Pancreatic beta cells are hyper-responsive to amino acids but have decreased glucose sensitivity after deletion of the sulfonylurea receptor 1 (SUR1) both in man and mouse. It was hypothesized that these defects are the consequence of impaired integration of amino acid, glucose, and energy metabolism in beta cells. We used gas chromatography-mass spectrometry methodology to study intermediary metabolism of SUR1 knock-out (SUR1−/−) and control mouse islets with D-[U-13C]glucose as substrate and related the results to insulin secretion. The levels and isotope labeling of alanine, aspartate, glutamate, glutamine, and γ-aminobutyric acid (GABA) served as indicators of intermediary metabolism. We found that the GABA shunt of SUR1−/− islets is blocked by about 75% and showed that this defect is due to decreased glutamate decarboxylase synthesis, probably caused by elevated free intracellular calcium. Glutaminolysis stimulated by the leucine analogue D,L-norbornane-carboxylic acid was, however, enhanced in SUR1−/− and glyburide-treated SUR1+/- islets. Glucose oxidation and pyruvate cycling was increased in SUR1−/− islets at low glucose but was the same as in controls at high glucose. Malic enzyme isoforms 1, 2, and 3, involved in pyruvate cycling, were all expressed in islets. High glucose lowered aspartate and stimulated glutamine synthesis similarly in controls and SUR1−/− islets. The data suggest that the interruption of the GABA shunt and the lack of glucose regulation of pyruvate cycling may cause the glucose insensitivity of the SUR1−/− islets but that enhanced basal pyruvate cycling, lowered GABA shunt flux, and enhanced glutaminolytic capacity may sensitize the beta cells to amino acid stimulation.

The pancreatic beta cells function as the predominant sensors and regulators of glucose, amino acid, and fatty acid levels of the mammalian organism, including man, by adjusting the minute to minute rate of insulin secretion such that these fuels are maintained at physiologically optimal blood concentrations under all nutritional conditions including feeding and fasting. This process of fuel-sensing and stimulation of insulin secretion requires that the various stimuli are transported into the beta cells and are metabolized to generate coupling factors that trigger and sustain the secretion of the hormone from large stores of insulin granules (1–4). The diverse specific pathways that allow access to metabolism for glucose, amino acids, and fatty acids converge to a complex network of intermediary metabolism represented by the citric acid cycle, a considerable variety of metabolite and cofactor shuttles including the GABA2 shunt, and the processes of electron transport and oxidative phosphorylation, to mention just a few outstanding features of the biochemical maze of the beta cell.

In the present study we have used uniformly labeled D-[U-13C]glucose and GC-MS methods to explore the role of intermediary metabolism of pancreatic islets isolated from normal and sulfonylurea receptor 1 (SUR1) knock-out (SUR1−/−) mice. SUR1−/− islets were chosen for comparison to control islets because they have profound changes of glucose and amino acid responsiveness, showing markedly reduced glucose but strikingly enhanced amino acid-induced insulin release (5–7). We used the conversion or incorporation of 13C carbon into CO2 and various amino acids, respectively, as readout for the integration of metabolic pathways. This approach allowed us to examine for the first time in isolated mouse islets and in one comprehensive study the concentration dependences of glucose oxidation and glutaminolysis, the operation of the so called “pyruvate cycling,” the nature of the glucose induced aspartate decrease (herein referred to as “aspartate switch”), the regulatory role of glutamate decarboxylase (GAD) in the operation of the beta cell GABA shunt, and glucose stimulation of glutamine synthesis. These studies provide new insights into the complex role of the metabolic network in fuel-stimulated authentic pancreas...
creatic islet cells as contrasted with studies using tumor-derived beta cell lines (8, 9).

**MATERIALS AND METHODS**

**Pancreatic Islet Source and Preparation**—SUR1 knock-out mice were obtained from Dr. Mark A. Magnuson. The knock-out procedure and genotyping were described by Shiota et al. (10). Both SUR1−/− mice and control (SUR1+/+) mice (B6D2F1) were maintained on a 12-h light/dark cycle and were fed a standard rodent chow diet. Islet isolation and culture were described previously (11). In brief, islets were isolated by collagenase digestion and cultured for 3–4 days in RPMI 1640 medium containing 10 mM glucose.

