Chapter 6

Characterization of TRPC Channels in a Heterologous System Using Calcium Imaging and the Patch-Clamp Technique

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Abstract

The family of transient receptor potential (TRPs) channels contains 28 mammalian members, each a unique cellular sensor that responds to a wide variety of external and internal signals. TRP channels are expressed by most mammalian cells, where they are involved in many different physiological functions. Canonical TRP channels (TRPCs) form a family of nonselective cationic channels, although with greater selectivity for Ca^{2+}. This family is made up of seven members (TRPC1-7), all of which contain a TRP box in the carboxyl terminal and 3-4 ankyrin repeats in the amino terminal. While these channels share some similar properties, they display diverse gating mechanisms and are involved in different signaling pathways (Gees M et al., Compr Physiol, 2012). The activation or inhibition of these channels has been studied using different approaches and techniques. Here, we characterize the activation of the TRPC5 channel expressed in a heterologous system, using calcium imaging and the patch-clamp technique in whole-cell configuration.

Key words Intracellular calcium recordings, Patch clamp, Osmomechanical stimulation, TRP channels

1 Introduction

Transient receptor potential (TRP) channels have been shown to have important properties and have been implicated in different diseases, although we still do not fully understand their behavior and gating mechanisms. Most TRP channels are plasma membrane channels that depolarize cells, increasing the intracellular concentrations of Na^{+} and Ca^{2+}. Moreover, intracellular Ca^{2+} itself can activate some mammalian TRP channels and it is known to modulate all TRP channels.

Canonical TRP channels (TRPCs) are cationic channels that participate in receptor-operated calcium influx and they include stretch-activated channels [2–8]. Ca^{2+} is an extremely important intracellular messenger that drives many cell functions; thus, quantitative measurements of cytosolic free Ca^{2+} (achieved by

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monitoring the fluorescence of an indicator such as Fura-2 AM) are an often-used method to characterize the activity of TRP channels in mammalian cells, including TRPC channels. Similarly, the patch-clamp technique in whole-cell configuration is also extremely useful to study the activity and physiological roles of ion channels. In this chapter, the protocols, and the calcium imaging and patch-clamp techniques used to characterize the activation of TRPC channels are described, in particular those used to study the TRPC5 channel. These measurements can be performed in cells expressing these channels natively or in human embryonic kidney (HEK) cells that transiently express heterologous constructs that encode the TRPC5 channel.

The human embryonic kidney cell line (HEK293) is used extensively as a vehicle for heterologous expression to carry out functional studies of TRP channels, or of other ion channels and proteins [9]. As well as a transient expression system, this cell line also represents a useful system for stable transfection. The endogenous expression of different ligand-gated and G-protein coupled receptors (GPCRs), as well as voltage-gated receptors, has been described in HEK293 cells, although using the HEK293 cell line as a host cell has the advantage of allowing the properties of ion channels to be studied in isolation from other proteins [9]. More recently, it was shown that members of the TRPC family of ion channels (TRPC1, 3, 4, 6, and 7) are endogenously expressed by HEK cells [2, 10–12]. However, this expression appears to depend on the source of the parental cell line, and the expression of these and other proteins is not always detected. As the expression of these channels cannot always be assumed in HEK293 cells, it is important to identify whether a protein of interest that is to be expressed in these cells is expressed endogenously to any extent.

We have used the techniques indicated above to investigate the role of a specific TRPC channel, the osmomechanical TRPC5 transducer channel. These same techniques and protocols can be used to study the modulation of other members of the TRPC family by physical or chemical stimuli, as well as for the studies of other TRP channels.

2 Materials

2.1 Cell Line

The human embryonic kidney (HEK) 293 cell line (Fig. 1) can be purchased from several suppliers, including the ATCC, European Collection of Authenticated Cell Cultures (ECACC) and Sigma-Aldrich. Cells can be purchased frozen or growing, and in both cases, the recommendations of the supplier should be followed to resuscitate or subculture the cells.

