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METHODS PAGE

Isolation of human pancreatic acinar cells from rat and human pancreas

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The pancreatic acinar cell is a specialised secretory unit, the physiology of which has been extensively studied over a number of decades principally using rodents, although recently significant progress has been made with respect to investigation of human samples. Due to significant difficulties in developing culture methods for this cell type, primary isolation procedures are essential in order to study function. The high quantity of dense-core vesicles and the clustered nature of acini, mean that low-speed centrifugation is extremely effective at isolating this cell type from less dense material such as cellular debris and other component cells of the pancreas. An isolation procedure was developed for murine tissue (1, 2) which was based on a series of purification steps following enzymatic dispersal of freshly dissected pancreas to yield isolated cells and small acinar clusters. We have recently modified this procedure for the isolation of human pancreatic acinar cells, taking into account differences between adult human and murine pancreas samples, most notably contamination with blood products and fat, and presence of fibrosis in the former (3). Here we describe a simple and effective procedure for the isolation of human pancreatic acinar cells using collagenase digestion, mechanical dispersion and low-speed centrifugation.

1. Materials

1. Extracellular solution (mM): 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 1 $CaCl_2$, 10 D-glucose, 10 HEPES (adjusted to pH 7.35 with NaOH)
2. Collagenase (CLSPA, 200 U/ml) (Worthington) to be dissolved in extracellular solution (above)
3. Polystyrene 15 ml tubes (Sarstedt) (*see* **Note 1**)
4. Tabletop centrifuge
5. Water bath (shaking if possible)
6. 70 μm nylon Cell Strainer (BD Biosciences),
7. Human pancreas (*see* **2.1 Human Pancreatic Tissue Sample Retrieval**)
8. *****Protease inhibitor tablets 1 per 10 ml (Roche Diagnostics)
9. *****Sodium Pyruvate 100 μM (100 mM stock solution, Sigma-Aldrich)

10. Soybean trypsin inhibitor (lyophilized powder, 0.01% w/v, Sigma-Aldrich)

*****These extra compounds are used to augment the extracellular solution for use during transport from theatre to the laboratory (see 2.1 point 3), but are omitted from the basic extracellular solution for all subsequent steps.

2. Methods

2.1 Human Pancreatic Tissue Sample Retrieval

Samples may only be taken from consenting patients. Human pancreatic tissue should be of high quality, which will normally only be available from surgery for left-sided pancreatectomy, resections for duodenal tumours, or non-obstructive right sided cancer resections in patients with no history of jaundice or chronic pancreatitis (3). Samples from patients with chronic pancreatitis or ductal obstruction are unsuitable due to the high levels of activated trypsin in this tissue (Fig. 1).

1. During surgery (pancreatic resection), a small piece (~1 - 3 cm^3) of pancreas should be cut from the transection margin of the remaining pancreas. This procedure should be carried out using a new scalpel blade to limit gross macroscopic cell damage from diathermy burns (*see* **Note 2**).
2. The piece of pancreas should be immediately washed by transfer between two 50 ml tubes of ice-cold extracellular solution to remove debris and blood products. These may contain neutrophils, macrophages etc. which have the potential to induce additional oxidative stress and tissue damage.
3. After washing, the sample should be added to a third, pre-prepared tube containing ~ 50 ml ice-cold extracellular solution plus soya bean trypsin inhibitor (*see* **Note 3**), protease inhibitors, and sodium pyruvate.
4. The sample is then immediately transported in this solution on ice to the laboratory. Crucially, the time from sampling to the start of cell isolation should be as brief as possible (ideally less than 10 minutes) to ensure good quality cell preparations. Failure to do so may result in poorly responsive cells that are either insensitive to agonists (cholecystokinin, acetylcholine) or require supramaximal stimulation to elicit responses e.g. oscillatory Ca^{2+} elevations.

2.2 Human pancreatic acinar cell isolation

1. Tissue with a high proportion of fat should be avoided. This is extremely important when loading with lipophilic dyes. Fatty tissue is elastic, pinkish and translucent, and will float in the extracellular solution. Using these identifying criteria, fatty tissue can be immediately recognised and removed to prevent lipid droplets from building up in the external solution / collagenase solution. Use a fresh surgical blade (size 15) or sharp scissors to remove all unwanted sections of the tissue.
2. Inject the resultant section of pancreas at several points with collagenase solution. In most cases, 1 ml of collagenase solution is used (*see* **Note** 4). Unlike the mouse pancreas, delivery to every portion of the pancreatic sample is impossible as the pancreatic ductal system is not intact following the initial surgical resection. To attempt to overcome this, the sample should be cut into fine pieces using a fresh surgical blade or sharp scissors so that the surface area exposed to the collagenase is as large as possible. The sample is then transferred into fresh collagenase solution to remove damaged cellular material and fat droplets.
3. Incubate the sample in a shaking water bath for 30 minutes at 37ºC.
4. Following digestion, add the pancreas sample to 5 ml standard extracellular solution. Manually agitate and disperse the suspension through pipette tips of progressively diminishing diameter. This is achieved by manually slicing the tip off 1 ml plastic pipette

