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Simultaneous Real-Time Measurement of the β -Cell Membrane Potential and Ca^{2+} Influx to Assess the Role of Potassium Channels on β -Cell Function

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Abstract

Stimulus-secretion coupling in pancreatic β -cells requires Ca^{2+} influx through voltage-dependent Ca^{2+} channels, whose activity is controlled by the plasma membrane potential (V_m). Here, we present a method of measuring fluctuations in the β -cell V_m and Ca^{2+} influx simultaneously, which provides valuable information about the ionic signaling mechanisms that underlie insulin secretion. This chapter describes the use of perforated patch clamp electrophysiology on cells loaded with a fluorescent intracellular Ca^{2+} indicator, which permits the stable recording conditions needed to monitor the V_m and Ca^{2+} influx in β -cells. Moreover, this chapter describes the protocols necessary for the preparation of mouse and human islet cells for the simultaneous recording of V_m and Ca^{2+} as well as determining the specific islet cell type assessed in each experiment.

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¹Shaking can be performed by hand; however, using a wrist-action shaker provides more consistency in islet preparations. At the end of shaking, 2–5 vigorous shakes by hand are usually needed to dissociate any remaining pieces of pancreas.

²Connective tissue and fat are resistant to collagenase P digestion, and have a tendency to “catch” islets and acinar cells during the isolation process. To improve yield and purity of islet preparations, it is helpful to remove floating fat from the suspension with clean forceps.

³Histopaque-1077 and islet isolation buffer with 10% FBS should be at room temperature. Underlay Histopaque-1077 by placing a glass Pasteur pipette into the digested pancreas suspension and adding Histopaque-1077 through the Pasteur pipette with a 2 mL serological pipette. Sometimes, it is necessary to briefly lift the Pasteur pipette to liberate air bubbles that prevent the Histopaque from flowing. Ensure that the MIB/Histopaque interface is minimally disrupted when removing the Pasteur pipette and transferring to the centrifuge.

⁴“Picking” islets in cold culture media will minimize clustering of islets and acinar tissue, improving cleanliness of the preparation. Islets should not be stored under these conditions for more than a few hours as they will begin to aggregate. If the islets must be stored for a longer period of time supplement islet culture media with 0.5 mg/mL bovine serum albumin to minimize aggregation.

⁵Slowly drop islets into the dish with a P20 pipette to ensure that they form a tight cluster.

⁶Transfer 1 mL of islet culture media from dish to a 1.5 mL Eppendorf tube, add 5 μL 1 mM Fluo-4 AM stock to media, remove the remaining media from the dish, and replace with media containing Fluo-4AM.

⁷The glucose concentration present in the KRB wash will change depending on the type of experiment being performed. However, for β -cells, incubation and starting experiments in a stimulatory glucose concentration (e.g., 11 mM glucose) facilitates obtaining a $\text{G}\Omega$ seal.

⁸Be very gentle when exchanging liquids in the plate, as cells have a tendency to detach from or relocate on the coverslip if fluid exchange is too forceful. Another important point is to avoid exposing the cells to air during the staining process—a small amount of liquid should remain on the cells when exchanging solutions.

Keywords

Calcium imaging; Pancreatic β -cell; Membrane potential; Potassium channels; Fluo-4 AM

1 Introduction

In electrically excitable insulin-secreting β -cells, Ca^{2+} influx through voltage-dependent Ca^{2+} channels (VDCCs) initiates stimulus-secretion coupling. Elevations in blood glucose and other nutrients accelerate β -cell metabolism, increasing the ATP: ADP ratio. This results in K_{ATP} channel inhibition and V_{m} depolarization, which triggers VDCC opening and action potential firing [1]. Thus, β -cells transduce nutrient metabolism into an electrical signal that stimulates Ca^{2+} influx and insulin secretion. Electrical excitability of human β -cells is modulated by the orchestrated function of several ion channels, which include VDCCs, voltagegated Na^{+} channels, as well as a number of K^{+} channels (e.g., voltage-gated K^{+} (K_{v}) [2], Ca^{2+} -activated K^{+} ($\text{K}_{\text{ca}}^{2+}$) [3], and two-pore domain (K2P) [4, 5] channels). K^{+} channels regulate the frequency and amplitude of glucose-stimulated oscillations in β -cell electrical activity and cytosolic Ca^{2+} ($\text{Ca}^{2+}_{\text{c}}$), which are important determinants of insulin secretion. Because electrical excitability stimulates activation of VDCCs, simultaneous measurement of $\text{Ca}^{2+}_{\text{c}}$ and β -cell V_{m} allows us to correlate how electrical activity influences Ca^{2+} influx in single cells as well as across intact whole islets. Here, we describe the use of Ca^{2+} indicators to examine rapid changes in $\text{Ca}^{2+}_{\text{c}}$ concurrently with measurements of the V_{m} . As demonstrated in Fig. 1, this technique allows the investigator to assess how changes in factors which regulate action potential firing (e.g., in response to manipulation of K^{+} channel function) affect β -cell Ca^{2+} influx.