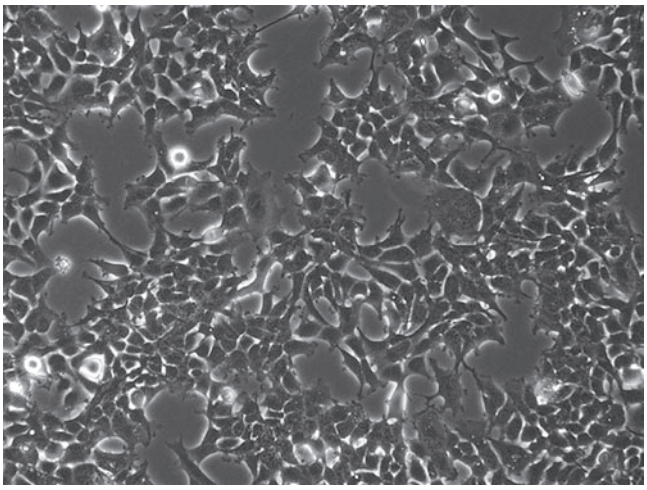

Fig. 1 A confluent culture of HEK293 cells

2.2 Cell Culture and cDNA Transfection Reagents

Store all the reagents at −20 °C or at 4 °C as indicated by the supplier.

1. Complete Dulbecco's modified Eagle's medium (DMEMc): DMEM + GlutaMAX, 10% fetal bovine serum (FBS), 1% penicillin/streptomycin.
2. Trypsin–EDTA 0.25%.
3. Dulbecco's phosphate-buffered saline (PBS).
4. Transfection kit. There are several commercially available kits (e.g., Lipofectamine 2000: ThermoFisher Scientific). Alternatively the DNA precipitation reaction using "calcium phosphate" can be used or that involving the formation of micelle structures (e.g., Effectene, Qiagen).
5. Poly-L-lysine.
6. Tissue culture flasks, tissue culture plates, 24- or 48-well plates, coverslips.
7. Neubauer chamber.
8. Centrifuge and Eppendorf tubes, and pipette tips.

2.3 Recording Solutions

The external solution can be prepared a day in advance and stored at 4 °C for several days.

2.3.1 Standard Extracellular Solutions (in mM)

1. Control: 140 NaCl, 3 KCl, 1.3 $MgCl_2$, 2.4 $CaCl_2$, 10 HEPES, 10 D-glucose. Osmolality ~300 mOsmol kg^{-1}.
2. Isotonic: 90 NaCl, 3 KCl, 1.3 $MgCl_2$, 2.4 $CaCl_2$, 10 HEPES, 10 D-glucose, 100 mannitol. Osmolality ~300 mOsmol kg^{-1}.
3. Hypotonic: 90 NaCl, 3 KCl, 1.3 $MgCl_2$, 2.4 $CaCl_2$, 10 HEPES, 10 D-glucose, 100 mannitol. Osmolality ~210 mOsmol kg^{-1}.

The pH of all the solutions is adjusted to 7.4 with NaOH and the osmolality is measured on a cryoscopic osmometer (Osmomat 030, Gonotec GmbH). The Osmometer determines the total osmolality of aqueous solutions.

2.3.2 Internal Pipette Solution (in mM)

A stock of 25 ml can be prepared, filtered (0.2 μm filter) and aliquoted into 1 ml Eppendorf tubes, and it can be stored at −20 °C for several months.

Internal solution: 135 CsCl, 2 $MgCl_2$, 5 Na_2-ATP, 10 HEPES, 1 EGTA. Adjusted to 300 mOsmol kg^{-1} with CsOH.

2.4 Calcium Measurement Indicator

1. Fura-2 AM Stock (Molecular Probes by Life Technologies): prepare a 1 mM stock solution of Fura-2 AM in DMSO and make 5 μl aliquots. The vials should be stored at −20 °C and they can be used for several weeks.
2. Pluronic acid stock: prepare a 20% solution of pluronic acid (Molecular Probes by Life Technologies) in DMSO (*see* **Note 1**) and store it at room temperature (*see* **Note 2**).
3. Fura-2 AM solution for cell incubations: prepare a 1 ml vial of Fura-2 AM at 5 μM with the control and pluronic acid solution at 2% (20% pluronic acid stock).
4. Fura-2 calcium Imaging Calibration kit (Molecular Probes by Life Technologies).

