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METHODS PAGE

Preparation of pancreatic fragments for studying the role of nerves and islets on pancreatic exocrine secretion

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Introduction

Pancreatic exocrine secretion is under neurohormonal regulation (1) **(Figure 1).** In order to completely assess the effects of bioactive agents on acinar cell secretion, it is imperative that the *in vitro* preparation employed should contain not only acinar cells, but also nerves and islets. Pancreatic lobules / fragments represent such a preparation. Nerve and islet function can be individually blocked by pharmacological agents to permit an appreciation of the indirect effects of the bioactive agent being studied on acinar cell secretion.

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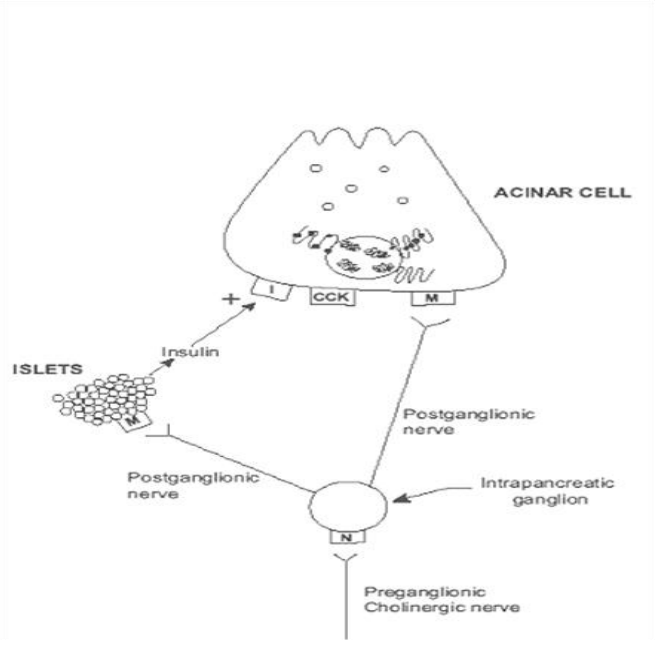

Figure 1. Diagrammatic representation of the neurohormonal regulation of pancreatic exocrine secretion. Preganglionic cholinergic nerves synapse with the nicotinic receptors (N) on intra-pancreatic ganglia. The postganglionic cholinergic nerves synapse with either muscarinic receptors (M) directly on the acinar cells or on the islets. Acetylcholine (Ach) functions as the predominant neurotransmitter in this system. Insulin exerts a potentiative effect (+) on amylase release from the acinar cells. Cholecystokinin (CCK) can act indirectly through the central nervous system or directly via receptors on acinar cells.

1. Materials

1. General reagents (e.g. NaCl) obtained from several reputable suppliers
2. Dextran (MW, 64,000-76,000; Sigma-Aldrich)
3. Soybean trypsin-chymotrypsin inhibitor, (T-9777, Sigma-Aldrich)
4. Protease inhibitor cocktail (P 8340; Sigma-Aldrich)
5. Bovine serum albumin (Fraction V (96-99%), RIA grade, Sigma-Aldrich)
6. Standard 24 well cell culture plate (Apogent, Roskilde, Denmark)

2. Methods

2.1 Pancreatic fragments

The protocol described here is for the preparation of mouse pancreatic fragments. It differs from the previously published protocols by Scheele & Palade (2), and Flowe *et al*. (3) in that the pancreatic lobules are not specifically harvested by dissection with the aid of a microscope. The main advantage of fragments over lobules is that a large amount can be prepared quickly without the need for careful dissection. The preparation of lobules requires dissection skill and still produces some damaged tissue. The procedure described here would be applicable to other species. The lobule is the functional unit present in the tissue fragments.

