Glucose-Stimulated Insulin Release by Individual Pancreatic β Cells: Potentiation by Glyburide (43180)

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Abstract. **To investigate the effect of glyburide on insulin secretion by individual β cells from normal rats, we employed a reverse hemolytic plaque assay. Pancreata were harvested from female Wistar-Furth rats, the pancreatic islets isolated, and the latter dispersed into single cells. These cells were mixed with protein A-coated ox erythrocytes, the mixture was placed in a Cunningham chamber in the presence of insulin antiserum, and the cells were exposed to the various test substances. Having developed hemolytic plaques around the insulin-secreting cells with complement, the percentage of plaque-forming cells was determined and the plaque areas (reflecting the amount of insulin secreted) were quantitated.**

For the purpose of validation, we demonstrated that (i) plaque-forming (but not non-plaque-forming) cells could be identifed as insulin secreting by an independent immunofluorescent technique, (ii), plaques did not form if insulin antiserum was deleted from the preparation, (iii) plaques failed to develop if insulin antiserum was preabsorbed with insulin, and (iv) incubation with non-protein A-coated RBC or omission of complement resulted in no plaque formation. In addition, both the percentage of plaque-forming cells and the mean plaque area increased upon exposure to glucose (0.75–20 m*M*) in a concentration-dependent manner at 5- and 60-min incubation times. Moreover, somatostatin suppressed the percentage of plaque-forming cells and diminished the mean plaque area of cells which continued to secrete insulin in response to glucose.

Exposure of cells to 100 n*M* glyburide in the presence of 5 m*M* or 20 m*M* glucose had no effect on the percentage of plaque-forming cells present at 5 min or 60 min. Similarly, glyburide did not alter mean plaque area at 5 or 60 min when cells were co-incubated with 5 m*M* glucose. However, mean plaque area was markedly enhanced at 5 and 60 min in response to glyburide and 20 m*M* glucose.

These results demonstrate that glyburide (i) does appear to enhance insulin secretion by an effect directly on the pancreatic β cell; (ii) does not act by recruiting previously noninsulin-secreting cells into a secretory pool; (iii) does not potentiate the effect of glucose, at fed concentrations, on insulin secretion by individual cells; but (iv) does augment insulin secretion by β cells stimulated with supraphysiologic concentrations of glucose. [P.S.E.B.M. 1991, Vol 196]

Although the sulfonylureas have been utilized for the treatment of NIDDM for over 30 years, major questions related to their mechanism(s) of action remain unresolved. It is widely accepted that these agents reduce blood glucose both by enhancing insulin secretion and by increasing the sensitivity of peripheral tissues to the action of insulin. With regard to the former, *in vitro* studies using dispersed pancreatic islet cell preparations in monolayer culture and perifusion systems and cultured insulinoma cells have demonstrated augmented insulin secretion upon exposure to sulfonylurea (1–4). Given that the process of cell dispersion disrupts the usual structural relationships among individual cells within the pancreatic islet, these studies suggest that the sulfonylureas may stimulate insulin release via a direct effect on the β (B) cell rather than through a paracrine process. To our knowledge,

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however, no one has documented that sulfonylureas act directly on B cells obtained from pancreatic islets of normal animals. Assuming that direct stimulation does occur, it is not known if these agents enhance insulin secretion by increasing the number of B cells actively secreting this hormone and/or by amplifying the secretory capacity of individual B cells. It is also unclear as to whether sulfonylureas alter insulin release by cells within a hyperglycemic milieu only or under both hyperglycemic and euglycemic conditions, an issue which has obvious clinical relevance.

Investigation of the above issues has been limited by the lack of a methodology with which to study pure populations of B cells from normal rats. Recently, application of a reverse hemolytic plaque assay (RHPA) has allowed for the characterization of hormone secretion by individual cells of known type (5–7) including the pancreatic B cell (8, 9). In the current study, we first sought to modify the RHPA methodology so that insulin secretion by B cells can be detected at very early time points following a glucose challenge. Having demonstrated that such cells secrete insulin in a concentration- and time-dependent fashion and exhibit suppressed hormonal release in the presence of somatostatin, we next addressed the question of how and under what conditions glyburide alters insulin secretion.

