Parallel Reduction of Pancreas Insulin Content and Insulin Secretion in 48-h Tolbutamide-Infused Normoglycemic Rats

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The overworked–β-cell hypothesis proposes that lowered glucose-potentiated insulin secretory responses in diabetes are secondary to hyperstimulated insulin secretion and depletion of the β-cell insulin stores. We tested this hypothesis in normal rats using a 48-h infusion of 200 mg · kg^{-1} · day^{-1} tolbutamide in 20% glucose. Insulin secretion was measured by in vitro pancreas perfusion. Twice daily blood glucose values were equal in the tolbutamide-infused and control rats. Pancreas insulin content was 47 ± 7% that of the controls (P < 0.004). Insulin responses to 16.7 mmol/l glucose, 16.7 mmol/l glucose/10 mmol/l arginine, and 5.5 mmol/l glucose/10 mmol/l arginine were reduced in parallel, except for the first phase response to 16.7 mmol/l glucose/arginine. Pancreas amylin content was unchanged in the tolbutamide-infused rats as was amylin secretion, resulting in higher than normal stored and secreted amylin-to-insulin molar ratios. Importantly, a raised amylin-to-insulin ratio and a relatively unimpaired first versus second phase insulin response for high glucose/arginine both occur in diabetic rats. Thus, our results support the overworked–β-cell hypothesis by showing chronic β-cell stimulation without hyperglycemia replicates part of the β-cell dysfunction found with diabetes. *Diabetes* 46:808–813, 1997

Diabetes is characterized by defective glucose-induced and glucose-potentiated insulin secretion (1,2). Restoring normoglycemia reverses much of the β-cell dysfunction (3,4), which has fostered the concept that some aspect of the diabetes environment such as hyperglycemia or hyperlipidemia is causative—so-called *glucose toxicity* or *lipotoxicity* (5–9). These hypotheses envision a direct detrimental effect on the expression and/or function of one or multiple β-cell proteins. We have proposed an alternative hypothesis—*the overworked–β-cell hypothesis*—whereby hyperstimulated insulin secretion causes impaired glucose potentiation (10). The proposed mechanism is lowered β-cell insulin stores, based on the pancreas insulin content being subnormal in diabetic states, plus our having shown maneuvers that lower insulin secretion, notably the inhibitor diazoxide (11) and a 40-h fast (12), paradoxically increased glucose-potentiated insulin responses and pancreas insulin content in an identical fashion. In contrast, glucose-induced insulin secretion was minimally affected, suggesting another etiology. Importantly, this effect of fasting (13) and diazoxide (14), which paradoxically augments insulin secretion, has also been observed in human NIDDM. Laedtke et al. (15) have also reported that insulin secretion and pulsatility were increased in NIDDM after an overnight infusion of somatostatin. Finally, hyperstimulated insulin secretion and depleted insulin stores were recently reported to cause the β-cell secretory dysfunction in rat β-cells cultured under high glucose conditions (16).

One approach to validating the overworked–β-cell hypothesis is to determine if chronic β-cell stimulation in normal rats replicates the insulin secretory dysfunction of diabetes. A method that dates back many years is sulfonylurea administration (17–20): Supporting our hypothesis, insulin responses are lowered in combination with reduced β-cell insulin stores, although the role of hypoglycemia has not been well worked out. Also unclear is the basis for the reduced insulin secretory responses.

The current study used 48-h tolbutamide-infused rats to explore the relationship between pancreas insulin content and quantitative insulin secretion. A secondary issue concerns amylin secretion. Amylin is synthesized in the β-cell, copackaged with insulin in granules, and co-secreted (21). Its importance is that it comprises the islet amyloid deposits that are found in NIDDM (22,23). The secreted amylin-to-insulin ratio is increased in diabetic animals, which may hold pathophysiological significance in terms of the amyloid deposition in NIDDM (24–26). The current study makes the same observation in the tolbutamide-infused rats, suggesting that a raised amylin-to-insulin ratio is another manifestation of the overworked–β-cell syndrome.

RESEARCH DESIGN AND METHODS

Tolbutamide-infusion model. Male Sprague-Dawley rats (Taconic, Germantown, NY) underwent 48-h intravenous infusions, as previously described, through jugular venous catheters that were implanted the day before (27). The protocol was 200 mg · kg^{-1} · day^{-1} tolbutamide (Upjohn, Kalamazoo, MI) in 0.45% NaCl/20% glucose at an infusion rate of 2 ml/h; glucose was added for the maintenance of normoglycemia. Control rats were parallel infused with 0.45% NaCl. Blood for plasma glucose was obtained from the tail vein before starting the infusion (time 0) and at 8, 24, 32, and 48 h.