tips, cut at a slight angle with a fresh surgical blade to give a sharp clean edge.

5. When the supernatant is cloudy, collect and transfer it to a fresh tube. The speed of this process is determined by many variations in tissue quality, collagenase digestion and ambient conditions but should be repeated until the supernatant stays clear upon agitation of the sample. The remaining bulk of the tissue should then remain white and fibrous. The collected supernatant now contains the final population of pancreatic acinar cells. Isolation of acini from other pancreatic cell types is achieved by two rounds of low-speed centrifugation.
6. Centrifuge the sample for 1 minute at 260 g and remove the supernatant by rapid inversion of the tube. 5 ml of fresh extracellular solution is then added and the pellet resuspended. Repeat this centrifugation step.
7. Resuspend the pellet in ~2 ml extracellular solution, filter using a 70 µm cell strainer to remove larger clumps of tissue, and centrifuge as before (*see* **Note 5).**
8. Finally, resuspend this pellet in ~2 ml of extracellular solution.
9. Cells can then be loaded with fluorescent dyes as required. Generally, loading conditions are similar to that of murine pancreatic acinar cells, but often require 35ºC.

2.3 Confirmation of human pancreatic acinar cell viability

Microscopic confirmation of the cells should always be performed in the first instance, as the quality of the cells will vary between patients (see all figures for examples of light transmitted images of human pancreatic acinar cells).

Cell viability was originally confirmed by the recording of transient cytosolic Ca^{2+} increases in response to cholecystokinin (10 pM), which were coupled to mitochondrial NAD(P)H increases (detected as autofluorescence) and amylase secretion (Figs. 2, 3); responses typical of freshly isolated murine pancreatic acinar cells. Ca^{2+} signals were shown to occur by direct stimulation of acinar cells by cholecystokinin and not via the indirect release of acetylcholine from neuronal tissue (that might have potentially adhered to the acinar cells during separation) by the addition of atropine and tetrodotoxin (3).

3. Notes

1. Avoid the use of polypropylene tubes for the isolation procedure. Polystyrene tubes increased the yield of the isolation significantly, possibly due to single cells and small clusters readily adhering to the polypropylene.
2. Wherever possible, the amount of time during which the pancreas is clamped should be kept to a minimum to prevent ischaemic damage.
3. Original protocols included the addition of 1 mM Benzamidine (Worthington), a cell membrane permeable trypsin inhibitor, to try to avoid any premature intracellular trypsinogen activation and features of acute pancreatitis. However, for simplicity this is no longer used in isolation procedures since if the tissue is of superior quality (as defined in 2.1), the cells should not show intracellular trypsin activation (Fig. 1).
4. During the injection, fat droplets can build up on the surface of the collagenase solution. In this case, the sample should be washed through external solution to remove any fat droplets and returned to a fresh aliquot of collagenase solution. Therefore it is advised to have surplus collagenase solution available.
5. Filtration will necessarily cause a reduction in the number of cells. If the final cell suspension tends to have little or no acinar cells, this step may be omitted to increase yield. In the case of confocal microscopy experiments, any remaining large clumps should not interfere with single cell recordings from acinar cells which can be easily identified visually.