Materials and Methods

Animals. Female Wistar-Furth rats (Harlan Sprague-Dawley, Indianapolis, IN) weighing 125–200 g were housed under a 12-hr light/12-hr dark schedule with lights on at 0700 hr. The temperature was maintained at 21–23°C, and standard laboratory chow and water were available *ad libitum*. The animals were fasted overnight prior to removal of the pancreas.

Isolation of Pancreatic Islets. Islets of Langerhans were isolated as described previously from the pancreata (10). Briefly, the whole pancreas was injected with Hanks' balanced salt solution containing 1.1% collagenase (Sigma Chemical Co., St. Louis, MO). The pancreas was then diced into 1-mm pieces and digested with 3.4% collagenase at 37°C for 20 min followed by 0.75% collagenase for 5 min. Tissue was removed after each timed exposure to the enzyme. The digested tissue was washed approximately 10 times with 1% bovine serum albumin in Hanks' balanced salt solution, layered on a 40/60 Percoll gradient, and the islets removed with some acinar tissue from the interface. Islets were hand-picked with a Lang-Levi pipette and washed with RPMI 1640 medium containing 1% bovine serum albumin.

Dispersion of Pancreatic Islets into Single Cells. The islets were dispersed in RPMI 1640 containing 2 m*M* EGTA (Sigma Chemical Co.) by gentle aspiration 7–10 times through a constricted blunt-ended Pasteur pipette (11, 12). The cell suspension was washed by centrifugation and resuspended in RPMI 1640 containing 10% fetal calf serum (Fisher Scientific Co., Columbia, MD), 400 units of penicillin/ml and 200 μg of streptomycin/ml. The cells were then incubated overnight at 37°C with 95% O_2 and 5% CO_2. A total cell count was performed prior to use in the plaque assay and the suspension adjusted to 2×10^5 cells/ml. Viability of the cells was determined by exclusion of trypan blue.

The Reverse Hemolytic Plaque Assay. *Assay procedure.* The reverse hemolytic plaque assay was performed as described previously (6) with minor modifications. The islet cell medium was changed to phosphate-buffered saline containing 25 m*M* Hepes, 5 m*M* $NaHCO_3$, 0.75 m*M* glucose, and 1% bovine serum albumin. One milliliter of this medium containing the dispersed cells was mixed with 1 ml of protein A (*Staphylococcus aureus*, no. p8143; Sigma Chemical Co.)-coated ox erythrocytes (Colorado Serum Co., Denver, CO). Fifty microliters of this mixture were infused into a chamber formed on a glass microscope slide previously coated with poly-L-lysine (0.25 mg/ml × 20 min; Sigma Chemical Co.). Cell attachment occurred during a 45-min incubation at 37°C. Test substances, diluted in the above phosphate-buffered saline mixture, were then introduced into the chamber in the presence of insulin antiserum (Arnel Laboratories, New York, NY) at a dilution of 1/150 and left for the required amounts of time as per the study design. Plaques were developed over 20 min following the addition of guinea pig complement (Colorado Serum Co.) in a 1/25 dilution. Cells were then fixed with 1% glutaraldehyde, stained with methyl green pyronine, and permanently mounted.

Quantitation. The percentage of plaque-forming cells was determined by systematically scanning each slide at a final magnification of 160. A plaque was defined as a concentric zone of RBC hemolysis with a diameter at least as wide as the pancreatic cell which it surrounds. On average, 350–400 pancreatic cells per slide were counted. The amount of insulin secreted was estimated by quantitating individual plaque areas. These were measured using the Bioquant System IV (R&M Biometrics, Inc., Nashville, TN) in a manner described previously (13). Typically, 100 plaque areas were determined for each slide and the mean plaque area per slide for each cell subsequently calculated. The relationship between plaque area and hormone released has been shown to be linear over a wide range of responses (6).

