Individual β Cells within the Intact Islet Differentially Respond to Glucose*

(Rceived for publication, July 28, 1997)

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Insulin production by the pancreatic islet is tightly coupled to the concentration of blood glucose. The mechanism by which glucose controls proinsulin biosynthesis in β cells is poorly understood. Analysis of insulin gene expression in individual cells within whole, living islets using adenovirus gene transfer and direct observation of insulin promoter-directed green fluorescent protein activity indicates that β cells are functionally heterogeneous. An increase in glucose concentration not only stimulates expression within individual β cells, but unexpectedly acts to increase the total number of positive cells. The net islet response to a given glucose stimulus reflects an integrated action of β cells with individually differing behaviors. This additional level of functional complexity may provide new insights into the pathophysiology and treatment of diabetes mellitus.

Glucose stimulates both insulin secretion and proinsulin biosynthesis through signals associated with its metabolism in the β cell of the pancreatic islet (1–3). Glucose metabolism increases the ratio of ATP/ADP in the β cell causing the closure of a tonically active potassium (K\textsubscript{ATP}) channel. Subsequent membrane depolarization opens L-type voltage-dependent calcium channels. The resulting calcium influx leads to secretion of stored insulin. Beyond the requirement for glucose metabolism, much less is known about the mechanisms by which proinsulin biosynthesis is regulated. Studies measuring aggregate responses of clonal, insulinoma cells or dispersed islet cells in culture have demonstrated that glucose stimulates insulin gene transcription through activation of the insulin promoter (4–11).

Subpopulations of dispersed β cells have been isolated on the basis of differing rates of glucose metabolism measured by NAD(P)H autofluorescence. Differences in the glucose concentration dependence of insulin biosynthesis and secretion among isolated β cells demonstrated that β cells are functionally heterogeneous (12–15). However, β cells are located within the 500–2,000 cell pancreatic islet, which provides potential interactions with other endocrine cell types: α (glucagon), δ (somatostatin), and PP (pancreatic polypeptide). Structural components such as intercellular matrix conform these cells to a specific microarchitecture (16). Therefore, the significance of studies using isolated β cells has been challenged by observations which suggest that β cells within the intact islet may function as part of a uniformly linked network. Glucose stimulation induced coupled oscillations of intracellular calcium through the entire mouse islet, suggesting that for insulin secretion, the entire β cell population acts in unison (17–19).

Examination of energy metabolism in the intact islet revealed a uniform, glucose-stimulated increase in cellular NAD(P)H autofluorescence (20). Moreover, within the islet, β cells appeared to be interconnected by gap junctions containing connexin 43 which may allow for extensive cell-cell communication via diffusion of small molecules (21, 22). We propose that regulation of insulin production in the islet may not be fully explained by the behavior of transformed islet cell lines or isolated native β cells. We have developed an experimental approach to study insulin promoter function within the microenvironment of the intact islet and at the resolution of a single cell. We have identified unique differences in the glucose response of individual β cells.

EXPERIMENTAL PROCEDURES

Construction and Preparation of Plasmids and Recombinant Adenovirus—Plasmids pJM17 (23), pbHG11 (24), and pACCMVpLpA (25) have been described. Plasmids pACCMV-β-gal, pACCMV-GFP, pACIns-β-gal, and pACIns-GFP were obtained by subcloning the genes coding for either β-galactosidase (β-gal) with an SV40 nuclear localization sequence or the Aequoria victoria green fluorescent protein (GFP), wild-type, or S65T variant (26–28) driven by cytomegalovirus (CMV) or rat insulin-I (Ins) promoter/enhancers into the shuttle plasmid, pACCMVpLpA.

Recombinant, replication-defective, adenoviruses (Ad) containing a single transgene at the viral E1 region were obtained as described previously (23, 29, 30) by homologous recombination upon cotransfection of human embryonic kidney 293 cells (HEK 293) with the viral genome-containing plasmid pJM17 and the appropriate transgene-containing pAC derivative shuttle plasmid.