**[U-13C]Glutamine Oxidation**—[U-13C]glutamine (PerkinElmer Life Sciences) oxidation in the presence or absence of 10 mM D,L-β-2-amino-2-norbornane-carboxylic acid (BCH) was examined in cultured SUR1−/− and control islets. Separate experiments were performed in control islets cultured with 0.3 μM gliburide for 3 days and then using the same concentration of gliburide throughout the experiment to mimic SUR1−/− conditions. The experimental procedures were described previously (12).

**Studies with [U-13C]Glucose**—Batches of 1000 cultured islets from SUR1−/− and SUR1+/+ animals were preincubated with 95% O2, 5% CO2-equilibrated Krebs-Ringer bicarbonate buffer (115 mmol/liter NaCl, 24 mmol/liter NaHCO3, 5 mmol/liter KCl, 1 mmol/liter MgCl2, 2.5 mmol/liter CaCl2, 10 mM HEPES, pH 7.4) with 0.25% bovine serum albumin for 60 min at 37 °C and then converted into the t-butyldimethylsilyl derivatives. Isotopic enrichment in glutamate isotopomers was monitored using ions at m/z 432, 433, 434, 435, 436, and 437 for M′+1, M′+2, M′+3, M′+4, and M′+5 (containing 1–5 13C-enriched atoms), respectively. Isotopic enrichment in aspartate isotopomers was monitored using ions at m/z 418, 419, 420, 421, and 422 for M′+1, M′+2, M′+3, and M′+4 (containing 1–4 13C-enriched atoms), respectively. Isotopic enrichment in alanine was monitored using ions at m/z 232, 233, 234, and 235 for M′+1, M′+2, and M′+3 (containing 1–3 13C-enriched atoms), respectively, and 13C enrichment in GABA was monitored using ions at m/z 274, 275, 276, 277, and 278 for M′+1, M′+2, M′+3, and M′+4 (containing 1–4 13C-enriched atoms), respectively.

The production of 13CO2 was monitored as follows. After 120 min of incubation, air samples were taken from the incubation tube. The latter was transferred into autosampler tubes for analysis. Isotopic enrichment in 13CO2 was determined by the product of (molar percent enrichment, which is the mol fraction percent of analyte containing 13C atoms above natural abundance (14, 15)). The production of 13C-labeled metabolites was calculated by the product of (molar percent enrichment/100 times concentration) (nmol/1000 islets) and is expressed as nmol of 13C metabolite/1000 islets. All the data are presented as the mean ± S.E. Student’s t tests were done when two groups were compared. Analysis of variances (one way analysis of variance) was used followed by the Bonferroni test when multiple groups were compared. Differences were considered significant when p < 0.05.

**Western Blot Analysis**—Monoclonal rabbit anti-mouse GS, rabbit, anti-GAD (against GAD 65 and 67), and mouse anti-GAPDH antibodies (all from Sigma) were used as the primary

**Metabolic Integration and Insulin Secretion**
Metabolic Integration and Insulin Secretion

TABLE 1

Concentrations of intracellular amino acid (nmol/1000 islets)

|                | G 0* | G 5* n = 3 | G 25* n = 6 | G 25/MSO* n = 6 | G 25/VGB* n = 3 |
|----------------|------|------------|-------------|-----------------|----------------|
| G0             |      |            |             |                 |                |
| Alanine        | 2.7 ± 0.2 | 2.6 ± 0.3 | 3.5 ± 3a   | 2.9 ± 0.3       | 4.6 ± 0.7b     |
| Aspartate      | 13.9 ± 0.4 | 14.1 ± 1.0 | 7.8 ± 0.4f | 8.3 ± 0.6b      | 5.1 ± 0.5d     |
| GABA           | 0.3 ± 0.0f | 1.8 ± 0.1  | 0.5 ± 0.0f | 1.8 ± 0.1       | 0.3 ± 0.0f     |
| Glutamate      | 11.5 ± 1.4d | 7.3 ± 0.6  | 11.5 ± 1.0 | 8.3 ± 0.9       | 11.6 ± 1.7     |
| Glutamine      | 1.0 ± 0.2  | 0.8 ± 0.1  | 2.2 ± 0.1bi | 1.3 ± 0.0f     | 2.6 ± 0.3d     |
| Glycerine      | 7.7 ± 0.5  | 9.4 ± 1.2  | 8.1 ± 0.3  | 12.0 ± 1.1      | 7.4 ± 0.8      |
| Isoleucine     | 0.5 ± 0.0  | 0.5 ± 0.0  | 0.4 ± 0.1  | 0.8 ± 0.3       | 0.5 ± 0.0      |
| Leucine        | 0.4 ± 0.0  | 0.3 ± 0.0  | 0.3 ± 0.0  | 0.3 ± 0.1       | 0.3 ± 0.1      |
| Serine         | 3.9 ± 0.3f | 1.8 ± 0.2  | 3.5 ± 0.3  | 2.2 ± 0.3       | 4.3 ± 0.7f     |

