

METHODS PAGE

Culture of pancreatic AR42J cell for use as a model for acinar cell function

Antonio Gonzalez, Patricia Santofimia-Castaño and Gines M. Salido

Department of Physiology (Cell Physiology Research Group), University of Extremadura, Avenida

Universidad s/n; E-10003; Caceres (Spain)

e-mail: agmateos@unex.es

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Introduction and approach

AR42J cells derive initially from a transplantable tumour of a rat exocrine pancreas (20). This line is tumorigenic in nude mice, and is the only cell line currently available that, in culture, maintains many characteristics of normal pancreatic acinar cells, such as calcium (Ca^{2+}) signalling, the synthesis and secretion of digestive enzymes, protein expression, growth and proliferation (7,

33). In fact, AR42J cell receptor expression and signal transduction mechanisms parallel those of pancreatic acinar cells. Thus, this cell line has been widely used as an “in vitro” model to study the exocrine pancreas (for references, please check table 1). We have recently used this cell line to study the effects of melatonin on Ca^{2+} signalling, mitochondrial physiology and cell viability (9, 12). In these studies we have applied the protocol described here.

Table 1. Examples and references for acinar cells' parameters and functions that can be studied employing pancreatic AR42J cells.

Parameter/Cell function	References
Calcium signalling	2, 9, 24, 47
Enzyme secretion	1, 4, 11, 17, 21, 28, 29, 30
Cell proliferation	4, 8, 16, 34, 35, 38, 43, 46
Protein expression	10, 18, 19, 31, 41, 42
Inflammation	5, 22, 27, 32, 40
Apoptosis	3, 5, 12, 23, 25, 36, 37, 39, 44, 45

But, as mentioned above, many other acinar cells' parameters and functions can be also studied employing this cellular type. For additional information see Table 1.

AR42J cells (Figure 1) can be easily cultured in a RPMI 1640 medium supplemented with 2 mM glutamine, 10 % fetal bovine serum and antibiotics (0.1 mg/mL streptomycin, 100 IU penicillin) at 37 °C under a humidified condition of 95 % air and 5 % CO₂. Cells are routinely plated

at a density of ~10⁵ cells/mL in 75 cm² flasks, and cultured for 7-10 days at 37 °C in a humidified incubator (5% CO₂). Twenty-four hours after isolation cells should be washed twice with standard phosphate-buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄; pH adjusted to 7.4) to remove dead cells; discard the washing PBS and add fresh culture medium to the flask. The experiments should be carried out after 7–9 days after plating of the cells.