Construction of the recombinant Ad containing two independent transcriptional units (AdIns-GFP/CMV-β-gal) was carried out by homologous recombination in HEK 293 cells that were cotransfected with the Ins-GFP-containing shuttle vector pACIns-GFP (that allows the rescue of this transcriptional unit in the viral E1 region) and a derivative of the viral genome-containing plasmid pbHG11 (Microbix, Toronto, Canada) in which a second transcriptional unit (CMV-β-gal) had been inserted into a unique Pac-I restriction site located at the deletion of adenoviral E3 region sequences.2

Clonal viral stocks were isolated by single plaque purification, their DNA analyzed by polymerase chain reaction to confirm the incorporation of the desired transgene insert, amplified in HEK 293 cells, and purified and concentrated to 10¹²–¹³ plaque-forming units/ml by CsCl ultracentrifugation.

Islet Isolation and Culture and Adenovirus Treatment—Islets from pentobarbital-anesthetized male Sprague-Dawley rats were isolated by pancreatic duct infiltration with collagenase, Histopaque gradient separations.

The abbreviations used are: β-gal, β-galactosidase; GFP, green fluorescent protein; Ad, adenovirus(es); CMV, cytomegalovirus; Ins, rat insulin-I; PI, propidium iodide.

1 The work was supported by National Institutes of Health Grants DK51111 and DK34447, a New England Health Care Foundation Grant to L. G. M., and National Institutes of Health GRASP Center Grant DK34928. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
2 Supported by National Institutes of Health Training Fellowship DK07704 and the Endocrine Fellows Foundation.

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This paper is available on line at http://www.jbc.org
**Evidence for β Cell Heterogeneity in Pancreatic Islets**

**Glucose-induced insulin promoter activity of individual β cells**

Isolated islets were treated with recombinant adenoviruses, either AdCMV-β-gal (open circles) or AdIns-β-gal (solid circles). After incubation for 48 h in medium with various concentrations of glucose (x axis), β-gal activity (y axis) were determined from whole islet extracts (see “Experimental Procedures”). Enzymatic activity is expressed as arbitrary units/mg of protein normalized to values from control islets treated with promoterless adenovirus. S.D. values from the mean (bars) are shown representing five experiments with a least 25 islets included per data point. Numbers in parentheses represent the ratio of the value at 20 mM glucose to that for the same virus at 2 mM glucose, i.e., fold stimulation.

**Assays of Reporter Gene Expression**

For determination of β-gal activity, islets were sonicated in small volumes, protein concentrations were determined, and extracts were assayed colorimetrically with o-nitrophenyl-β-D-galactopyranoside. For determination of GFP fluorescence, islets were imaged in 35-mm coverslip bottomed dishes (MatTek axis)-related changes in fluorescent intensity and wide range of islet (propidium iodide (PI) prior to imaging (488 nm excitation/610-nm long pass emission filter). Islets were stained with Vital staining of AdCMV-GFP transduced islets using PI (see “Experimental Procedures”).

**Confocal Microscopy and Immunofluorescence**
The GFP images were acquired using a Zeiss LSM410 confocal microscope (488 nm excitation line and 670-nm long pass emission filter). Individual GFP and anti-β-gal (Cy-5) fluorescence values for each GFP-positive cell were determined as above. Correlation analysis was performed with Statview 4.01.

**RESULTS**

**Glucose Induces Insulin Promoter Activity in Whole, Intact Pancreatic Islets**
We utilized attenuated adenovirus gene transfer to measure glucose-stimulated insulin promoter activity in cultured, intact rat pancreatic islets. This approach allows for the efficient expression of transgenes in islets without detectable perturbations of normal islet function (33, 34). Isolated rat islets were treated with a recombinant, type 5 adenovirus (see “Experimental Procedures”), which contains the rat Ins promoter (35) directing the expression of the lacZ (β-galactosidase) reporter gene (AdIns-β-gal). After a 48-h incubation period at differing glucose concentrations, extracts from islets that had been exposed to progressively higher levels of glucose yielded correspondingly higher levels of total β-gal activity (Fig. 1). We observed a 13-fold maximum stimulation from 2 to 20 mM glucose. In contrast, when a control adenovirus containing the CMV promoter-enhancer (AdCMV-β-gal) was utilized (Fig. 1), little effect of glucose on β-gal activity was observed. Our results are consistent with the observation that proinsulin mRNA abundance increases approximately 8–12-fold as a result of incubating islets in low or high glucose medium (36). Although these results established that the glucose induction of the insulin promoter could be detected after gene transfer into the whole islet, only the net islet response and not the contributions of individual β cells was measured.

