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Research Article

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Fluctuations of Calcium, Phosphorus, Sodium, Potassium, and Chlorine in Single Alpha and Beta Cells during Glucose Perfusion of Rat Islets

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ABSTRACT To study the relationship between islet hormonal secretion and intracellular content of five elements, a rat islet perfusion technique was used in 24 paired experiments. Control and experimental chambers each containing 100 islets, received 2.8 and 16.7 mM D-glucose, respectively. Effluent was collected frequently for hormone measurements. At eight different time intervals from 0–30 min islets were fixed and prepared for scanning electron microscopy. Over 900 unobscured alpha and beta cells were selected by size and shape criteria. Energy dispersive x-ray analysis was applied to each single cell to determine relative content of calcium (Ca), potassium (K), sodium (Na), chlorine (Cl), and phosphorus (P). Experimental chambers exhibited typical acute (0–9 min) and second phase (10–30 min) insulin secretion in association with suppression of glucagon release after 10 min. At 2 min an abrupt upward K spike in both alpha and beta cells was followed at 3–4 min with a 1.5- to 2-fold rise of Ca and a reciprocal decrease in K, Na, Cl, and P. From 3 to 30 min biphasic elevations of beta cell Ca content preceded biphasic insulin secretion. Reduced alpha cell calcium after 6 min preceded suppression of glucagon secretion. After 2 min K related inversely to Ca content in both alpha and beta cells. These results could not be reproduced when D-galactose was substituted for D-glucose. We conclude that sequential changes of Ca content that are reciprocally related to K are predictive of beta cell insulin release and suppression of alpha cell glucagon secretion.

INTRODUCTION

Numerous ions influence the secretory response of pancreatic islets to substrates. Potassium and calcium

are among the most important, because physiologic concentrations are absolute requirements for normal islet insulin secretion in response to glucose (1). Both ions also may stimulate release of the hormone independently of a rising glucose concentration (1–3).

The objective of the present study was to map sequential changes of calcium and potassium and three other important elements in single beta and alpha cells during perfusion of islets with glucose. This was accomplished by acutely fixing islet tissue at various time intervals throughout first and second phases of insulin secretion. By using energy dispersive x-ray analysis (4) in conjunction with single-cell selection during scanning electron microscopy, element changes could be directly correlated with secretory events up to the time of fixation.

The results of this study suggest that intracellular fluctuations of both potassium and calcium are important determinants of augmented beta cell secretion of insulin or suppressed alpha cell secretion of glucagon after a glucose challenge.

METHODS

Isolation of rat pancreatic islets. Virgin female rats weighing 225–250 g were fed liberal quantities of Purina rat chow (Ralston Purina Co., St. Louis, Mo.) and water and housed in air-conditioned quarters with a light source from 0600 to 1800 h. On the morning of a given experiment two rats were decapitated, pancreata were removed, pooled, and islets were isolated by a collagenase technique (5) as modified by our laboratory (6).

Perfusion of pancreatic islets. Buffer for perfusion consisted of Krebs-Henseleit bicarbonate buffer containing 16 mM HEPES [4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid] and 0.5% human serum albumin. The reservoir of buffer was maintained at pH 7.4 and constantly gassed with 95% O₂–5% CO₂ in a water bath at 37°C. Appropriate amounts of glucose or galactose were added.

The protocol of Lacy et al. (7) for perfusion was followed and our specific method is described in detail elsewhere (6). Briefly, 100 islets were transferred to one of two plastic peri-

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fusion chambers containing filters of 13-mm Diam and 5- μ m pore size (filter SMWP 01300, Millipore Corp., Bedford, Mass.). Both chambers were placed in a 37°C water bath and perfused with buffer containing 2.8 mM glucose for 30 min following which the control chamber was switched to the same medium and the stimulated chamber was switched to medium containing 16.7 mM glucose for an additional period of 2–30 min. The perfusion flow rate was 0.9 ml/min. Effluent was collected into graduated tubes containing 100 IU of Trasylol/ml for subsequent insulin and glucagon determinations.