In situ perfused pancreas and insulin content. Immediately following the infusions, rats underwent pancreas perfusion using a technique that has been described elsewhere (28). The perfusate was a Krebs-Ringer bicarbonate buffer (pH 7.4) plus 4% dextran T_{70} and 0.2% bovine serum albumin fraction

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RIA, radioimmunoassay.

TABLE 1
General characteristics of tolbutamide-infused and control rats

Animals	*n*	Body weight (g)	Plasma glucose (mmol/l)	Pancreas weight (g)	Insulin content (μg/pancreas)	Amylin content (μg/pancreas)
Controls	5	216 ± 12	8.1 ± 0.2	1.00 ± 0.06	71 ± 8	0.78 ± 0.02
Tolbutamide	5	224 ± 8	8.2 ± 0.2	0.99 ± 0.04	33 ± 5	0.76 ± 0.10
P		NS	NS	NS	0.004	NS

Rats underwent 48-h intravenous infusion of 200 mg · kg^{-1} · day^{-1} tolbutamide in 0.45% NaCl/20% glucose at 2 ml/h; controls received 0.45% NaCl.

V (Sigma, St. Louis, MO) at an infusion rate of 3.5 ml/min. Glucose and arginine were added as shown at the top of the figures. After a 17-min equilibration period, 1-min samples were collected in tubes containing 8 mg EDTA and stored at –20°C pending insulin and (where measured) amylin radioimmunoassay (RIA). At the end of each perfusion, pancreases were excised, blotted, weighed, and stored at –20°C in acid ethanol pending homogenization and assay for insulin and amylin contents.

Analytical methods. Plasma glucose levels were measured with a Beckman Glucose Analyzer II (Beckman, Fullerton, CA). The insulin RIA employed charcoal separation (29) and rat insulin standards (Lilly, Indianapolis, IN) with a sensitivity of 200 pg/ml. The amylin assay was a commercial kit (Peninsula, Belmont, CA) with a sensitivity of 30 pg/ml.

Data presentation and statistical methods. Data are expressed as means ± SE. Statistical significance was determined by the Student's unpaired *t* test. Mean insulin or amylin output during pancreas perfusion was calculated as the average hormone concentration of the samples collected at minute intervals during that perfusate condition times the flow rate. First and second phase responses represent the first 4 min and rest of that perfusate, respectively. The amylin-to-insulin molar ratio was calculated using the molecular weight for rat amylin of 3,918 and the mean value for rat insulins I and II of 5,767.

RESULTS

Characteristics of tolbutamide-infused rats. The overworked–β-cell hypothesis was tested in nondiabetic rats using a 48-h tolbutamide infusion (Table 1). The prevention of hypoglycemia was critical because of the well-known inhibitory effect on insulin secretion and biosynthesis. Twenty percent glucose was added to the infusate based on our experience with tolbutamide infusions (30,31). Figure 1 confirms the glycemia levels measured at several points during the infusion were equal to the control rats. Insulin content was 47 ± 7% of the controls ($P < 0.004$) with no change in pancreas weight. In contrast, the pancreas amylin content was unchanged.

Insulin secretion. Insulin secretory responses were quantified by pancreas perfusion. The protocol was a baseline of 5.5 mmol/l glucose, 15 min of 16.7 mmol/l glucose to reflect glucose-induced insulin secretion, then 15 min of 16.7 mmol/l glucose/10 mmol/l arginine to reflect glucose potentiation (Fig. 2). In the controls, high glucose caused the expected biphasic insulin response followed by a large increase when arginine was added. Both responses were blunted in the tolbutamide rats: high glucose to 41 ± 6% of control ($P < 0.007$) and high glucose/arginine to 64 ± 9% of control ($P < 0.017$). Table 2 quantifies the first and second phases of these responses. For 16.7 mmol/l glucose, both phases matched the insulin content. In contrast, for the arginine response, the first phase was unchanged as opposed to the second phase, which paralleled the insulin content. Finding the spared first- versus second-phase insulin response was of particular interest, since we previously made the same observation in neonatal streptozotocin (32) and 90% pancreatectomy (11) diabetic rats with arginine/high glucose.

