

# MONOPHASIC STATE OF INSULIN SECRETION IN ISOLATED RAT ISLETS OF LANGERHANS BY $K^+$ , $Ca^{2+}$ AND GLUCOSE

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## ABSTRACT

Isolation of rat islets of Langerhans was carried out by using collagenase and the destruction of exocrine tissue was performed through the injection of Hanks' solution into the common bile duct. The identification of the islets was done by techniques including the use of a stereomicroscope against a black background. Vital staining was done by an injection of neutral red solution through the abdominal aorta to observe the red tinted islets. Immunofluorescent staining with anti-insulin was done to identify islets and nuclear staining was done with propidium iodide. The microorgans were detected by a confocal microscope equipped with a laser source. The secretory activities of the islets was investigated by *in vitro* measurement of insulin and the effects of various concentrations of glucose, potassium and calcium ions were studied. It was found that the insulin secretion by these agents obeyed a monophasic trend which points to the depolarization effect on cytoplasmic membrane induced by these agents.

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## INTRODUCTION

In most animals, the cells of the endocrine section of the pancreas are grouped into numerous, small clusters called islets of Langerhans. These islets are approximately one million in human pancreas and about 2-3 thousand in most rodents and constitute 1% of the total gland's volume.<sup>1,2</sup> The components of pancreatic tissue, the acinar, tubular and the islets are held together by an extracellular matrix (ECM) in which the main constituent materials are collagen, glycoproteins and proteoglycans. To obtain the islets, the pancreatic tissue is separated by enzyme from ECM. Care should be taken to obtain the islets intact in this process.<sup>3</sup> For obtaining high yields of islets, it is essential to digest the collagen of connective

tissue by using collagenase.

Apparently the most reliable way to detect the vital capacity of the system is the response obtained in insulin secretion following the use of glucose. High concentrations of glucose and potassium ion can induce depolarization of the cytoplasmic membrane, which is a signal for the release of insulin from  $\beta$ -cells. Ion channels in the beta cell regulate electrical and secretory activity by controlling the permeability to  $K^+$  and  $Ca^{2+}$ .<sup>3</sup>  $Ca^{2+}$  influx through voltage-activated L-type  $Ca^{2+}$  channels serves as the major stimulatory signal in insulin secreting cells.<sup>6</sup>

One of the main goals of this research was to set up a method for identification of islets. This was performed by different techniques including vital staining, immunofluorescent staining with anti-insulin antibody, and nuclear staining.<sup>4</sup> The other purpose of this study was to use the static method as a simple procedure to study insulin secretion.

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# Effect of Glucose, K<sup>+</sup> and Ca<sup>++</sup> on Insulin Secretion

## MATERIALS AND METHODS

### Isolation of islets of Langerhans

Male rats with an average weight of 200-250 g were anesthetized by ether or chloroform or by subcutaneous injection of Nembutal. The common bile duct was cannulated by an angiocatheter and 15 mL of Hanks' solution was instilled to produce distention of the pancreas. The pancreas was immediately dissected out, immersed in a cold Krebs-Ringer-Bicarbonate-Hydroxyethyl Piperazine Ethanesulfonic acid (HEPES) buffer containing 2.8mM glucose (KRB-H-Glu) in an ice bath. The pancreas was then cut into 1mm pieces and centrifuged twice at 500 g for one minute. The precipitated tissue was then mixed with an equal volume of KRB-H-Glu (2.8mM) containing 500 units/mL collagenase. The mixture was mixed by swirling at 37°C in a water bath for complete digestion. The end point for the completion of digestion is to observe the disappearance of the tissue fibers and the adhesion of tissue to the walls of the centrifuge tubes. The end point could also be identified by stereomicroscope or a phase contrast microscope. The optimal time of digestion was about 20 minutes.

Immediately after digestion, the tissue was washed 3 times (150 g, 30 sec; 80 g, 30 sec and 80 g, 30 sec) with 15-20 mL of cold KRB-H-Glu containing 0.5% bovine serum albumin (BSA). This process could, on one hand, prevent the tissue from over-digestion and, on the other hand, separate the islets from the rest of the tissue and make them ready for the next steps.<sup>1,2</sup>

### Different techniques for the identification of islets

**A. Vital staining:** The rats were anesthetized, the abdominal aorta was cannulated below the renal arteries and the inferior vena cava (IVC) was cut. Subsequently 60 mL of neutral red (1: 15000 in NaCl) was injected into the aorta until the pancreas was distended. Isolation of islets was carried out and separated by hand-picking method.