Perfusion lag times. The total time elapsing for medium to pass from its reservoir through tubing to perfusion chambers was 0.78 min; from perfusion chambers to the collecting vessels, 0.67 min. Hence, measured hormonal concentrations in effluent represented secretory events in islets that occurred 0.67 min earlier.

Scanning electron microscopy (SEM).¹ At the end of the 30-min basal period (i.e., 0 min) and at 2, 3, 4, 6, 10, 20, and 30 min following the change to new buffered media, the perfusion chambers were quickly removed from the water bath, opened and the disc filters holding the islets were immersed in fixative containing 2% glutaraldehyde-1% acrolein for 30 min. Subsequently, the filters were placed in a Millipore filtration apparatus through which 15 ml of double distilled water was passed six times. Following the water washes, a gradient series of 5 ml of ethanol was passed through the chamber every 10 min (50, 70, 85% and three changes of 100% ethanol). The filter was removed and placed in a critical point dryer. After thorough drying a piece of the filter was cut out and attached to a spectroscopically pure carbon stub and a thin layer of carbon was evaporated onto the surface containing the islets.

SEM was performed with a JSM U-3 scanning electron microscope at a potential of 25,000 V. In some experiments photographs were taken at the time of SEM.

Cell selection and energy dispersive x-ray analysis. Selection of beta and alpha cells during SEM for subsequent single cell energy dispersive x-ray analysis (EDXA) was done by the same individual. All cells for analysis had to be attached to islet tissue clumps and unobscured by other cells above, below, or to the sides. Beta cells were recognized by their round to ovoid shape and larger size. The average of two measured diameters of each selected beta cell was limited to the 9.0–11.3- μ m range. Alpha cells were identified by their smaller, pyramidal shape. For selection the average diameter of each alpha cell was confined to a 5.2 to 8.0- μ m range. The basis for cell size criteria has been published previously (8).

EDXA was carried out on single cells with a Nuclear Semiconductor 155e V resolution Si (Li) retractable detector with a Tracor Northern NS 880 computer base data handling system, which is directly attached to the scanning electron microscope. All spectra were stored on magnetic tape for future reference. Five elements were measured: calcium, phosphorus, sodium, potassium, and chlorine. Results are expressed in spectral units for each element/100 seconds of analysis time. In all experiments background EDXA on portions of the filter not containing cells was performed to exclude spurious contamination. Background EDXA for each element was subtracted from EDXA values for single cells.

24 paired perfusions were performed with glucose, i.e., three paired experiments for each of the eight time intervals throughout the 30-min period. 10 beta and 10 alpha cells underwent EDXA in the control (2.8 mM glucose) and ex-

perimental (16.7 mM glucose) components of each pair. This represents 480 single alpha and 480 single beta cell determinations.

Galactose experiments. 16 additional paired studies were performed as described above except that in place of a 16.7-mM glucose challenge in one chamber, 13.9 mM galactose + 2.8 mM glucose was substituted and results were compared to the control chamber receiving 2.8 mM glucose. The intervals selected for EDXA analysis were 0, 2, 3, 4, 6, and 20 min. Each interval consisted of two experiments in which 20 control beta and alpha cell analyses were compared to 20 experimental beta and alpha cell analyses.

Hormone measurements. Insulin concentrations in collected effluent from perfusion chambers were measured by the technique of Morgan and Lazarow (9). Glucagon was determined by a charcoal separation method (10) using the 30 K glucagon antiserum. Hormone secretion was expressed as microunits insulin or picograms glucagon secreted per islet per minute.

Statistical analyses. Comparisons of hormonal secretory rates between control and experimental perfusion chambers at specific time intervals were performed with Student's *t* test for unpaired data. Similar analyses were done to assess the significance of the difference between mean cell diameters and mean cell element content of control alpha and beta cells at a given time interval with corresponding values for alpha and beta cells in paired chambers that were challenged with high glucose or galactose concentrations.

