Tolbutamide Perifusion of Rat Islets

SEQUENTIAL CHANGES IN CALCIUM, PHOSPHORUS, SODIUM, POTASSIUM, AND CHLORINE IN SINGLE BETA CELLS

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ABSTRACT Fluctuations of calcium, phosphorus, sodium, potassium, and chlorine in beta cells were followed during rat islet perifusion with tolbutamide and related to insulin secretion. In 24 paired experiments two chambers containing 100 islets were perifused with buffered medium containing 4.2 mM glucose alone or with added tolbutamide (200 μg/ml). Effluent was collected frequently for insulin determinations. At eight different time intervals from 0 to 20 min islets were acutely fixed, prepared for scanning electron microscopy and beta cells in islet tissue were identified. Element content in 480 single cells was measured by energy dispersive x-ray analysis. Tolbutamide elicited typical monophasic insulin release that exceeded control islet secretory rates from 2 to 6 min with a peak value at 3 min. This pattern was preceded by monophasic calcium accumulation in beta cells that abruptly rose 150% above control cells at 1 min and declined to base line by 4 min. The rapid ascent of calcium was associated with significant depressions of sodium and potassium content without alterations of cell phosphorus. Chlorine fell at 2 min and then rose >50% above control cells at 4 min. After 6 min insulin secretion and element content remained near control levels. We conclude that monophasic calcium accumulation in beta cells is the earliest, most predictive event of islet insulin secretion after a tolbutamide stimulus. Oscillations of beta cell sodium and potassium reciprocally relate to calcium, and an elevation of chlorine content is a relatively late phenomenon in the stimulus-secretion coupling process.

INTRODUCTION

It is well known that tolbutamide acutely stimulates monophasic insulin secretion by pancreatic islets in vitro (1). The importance of cytosolic accumulation of Ca^{++} as an initiator of tolbutamide action has been established by several reports of flux studies of this ion in whole islets (2, 3) as well as by other electrophysiologic investigations of the beta cell membrane (4). However, it is not clear how Ca^{++} movements relate temporally to other important cations, especially K^+ (3–7), in stimulus-secretion coupling.

The object of this study was to map sequential changes of calcium (Ca), sodium (Na), potassium (K), phosphorus (P), and chlorine (Cl) in single beta cells as opposed to whole islets during tolbutamide perifusion of isolated rat islet tissue. This was achieved by using energy dispersive x-ray analysis (EDXA)[1] after cell identification with scanning electron microscopy (SEM). In this study two assumptions must be made: (*a*) acute changes in total beta cell content of any given element are the product of transmembrane ionic movements, since EDXA cannot discriminate between free ions and those elements incorporated into intracellular compounds; (*b*) EDXA is done after perifusion, rapid fixation of islets, and processing for electron microscopy. A portion of elements are removed from islets by these procedures. We assume that the content of retained elements in tolbutamide-stimulated beta cells relative to corresponding control cells at any given moment is representative of the in vivo situation, even though precise quantitation is not possible. In support of this second assumption is our previous report of distinctive, sequential changes of these elements in both beta and alpha cells following D-glucose as opposed to D-galactose challenges (8).

Results of the present study suggest that tolbutamide evokes an acute rise of beta cell Ca that precedes per-

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

turbations of other intracellular elements as well as insulin secretion.

METHODS

Details of our experimental techniques have been published previously in this journal (8). Pancreatic islets from fed, female rats were isolated by a collagenase technique (9). Buffer for perifusion was composed of Krebs-Henseleit bicarbonate buffer containing 16 mM Hepes and 0.5% human serum albumin. Buffer was maintained at pH 7.4 and 37°C while being constantly gased with 95% O_2-5% CO_2. 100 isolated islets were transferred to one of two plastic perifusion chambers containing appropriate filters. They were submerged in a water bath at 37°C and perifusion was carried out according to the method of Lacy et al. (10) at flow rates of 0.9 ml/min. During a 30-min base-line period both chambers received buffer containing 4.2 mM glucose. Following this the control chamber was switched to a medium of the same composition; the experimental chamber was switched to the same medium containing 200 μg/ml of sodium tolbutamide (Upjohn Co., Kalamazoo, MI). Effluent was collected into graduated tubes at frequent intervals for insulin determinations (11). Perifusion lag time through tubing from reservoir to chamber was 0.78 min; from chamber to collecting tubes was 0.67 min.