2.2 Preparation of lobules / fragments

Euthanize 6 Swiss mice (25-30 g body weight) by cervical dislocation and harvest their pancreata (Note 1). The pancreas is then weighed, rinsed in ice-cold oxygenated modified Krebs solution (Krebs buffer - 133.4 mM NaCl, 4.7 mM KCl, 1.3 mM $NaH_2PO_4.2H_2O$, 16.3 mM $NaHCO_3$, 7.7 mM D-Glucose, 0.6 mM $MgSO_4.7H_2O$, 2.12 mM $CaCl_2.2H_2O$) supplemented with 1.5 % dextran (4), protease inhibitor cocktail (0.2 ml/l) and trypsin-chymotrypsin inhibitor (2 mg/l)(referred to as Dex-Krebs buffer) and transferred to a Petri dish on ice where it is finely minced with iris scissors (Note 2). The minced tissue is then transferred to a 50 ml container where it was washed 2-3 times in 20 ml of cold oxygenated Dex-Krebs buffer and then finally resuspended in 30 ml of cold oxygenated Dex-Krebs buffer. The process is repeated for each mouse and the minced tissue from all animals pooled.

2.3 Incubation

Approximately 40 mg wet weight of pancreatic fragment preparation suspended in 0.5 ml Dex-Krebs buffer is added to each well of a 24-well cell culture plate containing up to 10 µl of varying concentrations of bioactive agent (e.g. peptides), alone and in combinations and then incubated in the airtight, plastic box previously gassed with 5 % carbon dioxide / 95 % oxygen for 60 min at 37 ^{o}C in a shaking water bath (at 60 oscillations/min) (Note 3). Control incubations consist of fragments incubated in vehicle (e.g. Dex-Krebs buffer alone). In experiments where the effect of toxins or inhibitors (e.g. tetrodotoxin, atropine, hexamethonium, KCl, diazoxide) is to be assessed, the fragments are pre-incubated with the desired concentrations of these agents for 30 min before adding other peptides and the incubations continued for a further 60 min. All incubations are performed in at least duplicate. Fragments are sampled separately at 0 time to determine amylase release prior to the experimental period. These values are then subtracted from the final values in each experiment to derive the net amylase release for the incubation period. Following the incubation period, the fragments are transferred to 1.5 ml snap-top tubes on ice and centrifuged at 14,000 rpm for 30 sec at 4 ^{o}C. The supernatant is aspirated and used to measure amylase release. Lysis buffer (0.1 ml) containing 0.1 M NaH_2PO_4, 0.1 M Na_2HPO_4 and 0.1 % sodium dodecyl sulphate, is added to the pellet and the 1.5 ml snap-top tube is frozen (-80 ^{o}C) for 20-30 min and then thawed. The thawed pellet is then homogenized manually using a pestle and the homogenate is centrifuged at 14,000 rpm for 10 min at 4 ^{o}C. The supernatant (lysate) is transferred to another 1.5 ml snap-top tube. The pellet is re-homogenized after adding 0.1 ml of

saline and re-centrifuged as outlined above and this supernatant is combined with the previous one, mixed and stored at -20 ^{o}C prior to amylase assay (3, 5). With this protocol, amylase secretion is linear over 90 min **(Figure 2).**