RESULTS

Hormone secretion. Fig. 1 depicts insulin and glucagon secretory rates for the final 2 min of the 30-min basal period and during the 30-min interval following 2.8 mM (control) or 16.7 mM (experimental) challenges with glucose. The 16.7 mM glucose stimulus resulted in significantly greater insulin secretion at 5, 6, and 7 min of the acute phase (0–9 min) and at 15, 20, 25, and 30 min of the second phase (10–30 min) as compared to control islets perfused with low glucose concentrations.

In both control and experimental chambers there was an abrupt fall in glucagon secretion during the initial 5 min after switching stopcocks to respective low and high glucose concentration media. This was followed by an upswing in secretion of the hormone at 6 min. Thereafter, little change in secretory rates prevailed in control chambers whereas corresponding rates in chambers perfused with 16.7 mM glucose were suppressed below control values.

Alpha and beta cell size and identification. Fig. 2 shows two selected photographs taken of islet cells at the time of SEM. This illustrates the size and shape criteria for cell selection as defined in Methods.

Element changes in beta cells. Fig. 3 depicts the percentage change of the elements in beta cells receiving 16.7 mM glucose relative to element content in control cells exposed to 2.8 mM glucose. Asterisks indicating a significant difference, however, are based on statistics derived from comparing the means of 30 control cells in absolute EDXA units with those of 30

¹ Abbreviations used in this paper: EDXA, energy dispersive x-ray analysis; SEM, scanning electron microscopy.

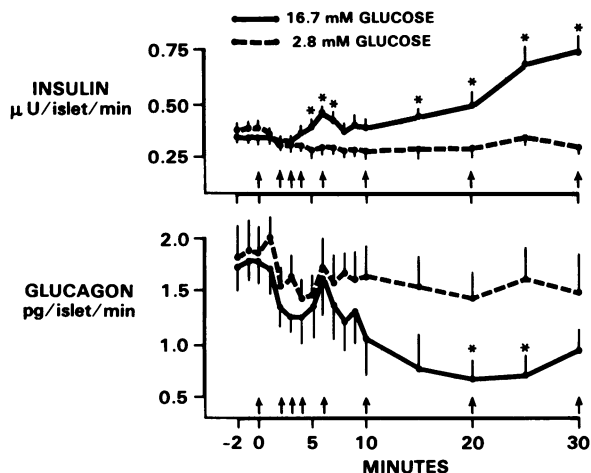


FIGURE 1 Insulin and glucagon secretory rates (mean \pm SEM) of isolated rat islets during 30-min perfusions with 2.8 mM (control) or 16.7 mM (experimental) glucose. Asterisks indicate the significance of the difference between control and experimental means, $P < 0.05$. Vertical arrows denote times of acute fixation of islets from three paired experiments in preparation for single cell analyses. The experiments consisted of 24 paired perfusions that were reduced by three pairs at each subsequent arrow.

experimental cells at each of the eight time intervals after applying Student's *t* test for unpaired data. These patterns, in turn, can be related to insulin secretory rates in the experimental chamber illustrated at the top of the figure.

The first event to occur at the 2-min interval was an abrupt increase in potassium content in beta cells stimulated with 16.7 mM glucose ($P < 0.001$). This was not accompanied by any changes in the other four elements. At 3–5 min a substantial rise in calcium content was observed ($P < 0.005$) that was associated with a reciprocal and significant fall in 3- and 4-min sodium as well as 4- and 6-min potassium levels below control values ($P < 0.005$). At 4 min significant suppression of phosphorus content was observed that was attended by a short-lived upward spike in chlorine ($P < 0.05$). All of these changes preceded any significant rise in acute phase insulin secretion that was first detected at 5 min (i.e., 4.3 min after adjusting for lag time between chamber and effluent collection).