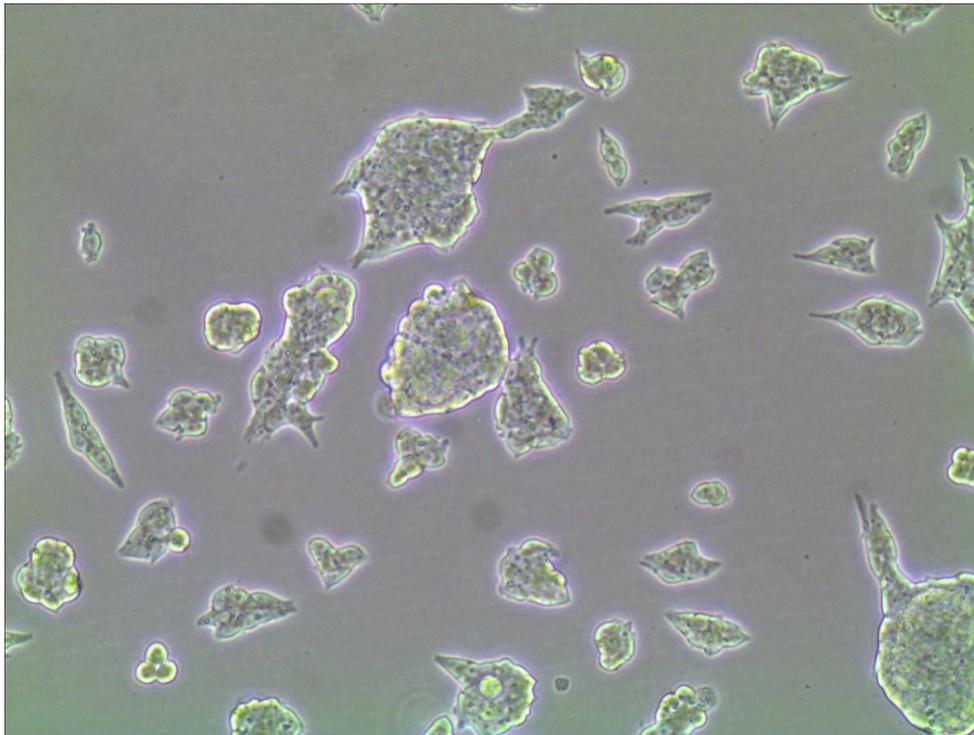


Figure 1. AR42J cells in culture. The photograph was taken using an inverted microscope.

Dexamethasone treatment has been found to convert these cells into exocrine cells (26). For this purpose cells must be incubated for 48-72 hours in culture medium supplemented with 100 nM dexamethasone (DEXA); then the cells are ready to be used for the experiments. We do not add DEXA to those cells that will be used for the propagation of the cultures, i.e., when cells are reseeded and grown to obtain new cultures, or

those that will be used to keep a stock of cells (frozen stock).

1. Materials

1.1. Laboratory equipment

1. Water bath (for incubation of solutions at 37 °).
2. Laminar flow chamber.
3. Humidified incubator (95 % air, 5 % CO₂).

4. CO₂ bottle.
5. Inverted microscope.
6. Automatic pipettors (for example a Pipetboy acu, from Integra[®]).
7. Centrifuge (cell suspensions will be centrifuged at 900 r.p.m., 90-100 xg; conditions usually achieved in a centrifuge with a 15 cm radius of rotation).
8. Freezer (at -80 °C).
5. NaOH (1-10 M)
6. HCl (1 M)
7. Bidistilled water, sterile (autoclaved).
8. Ethanol (EtOH), both absolute (100%) and 70%.
9. 2-isopropanol.

1.2. Cells

1. AR42J cell line (ECACC N^o 93100618); we purchased ours from The European Collection of Cell Cultures (ECACC). They can also be purchased from the ATTC (N^o CRL-1492).

1.3. Culture medium constituents

1. RPMI 1640 medium, can be obtained from BioWhittaker[®].
2. Fetal bovine serum (FBS), can be purchased from HyClone (Thermo Scientific[®]).
3. Glutamine, can be obtained from BioWhittaker[®].
4. Penicillin/streptomycin, can be obtained from BioWhittaker[®].
5. Dexamethasone (gamma-irradiated cell culture), can be obtained from Sigma Chemicals Co[®].
6. Trypsin-ethylenediaminetetraacetic acid (trypsin-EDTA), can be purchased from GIBCO[®] (Invitrogen[®]).
7. DMSO (Hybri-Max sterile filtered), can be obtained from Sigma Chemicals Co[®].

1.4. Other reagents (analytical grade)

1. NaCl
2. KCl
3. Na₂HPO₄
4. KH₂PO₄

1.5. Plastic and other materials

1. Syringe Filter 26 mm, 0.2 µm, sterile, can be obtained from Corning[®].
2. Cryotubes (1-2 mL), can be purchased from NUNC[®] (Thermo Scientific[®]).
3. Pipettes: 1-10 µL, 2-20 µL, 10-100 µL, 100-1000 µL.
4. Disposable sterile pipets (independent packaged-unitary bag): 5 mL, 10 mL, 50 mL.
5. Pipette Tips, sterile (autoclaved).
6. Sterile centrifuge tubes: 15 mL and 50 mL.
7. Culture flasks: 25 cm² and 75 cm², vent cap.
8. Multi-well plates and/or Petri dishes.
9. 22-24 mm coverslips (absolute ethanol plus flame sterilised).
10. 5 mL and 50 mL syringes (sterile, independent packaged).
11. Eppendorf vials (0.2-0.5 mL).
12. Squirt bottle with 70 % EtOH.
13. Kimwipes.
14. Screw-capped cup (urine container cup).
15. Cello-tape.
16. Permanent marker.
17. Disposable latex gloves.