SEM and EDXA. After the 30-min base-line period islets from paired chambers were rapidly fixed at different time intervals (0, 1, 2, 3, 4, 6, 10, and 20 min). This was accomplished by quickly removing the filter containing islets and placing it in 2% glutaraldehyde-1% acrolein. After 30 min of fixation a series of water and gradient ethanol washes were performed following which thorough critical point drying of islet tissue on the filters was done (8). A thin layer of carbon subsequently was evaporated onto the surface of the filter-containing islets. SEM utilized a JSM U-3 scanning electron microscope JEOL U.S.A., Peabody, MA at a potential of 25,000 V. Single, unobscured beta cells that were attached to islets were identified by size and shape criteria as previously described (8). After identification, the cell was subjected to EDXA utilizing an attached Nuclear Semiconductor 155e V resolution Si (Li) retractable detector (Nuclear Semiconductor, Mountain View, CA) and Tracor Northern NS 880 computer (Tracor Northern, Middleton, WI) base data handling system. All spectra were stored on magnetic tape for future reference. Measurements of total beta cell content of Ca, P, Na, K, and Cl are expressed in spectral units/100 s of analysis time. Background counts from areas on the filter not occupied by cells were subtracted from total counts of each element.

Each of the eight EDXA time points between 0 and 20 min involved measurements of 30 control and 30 tolbutamide-stimulated beta cells from three paired experiments. Thus, a total of 24 paired studies with EDXA on 240 control and 240 experimental beta cells were performed. Effluent for insulin determinations was collected every minute between 0 and 10 min and every 5 min between 10 and 20 min of the perifusion period up to the time of islet fixation.

Statistical analyses. Student's *t* test for unpaired data compared the significance of the difference between mean control and experimental data with respect to insulin secretory rates. Similar analyses compared mean content (spectral units) of the five measured elements in 30 control and 30 experimental beta cells at each EDXA time interval.

RESULTS

Fig. 1 illustrates insulin secretory rates of control perifusion chambers and corresponding rates in chambers perifused with tolbutamide, 200 μg/ml. The last 2 min of the 30-min basal period (−2 and −1 min, respectively) also are shown. Control chambers receiving 4.2 mM glucose had little change in insulin secretion throughout the 20-min period. After introducing tolbutamide plus 4.2 mM glucose into the experimental chambers, a typical monophasic insulin secretory pattern was observed. Rates of insulin release significantly exceeded control values at 2 through 6 min with a peak rate at 3 min ($P < 0.01$). After the 6-min interval tolbutamide-stimulated chambers secreted insulin at rates no longer differing from control values.

In Fig. 2 EDXA data for tolbutamide chambers are expressed as a percentage of corresponding mean content of elements in control cells. Statistics, however, compared average spectral units for each element in either group at each interval and not percent changes.

The earliest event to occur in these studies was an abrupt rise in beta cell Ca in islets perifused with tolbutamide. Peak values at 1 min exceeded control levels by > 150% ($P < 0.001$). A steady decline in Ca content rapidly ensued with 2 and 3 min values still significantly above control by factors of 91 and 60%, respectively ($P < 0.001$). Ca content returned to control levels between 4 and 6 min and remained unchanged thereafter.