At 10 min or the end of acute-phase insulin release, insulin secretory rates had decreased, a change that related to a return of potassium, calcium, sodium, and chlorine content to base-line values. Only phosphorus was significantly elevated ($P < 0.025$). During second-phase insulin secretion both calcium and phosphorus content increased ($P < 0.02$) whereas potassium, sodium, and chlorine remained at or below control values. Decreased potassium content relative to control cells was significant at 20 min ($P < 0.02$).

Element content in alpha cells. Fig. 4 reveals that

the initial, sole change in experimental alpha cells was also an acute, upward spike in potassium content at 2 min ($P < 0.001$). This was followed by an increased content of calcium at the 3- and 4-min intervals ($P < 0.005$) that was less pronounced and of shorter duration than in beta cells. Thereafter, there was a significant, sustained decrease in calcium levels ($P < 0.005$) in association with suppression of glucagon secretion. Potassium content was increased beyond 6 min and achieved significant elevations at 10 ($P < 0.01$) and 30 min ($P < 0.05$). Sodium tended to follow the pattern of change of potassium, rising to highest levels at the 20- and 30-min intervals ($P < 0.01$). Although some alterations of phosphorus and chlorine content were observed, none of the changes was significant.

Galactose experiments. Perfusion of the experimental chamber with 2.8 mM glucose + 13.9 mM galactose resulted in no significant change in insulin secretion throughout a 30-min period and secretory rates did not differ significantly from control chambers receiving 2.8 mM glucose alone (Fig. 5). Glucagon secretion, however, was acutely suppressed, reaching a nadir at 4 min and returning toward base-line values at 6 min. This was very similar to acute changes in glucagon secretion observed in islets perfused with 16.7 mM glucose shown in Fig. 1. However, after 6 min galactose failed to sustain further suppression of glucagon release unlike that observed with 16.7 mM glucose.

Element content was determined in 20 beta and 20 alpha cells from two paired experiments at 0-, 2-, 3-, 4-, 6-, and 20-min intervals. There were no significant differences between control and experimental chambers with respect to EDXA analyses for each of the five elements in question. In Fig. 5 only data for intracellular calcium content are depicted.

DISCUSSION

In beta cells and, to some extent alpha cells, there is evidence that stimulus-secretion coupling is contingent upon cytosolic accumulation of calcium ions (11–14). In some manner, this ionic change translates substrate-cell interaction into exocytotic extrusion of secretory granules (15, 16). Results of the present study suggest that intracellular calcium content of single cells does correlate with the pattern of hormonal secretion observed during glucose perfusion. Biphasic insulin release was attended by biphasic calcium accumulation in beta cells; significant suppression of glucagon secretion during the second phase was correlated with depressed calcium content in alpha cells after the 4-min interval. The early, attenuated elevation of alpha cell calcium did not stimulate glucagon release.

