Defective Glycolysis and Calcium Signaling Underlie Impaired Insulin Secretion in a Transgenic Mouse*

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Pancreatic beta cells from mice that overexpress the Ca\(^{2+}\)-binding protein calmodulin have a unique secretory defect that leads to chronic hyperglycemia. To further understand the molecular basis underlying this defect, we have studied signaling pathways in these beta cells. Measurements of cytosolic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{i}\)) using fura-2 or indo-1 revealed a markedly reduced response when glucose was the stimulus. However, eliciting membrane depolarization with 50 mM K\(^+\) or the addition of the ATP-sensitive K\(^+\) (K\(_{\text{ATP}}\)) channel antagonist tolbutamide restored [Ca\(^{2+}\)]\(_{i}\) transients to near normal levels. Electrophysiological analysis of the beta cell ion channels revealed that Ca\(^{2+}\) currents, delayed rectifier K\(^+\) currents, and K\(_{\text{ATP}}\) channel currents were similar in transgenic and nontransgenic cells, suggesting that these ion channels were able to function normally. However, whereas K\(_{\text{ATP}}\) channel currents in control cells were reduced by 50% by the presence of high glucose, those in transgenic cells were unaltered. Addition of tolbutamide inhibited this channel and enhanced the secretion of insulin in response to glucose for both control and transgenic cells. As these observations implicated a metabolic defect, glucose utilization, which is an indicator of glucose metabolism and ATP production in beta cells, was measured and found to be reduced by 40% in the transgenic cells. These data support the contention that excessive levels of calmodulin may compromise the ability of the beta cell to metabolize glucose and to modulate the state of the K\(_{\text{ATP}}\) channel, resulting in an inadequate control of the membrane potential, which collectively impair [Ca\(^{2+}\)]\(_{i}\), and thus insulin secretion in response to glucose.

Targeted overexpression of the ubiquitous Ca\(^{2+}\)-binding protein calmodulin to mouse pancreatic beta cells results in the generation of an early onset nonimmune hyperglycemic condition (1). This condition is manifested by the first postnatal day and progressively worsens with age (1, 2). Further analysis of these transgenic mice revealed that the hyperglycemic condition is the result of a reduced first phase and virtually absent second phase insulin secretory response when glucose is the secretagogue (2). These secretory defects are apparent even when pancreatic insulin reserves are at levels that should be sufficient to prevent the hyperglycemic condition (1, 3). However, insulin secretion in young animals can be restored to normal if agents that release Ca\(^{2+}\) from internal stores, such as muscarinic agonists or phorbol esters, are used together with glucose (2, 3). This led us to consider the presence of a Ca\(^{2+}\)/CaM-dependent pathway that might converge with the glucose-dependent stimulus-secretion coupling pathway in the transgenic cells.

It is well known that glucose-mediated stimulus-secretion coupling in pancreatic beta cells is the result of a complex intracellular signal transduction pathway that is designed to efficiently release insulin in a biphasic manner (4–6). During both phases of insulin secretion, the uptake and metabolism of glucose lead to signals that result in closure of a K\(^+\) channel located on the membrane that is regulated by ATP (7). The closure of these K\(_{\text{ATP}}\) channels leads to depolarization of the membrane and the opening of voltage-gated Ca\(^{2+}\) channels (8). The ensuing transient rise in [Ca\(^{2+}\)]\(_{i}\), leads to insulin secretion (9), but it is not clear exactly how the Ca\(^{2+}\) rise regulates the complex series of steps that culminate in hormone release. Evidence exists that implicates Ca\(^{2+}\)-binding proteins as being important primary and secondary mediators of the stimulus-secretion response in beta cells following the rise in [Ca\(^{2+}\)]\(_{i}\) (10, 11).