After the acute rise in Ca at 1 min there was a reciprocal, 20–25% fall in Na at 1–2 min ($P < 0.02$) as well as a 46% decrease in K content at 2 min ($P < 0.005$). As

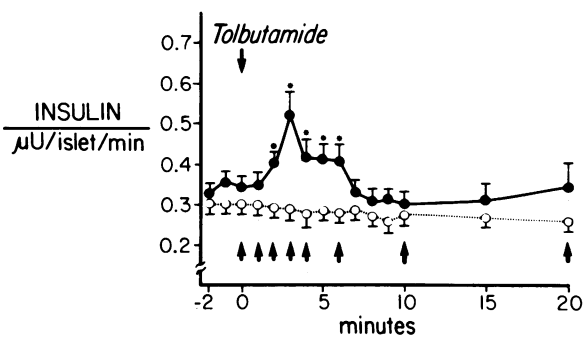

FIGURE 1 Insulin secretory rates (mean±SEM) of isolated rat islets during a 20-min perifusion with 4.2 mM glucose (control [O]) or 4.2 mM glucose + 200 μg/ml tolbutamide (●). Asterisks indicate a significant difference between control and corresponding experimental means (see Results section for *P* values). Vertical arrows denote times of acute fixation of islets from three paired studies in preparation for EDXA analyses of single cells. The experiments consisted of 24 paired perifusions which, after 0 min, were reduced by three pairs at each subsequent arrow.

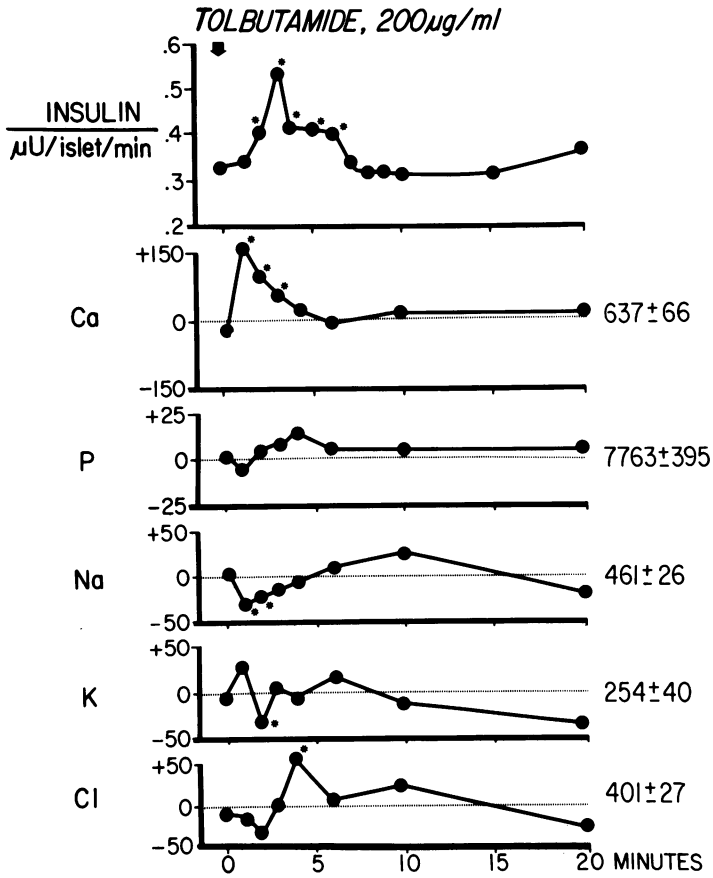

FIGURE 2 Changes in element content of beta cells during islet perifusion with tolbutamide, 200 µg/ml. The *upper* panel reproduces islet insulin secretory rates for reference. In subsequent panels horizontal zero percent lines represent base-line values for control beta cells. Mean EDXA spectral units±SEM for all control cell values are placed to the right of each zero percent line. Each point represents the average percentage change of element content of 30 beta cells stimulated by tolbutamide relative to 0 base line of 30 control cells. Asterisks indicate a significant difference between mean spectral units of control and experimental cells at a specific time interval (see Results section for *P* values).