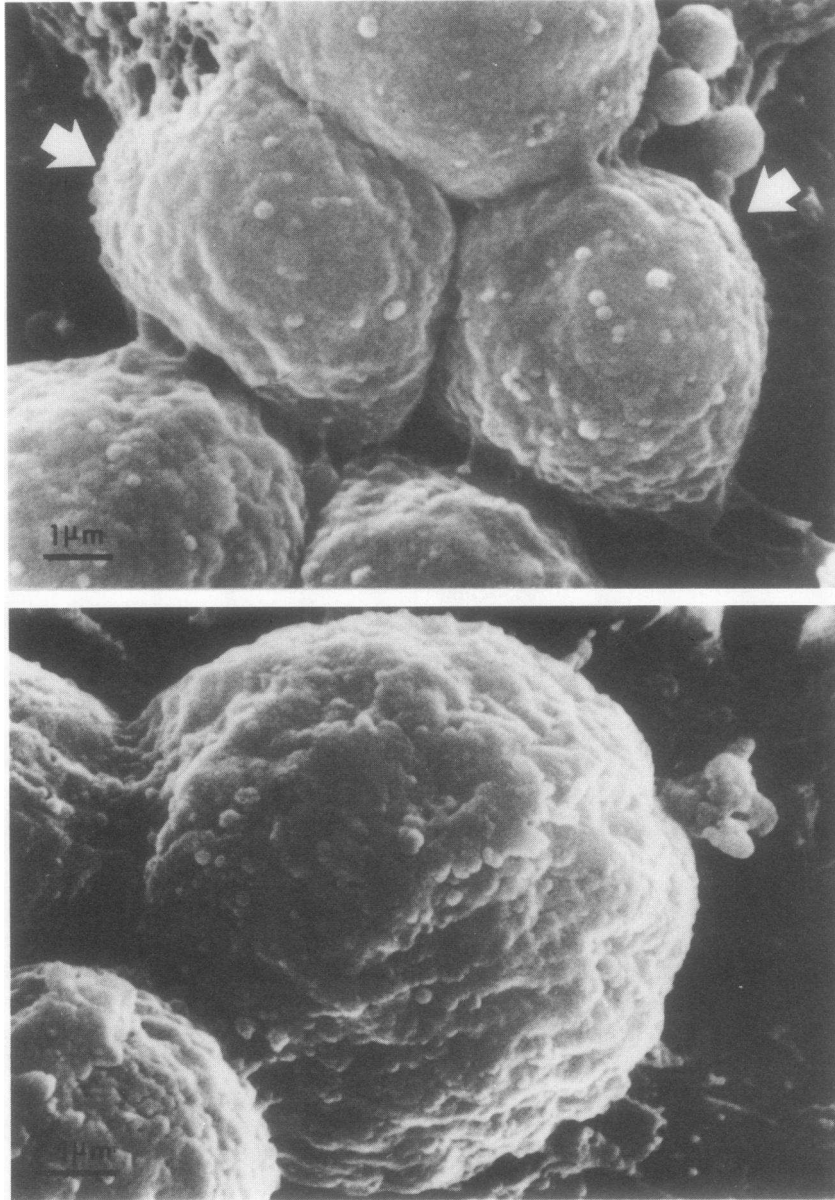


FIGURE 2 SEM of islet cells. White arrows in the upper photograph point to two alpha cells obtained from islets after 30 min perfusion with 16.7 mM glucose. The lower photograph shows a beta cell from an islet perfused for 30 min with 2.8 mM glucose. $\times 11,000$.

An initiating event in this process appeared to be an abrupt, isolated rise of potassium that occurred at 2 min before fluctuations of other elements were apparent. This is consistent with studies indicating that a glucose stimulus restrains potassium conductance out of pancreatic islet cells *in vitro* (17–19) and may represent an early step in stimulus-secretion coupling. The subsequent rise in intracellular calcium after the 2-min interval may be the consequence of potassium retention and the opening of voltage-dependent channels for calcium influx (20, 21). On the other hand, others

have provided evidence that increased intracellular calcium activates membrane permeability for efflux of potassium (22). Thus, it appears that the rise, fall, and secondary rise of potassium content in islet cells during acute phase insulin release (0–9 min) may be the consequence of the opposing actions of glucose (negative) and calcium (positive) on potassium efflux. The rise and fall of calcium, however, may be conditioned, in part, by the preceding rise and fall of intracellular potassium. The reciprocal relationship between calcium and potassium was also evident during the second phase