Since the primary response in beta cells to elevated levels of glucose is an increase in [Ca\(^{2+}\)]\(_{i}\), prior to the release of insulin and our transgenic mice did not respond to glucose, we have investigated the possibility that a defect in Ca\(^{2+}\) signaling is responsible. This was accomplished by systematically examining several key components of the glucose-induced secretion coupling pathway of beta cells. These components included glucose-induced changes in [Ca\(^{2+}\)]\(_{i}\), as well as beta cell ion channel properties and glucose metabolism. Our results suggest that the overexpression of CaM results in a metabolic defect that is distal to the membrane and apparently involves reduced metabolism of glucose, resulting in the inability to regulate the membrane potential via closure of the K\(_{\text{ATP}}\) channels and subsequent opening of the voltage-dependent Ca\(^{2+}\) channels.

MATERIALS AND METHODS

Animals and Preparation of Islets and Cells—6–8-Day-old control and CaM animals were taken from a colony of transgenic mice. Islets were used for perifusion and [Ca\(^{2+}\)]\(_{i}\) measurements were isolated by collagenase digestion, immediately placed in fully supplemented RPMI 1640 medium (Life Technologies, Inc.), and cultured for 24 h as described previously (2, 3). To obtain single beta cells, 10–20 acutely isolated islets were transferred into 1.5-ml sterile Eppendorf tubes and washed with 1.5 ml of Hanks’ balanced salt solution (Life Technologies, Inc.) containing 137 mM NaCl, 0.34 mM Na\(_2\)HPO\(_4\), 0.4 mM KH\(_2\)PO\(_4\), 5.3 mM KCl, 4 mM NaHCO\(_3\), and 5.5 mM glucose. The islets were pelleted at 600
rpm for 10 s and resuspended in 100 μl of the same Hanks' balanced salt solution. 100 μl of a 0.05% trypsin, 0.25 mM EDTA solution (Life Technologies, Inc.) was added, and the islets were mechanically triturated 100 times to disperse cell clumps. Trypsinization was stopped by addition of 1 ml of Dulbecco's modified Eagle's medium (Life Technologies, Inc.) containing 10% fetal bovine serum albumin, 11.1 mM glucose, 100 μg/ml streptomycin, and 100 μg/ml penicillin. Isolated cells were plated into 24-well plates (Becton Dickinson, Oxnard, CA) containing a 12-mm round glass coverslip (Fisher) and a ceramic cloning ring to concentrate the cells. Cells were then incubated at 37 °C in Dulbecco's modified Eagle's medium containing the same components as previously mentioned in a humidified chamber containing 95% O2, 5% CO2 for 24 h.

Secretion and Glucose Utilization Assays—General methods for measuring insulin secretion from intact islets have been previously described (2, 3). Islet radioimmunoassay measurements were done by the double antibody method as described previously (1) using rat insulin (Linco, St. Louis, MO) as a standard. 

Optical Measurements of $[Ca^{2+}]_i$—$[Ca^{2+}]_i$ was measured fluorometrically using the membrane-permeant $Ca^{2+}$ indicators (13) fura-2/AM (in whole islets) or indo-1/AM (in individual beta cells). Both dyes were obtained from Molecular Probes, Inc. (Junction City, OR). Dyes were dissolved in dimethyl sulfoxide and mixed into 1 μl of either fully supplemented RPMI 1640 medium for islets or fully supplemented Dulbecco's modified Eagle's medium for individual beta cells. Ester forms of the dye were loaded into the tissue by incubation at 20–22 °C for 1 h and subsequent incubation in dye-free medium for an additional 30 min at 37 °C. This loading procedure produced a uniform spatial distribution of dye, suggesting very little compartmentalization within intracellular organelles. Prior to each experiment, the islets or cells were allowed to equilibrate for 45 min at 30–37 °C while perfusing with Krebs-Ringer bicarbonate (133 mM NaCl, 2.5 mM CaCl2, 5 mM KCl, 1.2 mM MgSO4, 1.2 mM KH2PO4, and 5 mM NaHCO3) containing 2.8 mM glucose and 0.1 mg/ml bovine serum albumin and supplemented with 2 mM HEPES (pH 7.4) and preagitated with 95% O2, 5% CO2. For perfused cells, cells from CaM mice. Since Ca2+ is necessary for insulin secretion (9), this suggests that CaM overexpression produces a defect in some aspect of Ca2+ regulation of insulin secretion. We have systematically examined the possible sources of this beta cell defect in isolated islets and single cells from normal and transgenic mice.