2.5 Patch Pipettes

1. Borosilicate glass capillary with filament (O.D. 1.5 mm, I.D. 0.86 mm, World Precision Instruments).
2. Silver wire (Ag/AgCl) (*see* **Note 3**).

2.6 Calcium Imaging Recording Setup (Fig. 2)

1. Inverted or upright microscope.
2. Antivibration table.
3. Illumination system with dual 340 and 380 nm excitation filters, and emission fluorescence at 510 nm. Optical fiber, filter wheels (or monochromator).
4. CCD camera for imaging acquisition.
5. Perfusion system (gravity), manifold, and suction system (*see* **Note 4**).
6. Recording chamber (*see* **Note 5**).
7. Temperature controller (e.g., Heater/Cooler: Warner Instruments).
8. Computer and imaging software.

2.7 Patch-Clamp Recording Setup

1. Inverted or upright microscope (preferable inverted).
2. Antivibration table.

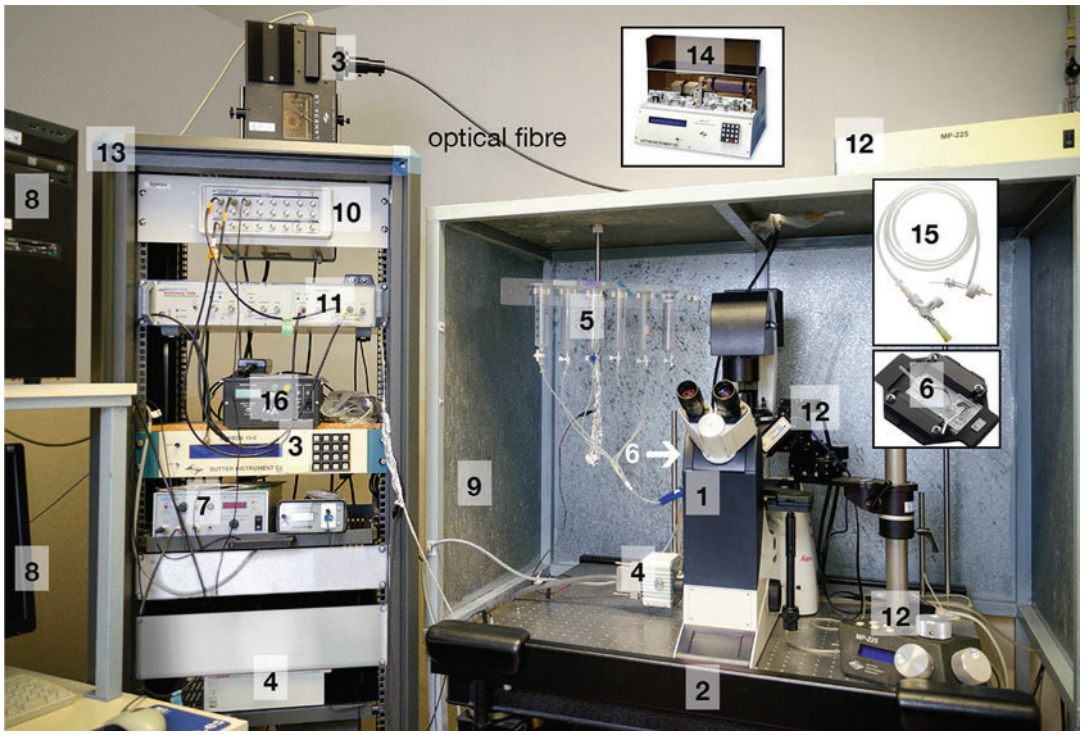

Fig. 2 The calcium and patch-clamp setup

3. Perfusion system (gravity), manifold, and suction system (*see* **Note 4**).
4. Recording chamber (*see* **Note 5**).
5. Temperature controller (e.g., Heater/Cooler: Warner Instruments).
6. Computer and patch-clamp recording software.
7. Faraday cage.
8. Digitizer.
9. Patch-clamp amplifier.
10. Micromanipulator.
11. Rack.
12. Pipette puller.
13. 1 ml syringe, rubber tube, multibarreled stopcock (mouth suction tube), recording pipette holder.