Note that in all 3G0a n d half of the G 25 and G 25/MSO experiments we used 300 µM NH_{4}Cl instead of NH_{4}Cl that was present in all the other experiments. Amino acids recorded in italics were not influenced by the experimental manipulations.

a p < 0.05 vs. G 0.

b p < 0.01 vs. G 0.

c p < 0.05 vs. SUR1*.
d p < 0.01 vs. G 25.
e p < 0.01 vs. SUR1*.
f p < 0.05 vs. G 25.

RESULTS

Profiles of Major Amino Acids in SUR1*– and Control Islets as Influenced by Glucose, MSO, and VGB—Earlier studies had indicated that incubation of cultured mouse pancreatic islets for 2 h in Krebs-Ringer bicarbonate buffer solution that lacked amino acids resulted in a substantial reduction of the intracellular amino acid pool by 1/2 to 2/3 (6, 13). This loss, amounting to as much as 10–15 mM intracellular amino acids, was not preventable by 5 or 25 mM glucose and was explained by a net efflux of amino acids due to the large downhill concentration gradient associated with nonphysiological incubation conditions. The present studies of intermediary metabolism of SUR1*– islets using [U-^{13}C]glucose were, therefore, performed in the presence of 3.5 mM concentrations of a physiological mixture of 18 amino acids plus 0.5 mM glutamine to approach physiological conditions. The medium also contained 300 µM ammonia to facilitate glutamine synthesis. Contents of the nine predominant amino acids that were reliably quantified in pancreatic islets of SUR1*– and control mice after incubation for 2 h with 4 mM amino acids present but with varying glucose and drug additions as dictated by the aims of the study are shown in Table 1. The total amino acid contents under the various experimental conditions were not different statistically, ranging from 30 to 42 nmol/1000 islets. This extrapolates to an ∼15–21 mM pool size. Three amino acids (glycine, isoleucine, and leucine) were virtually unchanged by the SUR1 knock-out or the incubation conditions. The other six were, however, markedly influenced by the experimental manipulations. Three changes are particularly striking: 1) the substantial drop of aspartate levels to as low as 1/3 that of baseline when glucose concentrations are increased, both in control and SUR1*– islets, 2) the striking depletion of the GABA content by about 75% in SUR1*– islets as compared with the controls whatever the conditions, and 3) the relatively high levels of alanine, glutamate, glutamine, and serine in SUR1*– as compared with the controls in many conditions studied here such that the

products were 151 bp (ME1), 249 bp (ME2), and 326 bp (ME3). The PCR products were confirmed by sequencing.
combined pool of these 4 amino acids was on average 52% higher in SUR1<sup>−/−</sup> islets than controls (i.e. 19 ± 1.7 versus 13 ± 1.1 nmol/1000 islets in glucose-free conditions). It is noteworthy that GABA was undetectable by HPLC in the incubation medium, which suggested the low intracellular GABA was not due to increased release. The careful comparison of the results of GABA and glutamate measurements in SUR1<sup>−/−</sup> islets with those in controls shows a classical “crossover” at the GABA decarboxylase step as illustrated in Fig. 1. The ratios of GABA/glutamate of SUR1<sup>−/−</sup> islets are at least 10-fold lower than those in the controls. The results strongly suggested that flux through the GABA shunt is greatly reduced in pancreatic islets of SUR1<sup>−/−</sup> mice. It is worth noting here that MO, an inhibitor of glutamine synthesis, had relatively little impact on the amino acid profile of isolated islets incubated in the presence of glucose (realizing the fact, however, that glutamine was not measurable because MO has the same chromatographic retention time as glutamine) and that VGB, an inhibitor of GABA transaminase, caused GABA to pile up as expected in the control and also in the SUR1<sup>−/−</sup> islets. Because of the marked inhibition of the GABA shunt, the possibility that glutamine and glutamate catabolism might also be impaired was tested by studying glucaminolysis. It was found that BCH-stimulated gluaminolysis was greatly enhanced in SUR1<sup>−/−</sup> islets, a phenomenon that was reproduced in model studies with glyburide-treated control islets (Fig. 2). The observation showed that the capacity of glutamine and glutamate oxidation is not reduced by the near complete block of the GABA shunt. In fact, both SUR1<sup>−/−</sup> and glyburide-treated control islets had increased BCH-stimulated glucaminolysis by 2- and 3-fold, respectively. The comprehensive analysis of the amino acid profiles of pancreatic islets as presented above is critical for the interpretation of tracer studies that use [U-<sup>13</sup>C]glucose because amino acids represent quantitatively a major sink for [<sup>13</sup>C]carbon.