2. Method

2.1 Safety

1. Wear disposable latex gloves at all times.

2. Dispose all used materials and media appropriately.

2.2 Composition of the culture medium:

1. RPMI 1640 (one bottle of 500 mL)
2. L-Glutamine (2 mM final concentration)
3. FBS (10 % final concentration)
4. Penicillin (100 Units/mL) plus streptomycin (0.1 mg/mL) (final concentration)

2.3. Preparing the culture medium

Wear disposable latex gloves at all times.

1. Mix, in a separate sterile container (two 50 mL centrifuge tubes can be used), L-Glutamine, FBS, and antibiotic mixture at the desired concentrations (to yield the final concentration required when added to the RPMI).
2. Steri-filter the mixture through a Syringe Filter (26 mm, 0.2 μ m) using a 50 mL syringe.
3. Add the mixture to the RPMI in the bottle and mix gently. Close the bottle and keep at 4-8 °C.
4. Another possibility to prepare the medium is to mix all constituents, by adding all of them directly in the RPMI bottle to yield the final concentration required, and then steri-filter the complete solution (this means more work to be done, i.e., more volume to be filtered).
5. Prior to use, warm media in 37 °C waterbath.

2.4. Preparing the standard phosphate-buffered saline (PBS) composition

1. Prepare, using bidistilled water, a buffer containing: 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄ (pH adjusted to 7.4).
2. Sterilise (autoclave or sterifilter).
3. Keep at 4-8 °C.

2.5. Preparing the cells

1. The cells are normally delivered or stored frozen (-80 °C).

2. Keep the vial 1 minute at room temperature (22-25 °C).
3. Bring the vial in a water bath at 37 °C for 2 minutes. Note: the cells should not be long time at this point in the freezing solution.
4. Disinfect the vial with EtOH 70% (a spray can be used; for example, that one used to clean the bottom in the laminar flow chamber).
5. Bring the vial into the laminar flow chamber. From now on we will work under sterile conditions.
6. Open the vial and add 500 μ L (0.5 ml) of culture medium. Gently mix by pipetting the cell suspension.
7. Transfer the cell suspension to a 15 mL centrifuge tube and add 11 mL of culture medium.
8. Centrifuge for 5 minutes at 900 r.p.m. (90-100 x g) and discard (aspirate) the supernatant
9. Add 6 mL of culture medium and resuspend (pipet) the pellet.
10. Transfer the cell suspension to a 25 cm² culture flask. Mark the flask, in order to identify the cell type growing, the passage of culture and the date of culture preparation.
11. Incubate at 37 °C, 5% CO₂.
12. Observe the culture after 24 hours of incubation, and check the general appearance.
13. 48 hours after seeding, replace 5 ml of culture medium.
14. Replace completely the culture medium 4 days after seeding with 5mL of fresh medium. If there are cells growing in suspension transfer the supernatant to a 15 mL centrifuge tube, and centrifuge for 5 minutes at 900 r.p.m. (90-100 x g). Add 1 mL of culture medium, resuspend the pellet, and add back to the flask with attached cells.

15. Incubate at 37 °C, 5% CO₂.
16. Culture medium should be renewed every 48 hours, depending on the rate of cell growth. Simply discard/aspirate the medium, and add fresh culture medium.
17. After one week of incubation a general growth is expected. The cells will be growing forming colonies, close to each other. At the microscope it should be observed an image similar to that shown in figure 1, but with many more colonies. The bottom of the flask should be pretty full of growing cells, and this could be observed at a glance.