Experimental Design. *Validation studies.* To validate the RHPA for insulin-secreting B cells, the following studies were undertaken: (i) All plaque-forming cells (i.e., presumed insulin-secreting cells) should be identifiable as B cells using an independent technique. Cells in the Cunningham chamber were exposed to an immunofluorescent insulin antiserum and the location of the resulting fluorescence compared with the locations

of the plaque-forming cells. (ii) If dispersed pancreatic cells are not exposed to insulin antibody, then no hemolytic plaques should form unless there is nonspecific activity. Therefore, insulin antiserum (developed in guinea pigs) was replaced with guinea pig serum and plaque formation monitored. (iii) If the insulin antibody were to be coupled to its specific antigen, then the antibody should not be available to interact with insulin being secreted by B cells and no hemolytic plaques would be expected to be formed. Insulin antiserum was preincubated with insulin for 2 hr before infusion into the Cunningham chambers and the slides were examined for the presence of plaques. (iv) Insulin antiserum was preabsorbed with glucagon and somatostatin, the secretory products of non-B islet cells, for 2 hr before infusion into the Cunningham chambers. If the antiserum was specific for insulin then plaques should be formed. (v) Given the fact that antiserum, protein A-coated erythrocytes, and complement are all necessary for the cascade of events required for the development of hemolytic plaques, omission of any one should prevent plaque formation. Each of these was systematically omitted from the procedure and the results documented. (vi) B cells identified with the RHPA should secrete insulin in response to glucose in a concentration- and time-dependent fashion. Therefore, cells were exposed to several concentrations of glucose including 0.75, 2.5, 5.0, 7.5, 10.0, 15.0, and 20 m*M* and allowed to incubate for 5 and 60 min before the addition of complement. The percentage of plaque-forming cells was calculated and the mean plaque area estimated as described above. (vii) Agents known to inhibit insulin secretion should suppress the number of plaque-forming cells and/or decrease plaque area. To test this prediction, cells were exposed to somatostatin in addition to 5 and 20 m*M* glucose and allowed to incubate for 5 min or 60 min before the addition of complement.

Effect of Glyburide on Glucose-Stimulated Insulin Secretion. Cells were exposed to medium alone or medium containing glyburide (100 n*M*) in the presence of 5.0 m*M* or 20.0 m*M* glucose and incubated for 5 min or 60 min before the addition of complement. Both the percentage of plaque-forming cells and the mean plaque area were calculated for each experimental group and the results compared.

Data Analysis. Student's *t* test was used for statistical analysis. Data analysis of plaque areas was based on the logarithmic mean for each treatment slide as these areas typically showed a log-normal distribution (13).

Results

Number of Islets and Pancreatic Cells. Using the methods mentioned above, 350 ± 51 islets (mean ± SD) were isolated from each rat pancreas. Upon dispersion, each islet in turn provided 800 ± 125 (mean ± SD) individual cells, of which over 80% were viable as judged by trypan blue exclusion just prior to use in the RHPA.

Validation of the RHPA Technique. In Figure 1 are shown four individual pancreatic cells, three of which are surrounded by hemolytic plaques. For the purpose of documenting that the plaque-forming cells were indeed insulin-secreting B cells, a series of experiments was undertaken as described in Materials and Methods. (i) When cells in the Cunningham chamber were exposed to an immunofluorescent insulin antiserum and the location of the resulting fluorescence compared with the locations of the plaque-forming cells, all cells surrounded by hemolytic plaques also were found to be labeled by the fluorescent tag. (ii) When insulin antiserum (developed in guinea pigs) was replaced by guinea pig serum, no hemolytic plaques were formed, thus eliminating the possibility of nonspecific hemolysis. (iii) When insulin antiserum was preincubated with insulin prior to infusion into the Cunningham chambers, no hemolytic plaques were developed. (iv) In contrast, when the insulin antiserum was preincubated with glucagon (up to 100 m*M*) or somatostatin (up to 5 m*M*), plaques were still formed. Therefore, the formation of plaques required an antibody specific for insulin. (v) When antiserum, erythrocytes not coupled to protein A, or complement was omitted from the procedure, no hemolytic plaques were developed, demonstrating the necessity for each of these reagents for the cascade of events required for the development of the plaques.