Ca content subsequently declined both Na and K rose again toward base line and no longer differed significantly from control values.

Measurements of Cl during tolbutamide stimulation revealed an initial, insignificant fall (2 min) followed by a substantial rise at 4 min that exceeded control values by 66% ($P < 0.001$). Beyond this interval Cl had returned to control levels.

No significant changes in P content were observed in beta cells receiving the tolbutamide stimulus.

DISCUSSION

In this study, the pattern of hormone release was closely aligned with significant perturbations of four out of five elements measured in beta cells. Thus, monophasic insulin secretion was preceded by an abrupt monophasic accumulation of Ca in single beta cells. In previous studies of this type with 16.7 mM glucose, biphasic insulin secretion was anticipated by biphasic accumulation of beta cell Ca and glucose suppression of glucagon secretion was attended by a significant reduction of alpha cell content of this element (8). These temporal, phasic relationships underscore the importance of cytosolic elevations of Ca^{++} as second messengers for the insulin secretory process, and, by direct measurement, support conclusions drawn by others utilizing flux measurements of labeled Ca in whole islets exposed to tolbutamide in vitro (2, 3, 5–7).

Fluctuations of Ca also appear to anticipate reciprocal changes of K and Na. For reasons previously discussed (8), the acute fall in beta cell Na at 1 min is consistent with the view that inward moving Ca^{++} exchanges with outward moving Na^{+} by a bidirectional Ca-Na exchange mechanism (12). In addition, cytosolic elevations of Ca^{++} increase K^{+} permeability (13) and explain our finding of a significant reduction in beta cell K content (2 min) shortly after maximal Ca concentrations were reached. The rapid decline of Ca in beta cells after the abrupt peak was reached may also be explained by the reciprocal fall of K, since the latter can provide repolarization of the beta cell membrane and closure of voltage-dependent membrane channels that control Ca^{++} influx (14). These reciprocal oscillations of Ca on the one hand and Na, K on the other, were also observed in our studies of glucose provocation using the same technique (8). The interdependence of Na, K, and Ca content on one another was also manifested when Ca returned to control levels at 4–6 min, at which time Na and K did as well.

Movements of Cl^{-} also have been implicated in beta cell exocytosis, since glucose-stimulated insulin release normally is associated with Cl^{-} efflux and nonpassive influx in pancreatic islets (15). In the present study tolbutamide stimulated a brief, though insignificant fall in Cl in beta cells at 2 min, which may represent a net enhancement of efflux. Others have offered evidence that Cl^{-} influx may be necessary for fusion of secretory granules with the beta cell membrane in the final steps of exocytosis (16). The steep rise in beta cell Cl at 4 min in our studies could also be an expression of this process. In our previous investigations with 16.7 mM glucose, acute phase insulin release also was attended by a significant fall followed by an abrupt elevation of Cl content in beta cells (8). To what extent these movements relate to other ionic fluxes remains to be defined.

Phosphorus was measured in beta cells, because nutrient stimuli like glucose trigger an acute phosphate "flush" or efflux during acute phase insulin release (17). Although this observation was documented in our previous study with glucose (8), tolbutamide failed to alter P in beta cells and is consistent with the report of Carpinelli and Malaisse (18) who observed unaltered efflux of ^{32}P from whole islets after tolbutamide provocation. This may represent one characteristic of sulfonylureas that distinguishes their action from those of nutrient secretagogues.

Controversy surrounds the mechanism whereby tolbutamide promotes net Ca accumulation in the beta cell. Unlike nutrient stimuli, it does not alter the basic energy metabolism of whole islets (19) nor influence the biosynthesis of insulin (20). Although earlier work suggested that tolbutamide stimulates cyclic AMP production either directly (21) or indirectly by inhibiting phosphodiesterase activity (22), these studies were carried out in whole islet homogenates. Since tolbutamide does not penetrate the beta cell membrane (23), the significance of these findings remains uncertain. It is possible that net intracellular Ca^{++} increments induced by tolbutamide per se may be responsible for cyclic AMP elevations (3).