2.6. Preparation of dexamethasone (DEXA) solution

The final concentration needed in the cultures is 100 nM. We have employed dexamethasone (gamma-irradiated cell culture) from Sigma® (D-8893; 1 mg vial). Steps in preparation of a stock solution of DEXA and dilution in culture:

1. Make a 1 mM stock solution in absolute EtOH plus culture medium
1. mixture.
2. Add 1 mL absolute EtOH to the vial (content 1 mg dexamethasone) and dissolve well.
3. Add 1.548 mL of culture medium, and mix. This yields a final volume of 2.548 mL (concentration 1 mM DEXA)
4. Split into 50 µL aliquots (0.2-0.5 mL vials; 50 vials). Mark the vials. Keep one aliquot (will be used later on), and freeze the others (at -20 °C) until use.
5. Prepare 10⁻⁴ M from 1 mM DEXA; 1:10 dilution by adding 450 µL culture medium to DEXA 1 mM in the vial. We obtain 500 µL of 10⁻⁴ M DEXA.
6. Split into 10 µL aliquots (0.2-0.5 mL vials; 50 vials). Mark the vials. Freeze (at -20 °C) until use.
7. When needed, prepare fresh culture medium

and add DEXA stock solution 10⁻⁴ M (1 vial per 10 mL of culture medium). 1:1000 dilution. The final concentration achieved is 100 nM.

8. Discard/aspirate the culture medium and add this DEXA medium. Incubate cells for 48-72 hours, and the cells will be ready for use in the experiments.

2.7. Propagation (subcultures) and maintenance of AR42J cells

At the present moment we have one flask with AR42J cells growing. We can prepare more flasks or dishes, in order to have more cells to perform our experiments, and also to have a pool of cells. Thus, cells can be detached, reseeded and/or frozen.

2.8. Subcultures

Cell passaging or splitting is a technique that enables an individual to keep cells alive and growing under cultured conditions for extended periods of time. Cells should be passed when they are 90 %-100 % confluent. Cells are normally purchased at passages 4-6. The number of passages increases once each time the cells are detached and reseeded.

One should bear in mind that over-subculturing changes cell lines' properties over time. Cell lines at high passage numbers experience alterations in cell morphology, response to stimuli, growth rates, protein expression, transfection and signalling, compared to lower passage cells. The degree of subculturing a cell line has undergone is often expressed as "passage number," which can generally be thought of as the number of times cells have been subcultured into a new vessel, usually within one lab.

For this process, simply follow the next steps (in sterile conditions, i.e., in laminar flow chamber):

1. Warm PBS and trypsin-EDTA in 37 °C waterbath.
2. Transfer the culture medium to a 15 mL centrifuge tube.

3. Wash the cells with PBS (6 mL).
4. Replace the PBS by 5 mL of HBSS medium containing trypsin-EDTA (1X).
5. Incubate the cells for 2-3 minutes in this medium (in the incubator).
6. Afterwards gently hit the flask laterally with one hand, once or twice. Most of the cells should be now in suspension.
7. Gently pipet the medium (with pipettor, and a 5 or 10 ml sterile pipette) to obtain the cells still attached; this manoeuvre can help to detach the cells.
8. Transfer the content of the flask to the 15 mL centrifuge tube containing the collected culture medium.
9. This step helps to stop trypsinization step. Avoid long period of cells in the presence of trypsin solution.
10. Centrifuge the cell suspension for 5 minutes (900 r.p.m.; 90–100 \times g).
11. Discard/aspirate supernatant.
12. Resuspend the pellet with 5 mL of fresh culture medium.
13. Finally, the cells can be reseeded as needed (we normally use $\sim 10^5$ cells), for example in culture flasks, petri dishes, multiwell plates, or onto glass cover slips, which can be placed in independent dishes (35 mm diameter). Mark the recipient, in order to identify the cell type growing, the passage of culture and the date of culture preparation.
14. Counting of cells is needed (use a cell counter, or do it in Neubauer chamber).
15. Transfer the desired amount of cells (i.e., the calculated volume in μ L of cell suspension) to the new support for the culture (flask, petri dish,...).
16. Add fresh culture medium (the amount to be added depends on the available volume).
17. Mark the recipient, in order to identify the cell type growing, the passage number and the date of culture preparation.
18. Incubate (in a humidified incubator at 37 °C and 5% CO₂).