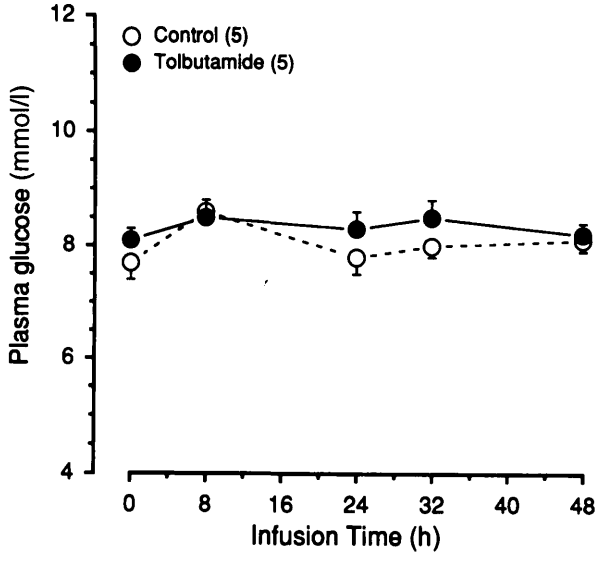

FIG. 1. Plasma glucose values during 48-h intravenous infusion of 200 mg · kg^{-1} · day^{-1} tolbutamide in 0.45% NaCl/20% glucose or 0.45% NaCl (controls).

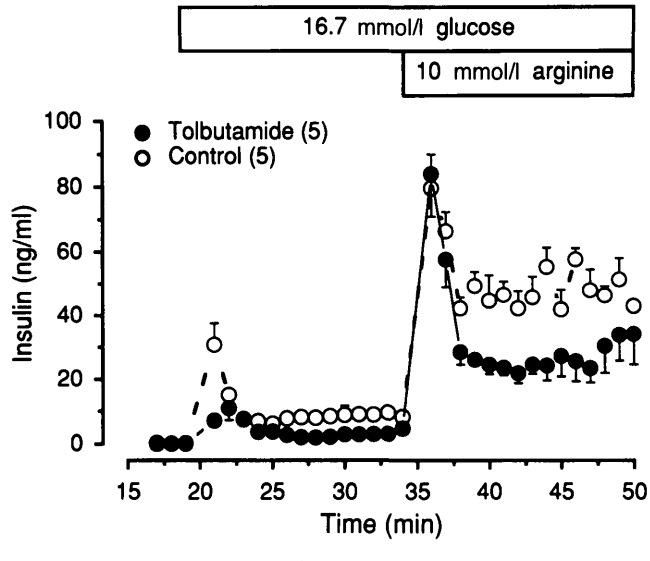

FIG. 2. Insulin secretion determined by in situ pancreas perfusion. Rats first underwent 48-h intravenous infusion of 200 mg · kg^{-1} · day^{-1} tolbutamide or 0.45% NaCl (controls).

TABLE 2
Phasic insulin secretion from tolbutamide-infused and control rats

Perfusate	Phase	Insulin secretion (ng/ml)		Tolbutamide (% controls)
		Control rats	Tolbutamide rats	
16.7 mmol/l glucose	First	17.8 ± 2.9	8.5 ± 2.2	48 ± 12
	Second	8.2 ± 1.7	3.0 ± 0.5	37 ± 6
16.7 mmol/l glucose + 10 mmol/l arginine	First	73.0 ± 8.0	70.8 ± 10.1	97 ± 14
	Second	47.1 ± 3.8	26.7 ± 4.2	57 ± 9
5.5 mmol/l glucose + 10 mmol/l arginine	First	20.5 ± 1.3	8.0 ± 2.5	39 ± 12
	Second	3.2 ± 0.8	1.1 ± 0.3	34 ± 9

Rats were infused 48-h with 200 mg · kg^{-1} · day^{-1} tolbutamide in 0.45% NaCl/20% glucose at 2 ml/h or 0.45% NaCl. Mean insulin secretion during in situ pancreas perfusion was calculated during minutes 1–4 (first phase) and 4–15 (second phase) for the perfusate conditions shown. Absolute data are shown in Figs. 2 (16.7 mmol/l glucose, 16.7 mmol/l glucose + 10 mmol/l arginine, $n = 5$ for both groups) and 3 (5.5 mmol/l glucose, 5.5 mmol/l glucose + 10 mmol/l arginine, $n = 4$ for both groups). The relative results in the tolbutamide rats (% controls) are compared in the text with the relative pancreas insulin content of 47 ± 7% of control.