To demonstrate that the B cells identified using the RHPA would respond to physiologic stimulation and inhibitors of insulin secretion, detailed concentration- and time-response experiments between glucose and insulin secretion were undertaken. The percentage of plaque-forming cells and the mean plaque area in response to the several concentrations of glucose utilized

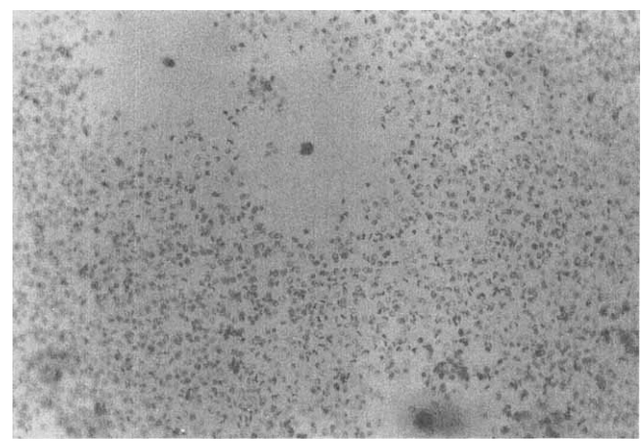

Figure 1. Photomicrograph of four islet cells, three of which are surrounded by a hemolytic plaque and a fourth which is not (original magnification ×160). The plaque-forming cells are presumably insulin-secreting B cells, given that the plaques were developed with a specific insulin antibody.

at 5- and 60-min incubation times are shown in Figure 2. Both the number of cells producing plaques and the mean area of the plaques increased progressively with increasing time of incubation at all glucose concentrations tested. The plaques were identified using glucose concentrations as low as 0.75 m*M*. There was a progressive increase in the percentage of cells forming plaques and plaque area as the glucose concentration was increased. Incubation beyond 1 hr (90 and 120 min) did not increase percentage plaque-forming cells at any glucose concentration (data not shown), and there was very little increase in plaque area beyond 7.5 m*M* glucose. Of importance to note is that plaques can be identified and quantitated as early as 5 min into the incubation period.

Having demonstrated augmentation of both the percentage of plaque-forming cells and the amount of insulin secretion by individual cells in response to glucose, the possibility that somatostatin (SRIF) would inhibit the number of glucose-stimulated plaque-forming cells and/or the amount of insulin secreted by the cells was investigated. As is shown in Table I, whereas a significant number of cells formed plaques at 5 and 60 min in the presence of 5 m*M* glucose, the number of plaque-forming cells was diminished at 5 min in the presence of 3 n*M* SRIF and abolished with ≥30 n*M* SRIF. In addition, the percentage of cells forming plaques at 60 min in the presence of glucose and ≥30 n*M* SRIF was significantly reduced. The mean plaque area of cells exposed to glucose and SRIF ≥30 n*M* for 60 min was significantly less than that of cells exposed only to glucose. Thirty nanomolar SRIF decreased the percentage of cells forming plaques in the presence of 20 n*M* glucose at 5 min but not 60 min and decreased plaque area at both time points.

Effect of Glyburide on Insulin Secretion. The results of exposure of the pancreatic cells to 100 n*M* glyburide are shown in Figure 3. As can be seen in A, the percentage of cells forming plaques in the presence of glyburide did not change at either glucose concentration at 5 min or 60 min of incubation. Similarly, as shown in B, the mean plaque area did not change at either 5 min or 60 min of exposure to glyburide in the presence of 5 m*M* glucose. However, at both time points there was a significant increase in the plaque area of these cells with glyburide in the presence of 20 m*M* glucose, approximating a 3-fold change at the latter time point.