**General Description of Pancreatic <sup>13</sup>C Labeling Profiles of Amino Acid Isotopomers Using [U-<sup>13</sup>C]Glucose as Substrate**—As a measure of pathway fluxes, we used [U-<sup>13</sup>C]glucose in an attempt to follow carbon flux into carbon dioxide and five prominent amino acids (alanine, aspartate, GABA, glutamate, and glutamine) of SUR1<sup>−/−</sup> and control pancreatic islets. To meet the sensitivity requirements of the GC-MS analysis of these islet constituents, we needed 1000 islets for each condition incubated in 1 ml of Krebs-Ringer bicarbonate buffer for 2 h to achieve significant <sup>13</sup>C labeling of amino acids. The results of limited ATP and ADP level determinations and of insulin and glucagon release rates demonstrated that the tissues were functionally well maintained (Figs. 3 and 4). The ATP levels (of 6–9 pmol/islet) and the ATP/ADP ratios (of 3–5) were within the expected ranges. To obtain more detailed quantitative information on the energy potential of islet cells dur-
Metabolic Integration and Insulin Secretion

FIGURE 3. Glucose oxidation and ATP contents of islets. $^{13}$CO$_2$ enrichments (atom % excess; APE) were used as measures of glucose oxidation of SUR1$^{-/-}$ (white bars) and SUR1$^{+/+}$ (black bars) islets incubated with low or high [U-13C]glucose ± VGB or MSO (panel A). Islets ATP contents (panel B) and ATP/ADP ratios (panel C) were measured in islet extract (n = 3).

FIGURE 4. Insulin and glucagon secretion. Insulin (panel A) and glucagon (panel B) secretion was measured using batches of 1000 incubated SUR1$^{+/+}$ (open triangles and white bars) and SUR1$^{-/-}$ islets (solid triangles and black bars). Statistical significance compared with SUR1$^{+/+}$ islets: *, p < 0.01; #, p < 0.05; compared with G0; a, p < 0.01; compared with G25; b, p < 0.01 (n = 3 for G0, G5, and G25/VGB, n = 6 for G25 and G25/MSO).

ing fuel stimulation would have required comprehensive measurements including ATP, ADP, AMP, inorganic phosphorus, phosphocreatine, and creatine to extrapolate to the effective free levels of these molecules, which was not possible in the present study.

The insulin release data are consistent with published results which demonstrate functional preservation of islets under the present experimental conditions (for further details on the hormone release data, see below). The [13C]carbon dioxide enrichment data (APE) show that SUR1$^{-/-}$ islets incubated at basal 5 mM glucose oxidize glucose twice as effectively as controls (Fig. 3A) and that the relative enhancement of glucose oxidation by increasing the substrate level to 25 mM is far less pronounced than in controls (i.e. the rate barely doubles in SUR1$^{-/-}$ islets but increases 4-fold in controls).