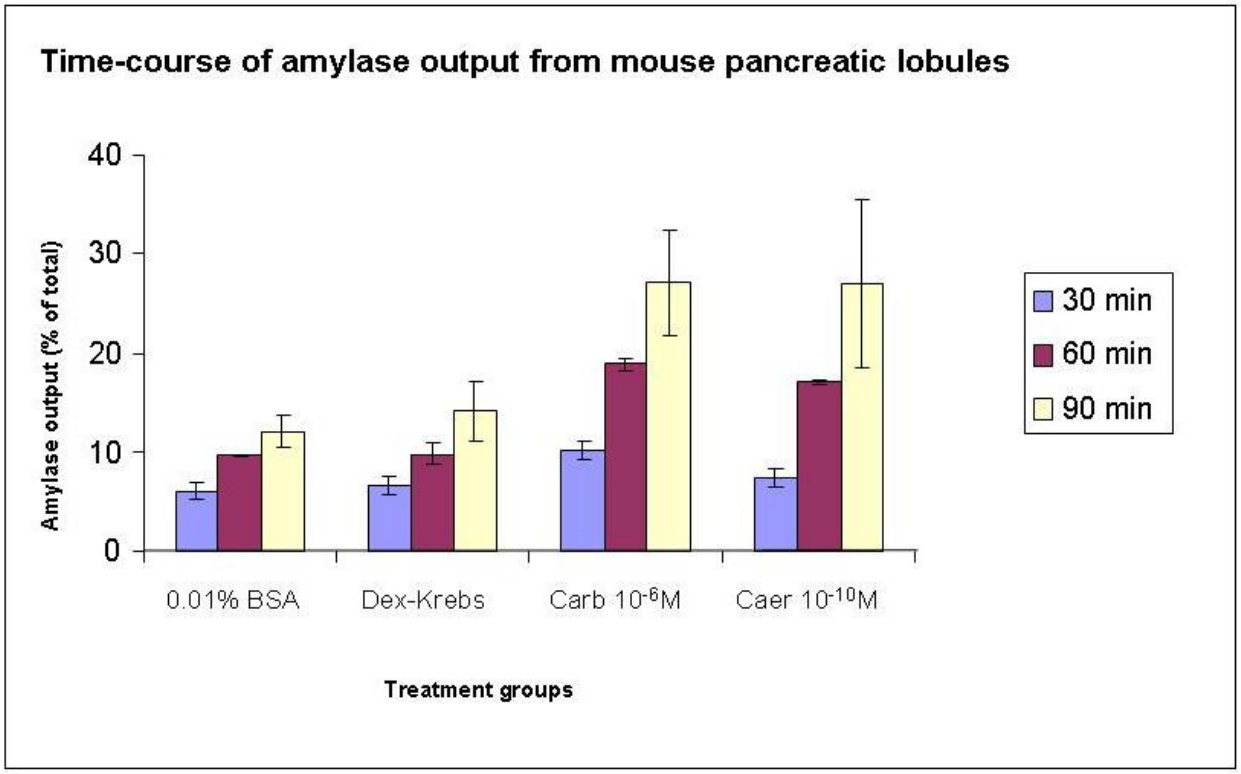

Figure 2. Time-course of amylase release from mouse pancreatic fragments using secretagogues like carbachol (Carb) and caerulein (Caer) as well as the vehicles for peptides (BSA) and the medium (Dex-krebs). n=5 per group.

2.4 Amylase Assay

The following method is designed for use with automatic analysers that are available in the hospital clinical chemistry department. The incubation medium is diluted (1 to 30) and the cell lysate is diluted (1 to 50) with saline containing 0.01 % bovine serum albumin (to stabilize enzyme activity) immediately prior assay. Samples are assayed for amylase activity (IU/l) by an enzymatic colorimetric assay using a Hitachi 917 automatic analyzer (Hitachi High-Technologies Corporation, Tokyo, Japan). The total lobule amylase activity (per well) is the sum of the incubation medium and lysate amylase activities. The amylase released for each well is expressed as a percentage of the total lobule amylase per well. The net amylase release (per 60 min) is then calculated by subtracting the pre-incubation amylase release (i.e. 0 time samples, expressed as % of total) from the amylase released per well. For graphical presentation, the data is expressed as % of control **(Figure 3).** The group data usually requires data from 4-5 experiments for adequate precision.