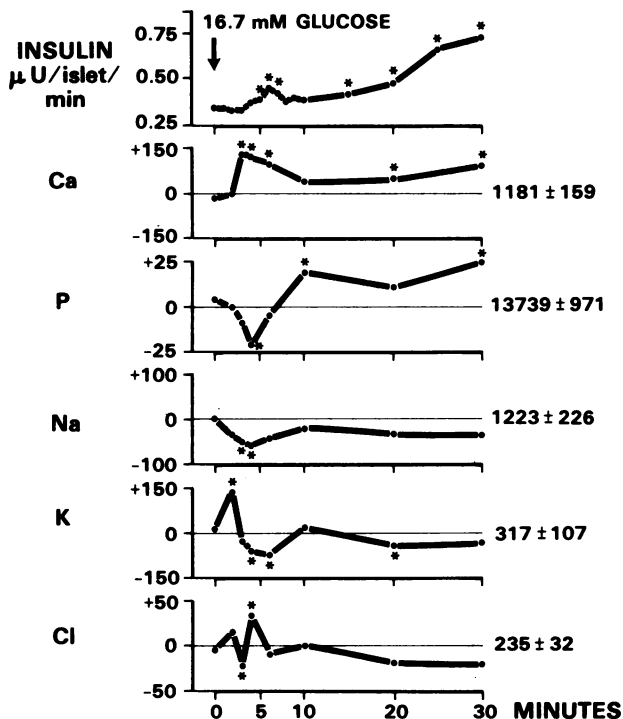


FIGURE 3 Changes in element content of beta cells during islet perfusion with 16.7 mM glucose. The upper panel reproduces islet insulin secretory rates for reference. In subsequent panels, horizontal zero percent lines represent baseline values for control beta cells. Mean EDXA units \pm SEM for all control cells are placed to right of each horizontal line. Each point represents the average percentage change of element content of 30 beta cells receiving 16.7 mM glucose relative to 0 base line of 30 control beta cells. Asterisks indicate a significant difference between mean EDXA spectral units of 30 control and 30 experimental cells at a given time interval (see results section for *P* values).

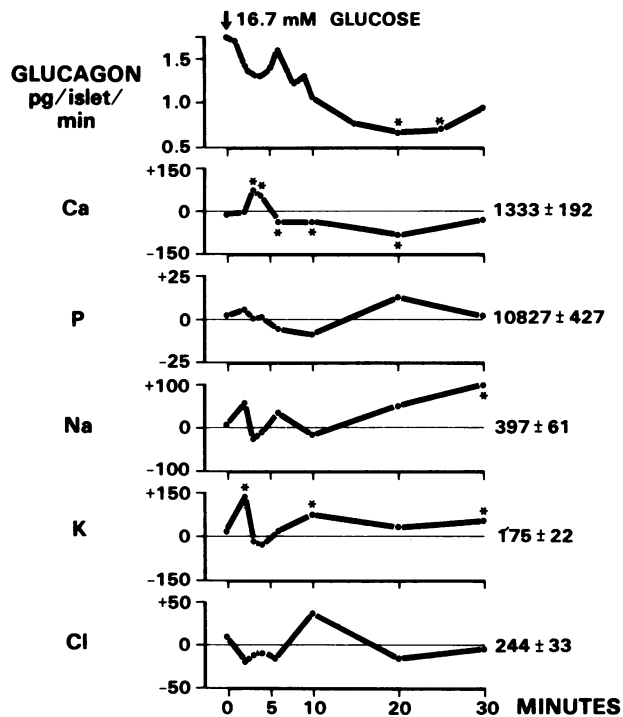


FIGURE 4 Changes in element content of single alpha cells during islet perfusion with 16.7 mM glucose. The upper panel reproduces islet glucagon secretory rates for reference. See legend under Fig. 3 for additional details.

of insulin secretion (10–30 min). The high Ca-low K in beta cells at a time when insulin secretion was increasing differed from the low Ca-high K in alpha cells in association with suppressed glucagon release. This fixed pattern is assumed to prevail when the glucose stimulus is prolonged.