The isotope labeling profiles of 5 predominant amino acids that are observed when isolated pancreatic islets from SUR1$^{+/+}$ and controls are incubated for 2 h with 5 or 25 mM glucose in a medium supplemented with amino acids, in some instances with MSO or VGB present, have several striking characteristics (Table 2). The alanine isotope labeling pattern is unique compared with that of the other amino acids. M+2 labeling is 3–4 times higher than M+3 labeling in all conditions. The intensity is lowest for the control at 5 mM glucose. High glucose has a significant effect on the control but not on the knock-out islets. The aspartate and glutamate isotope labeling patterns are comparable in that at 5 mM glucose controls and knockouts are practically indistinguishable, i.e. M+2 through M+5 have comparable labeling intensities. High glucose has practically no impact on M+2 for any of the conditions, but it increases labeling intensities for M+3 through M+5 nearly uniformly. The piling up of 13C in M+4 and M+5 in the presence of MSO is noteworthy. The GABA isotope labeling profile exhibits a highly differentiated phenotype. Labeling intensities are much lower in knockouts than in controls in all isotopomers for all conditions by factors of 2–4. High glucose has a significant effect on the control but not on the knock-out islets. The statistical significance of this effect is reached in some but not all instances, e.g. M+3 through M+5 in controls and KO samples. In what follows, attention is focused on specific topics: the GABA shunt, pyruvate cycling, the aspartate switch, and glutamine synthesis.

Tracer Studies with [U-13C]Glucose Demonstrate That the GABA Shunt in Beta Cells of SUR1$^{-/-}$ Islets Is Blocked—The results of the amino acid analysis (Table 1) focused our primary
TABLE 2

Intracellular $^{13}$C amino acid enrichments

ND, not detectable. $n$ = 3. G. glucose.

|          | G 5   | G 25  | G 25/MSO | G 25/VGB |
|----------|-------|-------|----------|----------|
| Alanine  |       |       |          |          |
| SUR1<sup>-/-</sup> |       |       |          |          |
| M<sup>+</sup>2 | 21 ± 1<sup>a</sup> | 9 ± 1<sup>b</sup> | 26 ± 2<sup>c</sup> | 26 ± 1<sup>b</sup> |
| M<sup>+</sup>3 | 5 ± 1<sup>d</sup> | 2 ± 0 | 7 ± 1 | 8 ± 0<sup>e</sup> |
| Aspartate |       |       |          |          |
| M<sup>+</sup>2 | 14 ± 2 | 13 ± 2 | 17 ± 0<sup>c</sup> | 16 ± 2<sup>f</sup> |
| M<sup>+</sup>3 | 18 ± 1 | 19 ± 2 | 31 ± 0<sup>c</sup> | 30 ± 3<sup>i</sup> |
| M<sup>+</sup>4 | 14 ± 1 | 15 ± 2 | 29 ± 0<sup>c</sup> | 30 ± 3<sup>i</sup> |
| GABA     |       |       |          |          |
| M<sup>+</sup>2 | 7 ± 2<sup>a</sup> | 18 ± 1<sup>j</sup> | 8 ± 2<sup>c</sup> | 21 ± 0<sup>j</sup> |
| M<sup>+</sup>3 | 5 ± 1<sup>k</sup> | 13 ± 1<sup>j</sup> | 4 ± 2<sup>c</sup> | 21 ± 0<sup>j</sup> |
| M<sup>+</sup>4 | 4 ± 0<sup>m</sup> | 10 ± 1<sup>j</sup> | 8 ± 2<sup>c</sup> | 30 ± 1<sup>j</sup> |
| Glutamate|       |       |          |          |
| M<sup>+</sup>2 | 16 ± 0 | 15 ± 1<sup>n</sup> | 15 ± 1<sup>n</sup> | 16 ± 1<sup>n</sup> |
| M<sup>+</sup>3 | 19 ± 1 | 15 ± 1<sup>n</sup> | 18 ± 0 | 20 ± 0<sup>n</sup> |
| M<sup>+</sup>4 | 21 ± 2 | 17 ± 1<sup>n</sup> | 24 ± 1 | 27 ± 0<sup>n</sup> |
| M<sup>+</sup>5 | 16 ± 1 | 12 ± 1<sup>n</sup> | 22 ± 0<sup>n</sup> | 29 ± 0<sup>n</sup> |
| Glutamine |       |       |          |          |
| M<sup>+</sup>2 | 2 ± 1<sup<n</sup> | 9 ± 1<sup<n</sup> | 4 ± 2<sup<n</sup> | 9 ± 0<sup<n</sup> |
| M<sup>+</sup>3 | 4 ± 1<sup<n</sup> | 9 ± 1<sup<n</sup> | 5 ± 2 | 13 ± 0<sup<n</sup> |
| M<sup>+</sup>4 | 7 ± 1<sup<n</sup> | 9 ± 0<sup<n</sup> | 11 ± 1<sup<n</sup> | 20 ± 0<sup<n</sup> |
| M<sup>+</sup>5 | 4 ± 0<sup<n</sup> | 7 ± 0<sup<n</sup> | 13 ± 0<sup<n</sup> | 14 ± 1<sup<n</sup> |