Recent work of Meissner et al. (4) has clearly shown that tolbutamide depolarizes the beta cell membrane to a plateau voltage with superimposed rapid voltage spikes. Since this electrical phenomenon and insulin secretion are abolished by blockade of voltage-dependent Ca channels, it reemphasizes the importance of Ca^{++} influx into beta cells for insulin secretion.

Henquin (3) has reported that tolbutamide, in the presence of threshold concentrations of glucose, suppresses K^{+} efflux while promoting rapid influx and efflux of Ca^{++}. He concludes, along with Meissner et al. (4), that depolarization of the beta cell membrane by tolbutamide relates, in part, to acute elevation of intracellular K^{+}. The subsequent opening of voltage channels allows influx of Ca^{++} with maintenance of a plateau potential.

Malaisse et al. (6) offer an alternative explanation. In the presence of 8.3 mM glucose tolbutamide excites immediate Ca^{++} fluxes in the absence of any restraint on K^{+} efflux. Additional work in the area suggests that tolbutamide interaction with the beta cell membrane promotes activation of what he terms native Ca^{++} ionophoretic material (5) that elicits Ca^{++} movement independently of K^{+} flux. It is clear, however, that tolbutamide itself is not an ionophore for several reasons discussed previously (24).

Findings in the present study suggest that tolbutamide has a very acute action on Ca^{++} flux in the absence of a preceding change in K content in beta cells. The rapidity of this effect is appreciated further by recalling the lag time for reservoir media containing tolbutamide to pass through tubing to the perifusion chamber containing islets (Methods). Thus, the 1-min spike in beta cell Ca content occurs within 13 s of tolbutamide exposure (1 min − 0.78 min lag time = 0.22 min or 13 s). Considering the relatively slow rate of perifusion (0.9 ml/min), this also suggests that tolbutamide effects on beta cell Ca^{++} flux in vivo are almost immediate. It is still possible that our study missed a transient K elevation during the first 12 s of the tolbutamide challenge, but it does not necessarily have to occur to explain the depolarizing action of this agent on the beta cell membrane, since Ca^{++} itself (7) may contribute to this phenomenon.

In summary, our studies reveal that tolbutamide

stimulates rapid accumulation of Ca in the beta cell, an event that overshadows fluctuations of other measured elements and anticipates monophasic insulin secretion. These properties of the sulfonylurea distinguish it from a slightly different, slower sequence of events observed with glucose under identical conditions (8).

