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INSULIN SECRETION FROM ISOLATED PURE BETA CELLS

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Summary - Isolation of pure beta cells of the rat pancreas was achieved employing counterflow sedimentation technique (CST) followed by density gradient centrifugation technique (DGCT). The proportion of non-endocrine cells to beta cells was minimal (1 acinar cell in 296 beta cells, and 1 duct cell in 300 beta cells) with total absence of alpha and delta cells. Oxidation of D-(U-^{14}C) glucose to $^{14}CO_2$ by the isolated beta cells was linear to time. Glucagon (1, 5, or 10 nM) or arginine (1, 5 or 10 mM) produced concentration dependent insulin secretion. Thus, a highly purified preparation of isolated beta cells of rat pancreas could be obtained with excellent morphologic, metabolic and functional integrity.

Although interaction between the adjacent cells of the endocrine pancreas has been suspected on the basis of studies of effects of various hormones (1-4), the exact role of adjacent cells in modulation of insulin secretion has not been clearly defined because of lack of preparations of pure pancreatic cells. The present report describes the technique of obtaining a highly purified preparation of beta cells of adult rat pancreas suitable for study of insulin secretion.

MATERIALS AND METHODS

Starting Sample Suspension: Sequential dissociation of the adult rat pancreas was achieved by incubation of lobules with collagenase (C-0130, sigma) 240 Units in 3 ml phosphate buffer (P.B., NaCl, 118 mM; KCl, 5.37 mM; $Na_2HPO_4 \cdot 2H_2O$, 1.9 mM; $NaH_2\ PO_4 \cdot H_2O$, 0.44 mM; and dextrose 3.9 mM per liter) then in 160 Units in 2 ml PB for 20 minutes, and finally in 240 Units in 3 ml PB for 20 minutes at 37°C with O_2 as a gas phase. The dissociated cells were pipetted after each incubation period, washed twice with 5 ml PB and resuspended in 2 ml PB plus 1% bovine serum albumin (BSA). The washed cells were pooled and the total volume adjusted to 7 ml.

Isolation of Beta Cells: The isolation of pure beta cells was achieved by two step procedure (5). In the first stage, predominantly endocrine fraction of the

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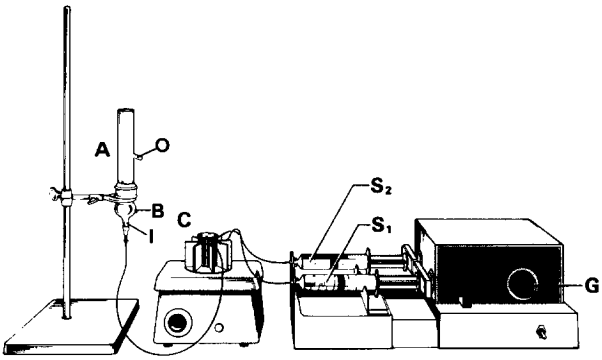


Figure 1: Diagramatic presentation of the equipment employed for counterflow sedimentation technique (CST). A - glass column, B - reservoir, I - inlet, O - side arm, G - gradient former, S_1 and S_2 - syringes containing KRB-I and 5% (w/w) Ficoll solutions, C - mixing chamber mounted on magnetic stirrer.

cell suspension was obtained at the exclusion of acinar cells and cell clumps employing counterflow sedimentation technique (CST). In the second stage pure beta cells were isolated employing density gradient centrifugation technique (DGCT).

Counterflow Sedimentation Technique (CST): The equipment employed for CST (Fig. 1) consisted of a glass column "A" with a reservoir "B" which was connected to the mixing chamber "C" at inlet "I" employing plastic tubing (2 mm diameter). Syringe "S_1" (50 ml) of Beckman gradient former "G" (Model 350 052) was filled with Kreb's ringer bicarbonate buffer without Ca^{++} and Mg^{++} (KRB-I) which was injected to displace air from the system up to the inlet "I". Syringe "S_2" (50 ml) was filled with 5% Ficoll solution (w/w, in KRB plus 1% BSA). The starting cell suspension was layered in the bulb "B" and the gradient former was switched on at low speed. After the formation of 31ml of continuous gradient (KRB & 5% Ficoll), the Beckman gradient former was switched off and the plastic tubing connecting inlet "I" to mixing chamber "C" was connected to the syringe (50 ml containing 5% Ficoll in KRB plus 1% BSA) of a constant speed infusion pump with a help of a two-way stop cock. 8 ml fraction of the cell suspension running out of the side arm "O" was collected, washed with KRB-I plus 1% BSA, and employed for further isolation of beta cells. This fraction of cell suspension contained 90-94% endocrine cells with remaining acinar and duct cells.