<sup>a</sup> $p < 0.01$ vs. SUR1<sup>-/-</sup>
<sup>b</sup> $p < 0.01$ (for alanine only) vs. M<sup>+</sup>3.
<sup>c</sup> $p < 0.01$ vs. G 5.
<sup>d</sup> $p < 0.05$ vs. SUR1<sup>-/-</sup>.
<sup>e</sup> $p < 0.05$ vs. G 25.
<sup>f</sup> $p < 0.01$ vs. M<sup>+</sup>4.
<sup>g</sup> $p < 0.05$ (only compared M<sup>+</sup>2 to M<sup>+</sup>4).
<sup>h</sup> $p < 0.01$ vs. G 25.
<sup>i</sup> $p < 0.05$ vs. G 5.

*Metabolic Integration and Insulin Secretion*

attention on the labeling pattern of GABA and glutamate (Table 2). The $^{13}$C enrichments and concentrations of the GABA isotomers of islet tissue clearly show that the GAD step is blocked in SUR1<sup>-/-</sup> islets; GABA labeling and actual $[^{13}C]$GABA contents are much lower in SUR1<sup>-/-</sup> islets than in the controls in all incubation conditions, whereas glutamate labeling and $[^{13}C]$glutamate contents are practically the same in both types of islets no matter what the treatment. Incubation with high glucose does not increase GABA labeling or contents of SUR1<sup>-/-</sup> islets but increases the labeling and contents of most glutamate isotomers in controls. MSO and VGB influence the $[^{13}C]$GABA and $[^{13}C]$glutamate data as one might anticipate from their actions; the $^{13}$C content of most GABA and glutamate isotomers is elevated by MSO in the controls but barely in SUR1<sup>-/-</sup> islets, and VGB enhances $^{13}$C enrichments and contents of practically all GABA isotomers in SUR1<sup>-/-</sup> islets and controls alike but does not alter glutamate in either case.

$[^{13}C]$Glucose as Carbon Source of Alanine, Aspartate, and Glutamine—The present study afforded an opportunity to address several highly intractable issues of pancreatic islet intermediary metabolism: 1) pyruvate cycling involving a variety of metabolite shuttles, 2) glucose-induced aspartate depletion, and 3) glucose regulation of glutamine synthesis. Considering the first question, $^{13}$C incorporation into alanine isotomers provides a measure of pyruvate labeling assuming equilibrium of the alanine/$\alpha$-ketoglutarate transaminase step (Table 2). The labeling patterns of the M+2 and M+3 alanine isotomers clearly show that carbon flux into pyruvate at low glucose levels is higher in SUR1<sup>-/-</sup> islets than in controls and that this difference is lost when glucose is raised to 25 mM (both in the absence and presence of MSO or VGB) corroborating the results of $[^{13}C]$carbon dioxide determinations (Fig. 3). It was observed that M+2 contains 3–4 times more label than M+3 under all conditions. We interpret this phenomenon as strong evidence for the existence of pyruvate cycling. Pyruvate carboxylase, malate dehydrogenase, fumarase, succinate oxidase, and malic enzyme (cytosolic and/or mitochondrial) are the likely reactions involved in the dilution of $^{13}$C by $^{12}$C. Considering the second point, namely aspartate depletion of islet tissue after glucose stimulation of islets, here referred to as the aspartate switch, the results of Table 2 show that aspartate turnover and, by extrapolation, oxaloacetate turnover are probably greatly enhanced because the net fall of aspartate is associated with a rise rather than a fall of specific labeling of $[^{13}C]$aspartate. It is noteworthy that the phenomenon occurs also in SUR1<sup>-/-</sup> islets, which are largely resistant to stimulation of insulin secretion by glucose when compared with controls. Considering the last issue, glucose regulation of glutamine synthesis, it was demonstrated that the process is indeed operative in normal and SUR1<sup>-/-</sup> islets and that high glucose enhances the rate of glutamine synthesis. We recall here that our attempts to demonstrate the reaction by using $^{15}$N-labeled ammonia had not been successful.