**B. Immunofluorescent staining with anti-insulin antibody:** The islets were incubated overnight in formalin solution prepared in siliconized tubes. The islets were then washed with phosphate buffered saline (PBS) solution containing 40 mM ammonium chloride. After centrifugation at 3000 rpm for 3 min. the precipitated islets were incubated in 500 µL of first specific antibody (anti-insulin) prepared with a dilution of 1: 100 in NET/gel (150 mM NaCl, 5 mM EDTA, 50 mM Tris pH= 7.4 and 0.1% gelatin and 0.1% triton X-100) for 60 minutes with racking. This step was eliminated in control tubes. The islets were washed (3X) with NET/gel and then incubated in 500 µL of second antibody (FITC-conjugated anti-guinea pig Ig antibody raised in rabbit) prepared with a dilution of 1: 500 in NET/gel for 60 minutes with racking.

The islets were washed similarly as the last step and the precipitate was then suspended in 40 mL distilled water.

The suspension was then spread onto a polylysine coated coverslip and placed onto a microscope slide with a drop of fluorescent preserving mounting fluid (FPM) to be studied in a laser confocal microscope.

**C. Nuclear staining with propidium iodide (PI):** As described in section (B), after incubation of the islets in a solution of NET/gel and centrifugation at 3000 rpm for 3 min. the precipitate was suspended in a 500 µL solution of PI/TERNase.\* The rest of the steps were similar to part B (after the application of the 2nd antibody).

### Effects of glucose, potassium and calcium ions on insulin secretion of islets of Langerhans via static method

In the static method, using hand-picking techniques, the collagenase digested pancreas tissue was transferred to a petri dish with a black background and subsequently viewed under a stereomicroscope. By using a Pasteur pipette clusters of 10 islets were collected in siliconized tubes of 12 × 75 mm.

Initially, they were washed 3 times with KRB-H-BSA-Glu (2.8 mM), and centrifuged each time at 500 g for 1 min. The islets were then incubated for a period of 90 minutes (3 × 30 min) in one milliliter of the base KRB-H-BSA containing 3 different glucose concentrations (0, 2.8 mM, and 16.7 mM). The incubation was carried out at 37°C in 5% CO<sub>2</sub> atmosphere. After each 30 minutes of incubation the tubes were placed in an ice bath with constant shaking followed by centrifugation at 1500 g for 10 minutes.

After each step 200 µL of the supernatant was preserved frozen for assay of insulin by RIA and 200 µL of the same buffer was added to it.<sup>1,7</sup>

Similar measurements were made for K<sup>+</sup> in a concentration of 5.94 mM and 40 mM and for Ca<sup>2+</sup> in concentrations of zero and 20 mM.

## RESULTS

Destruction of exocrine tissue by Hanks' solution instillation through the common bile duct resulted in an increase in the number of islets. This process is essential in the isolation of islets.<sup>2</sup>

Vital staining of the islets by neutral red dye and morphological study by stereomicroscope and phase contrast microscope facilitate the distinction between islets and exocrine tissue.

By immunofluorescent staining with anti-insulin antibody the islets could be identified as green spots at insulin sites of higher concentrations (Fig. 1). Nuclear

\*For preparation of 20 mL PI/TERNase: Tris-HCl (1M) + 40 µL EDTA (0.5M) + 40 µL RNase (10 mg/mL) + 200 µL PI (0.5 mg/mL) with dH<sub>2</sub>O to 20 mL volume.

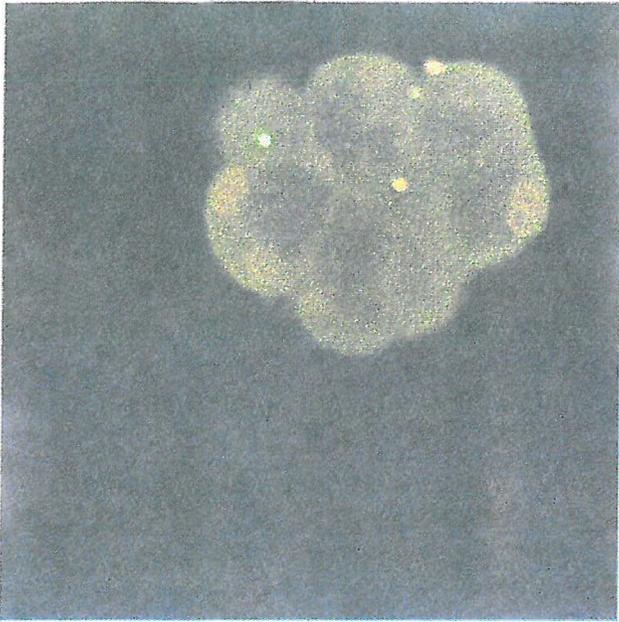


Fig. 1. Laser photograph (Argon source) of islets of Langerhans by confocal microscope. Intracellular staining with anti-insulin under permeabilization conditions.