2.8 Pressure Clamp (Fig. 2 (16))

Positive or negative pressure can be applied through the patch pipette using a high-speed pressure clamp system (HSPC-1: ALA Scientific Instruments, Westbury, NY, USA [7, 13]).

3 Methods

3.1 HEK293 Cell Culture and cDNA Transfection

All the procedures must be carried out in a flow cabinet under sterile conditions.

3.1.1 HEK293 Cell Culture [9]

Trypsinize the cells when they reach 70–80% cell confluence in a 25 cm^2 flask (Fig. 1):

1. Remove and discard the culture medium.
2. Rinse the cells with 3 ml DMEM + GlutaMAX to eliminate the traces of FBS that inhibits trypsin.
3. Add 1 ml of trypsin–EDTA 0.25% to the cells for 1–2 min at room temperature.
4. Add 4 ml of culture medium (DMEMc) to inactivate the trypsin.
5. Disaggregate the cells by gently pipetting and transfer the solution to a 15 ml tube for centrifugation.
6. Centrifuge at 202 RCF for 5 min.
7. Remove supernatant and resuspend the pellet in 5 ml in DMEMc.
8. Add an appropriate aliquot of the cell suspension to a 25 cm^2 flask in 5 ml DMEMc. The volume of the aliquot will condition the subculturing of the cells.
9. Incubate the cells at 37 °C, in 5% CO_2 and 95% humidified air.
10. Remove medium and add fresh DMEMc after 48–72 h.
11. Subculture cells when they reach 80% confluency.
12. Do not exceed ~30 cell passages.

3.1.2 Coating Coverslips

1. Use ethanol flamed or autoclaved 12 mm or 6 mm diameter coverslips, place them in a petri dish and add ~60 μl or 20 μl of poly-L-lysine (0.01%) to each coverslip for at least 20 min at 37 °C (*see* **Note 6**).
2. Rinse twice by filling the petri dish with PBS or sterile water, and swirling it around. Tip off the final wash.
3. Separate the coverslips and remove any droplets of PBS. Dry the coverslips by keeping the plate partially open to the air.

3.1.3 Seeding HEK293 Cells

1. Count the cells in the cell solution (Subheading 3.1.1, **step 8**) using a Neubauer chamber and seed at the required concentration: for calcium imaging experiments, the recommended final cell-volume per 24-well plate is 75,000–200,000 cells/ml per well; for individual cell patch-clamp recordings, the final

recommended cell-volume per 24-well plate is ~30,000 cells/ml per well.

2. Carefully place a coated and dry coverslip in each well of the culture plate and homogeneously distribute 1 ml of cell suspension on it.
3. Maintain the cells at 37 °C, gassed with 95% O_2/5% CO_2.
4. The cells can be transfected 5–6 h after seeding.