Defective $[Ca^{2+}]_i$ Signaling in Beta Cells from CaM Mice—We began by using the fluorescent calcium indicator dye fura-2 (13) to measure $[Ca^{2+}]_i$ in beta cells within islets taken from normal and transgenic mice. Fig. 1 is a representative tracing that clearly demonstrates that when the extracellular glucose concentration was 2.8 mM, $[Ca^{2+}]_i$ was similar in control and CaM islets. The average resting $[Ca^{2+}]_i$ levels (Fig. 2A) measured in control islets (n = 8) and in CaM islets (n = 6) were not statistically different from each other (p > 0.10). Increasing the external glucose concentration to 16.8 mM leads
to a rise in [Ca\(^{2+}\)], in both types of islets, although these rises were greatly reduced in the islets from CaM mice (Fig. 1). The rises in [Ca\(^{2+}\)], produced by the high glucose treatment were quantified by measuring the difference between the resting [Ca\(^{2+}\)], levels and the peak [Ca\(^{2+}\)], levels determined in the presence of 16.8 mM glucose (Fig. 2B). The glucose-induced rise in [Ca\(^{2+}\)], in islets from CaM mice (n = 6) was significantly smaller than that in islets from control mice (n = 7) (p < 0.05).

Because it has been suggested that the glucose-induced [Ca\(^{2+}\)], response of isolated beta cells differs from that of islets (16), we also measured [Ca\(^{2+}\)], in single dissociated beta cells loaded with Indo-I. As was found for isolated islets, individual beta cells from either control or CaM animals that were treated with low glucose (2.8 mM) solution showed similar resting [Ca\(^{2+}\)], values, while exposure to solutions containing 16.8 mM glucose resulted in robust rises in [Ca\(^{2+}\)], in control cells, but much smaller responses in CaM cells. The peak rise in [Ca\(^{2+}\)], produced by 16.8 mM glucose was 428 ± 85 nM in 29 control cells and 50 ± 7 nM in 31 CaM cells (p < 0.05). Because this defect in [Ca\(^{2+}\)], signaling could be responsible for the attenuation of glucose-sensitive insulin secretion in the CaM mice, we performed additional experiments to identify the source of the defect in [Ca\(^{2+}\)], signaling and the role of this defect in the secretory phenotype of the mice.

Ion Channels Are Normal in CaM Beta Cells—Given that voltage-gated Ca\(^{2+}\), channels are the primary source of [Ca\(^{2+}\)], that triggers insulin secretion (8), the defective glucose-induced [Ca\(^{2+}\)], response in the CaM beta cells suggested that opening of these channels might be impaired. We therefore examined the properties of voltage-gated Ca\(^{2+}\), channels to determine whether or not they permitted Ca\(^{2+}\), to enter the beta cell. Our initial measure of entry through voltage-gated Ca\(^{2+}\), channels was to examine the [Ca\(^{2+}\)], changes resulting from depolarization of the beta cell membrane potential by treatment with Krebs-Ringer bicarbonate containing an elevated (50 mM) K\(^+\) concentration. Treatment with high K\(^+\), in addition to glucose, should depolarize the membrane potential sufficiently to open voltage-gated Ca\(^{2+}\), channels (8, 17). K\(^+\)-induced depolarization of control islets loaded with fura-2 produced a prompt elevation of [Ca\(^{2+}\)],, as shown in Fig. 1. The peak rise in [Ca\(^{2+}\)], attained in control islets during exposure to the high K\(^+\) solution was ~400 nM above the resting level (Fig. 2C). Likewise, treatment of islets from CaM mice with solution containing 50 mM K\(^+\), produced comparable rises in [Ca\(^{2+}\)], (Fig. 1), with no significant difference between the mean responses recorded from the two populations of islets (Fig. 2C). Additional experiments performed on isolated beta cells from normal and CaM mice also yielded similar [Ca\(^{2+}\)], rises in response to treatment with the high K\(^+\) solution (data not shown). Thus, measurements of depolarization-induced [Ca\(^{2+}\)], signals suggest that the voltage-gated Ca\(^{2+}\), channels of beta cells from CaM mice are able to open and admit Ca\(^{2+}\),.