The Block of the GABA Shunt in SUR1<sup>-/-</sup> Islets Is Explained by Decreased GAD mRNA and Protein—The amino acid profiles and the results of the $^{13}$C tracer studies clearly showed that the GABA shunt of SUR1<sup>-/-</sup> islets is drastically inhibited. We hypothesized that GAD expression might be decreased in SUR1<sup>-/-</sup> beta cells (it should be remembered that the GABA
Metabolic Integration and Insulin Secretion

expression. To explore this possibility, we studied GAD expression in normal islets that had been cultured in the presence of the SUR1 inhibitor glyburide at a concentration of 0.3 \( \mu M \) known to increase intracellular calcium to the same extent as observed in SUR1 \(^{+/+}\) (5, 6) and causing a similar enhancement of BCH-stimulated [U-\(^{14}\)C]glutamine oxidation (Fig. 2) or by blocking the L-type calcium channels of SUR1 \(^{+/+}\) islets with nimodipine for 4 or 24 h. The results tend to support this working hypothesis; GAD protein and mRNA were reduced by \( \sim 30\% \) after treatment of normal islets by 0.3 \( \mu M \) glyburide, and nimodipine treatment of SUR1 \(^{+/+}\) islets resulted in a time-dependent partial recovery of GAD mRNA (Fig. 5).

Evidence for Malic Enzyme Expression in Mouse Islets—As shown in Fig. 6, malic enzyme isoforms 1, 2, and 3 were clearly expressed in islets as determined by reverse transcription-PCR \((n = 3)\). Similar expression patterns were observed in brain tissue. However, liver had a very low expression of ME2 and -3. ME1, -2, and -3 expression was also quantified by real-time PCR (in duplicate RNA preparations from three separate experiments). Malic enzyme expression of SUR1 \(^{+/+}\) and SUR1 \(^{+/+}\) was quantitatively the same. ME1 expression was comparable in islets, brain, and liver. Compared with islets, liver had \( 14 \pm 2 \) fold lower expression of ME2 and undetectable ME3.

Functional Correlates of Metabolic Data—Insulin and glucagon release was determined for all conditions to obtain some measure of the functional significance of the metabolic results (Fig. 4). Fuel stimulation of insulin release using the batch incubation procedure resulted in the secretion response expected according to pertinent published data; base-line insulin release in the presence of 4.0 mM of an amino acid mixture was 6-fold higher in SUR1 \(^{+/+}\) than controls and a combination of 5.0 mM glucose and the amino acid stimulus doubled hormone secretion in controls but had no effect in SUR1 \(^{+/+}\) islets. Combining 25 mM glucose with basal amino acids increased insulin release of control islets 20-fold compared with amino acids alone and 10-fold compared with stimulation by 5 mM glucose and 4.0 mM amino acids together. SUR1 \(^{+/+}\) islets merely doubled the insulin secretion rate when exposed to the combined amino acid/high glucose stimulus. MSO and VGB were without influence on the effect of high glucose in these two islet types. Measured

shunt is probably confined to the beta cells of the islet tissue, which facilitates the study of the phenomenon observed here) (16). Testing this hypothesis, it was indeed found with immunohistochemical methods that GAD protein in SUR1 \(^{-/-}\) islets was reduced by 75% and GAD mRNA by 80% compared with controls (Fig. 5). Immunodetection of glutamine synthetase was used as an internal control, and it was observed that this enzyme was not altered in the SUR1 \(^{-/-}\) islets. We further speculated that GAD expression might be regulated by the level of free intracellular calcium and that the elevated calcium levels of SUR1 \(^{-/-}\) beta cells might be the cause of the reduced enzyme
glucagon release rates were about 2 orders of magnitude lower than insulin release rates, and it is doubtful that the apparent differences in glucagon release between conditions are physiologically meaningful in view of the relative rates of insulin and glucagon release that are observed in the perfused pancreas, perfused islets, or the intact organism (7, 17, 18). Perhaps insulin levels are excessive and result in marked pharmacological inhibition of alpha cells under all conditions. Cognizant of these limitations, a statistically significant reduction of glucagon release by 5 mM glucose in controls and SUR1−/− islets alike deserves some attention.