Discussion

Although our understanding of the intracellular mechanisms that signal for insulin secretion by B cells and the agents that may affect insulin secretion has increased dramatically in the recent past, a number of issues of major importance remain unresolved. In part, certain questions related to insulin secretion remain unanswered because of the intrinsic limitations of the *in vitro* cell culture techniques which have been commonly employed for assessment of B cell function. Thus, most investigators have utilized intact or fragmented pancreases, whole islets, dispersed islet cells in monolayer culture or perifusion systems, or have relied upon tumor cell lines for such studies. In addition to the usual concerns related to necrosis of whole tissue blocks (14), the methods employing nondispersed cells do not allow for the exclusion of possible cell-cell interactions within a particular experimental setting. This consideration, which is of particular concern in the pancreatic islet, is addressed, at least on theoretical grounds, by dispersion of the islets into single cells.

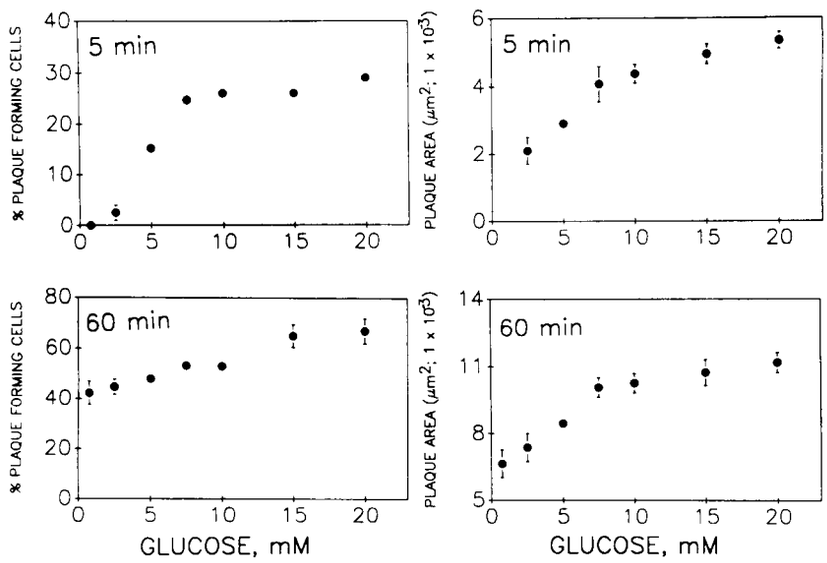

Figure 2. The percentage of plaque-forming cells and the mean plaque areas are shown in response to 0.75–20 m*M* glucose at 5 and 60 min. The data points are mean ± SE, $n = 4$.

Table I. Percentage of Plaque-Forming Cells and Plaque Area in Response to 5 and 20 m*M* Glucose ± 3, 30, or 100 n*M* Somatostatin at 5 and 60 Min of Incubation[a]

	% Plaque-forming cells		Plaque area (μm^2)	
	5 min	60 min	5 min	60 min
5 m*M* Glucose	18.7 ± 2.1[a]	45.5 ± 3.5	3892 ± 679	9014 ± 990
5 m*M* Glucose + 3 n*M* SRIF	7.0 ± 3.0[b]	40.2 ± 4.2	2856 ± 779	7861 ± 802
+ 30 n*M* SRIF	0	34.0 ± 2.4[b]	0	6481 ± 679[b]
+ 100 n*M* SRIF	0	31.0 ± 5.0[b]	0	6181 ± 789[b]
20 m*M* Glucose	31.0 ± 4.1	62.0 ± 5.0	4848 ± 416	12010 ± 1072
20 m*M* Glucose + 30 n*M* SRIF	22.0 ± 3.0[b]	51.0 ± 5.2	3566 ± 256[b]	10331 ± 876[b]

[a] Values are mean ± SE, $n = 3$–6.
[b] $P < 0.05$, Student's t test; glucose plus SRIF versus glucose alone.