Live-cell imaging of intracellular Ca^{2+} has contributed enormously to understanding basic cellular processes in a broad range of tissues. By using the appropriate Ca^{2+} indicator and detection methods, it is possible to define how individual cells or groups of cells tune Ca^{2+} signals to modulate physiological outputs. Since their introduction more than 30 years ago [6], synthetic Ca^{2+} -sensitive fluorescent indicator dyes have been widely used to investigate the molecular mechanisms that control cellular Ca^{2+} signals. Based on the spectral and Ca^{2+} -buffering characteristics of these dyes, investigators can measure fast changes in Ca^{2+} (e.g., action potentials), or use time-lapse imaging to determine changes in absolute Ca^{2+} levels. More recently, genetically encoded Ca^{2+} indicators (GECIs) have enabled measurement of Ca^{2+} signals in specific organelles such as the endoplasmic reticulum (ER) and mitochondria [1,7], as well as $\text{Ca}^{2+}_{\text{c}}$ changes of specific cells within a heterogeneous population (e.g., α -cells of the pancreatic islet [8]). Although we focus here on the use of dyes for these types of experiments, the described approaches are amenable to use with GECIs.

Accurate measurement of the V_{m} using patch clamp electrophysiology in β -cells requires intact cytosolic components that are lost using traditional whole-cell ruptured patch techniques. Therefore, it is necessary to use the perforated patch configuration, which preserves cytosolic elements required for regenerative action potential firing in β -cells. For this purpose, we include the pore-forming antifungal agent, amphotericin B [9], in the patch

pipette solution, which causes perforations in the cellular membrane within the patch pipette tip. These perforations allow monovalent cations to equilibrate across the patch, permitting excellent recordings of β -cell electrical activity. In combination with Ca^{2+}_c measurements, it is possible to assess how changes in the β -cell V_m affect Ca^{2+} influx. For example, with pharmacological inhibitors of K^+ channels, it is possible to resolve Ca^{2+} influx due to single-action potentials [3]. These data allow the investigator to dissect the ion channels that coordinate β -cell action potential firing. Although VDCC activity is required for insulin secretion, β -cell Ca^{2+}_c levels are also influenced by Ca^{2+} release from intracellular stores, primarily the ER. Thus, simultaneous measurement of the V_m and Ca^{2+}_c has also revealed important information about the mechanisms of β -cell Ca^{2+} influx [10]. Therefore, this technique permits examination of various stimuli that impact β -cell Ca^{2+}_c with concomitant assessment of their effects on the V_m (see Figs. 1 and 2).

2 Materials

2.1 Islet Isolation

1. Islet isolation buffer (500 mL): $1\times$ HBSS, divalent-free, 10 mM HEPES, 1 mM MgCl_2 (pH 7.4 with NaOH), (sterilize by autoclave); prior to use, add 1.25 mM CaCl_2 , 5 mM glucose, and 0.2 mg/mL BSA (this forms modified islet buffer, MIB); MIB supplemented with 10% fetal bovine serum, 7.5 mL per pancreas.
2. Collagenase P solution: prepare 0.25–0.5 mg/mL collagenase P (collagenase strength varies by lot, thus optimal concentration must be determined experimentally) in cold MIB (without FBS), 10 mL per pancreas.
3. 5 mL syringe and needle (30 G $\times$ ½ in.).
4. Histopaque-1077, warmed to room temperature.
5. Serological pipettes (2 and 5 mL) and pipettor.
6. 15- and 50 mL conical centrifuge tubes.
7. Glass Pasteur pipettes, 230 mm (e.g., Wheaton #357335).
8. Water bath set to 37 °C.
9. Wrist-action shaker (e.g., Burrell Model 75).
10. Centrifuge capable of accommodating 15- and 50 mL Falcon-style centrifuge tubes (e.g., Eppendorf 5810R).
11. Falcon 60 mm petri dishes.
12. Inverted tissue culture microscope (e.g., Olympus CK2).
13. Adjustable volume micropipettes capable of pipetting 20,200, and 1000 μL (e.g., Gilson Pipetman P20, P200, P1000).