Studies of membrane potentials of single, impaled beta cells also emphasize the importance of K^+ and Ca^{++} fluxes. Exposure to high glucose concentrations induces a series of electrical slow waves alternating between threshold and plateau potentials (23). The three components of the slow wave (depolarization, plateau voltage with superimposed rapid voltage spike activity and repolarization) have been linked, respectively, to beta cell potassium retention, calcium influx, and potassium efflux (23–25). Of major interest are those studies demonstrating that the duration of slow waves or period that the beta cell remains at its plateau voltage due to calcium influx is prolonged with

increasing glucose concentrations (23). Findings in the present investigation are in concert with these observations.

It is possible that biphasic inhibition of Ca^{++} efflux, as opposed to augmented uptake, also contributes to fluctuating calcium content in the beta cell. This is based on identification of Ca-ATPase in islets (26) and the known inhibitory effect of glucose on this system for pumping Ca^{++} out of the cell (27). However, other studies have established that glucose stimulation of islets is associated with brief inhibition of calcium efflux (28–30) followed by simultaneous augmentation of both influx and efflux of the ion (28–32). Calcium uptake is ostensibly more critical, because calcium efflux does not relate stoichiometrically to islet insulin secretion (32). From these observations we conclude that oscillating calcium influx is a more important factor in the biphasic net accumulation of calcium in beta cells in our studies.²

² Based on their studies with verapamil, an inhibitor of islet calcium influx, the Geneva group has concluded that calcium uptake in perfused islets is not necessary for acute, as opposed to second phase insulin secretion in response to verapamil + glucose (33). Others have not confirmed this observation when verapamil is added before the glucose stimulus (34).

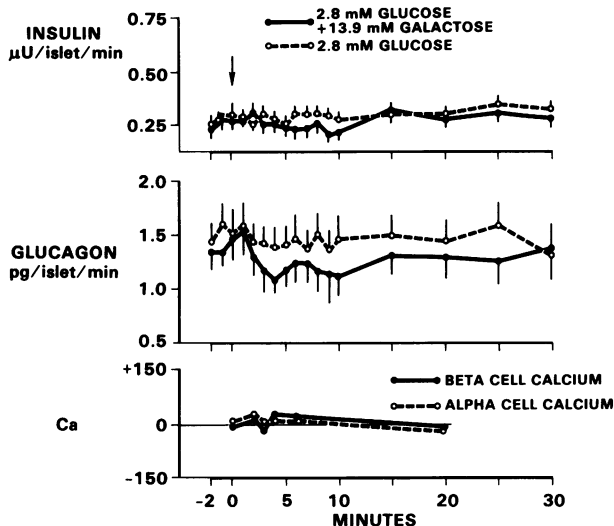


FIGURE 5 Changes in calcium content of single alpha and beta cells during islet perfusion with 2.8 mM glucose + 13.9 mM galactose (experimental) or 2.8 mM glucose alone (control). Upper panel illustrates insulin secretory rates and the middle panel, glucagon secretory rates (mean \pm SEM). The experiments consisted of 16 paired perfusions. Two pairs were removed for islet fixation at 0, 2, 3, 4, 6, and 20 min. Each point in the lower panel denotes the percentage change of cellular calcium in 20 beta cells and 20 alpha cells receiving galactose relative to content in 20 control beta and 20 control alpha cells receiving 2.8 mM glucose. Control values are expressed as 0 percent base line. No significant differences between any corresponding means were obtained.

Because glucose, potassium, calcium, and ouabain inhibit islet $\text{Na}^+\text{K}^+\text{ATPase}$ (27), and all are known to enhance insulin secretion, a role for sodium flux in stimulus-secretion-coupling has been sought. In the present study, Na tended to parallel falling beta cell K content after the 2-min interval and is consistent with a sustained Na efflux from islets that is observed following glucose stimulation (36). These results suggest that possible inhibitory effects of glucose on $\text{Na} + \text{K} + \text{ATPase}$, which would promote Na accumulation in the beta cell, are overshadowed and further modified by other events; that Na has a relatively passive role in acutely affecting glucose-induced islet hormonal secretion (37, 38). This position is strengthened by recent, detailed studies of membrane sodium channels by Pace (39). The present data suggest that calcium accumulation in the beta cell is attended by net sodium as well as potassium loss. Inward moving