A more direct measure of Ca\(^{2+}\), channel activity can be obtained from electrical measurements of beta cell Ca\(^{2+}\), currents (17, 18). We therefore used whole-cell patch clamp recording methods (19) to examine the currents resulting from Ca\(^{2+}\), channel gating. Cesium ions were added to the intracellular medium to block currents flowing through K\(^+\), channels and thereby allow measurement of Ca\(^{2+}\), channel currents in isolation. Families of Ca\(^{2+}\), currents recorded from both control and CaM beta cells are shown in Fig. 3A. These Ca\(^{2+}\), currents do not obviously differ between the two types of cells. In both cases, stepping the membrane potential from a holding level of −70 mV to potentials of −50 mV or more positive resulted in activation of an inward Ca\(^{2+}\), current. There were no consistent differences in the voltage dependence of these currents, as can be seen in the current-voltage curves shown in Fig. 3B. In both cases, peak current was attained at −0 mV, and the current reversed its polarity at approximately +50 mV. Peak current density, determined after dividing by the membrane capacitance to normalize for cell-to-cell variations in cell area, was not statistically different when comparing 23 control (18.1 ± 1.3 pA/pF) and 21 CaM (15.2 ± 1.43 pA/pF) beta cells (p > 0.10). Furthermore, there were no consistent differences in the kinetics of the Ca\(^{2+}\), currents between beta cells from normal and CaM mice (Fig. 3A). These data indicate that the deficit in glucose-induced [Ca\(^{2+}\)], signals in the CaM beta cells is not due to a change in the density or gating properties of Ca\(^{2+}\), channels.

It is possible that the CaM mutation affects Ca\(^{2+}\), entry indirectly by altering the K\(^+\), conductances responsible for re-
polarization of the membrane potential of the beta cell. We began our consideration of this possibility by characterizing delayed rectifier K⁺ currents. These currents were examined in isolation by including, in the intracellular patch pipette solution, EGTA (a Ca²⁺ buffer that blocks elevation of [Ca²⁺]ᵢ that would activate Ca²⁺-dependent K⁺ currents) and ATP to block ATP-sensitive K⁺ currents (20). The currents measured under these conditions were blocked by extracellular application of tetraethylammonium ions (data not shown), a well known blocker of delayed rectifier K⁺ currents in beta cells (20), providing pharmacological validation of the isolation procedure.

**Fig. 4.** Delayed rectifier K⁺ currents in control and CaM beta cells. A, families of K⁺ current recordings from both cell types. The membrane potential was held at −70 mV and stepped to the indicated voltages for 200 ms at 30-s intervals. B, voltage dependence of delayed rectifier K⁺ currents in both control (n = 25) and CaM (n = 15) beta cells.