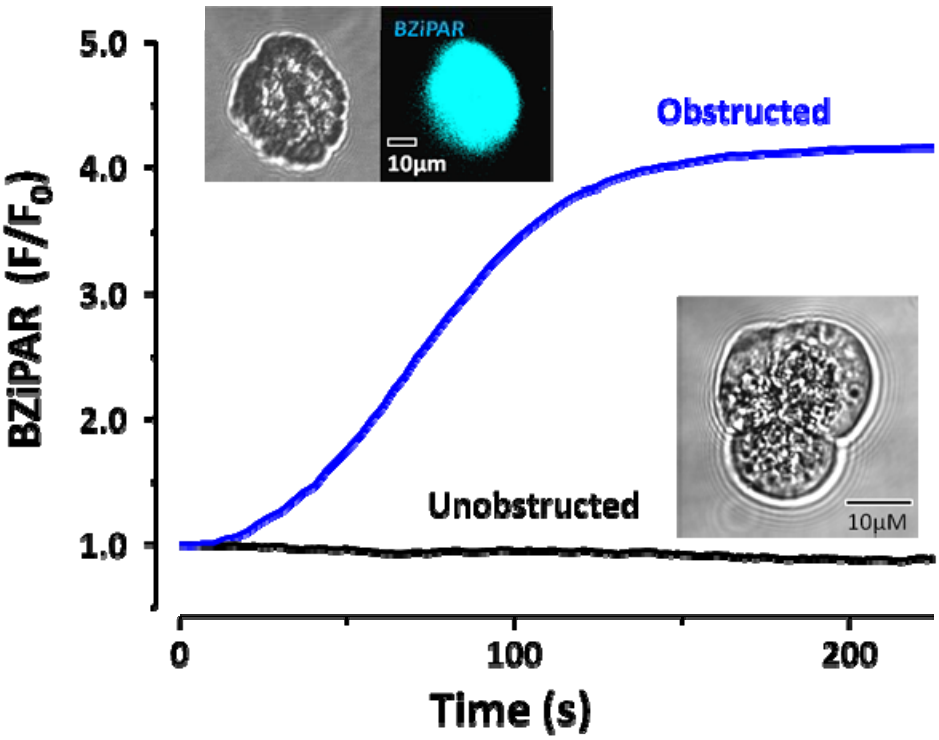

Figure 1. Morphology and integrity of isolated human pancreatic acinar cell clusters loaded with BZiPAR, a probe which fluoresces after cleavage by trypsin. Baseline BZiPAR trace (black) and accompanying inset shows no detectable trypsin activity and typical, well-preserved morphology of cells isolated from a sample of an unobstructed pancreas. Increased BZiPAR trace and inset display trypsin activity in cells with poorly defined morphology, typical of an acinar cell cluster isolated from an obstructed pancreas (Reproduced from ref 3).

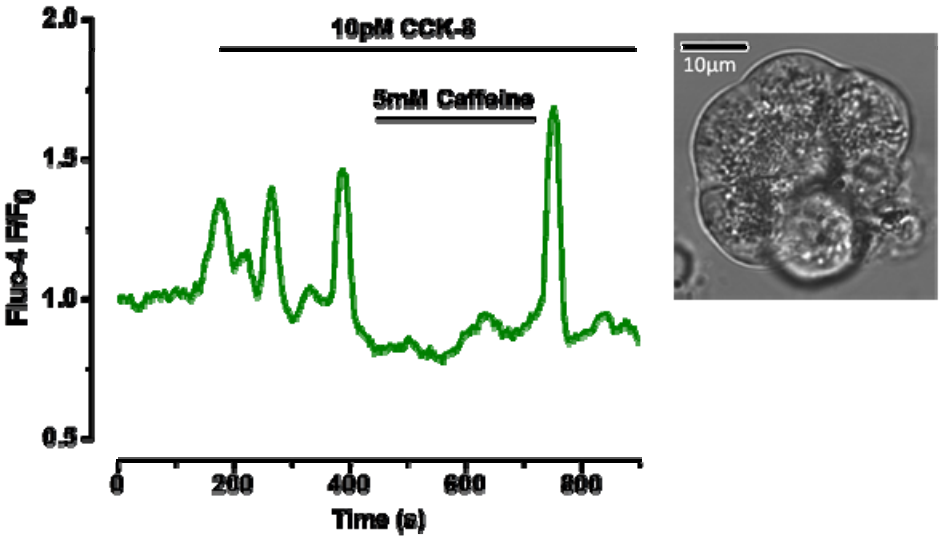

Figure 2. Direct cholecystokinin (CCK)-mediated cytosolic Ca2+ signals in isolated human pancreatic acinar cells. Application of a physiological concentration (10 pM) of CCK-8 evoked oscillatory increases of cytosolic Ca2+ within human pancreatic acinar cells. Experiments were performed in the presence of both atropine and tetrodotoxin (added to block any potential neural component to the response). Caffeine (5 mM), an IP3 receptor blocker, reversibly inhibited these signals (Reproduced from ref 3).