To further investigate the relationship between insulin content and insulin secretory responses in the tolbutamide rats, a second group of perfusions was carried out using 5.5 mmol/l glucose (Fig. 3). There was virtually no insulin output to 5.5 mmol/l glucose in the control rats, making the effect of tolbutamide unmeasurable. In contrast, the 5.5 mmol/l glucose/arginine response was clearly evident, averaging 13% of that to 16.7 mmol/l glucose/arginine from the previous protocol. Insulin release with this much weaker stimulus again perfectly agreed with the pancreas insulin content in the tolbutamide-infused rats (37 ± 11% of the control value, $P < 0.005$). There were no phasic differences between the tolbutamide and control rats (Table 2).

Amylin secretion. We investigated the basis for the lowered insulin secretory responses by quantifying secretion of another granule peptide to identify impaired mobilization of granules versus altered granule content of insulin. Amylin was chosen based on the pancreas amylin content being unchanged in the tolbutamide-infused rats (Table 1). Amylin is secreted in small amounts from the β-cell; the sensitivity of the assay allowed measurement only of the samples to 16.7 mmol/l glucose/10 mmol/l arginine from protocol 1. Shown in Fig. 4, amylin secretion was normal to increased in the tolbutamide rats (integrated response, 11.1 ± 2.0 ng · 15 min^{-1} tolbutamide-infused rats vs. 5.4 ± 1.9 ng · 15 min^{-1} in the controls, $P < 0.07$). The unchanged amylin secretion/content with the lowered insulin values resulted in increased amylin-to-insulin ratios for both measures (Fig. 5).

DISCUSSION

The current study shows striking similarities between the β-cell functional characteristics of tolbutamide-infused rats and diabetic rats. Clearest was the linear relationship between insulin secretion and pancreas insulin content, which parallels the identical fractional increases in 90% pancreatectomy rats after diazoxide (11) and fasting (12). The one disagreement, the spared first phase response to 16.7 mmol/l glucose/arginine, also supports linking the models, since we

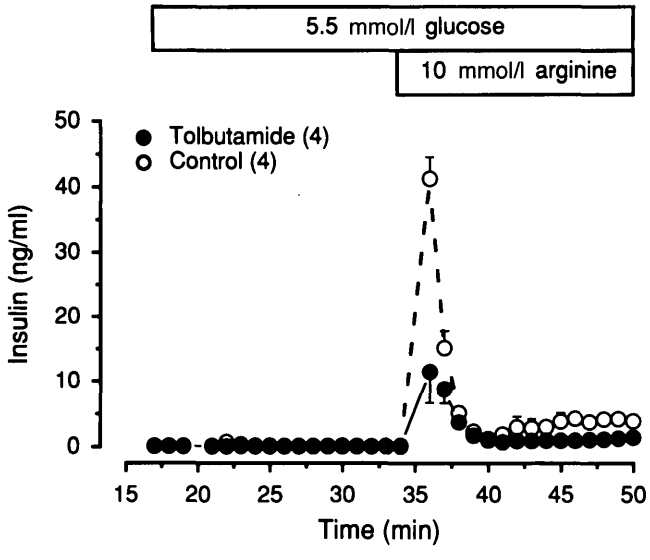

FIG. 3. Insulin secretion determined by in situ pancreas perfusion. Rats first underwent 48-h intravenous infusion of 200 mg · kg^{-1} · day^{-1} tolbutamide or 0.45% NaCl (controls).

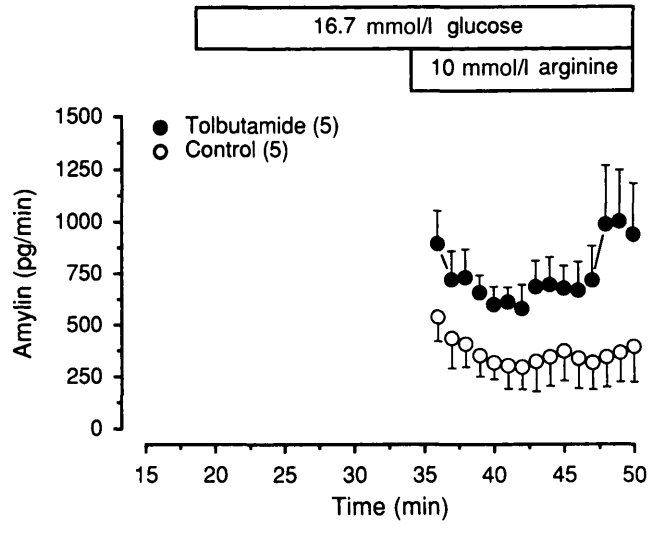

FIG. 4. Amylin secretion determined by in situ pancreas perfusion. Rats first underwent 48-h intravenous infusion of 200 mg · kg^{-1} · day^{-1} tolbutamide or 0.45% NaCl (controls). The samples were the same as those shown for insulin secretion in Fig. 2.