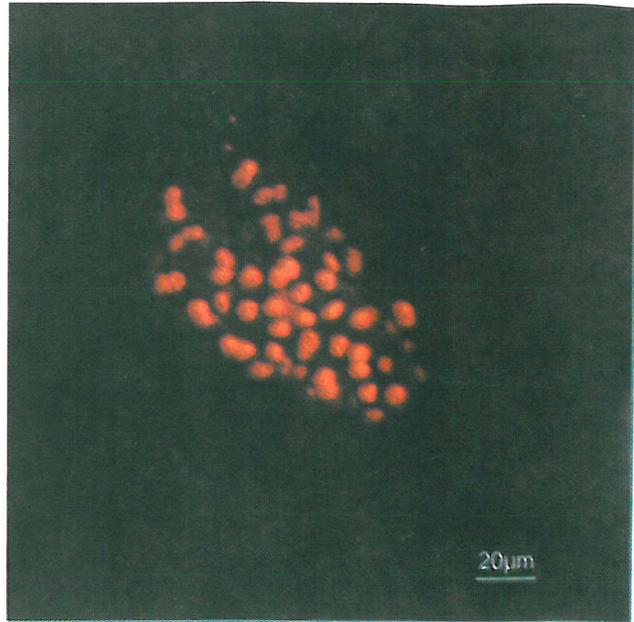


Fig. 3. Laser photograph (Argon source) of the nuclei of an islet (oval) by confocal microscope, principally stained as in Fig. 1.

tion of insulin secretion is due to an increase in the concentration of each of the effectors.

## DISCUSSION

The isolation of islets by hand-picking method was found to be a simple and fast route to obtain islets. The best incubation time for enzyme digestion was estimated to be about 20 minutes with 500 units/mL of the enzyme plus KRB-HEPES. At the end of this period, the islets were found to be completely separated and could be isolated under a stereomicroscope.

Immunofluorescence and nucleus staining of islets under intracellular and permeabilization conditions permit staining components to penetrate this micro-organ and react with antigen (insulin) and cellular DNA, respectively. This causes a three-dimensional and clear picture of different layers of islets under laser confocal microscopy. The effects of glucose, calcium and potassium ion on insulin secretion using the static method showed that not only could these effectors act as activators of insulin secretion but also they could point to the fact that the islets had remained intact. Similar to other endocrine cells, calcium acts as a trigger of the exocytosis process in  $\beta$ -cells of islets.<sup>8,9</sup> By controlling cytosolic free- $\text{Ca}^{2+}$  levels, calcium channels play an important role in transducing the initial stimulus to the effector systems that modulate secretion.<sup>10</sup> High concentrations of glucose and  $\text{K}^+$  that cause depolarization of the cytoplasmic membrane could signal the release of insulin from  $\beta$ -cells.<sup>11</sup>

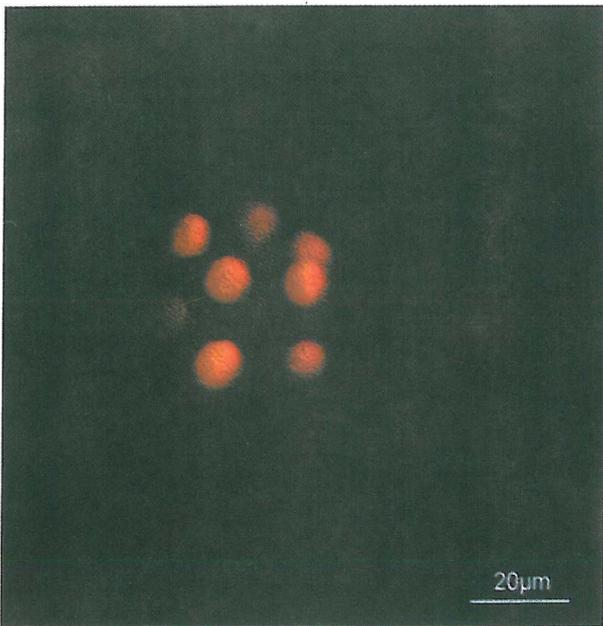


Fig. 2. Laser photograph (Argon source) of the nuclei of an islet (circular) by confocal microscope, stained as in Fig. 1.

staining by propidium iodide (PI) was performed as an alternative way for identification of the islets (Figs. 2 and 3).