Because the K⁺_/ATP_ channel plays a key role in the membrane depolarization produced by glucose (21), we next examined whether the defect in CaM cells was caused by changes in K⁺_/ATP_ channels. We first examined the actions of the sulfonylurea drug tolbutamide. This drug acts by blocking ATP-sensitive K⁺ channels; the resulting depolarization of the beta cell membrane potential normally results in an increase in [Ca²⁺]ᵢ, and triggering of insulin secretion (8). We measured [Ca²⁺]ᵢ in islets loaded with fura-2 to determine whether tolbutamide causes an elevation of [Ca²⁺]ᵢ in CaM islets. Addition of tolbutamide (1 mM) to the perfusate of resting islets resulted in sustained and reversible increases in [Ca²⁺]ᵢ in both control and CaM mice (Fig. 5A). These increases in [Ca²⁺]ᵢ, presumably are due to tolbutamide depolarizing the membrane by blocking ATP-sensitive K⁺ channels. The peak rises in [Ca²⁺]ᵢ produced by tolbutamide did not differ significantly (p > 0.05) in control
and CaM islets (Fig. 5B). Likewise, tolbutamide restored the ability of CaM islets to secrete insulin. At resting conditions (2.8 mM glucose), both the CaM and control islets secreted insulin at about the same rate (Fig. 6). Addition of 1 mM tolbutamide increased the sensitivity of the islets to 2.8 mM glucose in both the control and CaM islets, with secretion levels attaining a 4-fold increase over basal rates. Further addition of 16.8 mM glucose potentiated the tolbutamide response in both lines by 15-fold over low glucose alone and 3.5-fold over low glucose plus tolbutamide. These data indicate that the tolbutamide block of K\textsubscript{ATP} channels bypasses the defects in [Ca\textsuperscript{2+}]\textsubscript{i} signaling and insulin secretion characteristic of CaM islets.

To further understand the role of K\textsubscript{ATP} channels in the CaM phenotype, we next measured currents flowing through these channels. This was done by using the patch pipette to lower intracellular ATP concentrations while measuring the resultant changes in K\textsubscript{ATP} conductance (21). The procedure used in our experiments on beta cells from both control and CaM mice is illustrated in the current recordings shown in Fig. 7A. Establishment of diffusional contact between the patch pipette and the inside of the cell, evident as a large increase in membrane capacitance (19), occurred at the times indicated by arrows. Because the patch pipette contained only a minimal concentration of ATP (0.3 mM), the K\textsuperscript{+} conductance of the membrane grew larger as ATP was dialyzed out of the cytosol. During this time, the membrane potential was repeatedly alternated between three values, -60, -70, and -80 mV. Because the equilibrium potential for K\textsuperscript{+} ions is approximately -77 mV under our experimental conditions, the increase in ATP-sensitive K\textsuperscript{+} conductance was evident as a gradual increase in outward current when the potential was at -60 mV and as an increase in inward current when the potential was at -80 mV. These currents reached peak levels within 10 min of starting intracellular dialysis and declined at later times because of rundown (evident at the end of each of the two traces). Addition of tolbutamide (0.1 mM) to the external solution blocked the currents measured in both the CaM and control beta cells, showing that these currents were carried by the K\textsubscript{ATP} channel (Fig. 7A). These K\textsubscript{ATP} channel currents appeared similar in magnitude in both types of cells. To quantify the conductance of the K\textsubscript{ATP} channels of these two cell types, peak currents were measured at all three membrane potentials, and the conductance was calculated as the slope of the current-voltage relationship. These conductances, when normalized for variations in membrane area, were very similar for the two types of cells (Fig. 7B). These data indicate that the density, ATP sensitivity,
FIG. 7. ATP-sensitive K⁺ conductance in beta cells from control and CaM mice. A, currents recorded while the membrane potential was alternated between −60 and −70, and −80 mV in 2-s intervals. The patch pipette contained 0.3 mM ATP to dialyze out cytosolic ATP. Removal of intracellular ATP beginning at the time indicated (Breakthrough) caused a progressive increase in K⁺ conductance, evident as increased outward current at −60 mV and increased inward current at −80 mV. Addition of 0.1 mM tolbutamide (bar) caused inhibition of these currents, confirming that they result from K⁺ATP channels. Also note the variable amplitude and speed between the traces due to variations in the series resistance of the pipette and normal cell to cell differences. B, normalized membrane conductance for control (0.53 ± 0.12 nS/pF) and CaM (0.68 ± 0.15 nS/pF) cells. Data are from 27 control and 24 CaM cells (p > 0.20).

and pharmacological properties of K⁺ATP channels are similar in both types of cells. When combined with the observations that tolbutamide is able to activate [Ca²⁺], signaling and insulin secretion in the beta cells from CaM mice, these data further indicate that the defect in glucose-induced insulin secretion in CaM mice is neither due to changes in the intrinsic properties of K⁺ATP channels nor at steps that follow closure of these channels. Instead, the defect must be at, or proximal to, the glucose-induced rise in intracellular ATP concentration that normally leads to blocking of the K⁺ATP channels.