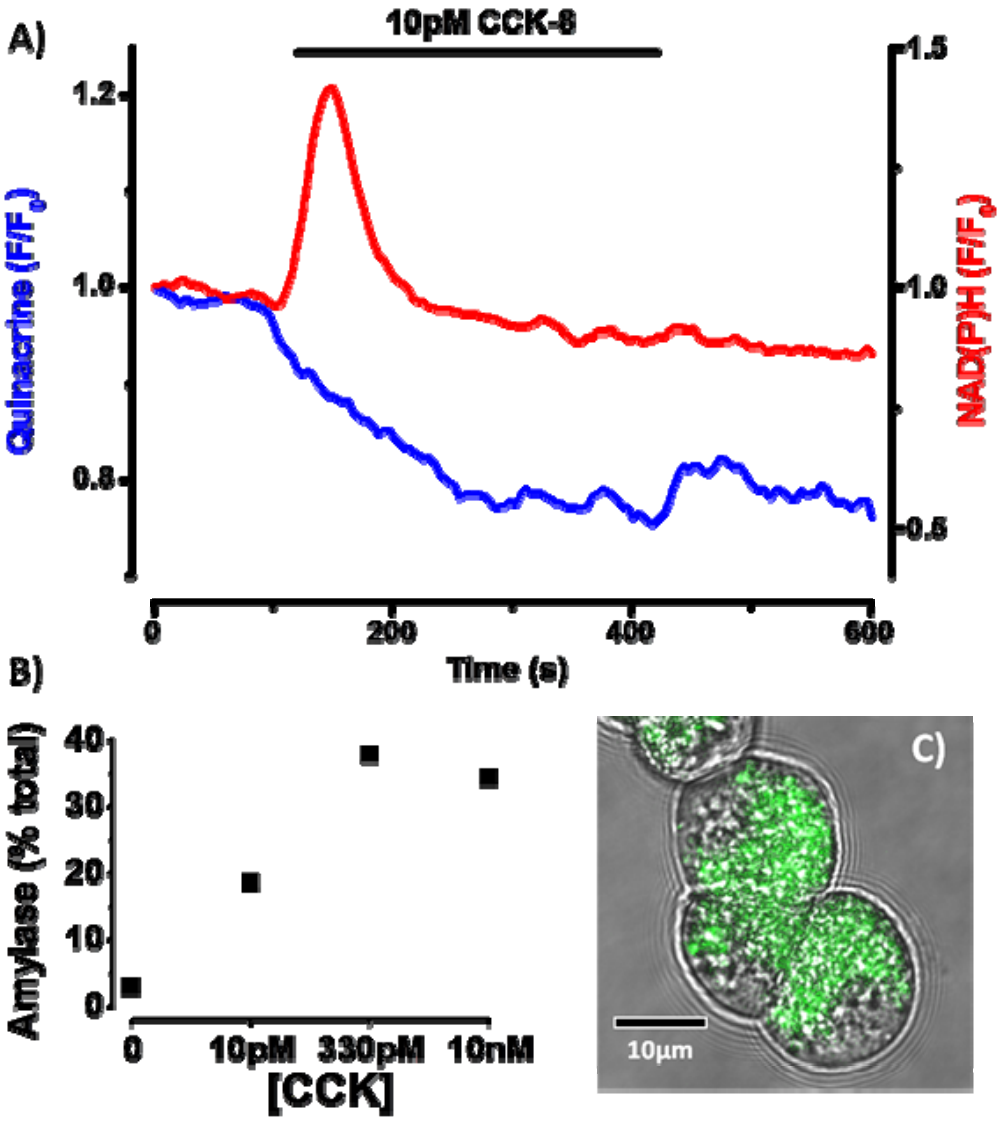

Figure 3. Secretory responses to cholecystokinin in isolated human pancreatic acinar cells. (A) Exocytosis was observed upon stimulation of isolated human pancreatic acinar cells with a representative physiological concentration of CCK-8 (10 pM), seen as a prompt decrease of quinacrine fluorescence from a steady baseline (blue), reflecting loss of quinacrine-containing zymogen granules; such decreases were not observed without stimulation. Inset shows typical quinacrine staining of zymogen granules. A coordinated increase in NAD(P)H autofluorescence (red) occurred at the start of stimulation, indicative of increased ATP production to fuel secretion. These experiments were performed in the presence of atropine and tetrodotoxin to prevent neurotransmitter release or effects that might theoretically occur from possible adherent nerve endings. (B) Concentration-dependent amylase secretion, expressed as a percentage of total amylase, evoked from freshly isolated human pancreatic acinar cells by stimulation with CCK-8 (10 pM – 10 nM) (Reproduced from ref 3).

4. References

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