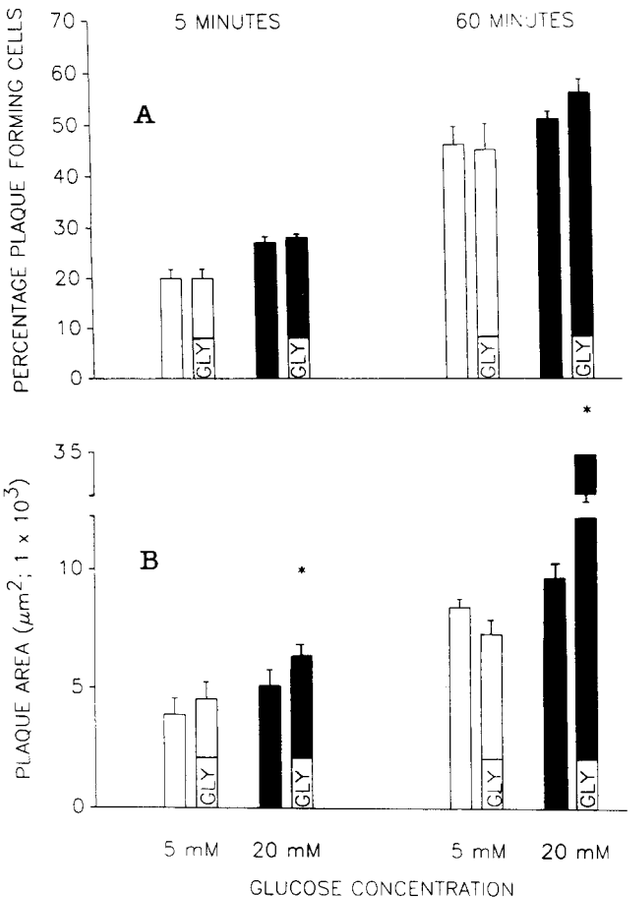

Figure 3. In A are shown the percentages of plaque-forming cells and in B the plaque areas measured in response to control medium or 100 n*M* glyburide (GLY) in the presence of 5 m*M* or 20 m*M* glucose at 5 or 60 min of incubation time. Values indicate mean ± SE, $n = 6$. *$P < 0.05$ (Student's t test).

However, the techniques of monolayer culture and perifusion of such dispersed cells also have inherent limitations. In particular, these techniques do not allow for adequate assessment of the relative contributions made toward an overall response by the individual cell types within these mixed cell populations. This problem has served to focus attention on the possibility of obtaining a pure population of B cells and has resulted in the identification and utilization of certain pancreatic tumor cell lines (15–17). Unfortunately, such cell lines may have their own limitations, given that they are by definition nonphysiologic and that they may fail to respond to glucose stimulation and somatostatin inhibition.

Very recently, we (7, 13, 18) and others (5, 6) working with nonpancreatic endocrine systems and faced with similar concerns as discussed above have adapted the so-called RHPA for the characterization of hormone secretion by individual cells of known type. Indeed, the laboratories of Salomon and Meda (9) and Hiriart and Matteson (8) have reported the application of RHPA technology for the study of pancreatic B cells from normal rats. Unfortunately, both groups were hampered by an inability to detect changes in insulin secretion at early time points after challenge with a secretagogue. Nonetheless, believing that this assay method would prove ideal for the investigation of questions related to the effect of glyburide on insulin secretion, we sought to validate and apply a modified RHPA for this purpose.