Impaired Activation of ATP-sensitive K⁺ Channels in CaM Beta Cells—To determine whether the excess CaM affects the glucose-induced rise in intracellular ATP concentration that normally leads to blocking of the K⁺ATP channels, we next measured the utilization of glucose by control and CaM islets. Glucose utilization measurements are an indicator of the ability of the beta cell to metabolize glucose and to produce ATP (12). Fig. 8 shows that under basal conditions (2.8 mM extracellular glucose), the utilization of glucose for the control islets was 5.0 ± 0.7 pmol/h/µg of protein, while the CaM animals had a lower but not significantly different basal utilization rate of 3.2 ± 0.6 pmol/h/µg of protein (p > 0.05). Elevation of glucose to a concentration (16.8 mM) that stimulates insulin secretion increased the utilization of the glucose in control islets to nearly three times the rate seen at 2.8 mM glucose (17.8 ± 1.6 pmol/h/µg of islet protein). CaM islets also increased their utilization rates at this higher glucose concentration, attaining a rate of 10.6 ± 2.2 pmol/h/µg of islet protein. This rate was, however, 40% lower than that of control islets (p < 0.05). Thus, it appears that glucose cannot activate [Ca²⁺], signaling or insulin secretion in CaM islets because these islets are less capable of converting glucose to ATP.

If the CaM mutation produces a defect in production of ATP from glucose, then glucose should be less capable of closing K⁺ATP channels even though these channels appear normal in CaM cells (see above). This prediction was tested by measuring the ability of high glucose to close these channels in single beta cells. While whole-cell patch clamp methods are the ideal way to measure such currents, dialysis of the intracellular medium during such recordings would necessarily perturb intracellular ATP generation during glucose treatment. To avoid this complication, we turned to perforated patch recordings as a means of measuring the K⁺ATP channel current while keeping intracellular metabolism intact (15, 21). Fig. 9A shows an example of the recording of the K⁺ATP channel currents activated while treating a control beta cell with an extracellular solution containing 16.8 mM glucose. In control cells, this treatment consistently produced a large decrease in the conductance associated with the K⁺ATP channels; this conductance decrease is reflected in a progressive decrease in current during glucose exposure. The resting conductance of cells from CaM islets (0.24 ± 0.05 nS/pF), measured in low (2.8 mM) glucose conditions, was similar to that of control cells (0.24 ± 0.04 nS/pF) presumably because, as in control cells, intracellular ATP levels were low and the K⁺ATP channels were open. However, elevation of glucose concentration to 16.8 mM produced much smaller decreases in conductance in cells from CaM islets (Fig. 9A). This difference in conductance sensitivity to glucose was most evident after the K⁺ATP channel conductance was calcu-
lated, as described above, using measurements of currents recorded at three potentials and normalized for variations in cell area (Fig. 9B). In control cells, glucose treatment produced a large decrease in conductance (to 0.13 ± 0.03 nS/pF), while there was no consistent decrease in conductance (0.24 ± 0.05 nS/pF) in beta cells from CaM mice (p < 0.05). These data are consistent with the hypothesis that the metabolism of glucose is impaired in CaM cells, so that glucose metabolism does not result in the usual elevation of ATP concentration and subsequent blocking of K$_{\text{ATP}}$ channels in these cells.