**Glucose Stimulates Insulin Promoter Activity of Individual β Cells within the Intact Islet**
We generated a recombinant CMV GFP adenovirus (AdCMV-GFP) to provide a means for detection and quantification of promoter activity of individual cells within the intact, living islet. GFP fluorescence was detected by confocal microscopy imaging in a high percentage of living cells (Fig. 2A). Whereas transduction efficiencies approached 100% for small islets, they were lower for large islets. Vital staining of AdCMV-GFP transduced islets using PI (see “Experimental Procedures”) revealed that this decreased efficiency was often associated with islets exhibiting central ne-
Evidence for β Cell Heterogeneity in Pancreatic Islets

Differential Activation of β Cell Subpopulations with Varying Sensitivities to Glucose—To evaluate the effect of glucose on Ins-GFP expression, isolated islets were transduced with AdIns-GFP as a single pool and divided into separate dishes. Incubation proceeded for 48–72 h at glucose concentrations ranging from 2 to 20 mM. The islets were then imaged for GFP fluorescence by optical sectioning (see “Experimental Procedures”). Green fluorescence corresponds to the GFP signal. A faint red background autofluorescence signal was co-imaged to outline the islet boundaries. C, the number of total GFP-positive cells (y axis) from AdCMV-GFP treated islets (open squares) or AdIns-GFP-treated islets (closed circles) in an area-normalized z axis section of islets was plotted against glucose concentration (x axis). Positive cells were defined as signals at least 2-fold above the islet background intensity. D, the relative fluorescent intensities of GFP-positive cells (see “Experimental Procedures”) were determined (y axis) in islets treated with AdCMV-GFP (open squares) or AdIns-GFP (closed squares) and plotted against glucose concentration (x axis). For both graphs standard deviations from the mean (bars) are shown representing four experiments with a least 50 islets included per data point. Fold stimulation induced by incubation at 20 mM versus 2 mM glucose is indicated in parentheses.

Thus, the inherent lack of uniformity when using primary islet preparations but was not related to adenovirus exposure. Moreover, a comparison of relative cell intensities revealed that the average GFP fluorescence per positive cell also increased as a function of glucose concentration (Fig. 3D). In contrast, no significant difference in the number of GFP-positive cells and only a slight increase in average fluorescence intensity were detected in AdCMV-GFP-treated islets incubated in high or low glucose medium (Fig. 3B). These results clearly indicate that glucose stimulation does not result in a uniform β cell response. Glucose induction of the insulin transcriptional promoter involves the differential activation of β cell subpopulations with varying sensitivities to glucose. Insulin promoter activity within most β cells exhibits a graded dose response to glucose rather than a simple on/off behavior.

Individual β Cells within the Intact Islet Differentially Respond to Glucose—To confirm that the differences among individual cells in insulin promoter function were not due to cell cell variability in adenoviral transduction, we designed an adenovirus containing a second transcription unit to serve as an internal control (see “Experimental Procedures”). Fig. 4A shows a schematic representation of the AdIns-GFP/CMV-β-gal generated by insertion of a CMV-nlsLacZ expression cassette at the site of a deletion in the viral E3 region approximately 25 kilobase pairs rightward from Ins-GFP at ΔE1 (24). We performed simultaneous measurement of anti-β-gal immunofluorescence and GFP fluorescence on fixed islet sections to compare respective levels of expression. Fig. 4, B through D, depict a section of an Ins-GFP/CMV-β-gal-transduced islet incubated at a half-maximal stimulatory concentration of glucose (6 mM) to generate a wide range of Ins-GFP signal intensities. More cells expressed β-gal than GFP, presumably representing...
transduced non-β cells or β cells whose GFP expression was below detection threshold. No cells exhibited GFP fluorescence in the absence of β-gal immunostaining. When averaged over the whole islet, the total number of GFP-positive cells correlates with the number of β-gal-positive cells with the same relationship holding for estimates of total fluorescence in a whole islet section. Thus, this construct provides a means to correct for overall transduction efficiency. The values of the relative signal intensities for GFP fluorescence and β-gal immunofluorescence were compared within a single islet section for every cell that expressed both transgenes (see “Experimental Procedures”). The plot of relative intensities in Fig. 4E failed to demonstrate any general correlation between Ins-GFP and CMV-β-gal signals from individual cells. Therefore, even though transgene copy number undoubtedly affects the signal of a given cell, it cannot account for the heterogeneity of β cell response with respect to glucose stimulation of insulin promoter activity. This result reinforces other experimental data,3 suggesting that promoters inserted at these distant sites within the adenovirus genome can be shown to function independently.