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REFERENCES

1. Curry, D. L., L. L. Bennett, and G. M. Grodsky. 1968. Requirement for calcium ion in insulin secretion by the perfused rat pancreas. *Am. J. Physiol.* 214:174–178.
2. Hellman, B., S. Lenzen, J. Sehlin, and L. B. Taljedal. 1977. Effect of various modifiers of insulin release on the lanthanum-nondisplaceable $^{45}Ca^{++}$ uptake by isolated pancreatic islets. *Diabetologia.* 13:49–53.
3. Henquin, J. C. 1980. Tolbutamide stimulation and inhibition of insulin release: studies of the underlying ionic mechanisms in isolated rat islets. *Diabetologia.* 18:151–160.
4. Meissner, H. P., M. Preissler, and J. C. Henquin. 1979. Possible ionic mechanisms of the electrical activity induced by glucose and tolbutamide in pancreatic beta cells. *In* Diabetes 1979. Proceedings of the 10th Congress of the International Diabetes Federation. W. K. Waldman, editor. Excerpta Medica, Amsterdam. 166–171.
5. Courtier, E., and W. J. Malaisse. 1980. Insulinotropic effects of hypoglycemic and hyperglycemic sulfonamides: the ionophoretic hypothesis. *Diabetologia.* 19:335–340.
6. Malaisse, W. J., A. R. Carpinelli, and A. Herchulez. 1980. Tolbutamide stimulates Ca^{++} influx in islet cells without reducing K^+ conductance. *Diabetologia.* 19:85.
7. Henquin, J. C. 1980. Mechanisms of tolbutamide-stimulation of pancreatic B cells—A reply. *Diabetologia.* 19:162.
8. Kalkhoff, R. K., and K. A. Siegesmund. 1981. Fluctuations of calcium, phosphorus, sodium, potassium, and chlorine in single alpha and beta cells during glucose perifusion of rat islets. *J. Clin. Invest.* 68:517–524.
9. Lacy, P. E., and M. Kostianovsky. 1967. Method for isolation of intact islets of Langerhans from the rat pancreas. *Diabetes.* 16:35–39.
10. Lacy, P. E., M. M. Walker, and C. J. Fink. 1972. Perifusion of isolated pancreatic islet cells. *Diabetes.* 21:987–998.
11. Morgan, C. R., and A. Lazarow. 1963. Immunoassay of insulin: two antibody system. *Diabetes.* 12:115–126.
12. Carafoli, E., and M. Crompton. 1978. The regulation of intracellular calcium. *Curr. Top. Membr. Trans.* 10:151–216.
13. Henquin, J. C. 1979. Opposite effects of intracellular Ca^{++} and glucose on K^+ permeability of pancreatic islet cells. *Nature (Lond.).* 280:66–68.
14. Ribalet, B., and P. M. Biegelman. 1979. Cyclic variations in pancreatic beta cells: Ca^{++} and voltage dependence. *Am. J. Physiol.* 237:C137–C146.
15. Sehlin, J. 1978. Interrelationship between chloride fluxes in pancreatic islets and insulin release. *Am. J. Physiol.* 235:E501–E518.
16. Orci, L., and W. J. Malaisse. 1980. Single and chain release of insulin secretory granules is related to anionic transport at exocytotic sites. *Diabetes.* 29:943–944.
17. Freinkel, N., C. E. Younci, J. Bonnar, and R. M. C. Dawson. 1974. Rapid transient efflux of phosphate ions from pancreatic islets as an early action of insulin secretagogues. *J. Clin. Invest.* 54:1179–1189.
18. Carpinelli, A. R., and W. J. Malaisse. 1980. The stimulus-secretion coupling of glucose-induced insulin release. XLIV. A possible link between glucose metabolism and phosphate flush. *Diabetologia.* 19:458–464.
19. Krzanowski, J. J., R. Fertel, and F. M. Matschinsky. 1971. Energy metabolism in pancreatic islets of rats. Studies with tolbutamide and hypoxia. *Diabetes.* 20:598–606.
20. Taylor, K. W., and D. G. Parry. 1967. Tolbutamide and the incorporation of ^3H-leucine into insulin in vitro. *J. Endocrinol.* 39:457–458.
21. Kuo, W. N., D. S. Hedgins, and J. F. Kuo. 1973. Adenylate cyclase in islets of Langerhans. Isolation of islets and regulation of adenylate cyclase activity by various hormones and agents. *J. Biol. Chem.* 248:2705–2711.
22. Goldfine, J. D., R. Pearlman, and J. Roth. 1971. Inhibition of cyclic 3′,5′-AMP phosphodiesterase in islet cells and other tissues by tolbutamide. *Nature (Lond.).* 234:295–297.
23. Hellman, B., J. Sehlin, and I. B. Taljedal. 1973. The pancreatic β-cell recognition of insulin secretagogues. VI. Islet uptake of sulfonylureas. *Diabetologia.* 9:210–216.
24. Hellman, B. 1981. Tolbutamide stimulation of ^{45}Ca fluxes in microdissected pancreatic islets rich in β-cells. *Mol. Pharmacol.* 20:83–88.