Density Gradient Centrifugation Technique (DGCT): A continuous density gradient of Ficoll (5% - 30% w/w in KRB plus 1% BSA) was prepared in cellulose nitrate tube employing Beckman gradient former. The endocrine cell suspension (7 ml) was layered on the gradient and it was centrifuged at 13,200 g for 30 minutes in Beckman L2-65B ultracentrifuge with SW27 rotor. The cells were observed to be distributed in four zones. A thin, cloudy uppermost layer containing alpha cells, delta cells, cell debris, and duct cells and the second thin layer containing cell debris were discarded. Subsequent 12 ml fraction of the gradient containing pure beta cells was removed, washed twice with KRB-I at 550 g for 10 minutes, resuspended in KRB-II (KRB-I plus Ca^{++} and Mg^{++} and 1% BSA) and employed for further studies. The acinar cells and few beta cells were observed in the fourth zone.

Morphology

The cell pellets were prepared in 2 ml plastic conical tubes by centrifugation, fixed in Bouin's solution, dehydrated and embedded in paraffin. Sections (5 μ) were mounted, rehydrated and employed for differential staining with aldehyde

fuchsin stain. Modified Karnovsky fixative was layered on cell pellets and the cells processed for electron microscopy (EM) (6). Antiinsulin, antiglucagon, antisomatostatin, and antigastrin antibodies were employed for indirect immunofluorescence staining (7) of the paraffin sections of the beta cells. The sections were examined under Leitz Dialux 20 EB fluorescence microscope with K2 filter.

Hormone and Enzyme Content:

Acid-ethanol extracts of the cell pellets were prepared by adding 1 ml acid-ethanol solution to the cell pellets and content of insulin, glucagon and gastrin was determined by radioimmunoassay (RIA). Lyophilized acetic acid extracts of cell pellets were prepared and sent to Dr. Akira Arimura, M.D., Professor of Medicine, Tulane University School of Medicine, New Orleans, Louisiana for determination of somatostatin content by RIA. Amylase content of the supernatants of the cell preparations ruptured by sonication was determined (8).

D-(U-^{14}C) glucose oxidation: 1 ml aliquots of beta cells preincubated for 60 minutes were distributed in 6 flasks containing glass vials in central wells. One μci in 0.1 ml D-(U-^{14}C) glucose (sp. activity 4.5 MCi/mM) was added to each flask. The flasks were capped and incubated for 15 minutes at 37°C and 70 oscillations per minute. The contents of the flask were acidified and $^{14}CO_2$ collected by injecting hyamine in the glass vials. After one hour the radioactivity of the aliquots obtained from glass vials was determined.

Insulin Secretion:

In five experiments, 0.5 ml cell suspension was distributed in 8 flasks. Arginine in 0.5 ml KRB-II was added to each flask to achieve a final concentration of 1,5 or 10 mM. The flasks were incubated at 37°C, 70 oscillations per minute with 95% O_2 and 5% CO_2 as a gas phase for 15, 30 or 60 minutes. The incubation media were separated from the cell pellets and acid ethanol extracts of cell pellets were prepared. Insulin content of the incubation media and the acid ethanol extracts was determined by RIA. The results were expressed as percent of the total cellular insulin released into the media.

In five experiments each insulin secretion was studied with glucagon and arginine employing perifusion technique.