2.2 Islet Culture

1. Poly-D-lysine coated 35 mm glass-bottom dishes (CellVis #D35–14–1.5–N).

2. 0.05% Trypsin-EDTA.
3. Versene solution.
4. 1.5 mL Eppendorf tubes.
5. Islet culture medium: RPMI 1640 supplemented with 15% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 mg/mL streptomycin.
6. Cell culture incubator (37 °C, 5% CO₂).

2.3 Imaging and Electrophysiology: Solutions

1. Krebs-Binger-based buffer containing (in mM): 119 NaCl, 2.5 CaCl₂, 4.7 KCl, 25 HEPES, 1.2 MgSO₄, 1.2 KH₂PO₄, (pH 7.35 with NaOH); glucose added as needed.
2. Fluo-4AM (Molecular Probes), dissolved in DMSO (1 µg/µL).
3. Intracellular recording solution containing (in mM): 140 KCl, 5 HEPES, 1 EGTA, 0.5 CaCl₂, (pH 7.22 with KOH); supplemented with ~0.025 mg/mL amphotericin B.

2.4 Imaging and Electrophysiology: Equipment

1. An inverted microscope equipped with at least a 20 × objective (e.g., Nikon Eclipse TE2000-U).
2. An object marker (e.g., Nikon MBW10020).
3. Borosilicate glass capillaries (e.g., World Precision Instruments #1B150F-4).
4. Micropipette puller (e.g., Sutter P-97).
5. 1 mL syringe (e.g., BD #309659).
6. Needle for filling patch pipettes (e.g., WPI #MF28).
7. Micromanipulator (e.g., Burleigh PCS-5000).
8. Patch clamp amplifier (e.g., Axon 200B).
9. Analog signal digitizer (e.g., Axon Digidata 1440).
10. Waveform generator (e.g., BK Precision Model 4045).
11. Wide-field fluorescence light source (e.g., Excelitas X-Cite 120Q).
12. Appropriate excitation/emission bandpass filters (e.g., 485/530 nm for Fluo-4).
13. Photomultiplier tube (PMT) equipped with apertures (e.g., PTI D-104 microscope photometer).
14. Data acquisition and analysis software (e.g., pCLAMP suite of software, GraphPad Prism).

2.5 Post-staining of Islet Cells

1. 1× Phosphate-buffered saline (PBS).
2. Paraformaldehyde, 4% in PBS.
3. Bovine serum albumin (BSA).
4. Normal donkey serum (NDS).
5. Triton X-100.
6. Primary antibodies: anti-insulin (e.g., Dako #A0564), antiglucagon (e.g., Proteintech #15954), anti-somatostatin (e.g., Santa Cruz #D-20).
7. Secondary antibodies (e.g., Alexa Fluor 594-conjugated anti-guinea pig, Alexa Fluor 488-conjugated anti-rabbit, Alexa Fluor 647-conjugated anti-goat).

3 Methods

3.1 Islet Isolation

1. Distend pancreas from 6 to 10 week old mouse with 4 mL cold collagenase P solution [11].
2. Transfer pancreas into 6 mL of cold collagenase P solution in a 15 mL conical tube and shake at 37 °C for 10 min (*see Note 1*) followed by 2–5 vigorous shakes by hand.
3. Transfer digested pancreas suspension into a 50 mL conical tube filled with approximately 25 mL cold MIB.
4. Add cold MIB to bring the total volume to 50 mL and gently mix by inverting several times (*see Note 2*).
5. Centrifuge for 2 min at $150 \times g$, 25 °C.
6. Aspirate MIB, being careful not to disturb the pelleted tissue.
7. Repeat **steps 4–6**.
8. Suspend the digested pancreas pellet in 15 mL of cold MIB by triturating with a 5 mL serological pipette, and transfer to a 15 mL conical tube.
9. Centrifuge 2 min at $150 \times g$, 25 °C.
10. Aspirate MIB, being careful not to disturb the pelleted tissue.
11. Suspend the digested pancreas pellet in 7.5 mL of MIB supplemented with 10% FBS.
12. Carefully underlay the digested pancreas/MIB suspension with 5 mL of Histopaque-1077 (*see Note 3*) and centrifuge for 15 min at $450 \times g$, 25 °C.
13. Transfer Histopaque-1077 layer containing islets to a 50 mL conical tube.
14. Add 40 mL of cold MIB and gently mix by inverting several times.