**DISCUSSION**

The early stages of the CaM phenotype are characterized by a reduction in the first phase of glucose-induced insulin secretion and the abolition of the second phase of insulin secretion (2, 3). In this report, we have considered the molecular mechanisms responsible for the defects in insulin secretion resulting from targeted overexpression of CaM in beta cells. Our most striking finding is that these cells have a greatly attenuated ability to elevate [Ca$^{2+}$]$_i$ in response to glucose stimulation. Because the values of CaM beta cells were indistinguishable from those of normal beta cells, it appears that the reduction in [Ca$^{2+}$]$_i$, in response to glucose stimulation, is due to a defect that is distal to the beta cell membrane. This defect, however, appears to be directly linked to the modulation of the ion channels that control membrane potentials, ion fluxes, and insulin secretion. The fact that glucose utilization and glucose-induced closure of ATP-sensitive K$^+$ channels are decreased in CaM cells suggests that the defect apparently is due to an impaired ability of these beta cells to metabolize glucose into intracellular ATP. Thus, we conclude that beta cells from CaM mice probably produce less ATP when exposed to glucose, and this defect prevents the cells from depolarizing their membrane potential, opening voltage-gated Ca$^{2+}$ channels, or elevating [Ca$^{2+}$]$_i$, sufficiently to produce the phasic secretion of insulin responses characteristic of normal beta cells (22).

Elevation of [Ca$^{2+}$]$_i$ is necessary for producing glucose-induced insulin secretion (23). Therefore, it is likely that the impaired ability of glucose to elevate [Ca$^{2+}$]$_i$ (Fig. 1) underlies this defective insulin secretion in CaM beta cells. Direct support for this contention comes from experiments demonstrating that conditions that elevate [Ca$^{2+}$]$_i$ by depolarizing the membrane with agents such as tolbutamide (Fig. 6) or by increasing the external K$^+$ concentration (2, 3) are able to restore insulin secretion from CaM islets. In addition, agents that release internal stores of Ca$^{2+}$, such as phorbol esters (12-O-tetradecanoylphorbol-13-acetate) or muscarinic agonists (carbachol), also restore insulin secretion in the transgenic beta cells (2, 3).

Thus, the phenotype exhibited by the CaM mice appears to result secondarily from impaired Ca$^{2+}$-sensing within the beta cell.

We have also demonstrated that there is an absence of any changes in the intrinsic properties of voltage-gated or ATP-sensitive ion currents in CaM beta cells (Figs. 3, 4, and 7). It is also clear that the K$_{\text{ATP}}$ channel currents of these cells can be inhibited by compounds that interact directly with this channel (Fig. 7). This defect in [Ca$^{2+}$]$_i$, signaling also means that Ca$^{2+}$-activated K$^+$ channels, which normally would aid in the resetting of the membrane potential (8), should not be activated by glucose in the CaM mice. It is possible that excessive CaM alters the properties of the Ca$^{2+}$-activated K$^+$ channels, but because the secretory defect can be rescued by providing internal Ca$^{2+}$, this is not likely to be a significant part of the CaM phenotype. Taken together, these findings demonstrate that the usual complement of ion channels are present in the CaM beta cell and are functional. While there is some evidence that CaM-regulated pathways may modulate ion channels in beta cells (24, 25), the 5-fold overexpression of CaM in these beta cells (1) appears to have no direct effects on the ion channels.

Increasing the intracellular concentration of anything that will bind Ca$^{2+}$ will slow and attenuate transient rises in [Ca$^{2+}$]$_i$ (26). Given that CaM binds 4 Ca$^{2+}$ ions and that there is a much higher concentration of CaM in beta cells from CaM mice (1), it is possible that Ca$^{2+}$ signaling is defective in CaM cells simply because the extra CaM acts as a Ca$^{2+}$ buffer. Previous calculations alluded to the possibility that up to 1 mM Ca$^{2+}$ might be bound during stimulatory Ca$^{2+}$ transients in these cells (1). To test this possibility, we reported that overexpression of the beta cells from mice of an inactive CaM that only binds Ca$^{2+}$ also impairs insulin secretion (3). However, more recent studies indicate that this secretory defect is the result of a different molecular mechanism than the secretory defect seen in the CaM mice, suggesting that Ca$^{2+}$ buffering is not the cause of the phenotype in the CaM mice. Our observa---