2.9. Keeping a pool of AR42J cells. Freezing of cells

A pool of cells can be frozen, and stored (at -80 °C), in order to be used in the future. The preparation procedure must be done in sterile conditions (i.e., in laminar flow chamber). A freezing medium will be needed. Use 1 mL of this medium to freeze $1.5-2.0 \times 10^6$ cells as a 1 mL aliquot of cells. Thus the volume of freezing medium that will be used must be calculated. Wear disposable latex gloves at all times.

2.9.1. Composition and preparation of the freezing medium

1. Dissolve DMSO (Hybri-Max sterile filtered) in FBS at a final concentration of 5% (v/v).
2. Sterifilter the solution.
3. Keep in laminar flow chamber until use.

2.9.2. Procedure for freezing the cells

1. We start from a cell suspension (once detached), with known number of cells per mL in culture medium.
2. Calculate the amount of freezing medium (in mL) that will be needed, and prepare.
3. Set ready the number of cryotubes needed (depending on the number of aliquots that will be frozen).
4. The cryotubes must be identified, indicating the type of cell that contains, the passage number, the date of preparation, and the number of cells. Use a permanent marker, and protect with cello-tape.
5. Put 5-10 mL of 2-isopropanol in a urine container cup.

6. Centrifuge the cell suspension (5 minutes, at 900 r.p.m., 90-100 × g).
7. Discard/aspirate the supernatant (under the laminar flow chamber).
8. Resuspend the pellet in the volume needed of freezing medium.
9. Add 1 mL of cell suspension in each cryotube and seal the vial.
10. Put the vial/vials in 2-isopropanol in the urine container cup and seal.
11. Quickly put the cup in the freezer at -80 °C.

3. Experiments

Cultured cells are ready to be used for the experiments, once 90 %-100 % confluence has been reached. Here we will describe how the cells can be loaded with different fluorescent dyes.

3.1. Dye loading

Let's consider that we are growing the cells on a glass coverslip that will be used for fluorescence imaging or confocal microscopy. The protocol is as follows:

1. Replace the culture medium by a physiological solution (Na-HEPES buffer) containing: 130 mM NaCl, 4.7 mM KCl, 1,3 mM CaCl₂, 1 mM MgCl₂, 1.2 mM KH₂PO₄, 10 mM glucose, 10 mM Hepes, 0.01 % trypsin inhibitor (soybean) and 0.2 % bovine serum albumin (pH = 7.4 adjusted with NaOH).
2. Add the fluorescent indicator of choice to yield the final concentration required, and protect from light.
 - 2.1. For intracellular free Ca²⁺ concentration determination, add the fluorescent ratiometric Ca²⁺ indicator fura-2/AM (4 μM final concentration). Incubate at room temperature (23-25 °C) for 40 minutes. Gently stir every 5-8 minutes (15). Figure

2 shows a typical trace of changes in [Ca²⁺]_c of AR42J cells in culture. Cells were stimulated with 1 nM CCK-8, and fura-2-derived fluorescence was monitored employing single cell fluorescence microscopy.