3.1.4 Transfection of HEK293 Cell with TRPC5 cDNA in the Culture Plate Well

1. Prepare the solution for plasmid DNA transfection following the kit instructions (or alternatively follow the protocol below). The amounts and volumes are based on the use of a 24-well plate and the DNA concentration depends on the plasmid's characteristics: for TRPC5 the optimal concentration is 1–2 μg in a 500 μl volume. To identify cells expressing the channel, TRPC5 is cotransfected with green fluorescent protein (GFP: Life Technologies) at a concentration of 0.5 μg in a 500 μl well volume. Alternatively, a TRPC5 construct fused to the GFP protein can be transfected.
2. Transfection solutions for 24-well and calcium imaging experiments:
   i. Prepare two sterile Eppendorf tubes and add 50 μl of serum-free DMEM (or other medium) to each tube.
   ii. Dilute cDNA (1–2 μg) in 50 μl of serum-free DMEM (tube 1) or other medium and mix gently.
   iii. Gently mix the Lipofectamine 2000 tube and add the necessary amount to 50 μl of serum-free DMEM (or other medium) (tube 2). Proportion of Lipofectamine 2000 2–3 μl/μg DNA Incubate for 5 min at room temperature.
   iv. After the 5 min incubation of Lipofectamine 2000 with the medium, combine the DNA (tube 1) with the Lipofectamine 2000 solution (tube 2). Mix these two solutions gently and incubate for 20–25 min at room temperature.
   v. Remove 600 μl of the medium from the wells containing the cells attached to a coverslip (Subheading 3.1.3, **step 2**) and add the 100 μl of the cDNA–Lipofectamine 2000 complexes (**step iv**). Mix gently by gently shaking the plate and then incubate the cells at 37 °C in a CO_2 incubator for 4–6 h. Transfect as many wells in the culture plate as necessary.
   vi. After the 4–6 h incubation, remove the medium and add 1 ml of DMEMc to each well culture plate.
   vii. After a further 4–6 h incubation, test the efficacy of the transfection by assessing the expression of GFP under a fluorescence microscope. The expression from the plasmids

(GFP and TRPC5) will be stronger 24 h after transfection. Recordings can be performed 24–48 h after transfection.

viii. Optional: a confluent transfected well culture plate (without coverslips) can be trypsinized 4–6 h after transfection:

(a) Remove the medium from the well and rinse the cell layer with 1 ml DMEM + GlutaMAX to eliminate any traces of serum that will inhibit the trypsin.

(b) Add 200 μl of trypsin–EDTA 0.25% for 1–2 min at room temperature (check under the microscope when the cells detach).

(c) Add 1 ml of culture medium (DMEMc) to inactivate the trypsin.

(d) Disaggregate the cells by gently pipetting and transfer the solution to a tube. Add 5–10 ml of DMEMc to dilute the cells and plate 1 ml on coverslips in the wells of a culture plate.

(e) Incubate the cells at 37 °C in a CO_2 incubator until they reach the desired confluence (*see* **Note 7**).

3. Transfection solutions for 24-well and electrophysiological experiments. Follow the **steps 2i–vii** and at this point, trypsinize the cells, dilute and seed them onto coverslips in culture plate wells to obtain isolated cells (from **step 2viii-d**). Recordings can be obtained 3–24 h after trypsinization. It is also possible to generate a HEK293 cell line that stably expresses the specific protein (e.g., TRPC5 [14]).

3.2 Calcium Imaging Recordings

3.2.1 Incubation the Cells with Fura-2 AM

1. Remove the medium from the well.
2. Using fine tweezers, transfer one or several coverslips to a petri dish (35 mm) containing 1 ml of Fura-2 AM (5 μM final concentration, *see* Subheading 2.4).
3. Incubate at 37 °C in a CO_2 incubator for 30–45 min.
4. Remove the Fura-2 AM and add 1 ml of the external recording solution, maintaining the cells protected from light.
5. Use a scalpel blade to cut the coverslip into 3–4 pieces (*see* **Note 8**).

3.2.2 Perfusion

The gravity perfusion system consists of 50 ml syringes (the number of syringes used depends on the different solutions to be tested) connected by silicone tubes (3 mm ID) and a multibarreled stopcock. The tubes are connected to a manifold.

1. Fill the syringes with control, isotonic, hypotonic and any other test solution, and purge the lines to eliminate air bubbles (Fig. 2 (5)).

2. Fill the recording chamber of the microscope with the control solution and place a piece of a coverslip in the chamber (Fig. 2 (6)).
3. Cells should be perfused continuously (1–2 ml/min), yet the perfusion can be switched to any of several test solutions using the multibarreled stopcock (Fig. 2 (5)).
4. Elimination of the chamber solution can be achieved by gravity or using vacuum pressure.