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3 T. J. Ribar, C. R. Jan, G. J. Augustine, and A. R. Means, unpublished results.
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Ca\textsuperscript{2+} that depolarization of the membrane with tolbutamide, elevated concentrations of K\textsuperscript{+}, or agonists of internal Ca\textsuperscript{2+} release restore [Ca\textsuperscript{2+}], signaling and insulin secretion in CaM islets is a more direct indication that Ca\textsuperscript{2+} buffering is not altered in the CaM beta cells. It is therefore unlikely that overexpression of CaM impairs Ca\textsuperscript{2+} signaling in the beta cell simply by buffering Ca\textsuperscript{2+} ions.

Our results instead indicate that CaM overexpression causes a metabolic defect in beta cells. The clearest indication of this is that K\textsuperscript{+}\textsubscript{ATP} channels were not closed during exposure of CaM cells to high extracellular glucose concentrations. This could result from attenuation of ATP production from glucose metabolism or from changes in the ability of the K\textsuperscript{+} channel to bind ATP. The former possibility is more likely for two reasons. First, tolbutamide appears to bind and close the K\textsuperscript{+}\textsubscript{ATP} channel similarly in both control and CaM cells, suggesting that the channel is conformationally correct and can bind ATP. Second, our measurements of glucose metabolism (Fig. 8) indicate low rates of glucose utilization in the beta cells from CaM mice.

Decreased glucose utilization in the CaM beta cells points to a defect somewhere in the glycolytic pathway. It is unlikely that this defect occurs at the most proximal steps of glycolysis, such as glucose transport, because down-regulation of the primary glucose transporter (GLUT2) does not result in hyperglycemia (27). Furthermore, the glucose-sensing mechanism of the beta cell is still functional in the CaM mice because elevated glucose still increases utilization rates (12, 28). Thus, it is more likely that the defect of the CaM beta cells lies in the distal steps of glycolysis.

It is not yet clear which distal step in glucose metabolism is responsible for the impaired ATP production in the CaM mice. In neonatal streptozotocin-induced diabetic rats, impaired insulin secretion is linked to reduced ATP production resulting from defective glucose metabolism in the mitochondria of beta cells (29, 30). Furthermore, up to 40% of the total glucose utilized by beta cells during the stimulus-secretion response can be shunted through this mitochondrial pathway to produce ATP (31). The CaM mice are markedly similar to these neonatal streptozotocin-induced diabetic rats because they have an approximately 40% lower glucose utilization rate (Fig. 8) than control mice. In addition, ketosaccharic acid, which can be readily metabolized directly in the mitochondria and induce secretion via direct effects on the K\textsuperscript{+}\textsubscript{ATP} channel (32), failed to do so in the CaM mice (2). This indicates a defect in mitochondrial metabolism in the CaM beta cells.

Dukes et al. (33) have suggested that in beta cells, ATP is produced by two interrelated metabolic pathways in the mitochondria. While one of these pathways does not appear to have any CaM-regulated reactions, the second pathway uses a FAD-linked glycerol-3-phosphate dehydrogenase (33) that possesses an EF hand motif in its carboxyl terminus and thus could be regulated by Ca\textsuperscript{2+}, Ca\textsuperscript{2+}, or some CaM-dependent event (34). Thus, overexpression of CaM might somehow prevent the glycerol-3-phosphate dehydrogenase from generating the reduced intermediates required for the generation of ATP in mitochondria. In addition, any inhibition of this FAD-linked shuttle would also result in lower yields of NAD\textsuperscript{+}, a metabolite needed for augmentation of glycolysis (33).

In summary, the phenotype seen in the CaM mice apparently is associated with a defect within the glycolytic pathway. Other transgenic mice overexpressing proteins in the beta cell do not have similar metabolic abnormalities (35), demonstrating that the reduction in glucose utilization and ATP production is a specific consequence of overexpressing CaM. These data also suggest that this defect is associated with mitochondrial production of ATP, although it is not yet clear why overexpression of CaM leads to this diminished metabolic response.

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