*In vitro* studies show the release of insulin from islets of Langerhans employing the static method of determination with different concentrations of glucose (Fig. 4a), potassium (Fig. 4b), and calcium (Fig. 4c). As shown, the eleva-

## Effect of Glucose, K<sup>+</sup> and Ca<sup>++</sup> on Insulin Secretion

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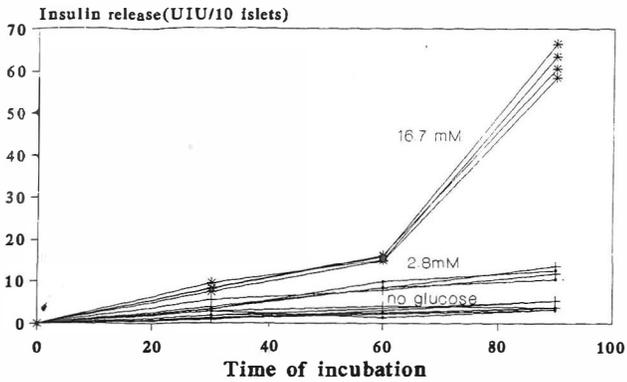


Fig. 4, a. Insulin release by islet incubation in the presence of different concentrations of glucose.

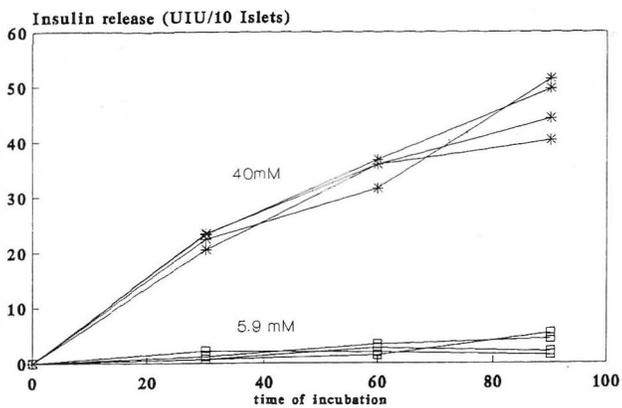


Fig. 4, b. Insulin release by islet incubation in the presence of K<sup>+</sup>.

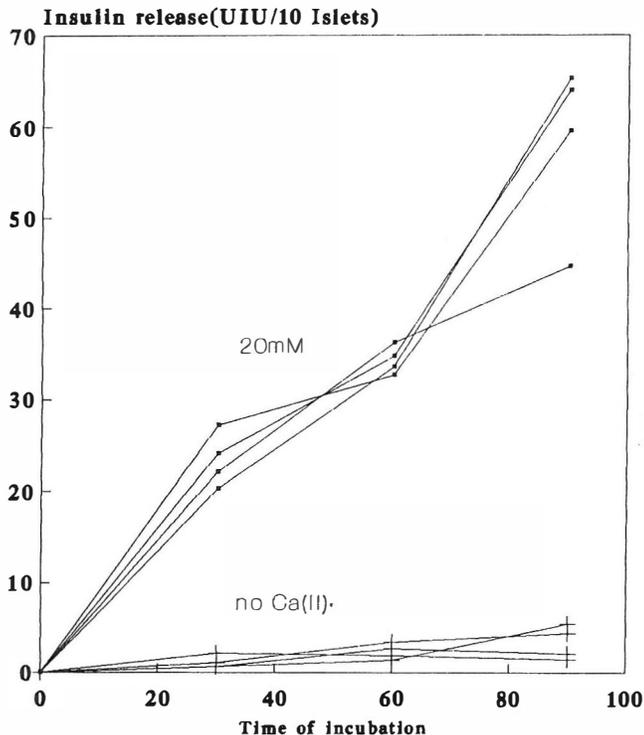


Fig. 4, c. Insulin release by islet incubation in the presence of Ca<sup>2+</sup>.