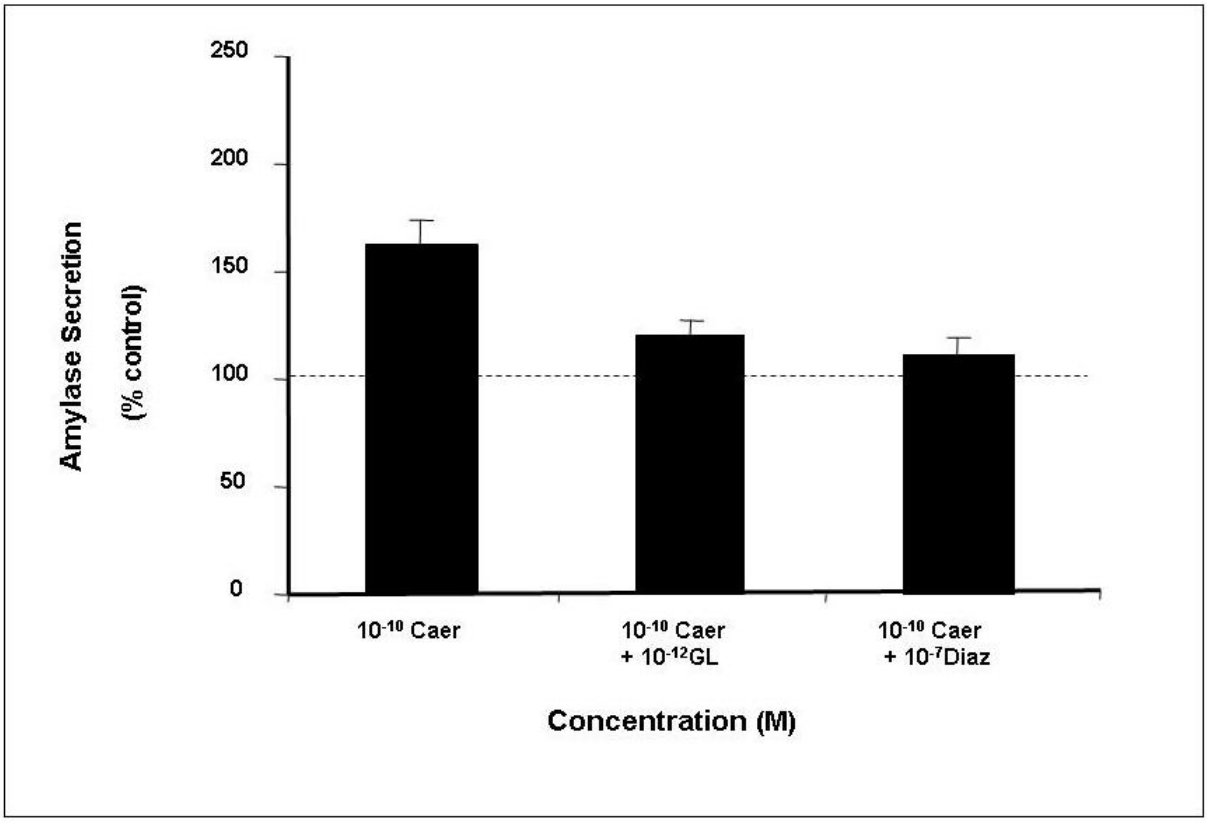

Figure 3. **Graphical presentation of data showing inhibition of caerulein-stimulated amylase release by galanin and diazoxide. The concentrations of caerulein (Caer 10^{-10} M), galanin (GL10^{-12} M) and diazoxide (Diaz 10^{-7} M) are represented on the X-axis.** Amylase release as a % of control is expressed on the Y-axis. Control (100 %) is indicated as a dashed line (--). n=4 per group. (Figure modified from Barreto SG, et al. Am J Physiol Gastrointest Liver Physiol 2009; 297: G333-339).

3. Notes

1. We use cervical dislocation for euthanasia since agents injected into the abdomen might compromise some tissue viability. An alternative could be carbon dioxide asphyxia, although we have not tested this.

2. Our experience indicates that it is best not to mince the tissue into very small pieces. Our guide was the pipette tip internal diameter used to aliquot the suspension. We used a regular 1 ml pipette tip and cut off the end about 1 cm from the original tip to give a bigger tip internal diameter of about 2 mm. The mince tissue suspension should be able to be aliquoted with this cut pipette tip.

3. The box we use to hold the 24-well plate is a plastic lunch or storage box that has a gas-tight seal. We add distilled water (about 100 ml) to the box such that when the 24-well plate is placed in the box the water level should not exceed half the height of the culture plate. This will ensure that water inadvertently does not enter the culture plate at anytime during the incubation period. The box containing the water is pre-warmed (37 ^{o}C at 60 oscillations/min for a minimum of 30 min) prior to the insertion of the 24-well plate containing the suspension of pancreatic fragments. We place inert material around and on top of plate to minimize movement of the plate within the box during the incubation period.

4. References

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