Our overall approach was to combine a well-established method for the dispersion of pancreatic islet tissue into single cells (11, 12) with a RHPA which had been successfully applied to the study of dispersed rat anterior pituitary cells (13, 18). Using these technologies, we have shown that the majority (i.e., over 80%) of pancreatic cells are viable following dispersion and that approximately 45% secrete insulin when incubated with medium containing a concentration of glucose (5 m*M*) which simulates that observed in the fed rat. It has been demonstrated convincingly that the amount of hormone secreted by a cell is directly and positively correlated with the logarithm of the plaque area surrounding that cell (22) and that the relationship between hormone released and plaque area is linear over a wide range of responses (6). Therefore, we have used the logarithmic mean plaque areas as an index of the quantity of insulin released by isolated B cells of the

pancreas. The B cells identified by the RHPA respond in a concentration- and time-dependent manner to stimulation by glucose and exhibit inhibition by somatostatin. The secretion of insulin can readily be detected as early as 2.5 min (data not shown), and easily estimated at 5 min after stimulation of the cells with glucose, allowing for the design and implementation of studies which require that secretion be promptly monitored following the application of a physiologic or pharmacologic probe. In addition, the relationships between glucose concentration and insulin secretion over a relatively wide range of glucose concentrations will allow for investigations focused on B cell secretory capacity within glucose milieus simulating hypoglycemic, euglycemic, and hyperglycemic conditions. This method is specifically designed to study single B cells, removed from contact with other B cells and from paracrine influences, unlike previously described methods of studying single B cells (23). Therefore, comparisons of the RHPA with other cell culture methods is difficult. However, it is known that purified single B cells release at least 30-fold less insulin than cells within intact islets (24). Thus, some of our responses, such as the small increase in plaque area beyond 7.5 m*M* glucose (the observed minimum glucose concentration required to cause plaque formation) and the maximum of 62% of islet cells forming plaques with 20 m*M* glucose are in all probability due to the singularity of the B cells in our system. This modified and validated technique will permit, in addition to assessment of insulin secretion by individual B cells, monitoring of certain intracellular events (e.g., changes in intracellular calcium) in response to a variety of physiologic and pharmacologic challenges.

Using a RHPA we have, in the current study, addressed the issue of whether glyburide stimulates the secretion of insulin by a direct effect on the B cell and, if so, whether it does so by recruiting previously nonsecreting B cells and/or by enhancing the insulin secretory capacity of previously secreting cells. Our results failed to demonstrate any effect of this pharmacologic agent on the percentage of cells secreting insulin when exposed to 5 m*M* or 20 m*M* glucose concentrations. Similarly, the amount of insulin secreted by the single B cells exposed to 5 m*M* glucose was not augmented by glyburide. This finding is somewhat at variance with other data (25, 26) which have demonstrated sulfonylurea effects independent of glucose. However, concentrations of drug used were higher than those employed in the present study and may have been high enough to mimic the effects of glucose stimulation on B cell depolarization (27). Glyburide did significantly enhance the amount of insulin secreted by cells in the presence of 20 m*M* glucose, an observation consistent with the glucose-dependent permissive factor possibly required for the normal pattern of depolarization seen in B cells (27). It is unlikely that this augmentation represents cell lysis as it did not occur in the presence of 5 m*M* glucose and incubation of islet cells with glyburide in the presence of 20 m*M* glucose for 2 hr did not alter the percentage of cells identified with trypan blue staining (data not shown). That glyburide enhances insulin secretion in the presence of elevated glucose at the cellular levels is of interest given that it has been proposed that *in vivo*, glyburide signals for enhanced insulin release only within the setting of hyperglycemia.

Although the current study clearly documents a direct effect of glyburide on glucose-stimulated insulin secretion by individual B cells, the mechanism(s) subserving this effect remains unclear. Previous studies have suggested that glyburide may affect ATP-sensitive K^+ channels and increase intracellular calcium concentrations (4, 20, 21). We believe that the use of the RHPA, in combination with currently available fluorescent dye techniques for detecting changes in intracellular calcium concentrations, will allow for more detailed studies concerning the hypothesis that glyburide alters the glucose-stimulated calcium signal which ultimately results in insulin secretion.

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