3.2.3 Calcium Measurement [7]

1. Transfected cells are identified by GFP fluorescence, at an excitation wavelength of 470 nm.
2. Select a field with a good confluence of GFP-positive cells, and take images of the cells in white light and at the excitation wavelength for GFP fluorescence (Fig. 3a).
3. Switch to a Fura-2 fluorescence protocol and start acquiring images. Fluorescence images of HEK293 cells were obtained using paired exposures to excitation wavelengths of 340 nm and 380 nm, and fluorescence emission was monitored at wavelengths longer than 510 nm [7, 15]. Images were acquired and stored at 0.2 Hz (*see* **Notes 9** and **10**).
4. The background fluorescence is measured in a region of the culture dish devoid of cells and this is subtracted from the cellular fluorescence signals on-line.
5. Expose the cells to the control solution for 1 min, to the isotonic solution for 1 min and to the hypoosmotic solution for 3–4 min (Fig. 3b, c). Finally, switch to the control solution for washing out and recovery.
6. After recovery, it is useful to apply a muscarinic receptor agonist (e.g., carbachol, 10 μM), as these receptors are endogenously expressed by HEK293 cells. This produces significant Ca^{2+} responses that can be used as controls in both transfected and untransfected cells.
7. Perform recordings at room temperature or fix a temperature of interest using the solution heater/cooler (temperature controller).
8. Changes in calcium concentration are shown in Fig. 3d, with recordings taken alternately from coverslips with transfected and nontransfected cells.
9. The increase in calcium can be displayed as arbitrary units of fluorescence that reflect the changes in the ratio between 340 and 380 nm, or as changes in the intracellular calcium concentration. The apparent concentration of free intracellular calcium is calculated from the ratio of the fluorescence emission at the two excitation wavelengths, using the formula $[Ca^{2+}]_i = K_{eff}{}^{*}[(R - R_{min})/R_{max} - R)]$ [15, 16]. R_{min} and R_{max} are obtained in vitro using the Fura-2 calcium Imaging Calibration kit (Molecular Probes by Life Technologies).

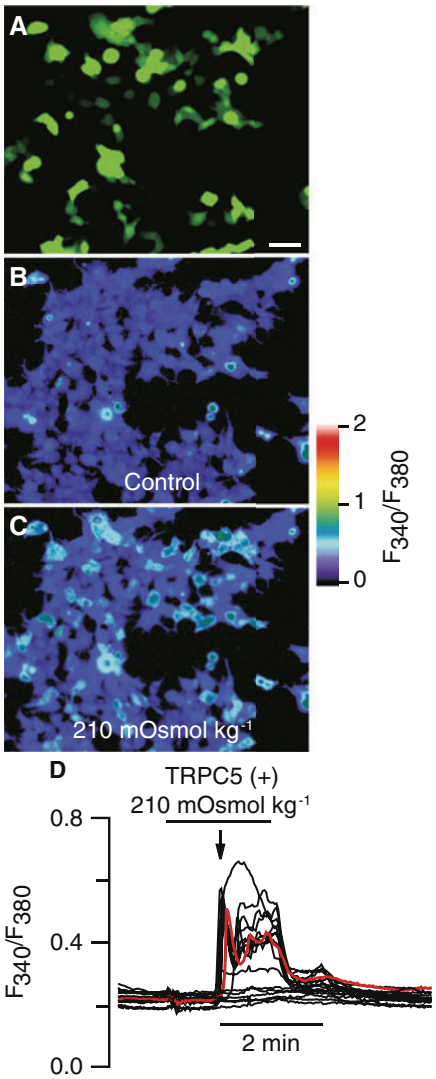

Fig. 3 Pseudocolor images of TRPC5-GFP transfected cells showing GFP fluorescence (**a**) and ratiometric $[Ca^{2+}]_i$ responses to control (**b**) and hypoosmotic solutions (**c**). Changes in $[Ca^{2+}]_i$ are reflected by the ratio of Fura-2 emission at 340 to 380 nm excitation (see color bar). Scale bar, 50 μm. (**d**) Time course of the calcium changes in TRPC5 transfected cells in hypoosmotic solution. Image taken in a 210 mOsmol kg^{-1} solution corresponds to the time course of the calcium increase indicated by the arrow. The red trace shows an example of an individual response to hypoosmotic stimulation (Adapted with permission from Jemal et al., 2013 [19])