15. Centrifuge for 1 min at $450 \times g$, 25 °C.
16. Aspirate MIB, being careful not to disturb the pelleted tissue.
17. Repeat **steps 14–16**.
18. Resuspend islets in 8 mL cold islet culture media and transfer to a 60 mm petri dish (*see Note 4*).
19. Transfer islets into a second 60 mm petri dish containing 8 mL islet culture media on ice using a P200 pipette and an inverted tissue culture microscope.
20. Disperse islets by gently rocking the petri dish then incubate at 37 °C, 5% CO₂ until ready to plate (*see Note 4*).

3.2 Islet Plating and Culture (Mouse or Human Islets)

3.2.1 Whole Islets

1. Under aseptic conditions, dilute 2 mg/mL poly-D-lysine stock to 80 µg/mL with sterile deionized H₂O (diH₂O) and add 100 µL to each 35 mm glass-bottom dish and incubate at room temperature for at least 2 h.
2. Wash glass-bottom dishes twice with diH₂O and keep dishes open in hood until dry.
3. Add 3 mL of pre-warmed (37 °C) islet culture media to each glass-bottom dish and transfer the desired number of islets into each dish using a P20 pipette (*see Note 5*).
4. Incubate islets at 37 °C, 5% CO₂ for 24–48 h prior to experimentation.

3.2.2 Islet Clusters and Single Cells

1. Prepare poly-D-lysine-coated 35 mm glass-bottom dishes as detailed in Subheading 3.2.1.
2. Transfer islets from the petri dish to a sterile 1.5 mL Eppendorf tube on ice using a P20 pipette.
3. Allow islets to settle to the bottom of the Eppendorf tube and carefully remove islet culture media.
4. Add 850 µL of Versene to the Eppendorf tube followed by 150 µL of 0.05% trypsin-EDTA.
5. Triturate islet suspension by pipetting up and down with a P1000 pipette approximately 20 × to prepare islet clusters, or 30 × to prepare single cells.
6. Centrifuge islet cell suspension 1.5 min at $250 \times g$, 25 °C.
7. Remove Versene/trypsin-EDTA solution and resuspend islet clusters/single cells in pre-warmed islet culture media.
8. Pipette 100–125 µL cell suspension into the middle of the glass-bottom dishes forming a liquid “bubble.”

9. Incubate islet clusters/single cells at 37 °C, 5% CO₂ for 6–18 h to allow them to adhere to poly-D-lysine-coated surfaces.
10. Supplement with 3 mL of pre-warmed islet culture media and incubate at 37 °C, 5% CO₂.

3.3 Measurement of V_m and Intracellular Ca^{2+} : Loading with Ca^{2+} Dye

1. Load cells with 5 μ M Fluo-4AM in 1 mL of islet culture media and incubate for 25 min at 37 °C, 5% CO₂ (see Note 6).
2. Wash twice with KRB supplemented with the desired glucose concentration (see Note 7).
3. After washing the cells, add approximately 2 mL of KRB to the plate.
4. Load the plate onto the microscope, and identify the desired cell(s) to record. Position the bath electrode, any perfusion lines, vacuum line, and PMT apertures (if equipped) to capture fluorescence signal from cell(s) to be recorded.
5. Proceed to patch clamp setup.

3.4 Patch Clamp Setup

1. Use a 1 mL syringe and MicroFil filament (WPI) to fill patch pipette with intracellular recording solution. Ensure there are no bubbles present in the pipette by grasping it between the thumb and forefinger and gently “flicking” the patch pipette.
2. Place the filled patch pipette in the electrode holder.
3. Apply positive pressure using an air-filled syringe attached to the pipette holder to minimize debris entering the patch pipette. Lower the patch pipette into the KRB bath and position over the cell to be recorded using the micromanipulator.
4. Adjust offset potential to zero.
5. Apply a test potential (e.g., a 5 mV voltage step at 10 Hz) to monitor the resistance of the patch pipette.
6. Using the micromanipulator, slowly guide the patch pipette toward the cell until contact is made with the plasma membrane, which is indicated by a sudden reduction in the amplitude of the square wave produced by the voltage step.
7. Rapidly release positive pressure on patch pipette and apply slight negative pressure to facilitate seal formation. As the seal between the patch pipette and the plasma membrane improves, the amplitude of the square wave will decrease.
8. Activate the external command function of the patch clamp amplifier to hold the command voltage at –80 mV and turn off the waveform generator used to apply the test potential.
9. Release negative pressure on patch electrode and monitor leak current until less than –15 pA.