made the same observation in neonatal streptozotocin and 90% pancreatectomy diabetic rats (11,32). Finally, a raised ratio of amylin to insulin occurs in diabetic rats (24–26). However, a note of clarification is needed. Not all the β-cell defects that occur with diabetes were found in the tolbutamide rats, in particular the hyperstimulated insulin response to arginine at a basal glucose level (32,33), the paradoxical increase of insulin release after lowering of the glucose concentration (34), and the profound impairment of glucose-induced insulin secretion that occurs even when pancreas insulin stores are minimally reduced (35,36). Thus, our results support the overworked–β-cell hypothesis by showing that chronic β-cell stimulation in nondiabetic rats replicates some of the β-cell functional defects that characterize the diabetic state and by focusing on the important relationship between insulin content and secretory responses. However, they also show that the diabetic state has complex pathophysiological effects on the β-cell that are mediated through more than one mechanism.

The proposed cause of the lowered insulin secretory responses according to the overworked–β-cell hypothesis is reduced insulin stores. However, it must be emphasized this idea is based on correlative data (including the current study). How were the lowered insulin content and secretory responses linked in the tolbutamide-infused rats? Insight into this question was obtained from the preserved amylin secretion. We interpret this finding as evidence that the granule composition of insulin is altered rather than defective granule mobilization. Alternatively, impaired mobilization of amylin-enriched granules cannot be excluded. Intriguing from this second point of view is the differential phasic insulin response to the high glucose/arginine. Zawalich (37) postulated that impaired phospholipase C and protein kinase C activation underlies the impaired insulin secretion with hyperglycemia, based on substantial in vitro data. Of particular interest, their data have shown these enzymes act primarily in the second phase of glucose-induced insulin secretion, so the first phase is relatively unimpaired versus the second phase with chronic β-cell stimulation, analogous to our findings. However, the current results showed differential phases only for the arginine response, not glucose-induced insulin secretion, which argues against a causative role for

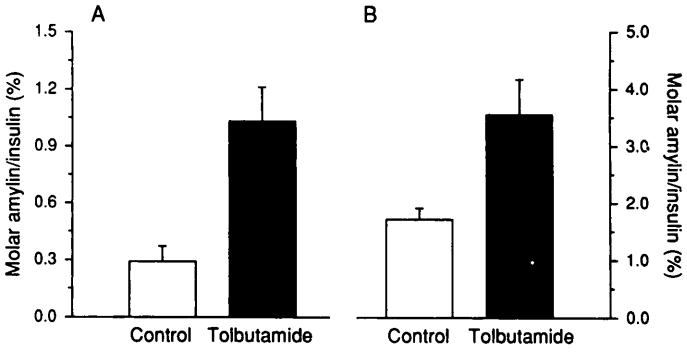

FIG. 5. Amylin-to-insulin molar ratio (%) secreted and stored from the pancreas of rats infused 48-h with 200 mg · kg^{-1} · day^{-1} tolbutamide or 0.45% NaCl. *A* is the ratio of amylin to insulin secreted during 15 min of 16.7 mmol/l glucose/10 mmol/l arginine, based on the values shown in Figs. 4 and 2, respectively. *B* is the ratio of the pancreas contents from Table 1. Both measures were significantly increased in the tolbutamide rats: $P < 0.006$ in *A*, and $P < 0.02$ in *B*.

these enzymes. Additional contrary evidence is the absence of phasic dichotomy in the lowered insulin secretory response to 5.5 mmol/l glucose/arginine. Thus, our results most agree with the granule insulin content being lowered. Having said that, the differential effect of tolbutamide and diabetes on phasic insulin secretion with arginine/high glucose is an intriguing finding that needs additional investigation.

Whether our conclusion of a subnormal granule insulin content is applicable to diabetic states is presently unknown. Sulfonylureas stimulate insulin secretion without increasing proinsulin synthesis and may inhibit it (18,38,39). As such, the reduction of granule insulin content is not a surprise. The situation with regard to hyperglycemia is less predictable. Supporting a common pathogenesis is the raised amylin-to-insulin ratio in diabetic rats (24–26). Possibly explaining this finding is an inhibitory effect of hyperglycemia on proinsulin gene transcription, a hypothesis that has been suggested by several in vitro studies (40,41) and a recent abstract in markedly diabetic rats (42).

