP-Mg salt
   6.6 mM Phosphocreatine
   0.04 mM $CaCl_2$
   10 mM HEPES
   Adjust the pH to 7.2 with KOH
4. Whole-Cell Current-Clamp Intracellular solution
   130 mM K-Gluconate
   20 mM KCl

0.5 mM $MgCl_2$
2.0 mM ATP-Mg salt
0.3 mM GTP-Na salt
5.0 mM Phosphocreatine
0.05 mM EGTA
10 mM HEPES
Adjust the pH to 7.4 with KOH
Note: KOH is used instead of NaOH in the intracellular solution so as not to significantly change the internal (in the cell) Na^+ concentration, which would then change action potential properties.

5. Laminin-coated coverslips
   a. Place coverslips in individual wells of a 12-well plate. On each coverslip place 1 µl of 1 mg/ml Laminin
   b. Spread the Laminin evenly around the coverslip using a P200 pipette tip
   c. Allow to dry for at least an hour before use

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Competing interests

The authors have no competing interests.

Ethics

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the British Home Office regulations (covered by project license PE9055EAD) and by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

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