- 2.2. Localization of mitochondria can be assayed by incubation of cells in the presence of MitoTracker™ Green FM. This dye has been widely employed as a mitochondrial marker. Loading of cells with MitoTracker™ Green FM (100 nM final concentration) can be performed at room temperature (23–25 °C) for 30 minutes. Gently shake every 5-8 minutes (13).
- 2.3. Determining changes in mitochondrial Ca²⁺ signals can be performed after loading of cells with rhod-2/AM (8 μM final concentration) at 4°C for 15 min. After incubation with rhod-2/AM, replace the extracellular medium by fresh Na-HEPES buffer (containing -in mM-: 140 NaCl, 4.7 KCl, 1,3 CaCl₂, 2 MgCl₂, 10 Hepes, 10 glucose; pH adjusted to 7.4), and further incubate the cells at room temperature (23–25°C) for 30 minutes. Gently shake every 5-8 minutes (14).
- 2.4. Changes in mitochondrial membrane potential (Ψ_m) can be recorded using the dye TMRM as described previously (13). Incubate the cells during 30 min in the presence of 50 nM TMRM at 37 °C. Gently shake every 5-8 minutes. At the concentration used in our conditions, TMRM accumulates within mitochondria driven by the membrane potential but autoquenching is negligible. A decrease in TMRM fluorescence reflects depolarization of Ψ_m, due to diffusion of the dye to the cytosol.
3. After dye loading of cells, replace the medium by a Na-Hepes buffer containing (in mM): 140

NaCl, 4.7 KCl, 1,3 CaCl₂, 2 MgCl₂, 10 Hepes, next.
10 glucose (pH adjusted to 7.4). Use the cells

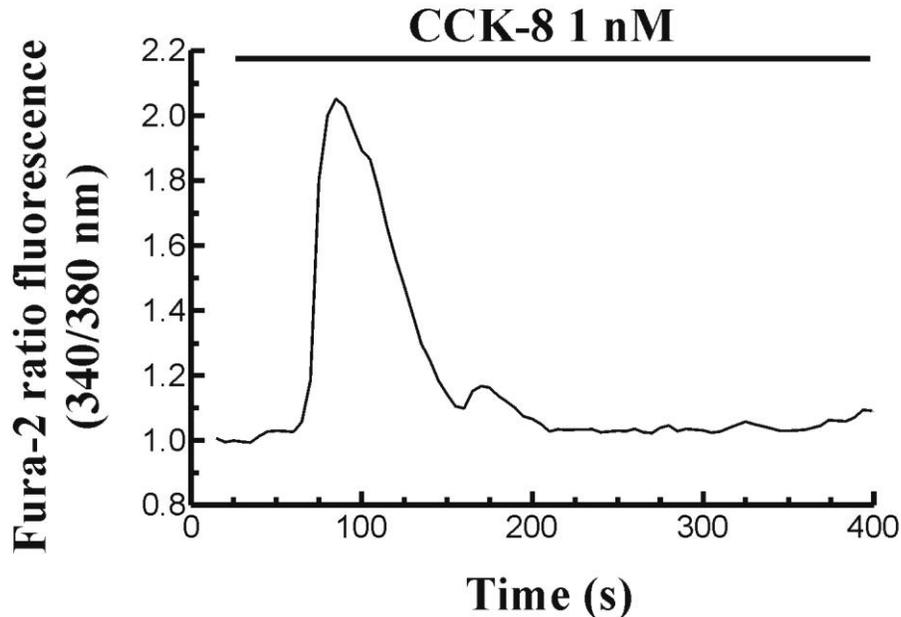


Figure 2. Changes in [Ca²⁺]_c of AR42J cells in response to CCK-8. The graphic shows a typical trace of changes in [Ca²⁺]_c of AR42J cells in culture in response to 1 nM CCK-8. Experiments were carried out in the presence of extracellular Ca²⁺. Horizontal bar shows the time during which CCK-8 was applied to the cells.

Other examples of use: if cells are to be used for western blot, incubation with stimuli/drugs can be performed in the flasks. Then the cells must be detached, and used appropriately. Cells can be also used to study enzyme secretion. Amylase release can be carried out using cells grown in

multiwell dishes (usually 24 or 28 wells). Further studies that can be performed using AR42J cells are summarized in table 1. Thus, the substrate where cells are to be grown depends on the final use one will do of the cultures.

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