10. This calcium imaging protocol (Subheading 3.2) can also be used when testing other possible modulators of TRPC5, or any other TRPC channel expressed endogenously or transiently in HEK293 cells.

3.3 Whole-Cell Patch Clamp

For a more extensive description of the patch-clamp technique *see* [17, 18].

1. Pull the patch pipettes (an electrode resistance of 3–5 MΩ is desirable).
2. Using fine tweezers transfer one or several coverslips to a petri dish 35 mm with control solution and use a scalpel blade to cut the coverslip in 3–4 pieces (*see* **Note 8**). Select a single GFP-positive cell with no visible connection to other cells.
3. Fill a patch pipette with the internal solution (*see* **Note 11**) and connect the patch pipette to a holder, applying a mild positive pressure through the mouth suction tube (Fig. 2 (15)). Alternatively, attach a 1 ml syringe to the yellow tip (*see* **Note 12**).
4. Move the pipette tip down into the bath solution and in voltage clamp mode, apply a test pulse to check that the ideal pipette resistance at this stage is 3–5 mΩ.
5. At 40× magnification, visualize the tip of the pipette and approximate the patch pipette to the selected cell. Release the positive pressure and very gently touch the cell's membrane, applying continued suction until the seal resistance reaches ~100 MΩ. Apply a negative potential (~−60 mV) through the voltage clamp command to form a GΩ seal.
6. To establish the whole-cell configuration, apply strong suction pulses to break the membrane, either orally or using a syringe. Then allow about 15–30 s to equilibrate the internal solution.
7. Record in the voltage clamp configuration at room temperature or fix a different temperature using the solution heater/cooler. In the voltage clamp mode, the recorded signal is the transmembrane current and the controlled input is the membrane voltage.
8. To study the activation of TRPC5 by osmotic stimulation, we measure the whole-cell ionic currents in response to the application of hypoosmotic solutions.
9. Cells are held at a potential of −60 mV and the current–voltage (*I–V*) relationships are obtained using voltage ramps from −100 mV to +100 mV, with a duration of 400 ms applied every 3–5 s. The currents are sampled at a frequency of 20 kHz and filtered at 5 kHz. A series resistance compensation of >50% is applied.
10. Apply voltage ramps in control solution over 1 min, switch to an isotonic solution for 1 min and then to a hypoosmotic solution for 3–4 min. Finally, switch to a control solution for the washout and recovery. Applying a hypoosmotic stimulus induces a large, slow transient whole-cell current (Fig. 4a, b [7, 19]. The current displays the characteristic doubly rectifying shape of TRPC5-mediated currents (Fig. 4b).