The Glucose Stimulation of Insulin Promoter Activity Is Insensitive to Inhibitors and Inducers of Insulin Secretion—We then investigated whether inhibition of signaling pathways

3 L. Moitoso de Vargas, R. Siegel, and L. G. Moss, unpublished observations.
that are known to couple glucose metabolism with insulin secretion would affect the glucose response of insulin promoter activity. Treatment of islets with nifedipine or verapamil (inhibitors of L-type voltage-dependent calcium channels) at concentrations that inhibit insulin secretion by >90% did not block insulin promoter induction by 20 μM glucose (Fig. 5). Furthermore, treatment with the sulfonylurea glyburide, which stimulates insulin secretion through inhibition of the ATP-sensitive K⁺ channel, failed to increase insulin promoter activity at low glucose (2 mM). Similar results were obtained using both reporter systems. Fig. 5A indicates the total β-gal activity after transduction with AdIns-β-gal. The number and intensity of fluorescent cells after treatment with AdIns-GFP is shown in Fig. 5B. The absence of insulin promoter activation by the sulfonylurea and the failure of calcium channel antagonists to block glucose stimulation of the insulin promoter indicate that insulin release and insulin promoter activation must utilize different signaling pathways. Although the response of gene expression to glucose in individual β cells can be visualized in the intact islet, other differences, such as secretory behavior, may be masked by networked cell depolarization or the rapid diffusion of small molecule signals.

**DISCUSSION**

Our studies suggest that, in terms of insulin promoter activity, functional β cell heterogeneity in the context of the intact islet provides an additional level of organizational complexity. The net β cell response may result from a unique integration of cells with individually specific stimulus-response characteristics. For example, even at very low glucose levels some β cells appear to be tonically “on.” These GFP-positive β cells could represent the insulin source which fulfills the basal, non-glucose requirements of the organism. Functional differences between β cells may arise by random phenotypic variation or by specific programmed assignment. No specific pattern relating β cell behavior and geographic location within the islet has yet been discerned. However, when dispersed islet cells were transduced with Ins- or CMV-GFP Ad and cultured under identical conditions as intact islets, we observed the same overall glucose response patterns as in Fig. 3 (data not shown). This suggests that the variation of insulin promoter activation by glucose observed in β cells may represent a cell autonomous phenomenon.

Further investigation will be required to elucidate the mechanisms responsible for β cell heterogeneity. Our ability to measure gene expression in individual β cells in the living islet will allow for the direct analysis and manipulation of cellular signaling pathways. In addition, GFP fluorescence tagging based on promoter function will provide a means by which behaviorally disparate populations of β cells can be obtained and regulatory factors that mediate glucose responsiveness characterized. For example, glucokinase/hexokinase activity dictates the glucose concentration dependence of insulin secretion in the islet by serving as the rate-limiting step for β cell glycolysis (37). Variations in glucokinase activity, that could provide an explanation for these differences in β cell glucose responses, could be directly measured. A more complete understanding of islet function may aid in the development of bio-prosthetic approaches to insulin replacement in diabetes mellitus and shed light on the origins of the β cell dysfunction seen in non-insulin-dependent diabetes (38).

Finally, other apparently homogeneous cell types such as neurons can exhibit heterogeneous behaviors. Recently, prolactin-secreting pituitary cells with differing hormonal responses have been characterized (39). Thus, our findings sound an additional note of caution, not only for the extrapolation of data regarding physiological function obtained from transformed, clonal cell lines to native tissues, but also for the need to evaluate the contributions of individual cells within those tissues.

**Acknowledgments**—We thank T. Becker and R. Noel for adenovirus technology and J. Moss, M. Brown, and A. Leiter for helpful discussions and reading of this manuscript.

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