After subsequent comparative studies of glucose and ouabain, they proposed that acute phase insulin release may be induced by glucose inhibition of calcium efflux rather than by stimulation of calcium influx (35). This information is placed in a footnote to highlight a viewpoint that is opposite to that enumerated above.

calcium may exchange with outgoing sodium via a bi-directional Ca-Na exchange mechanism (40, 41) and may concomitantly promote K efflux by enhancing calcium-sensitive membrane permeability to potassium (22).

A series of investigations by Freinkel and co-workers (42) have defined an acute phosphate "flush" or efflux of labelled inorganic phosphate out of islets perfused with glucose (42–44). Our findings provide direct evidence that acute phosphorus loss does occur specifically in the beta cell and achieves significance (4 min) after the potassium spike (2 min) and the first significant elevation of calcium (3 min). In contrast to first phase insulin release, there was retention of phosphorus during the second phase of insulin secretion. Because our results cannot distinguish between phosphorus in inorganic or organic forms, it is conceivable that some of the change in its content may reflect net breakdown and synthesis of organophosphate compounds during acute and second phase insulin release, respectively. The role of phosphorus in islet hormonal secretion remains undefined, since the "flush" relates to beta cell glucose metabolism and can be dissociated from actual exocytosis (42).

There appeared to be an inverse relationship between content of phosphorus and chlorine in the beta cell. In the acute phase chlorine levels rose abruptly at 4 min when phosphorus reached its lowest point. Throughout the second phase of insulin secretion, phosphorus retention was attended by chlorine levels that were at or below control values. Findings in the acute phase may have relevance to recent studies of Orci and Malaisse (45) who suggest that exocytosis is dependent on influx of chloride into the beta cell which, in turn, promotes fusion of membranes of insulin secretory granules held in tandem near the cell surface membrane. As a corollary to this report, others have observed defective chloride ion movements in beta cells of rodents with genetic diabetes (46). Further studies are necessary to ascertain the importance of anion fluxes in the secretory process and whether a meaningful physiologic relationship exists between phosphate and chloride ions.

Our investigation would not have been complete without further establishing the specificity of the glucose effect on elements measured in single islet cells. Perfusion of pancreatic islets with D-galactose, a hexose not metabolized by islets, failed to stimulate insulin secretion, had no significant suppressive effect on glucagon release, and did not significantly alter calcium or other trace element content in beta or alpha cells during acute and second phase periods. It is concluded that the pattern of fluctuation of these five elements during stimulation with 16.7 mM glucose is not spurious, but very relevant to the stimulus-secretion process. The evanescent, slight fall in

glucagon secretion during the early phase of perfusion with either glucose or galactose appears to be non-specific and unrelated to the observed changes in elements.

Although EDXA is a new method for studying important elements in single cells as opposed to whole islets, there are limitations to this approach. EDXA cannot distinguish free ions from bound ions and those incorporated into compounds within tissues, nor can it determine whether a specific change in content is the product of altered ionic influx or efflux or both. In addition, the perfusion with artificial media as well as the water and gradient ethanol washes required after islet fixation very likely resulted in leeching a portion of free ions out of islet cells before the EDXA. For this reason, EDXA measurements with this technique as we used it do not precisely quantitate intracellular content of these elements. Nevertheless, the patterns of change are decidedly different between control and glucose-stimulated cells of the same type as well as distinctive in nature when beta and alpha cells are compared at specific time periods after glucose stimulation. In addition, these profiles cannot be reproduced during islet perfusions with galactose.

The present study places emphasis on the apparent reciprocal relationship between intracellular content of calcium and potassium in islet tissue, since this relationship appears to anticipate secretory responses of alpha and beta cells to glucose.

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