RESULTS

The isolated pure beta cells were found to be round, dense with clear sharp cell membranes by light microscopy and stained uniformly purple by differential staining procedure. The beta cell to acinar cell proportion was 296:1 and to duct cells it was 300:1. Other endocrine cells were totally absent. The beta cells were morphologically intact by EM (Fig. 2). Indirect immunofluorescence staining of the cells indicated cells stained uniformly with antiinsulin antibodies but not with antiglucagon, antisomatostatin or antigastrin antibodies. On an average 40 ± 5 X 10^3 (M ± SE, N=10) beta cells were obtained per preparation. The mean insulin content of the preparation was 19,160 μu (range 14,800 to 21,000 μu, N=10). The glucagon content was less than the sensitivity of the assay system and

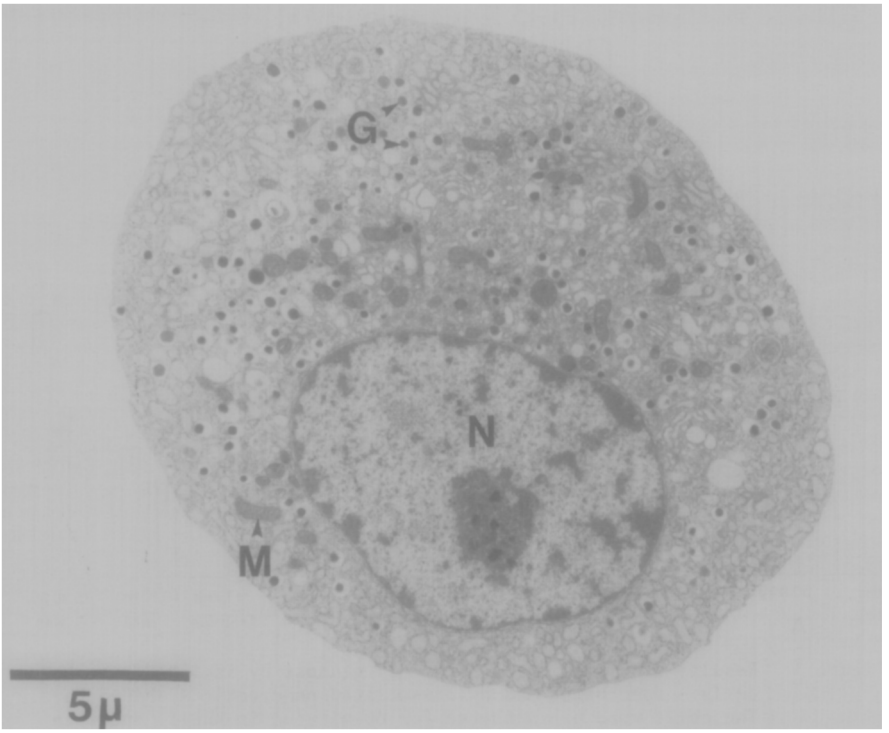
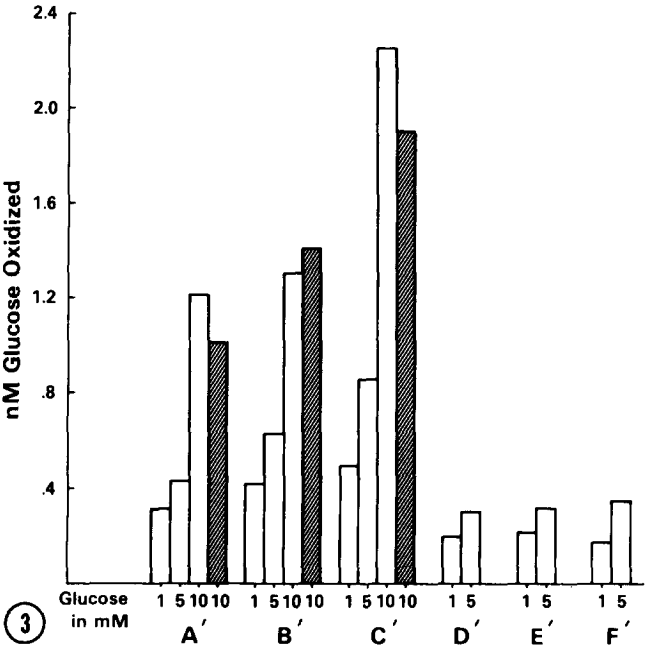


Figure 2: Electronmicrograph of the isolated beta cell (x 6000). N - nucleus, G - granules, M - mitochondrion.

the mean somatostatin content was 0.273 ng (range 0.148 to 0.575 ng). Amylase was not detected in the preparation. The utilization of D-(U-^{14}C) glucose and release of $^{14}CO_2$ was progressive with time (Fig 3).