An unexpected finding was the dichotomy in the amylin-to-insulin content and secretion. Unclear from the overworked–β-cell schema is why upregulating insulin secretion

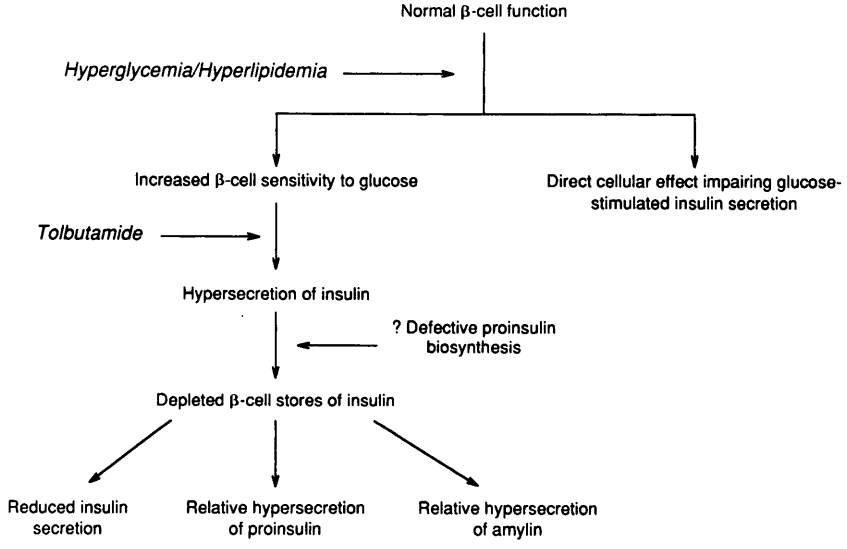

FIG. 6. Model of β-cell dysfunction induced by chronic hyperglycemia/hyperlipidemia. The left part depicts the "overworked β-cell" cascade. The right represents an undefined direct inhibitory effect of the diabetic environment on glucose-induced insulin secretion. The model is based on extensive animal and human experimental data (10).

per se should separately affect the cellular content of these peptides. That result suggests differential regulation of how these peptides are packaged into insulin granules. Indeed, a known difference is at the level of gene expression (43–45). Whether it underlies our finding versus differences in cellular trafficking, processing, and/or degradation remains to be determined. An interesting speculation from these results is that an increased blood amylin-to-insulin ratio may be a marker of β-cell decompensation in early NIDDM.

Based on the current results plus our previous studies in diabetic rats (10), our working model for the β-cell dysfunction with diabetes is depicted in Fig. 6. It proposes a dual inhibitory pathway on insulin secretion. The left-hand cascade is the overworked component, with the initiating event being a β-cell hypersensitivity to glucose, which we have postulated is secondary to the upregulated activity of hexokinase (10,46). The resulting insulin hypersecretion outstrips the synthetic capacity, causing insulin content to fall, mediating three functional consequences: reduced insulin secretion with glucose potentiation being primarily affected, and increased ratios of proinsulin to insulin and amylin to insulin in the stored and secreted material. The link between β-cell hyperfunction and the raised proinsulin-to-insulin ratio, which typifies the diabetic state (47) has previously been validated by our studies in diabetic and tolbutamide-infused rats (30,48,49). The right-hand side represents additional undefined mechanisms that mediate other secretory defects including the impaired glucose-induced insulin secretion.

In summary, chronic β-cell stimulation independent of the level of glycemia is sufficient to replicate many of the β-cell functional characteristics of diabetes. This finding, combined with our diazoxide (11) and fasting (12) results in diabetic rats and similar results in NIDDM (13–15) and cultured β-cells (16), provides evidence for an overworked β-cell being instrumental in the impaired glucose-potentiated insulin secretion that characterizes diabetes. The postulated cause, based on correlative data, is reduced β-cell insulin stores, although some other intracellular β-cell defect may eventually be proven as causative. Also, the inhibitory effect of tolbutamide on proinsulin biosynthesis (18,38,39) causes significant shortcomings for this model in terms of exploring the cellular mechanism of the impaired insulin secretion in diabetic states. Thus, direct investigation of diabetes models is required to clarify the role of altered granule composition versus the degranulation and impaired granule mobilization in the insulin secretory dysfunction.

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