10. Allow perforations to form for ~10 min.
11. Turn off the external command function and switch to current clamp mode to monitor cell V_m .
12. To assess the role of potassium channels on V_m and intracellular Ca^{2+} (see below), apply pharmacological agents targeting specific potassium channels in the bath solution by perfusion during the recording. Alternatively, the role of a specific potassium channel can be assessed by comparing islets from wild-type mice and islets from mice lacking the channel, overexpressing the channel, or expressing a mutant form of the channel.

3.5 Simultaneous Measurement of V_m and Intracellular Ca^{2+}

1. After successfully patching an electrically excitable cell, activate the fluorescence light source. If necessary, change the optical path on the microscope to direct the emitted fluorescence to the PMT.
2. Optimize the fluorescence light source and microscope apertures as well as PMT sensitivity to produce detectable signals with the minimal amount of fluorescence excitation light intensity required.
3. Begin recording protocol. Exemplary data that were obtained using this approach are provided in Fig. 2.

3.6 Post-staining of Islet Cells

1. After the completion of the experiment mark the region of the dish containing recorded cell(s) using a microscope object marker.
2. Add approximately 1 mL of ice-cold 4% PFA. Incubate for 30 min on ice.
3. Remove PFA, then wash cells twice with PBS (*see Note 8*).
4. Prepare protein blocking solution in PBS: 0.2% w/v BSA, 2% v/v NDS, 0.05% v/v Triton X-100.
5. Remove any remaining PBS and then apply the blocking solution to the cells and incubate for at least 1 h at room temperature. No wash step is necessary after blocking.
6. Prepare primary antibody solution in PBS: 0.2% w/v BSA, 1% v/v NDS, 0.1% Triton X-100 with primary antibodies diluted as follows (insulin: 1:400, glucagon: 1:400, somatostatin: 1:250).
7. Remove the blocking solution and then apply the primary antibody solution to the cells. Incubate in a humidity chamber overnight at 4 °C.
8. Remove the primary antibody solution and then wash with PBS. Leave PBS on cells for 10 min. Aspirate PBS and repeat this wash step.
9. Remove PBS and add the secondary antibody solution to the cells: 1% v/v NDS, with 1:500 dilutions of secondary antibodies (e.g., Alexa Fluor 594-conjugated

anti-guinea pig, Alexa Fluor 488-conjugated anti-rabbit, Alexa Fluor 647-conjugated anti-goat); incubate between one and two hours at room temperature.

10. Remove the secondary antibody solution and wash with PBS. Leave PBS on cells for 10 min. Aspirate PBS and repeat this wash step. While not required, further incubation of the cells overnight in PBS at 4 °C helps to reduce nonspecific staining.
11. Proceed to imaging/identification of stained cells. Representative staining is shown in Fig. 3.

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Fig. 1.

Simultaneous monitoring of β -cell electrical activity and islet Ca^{2+} influx. (a) Representation of a Fluo-4-loaded islet attached to a glass coverslip, with an electrode recording the V_m of an islet cell in perforated-patch whole cell current clamp mode. The islet is exposed to constant 488 nm light through a 20 $\times$ objective and the emission signal at 520 nm is monitored with a PMT and sampled at 10 kHz. The electrode is used to monitor electrical activity in whole cell perforated-patch current clamp mode at 20 kHz. (b) Representative recording of Ca^{2+} influx measured from the bottom of an intact C57BI6 mouse islet simultaneously with a V_m recording of a β -cell on the top of the islet in response to the indicated glucose stimulation, (c) Representative recording of Ca^{2+} and V_m from a Kv2.1 knockout C57BI6 mouse islet in response to the indicated glucose stimulation

Fig. 2.

Simultaneous monitoring of islet Ca^{2+} and V_m in response to GLP-1 receptor activation. Simultaneous monitoring of β -cell electrical activity (*top portion* of each panel) recorded from an intact whole islet in response to 200 μM tolbutamide and 2 mM glucose with (*black line*) and without 100 nM liraglutide (GLP-1 analogue), with fast-acquisition Ca^{2+} traces recorded from the entire islet (*lower portion* of each panel). The islets were loaded with Fluo-4AM and imaged at 10 kHz. *Inset lines* represent the section of the top tracings expanded below

Fig. 3. Identification of the human islet cell type recorded or imaged. The data show a typical experiment where the plate is marked and stained for glucagon to identify imaged alpha-cells, (a) The MatTek plate is marked with a Nikon Object Marker leaving a *purple ink circle* on the dish representing the field of view (*arrow*), marks are also placed on one side of the *X* and *Y* axes for orientation, (b) The cells (Fura-2 fluorescence) are fixed in **4%** paraformaldehyde and stained for islet hormones, panel (c) shows glucagon staining of the cells in panel (b)