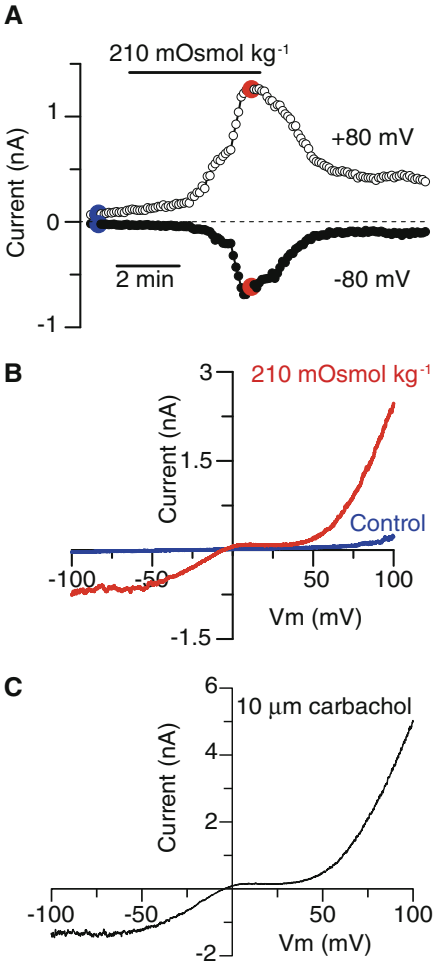

Fig. 4 (**a**) Representative experiment showing the peak whole-cell current, measured at ±80 mV, obtained with a 400 ms voltage ramp from −100 to +100 mV in a cell expressing TRPC5 maintained in a 210 mOsmol kg^{-1} solution. (**b**) Current–voltage relationships obtained in a control solution and at maximal current from the same cell as in A (adapted with permission from Gomis et al., 2008 [7]). (**c**) Current–voltage relationship from HEK293 cells coexpressing TRPC5 evoked by a 400 ms voltage ramp from −100 to +100 mV applied in the presence of carbachol (10 µM: adapted with permission from Arcas and Gomis, 2018 [20])

11. After recovery, a muscarinic receptor agonist can be applied, as these receptors are expressed endogenously by HEK293 cells (e.g., carbachol, 10 µM; Fig. 4c). This produces consistent inward and outward currents in TRPC5 transfected cells, and these TRPC5 channels are known to activate following the stimulation of GPCRs. The current displays the characteristic doubly rectifying shape of TRPC5-mediated currents (Fig. 4c).

12. To study TRPC5 activation by pressure clamp, we measure whole-cell ionic currents in response to application of positive pressure through the patch pipette. The patch pipette is connected to the high-speed pressure clamp device that applies controlled pressure steps in whole-cell configuration (*see* **Note 13**). Membrane stretching was induced by positive pressure applied to the patch pipette. Application of repeated positive pressure pulses that increase in amplitude from 10 to 140 mmHg activate a double rectifying TRPC5-mediated current with a reversal potential close to 0 mV [7].
13. This protocol (Subheading 3.3) can also be used when testing any other possible modulator of TRPC5 or any other TRPC (or even other TRP channels) expressed endogenously or transiently in HEK293 cells.

4 Notes

1. Shake vigorously or sonicate to dissolve the pluronic acid.
2. The solution may solidify at cold temperatures so warm it for several minutes until it becomes transparent. The solution can be stored at room temperature for several weeks.
3. Place about half the length of a silver wire into an Eppendorf tube with bleach. The wire should become duller and slightly darker as silver chloride is formed. Rinse with distilled water and dry.
4. Elimination of the chamber solution can be achieved by gravity or using vacuum pressure.
5. Perfusion chambers are commercially available (e.g., Warner instruments).
6. Poly-L-lysine can be reused up to ten times. Use a standard fine tweezers to manipulate the coverslips (e.g., #5).
7. Wait 2–3 days until the cells are sufficiently confluent to carry out the calcium experiments. After longer periods, the overexpression of the channel and the GFP might become toxic and possibly damage the cells.
8. The size of the piece of coverslip depends on the size of the recording chamber. It is also possible to use the whole coverslip if the chamber is large enough, but breaking the coverslip allows more experiments to be performed with each coverslip.
9. Excitation times of 20–200 ms can be used. This depends on the Fura-2 loading of the cells and the power of the illumination system. One image/3 s is a good sampling rate for the study of TRPC5. It takes several seconds for the calcium increase to peak and at this sampling rate, the size of the files are not too large.

10. Depending on the calcium imaging software, it may be possible to select the cells and visualize the temporal course of the calcium changes on line. With other software the images are only visible on line and the temporal course must be analyzed off line.
11. Keep the Eppendorf tube with the internal solution cold.
12. Fix the portion of the tube close to the holder of the microscope to avoid movement.
13. The pressure clamp system used for stimulation is also used to obtain the membrane seal in the whole-cell configuration.

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