In incubation studies, arginine produced concentration dependent insulin release (1 mm, 5 mm and 10 mm produced 9.3% ± 0.83, 12.27% ± 0.21 and 21.17% ± 2.91, M ± SE after 15 minute incubation, Fig. 4).

Similar results were obtained after 30 and 60 minutes incubation. The response to arginine was progressive with time (1 mM released 9.3 ± 8.3%, 10.47 ± 1.2% and 14.17 ± 1.58%, M ± SE insulin after 15, 30 and 60 minutes). In perifusion studies arginine (1, 5 or 10 mM) stimulated insulin release (7.5%, 29.7%, 79.5% and 71.5%) above basal levels. Glucagon (1, 5 or 10 nM) produced concentration dependent secretion of insulin (26.25%, 58% and 77% respectively) above the basal level (Table 1).



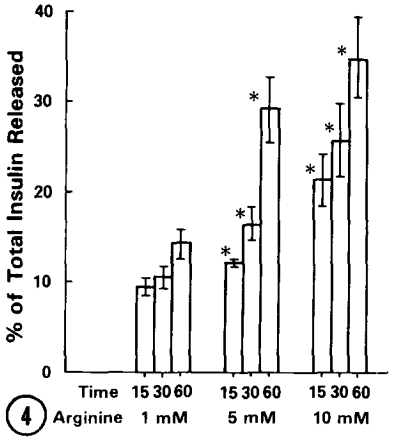


Figure 3: Results of oxidation of D-(U-^{14}C) glucose by isolated pure beta cells - open bars, and by dissociated pancreatic - shaded bars. The cells were preincubated for 60 minutes in KRB-II and then incubated in KRB-II for 15 min. - A; 30 min. - B; or 60 min. - C. They were preincubated for 60 min. in M-199 and then incubated in M-199 for 15 min. - D; 30 min. - E; and 60 min. - F.

Figure 4: Results of the study of insulin release from isolated beta cells incubated with arginine 1, 5, or 10 mM for 15, 30 or 60 minutes. * $p < 0.05$ when compared to the corresponding 1 mM value. I - M ± SE.

DISCUSSION

The preponderance of acinar cells over the small number of endocrine cells, a rapid aggregation of the dissociated cells and the streaming of cells in the gradients had made isolation of pure endocrine cells unsuccessful by commonly

TABLE 1

ARGININE & GLUCAGON STIMULATED INSULIN SECRETION FROM PERIFUSED BETA CELLS

ARGININE		GLUCAGON	
1 mM	7.2 ± 2.2	1 nM	26.25 ± 4.5
5 mM	29.7 ± 3.4	5 nM	58.0 ± 5.6
10 mM	79.5 ± 7.5	10 nM	77.0 ± 6.5
20 mM	71.5 ± 6.5		

Results expressed as % increase over the basal insulin secretion with respective concentrations of the stimulant.

employed techniques of cell isolation. Retrograde development of gradient of CST prevented reaggregated cells and a large number of acinar cells from interfering in separation of endocrine cells. Thus, suspension of predominantly endocrine cells could be obtained. Further purification of beta cells could then be achieved easily employing DGCT. In the past technique of isolating various pancreatic endocrine cells was described by us (9, 10) employing density gradient centrifugation technique. Improved cell yield, high purity of preparation and simplicity prove the technique superior to the one described previously.

The isolated beta cells were morphologically and functionally intact. Arginine stimulated insulin secretion. Glucagon in the concentrations employed stimulated insulin secretion. Higher concentrations of glucagon have not been tried to determine if it inhibits insulin secretion as observed in other preparations and in intact animals. Employing this technique sufficient number of cells could be obtained from 2 to 4 rat pancreases for studies of insulin secretion by incubation or perifusion, or for studies of cellular interactions.

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