DISCUSSION

Methodological Considerations—Pancreatic islets isolated by the widely used collagenase procedure were cultured for 3–4 days in RPMI 1640 with fetal calf serum and with 10 mM glucose present to eliminate poorly defined in vivo influences of the SUR1−/− and control animals, for example the possibility that SUR1−/− are abnormally primed by acetylcholine or GLP1 because of their higher sensitivity to these potent modifiers of beta cell function (6, 7). The two types of islets are, however, of comparable size and have comparable relative numbers of the various islet cell types as demonstrated in earlier studies of insulin release and metabolism (10, 19). The incubation medium of the tracer studies contained 4.0 mM concentrations of a physiological mixture of 19 amino acids (AAM) with glutamine contributing 0.5 mM to avoid substrate depletion due to the large extracellular sink and extended incubation period. A 4.0 mM AAM is not stimulatory in control islets but causes insulin release in SUR1−/− islets even though stimulation of respiration was shown to be comparable (6, 7). Fatty acids were not included in the incubation medium because they have poorly defined effects on isolated islets and because the intracellular lipid pools are probably large and not substantially modified by the 3-h experiment. Respiration in the absence of external fuel is comparable in SUR1−/− islets and controls, but stimulation of respiration by high glucose is substantially less pronounced in SUR1−/− islets as compared with controls (i.e. it is reduced by more than 50%) (7). The reduction of glucose-induced respiration is probably not caused by decreased glycolysis because the pacemaker enzyme glucokinase is not altered but more likely results from dysregulation of nonglycolytic processes farther downstream (5, 6). Even though intact islets of Langerhans contain at least 4 different cell types, the glucose-dependent metabolic phenomena are primarily attributed to the 60–70% beta cell compartment because its glucose metabolism is prominent and has highly distinguishing features including effective transport and glucokinase-dependent phosphorylation and oxidation. Most GABA shunt-related observations are considered beta cell-specific based on the strength of available evidence (16). We are using the labeling of the amino acids alanine, aspartate, and glutamate as proxies for the labeling of pyruvate, oxaloacetate, and α-ketoglutarate assuming that the corresponding transaminases achieve rapid equilibrium. The interpretation of amino acid labeling is further based on the assumption that eight of the critical reactions considered here are irreversible (pyruvate dehydrogenase, pyruvate carboxylase, citrate synthase, α-ketoglutarate dehydrogenase, glutamate dehydrogenase, GAD, glutaminase, and GS), whereas all other enzymatic reactions are at equilibrium or near equilibrium including succinate dehydrogenase and the cytosolic and mitochondrial forms of malic enzyme. Glutamate dehydrogenase is considered irreversible based on earlier studies showing that the enzyme operates predominantly in the oxidative direction (13). It should be realized that each data point required about 1000 mouse islets, which precluded time dependence studies. The single 120-min time point was chosen based on prior experience with the expectation of achieving significant isotope incorporation in all critical metabolites and approaching the steady states.

Significance of Alanine, Aspartate, Glutamate, and Glutamine Labeling—The 13C labeling pattern of alanine is exceptional for SUR1−/− and control islets alike and is observed under all incubation conditions. The heavy isotope is incorporated 3–4 times more effectively into the M+2 than the M+3 isotopomer, in striking contrast to the labeling profiles of all other indicator amino acids which show preferential incorporation into the heavier isotopomers. One plausible explanation is that unlabeled CO2 is incorporated into pyruvate by pyruvate carboxylase or by the malic enzymes (in the reducing direction). The products oxaloacetate and malate are in rapid equilibrium with fumarate, which would then allow a loss of the heavy isotope in the oxidative malic enzyme reaction(s). The explanation would apply only if one assumes that the process is very fast compared with the operation of the cycle. The data are consistent with reports which provide evidence for the operation of a highly active pyruvate cycle in pancreatic beta cells (9, 20, 21). It is now widely considered that generation of NADPH, a potential metabolic coupling factor, is the main purpose of this process which results in net transfer of hydrogen from NADH to NADP. It is well known that both NAD and NADP are more reduced when glucose levels rise (9, 20, 22). Because the pentose-P pathway is slow in beta cells, it would be left to malic enzyme and isocitrate dehydrogenase to generate NADPH. The mitochondrial energy-dependent NADP reductase, which transfers hydrogen from NADH to NADP, is not in equilibrium with the cytosolic NAD(P)/NAD(P)H system because of absolute permeability barriers for pyridine nucleotides (23, 24). It is striking that pyruvate in SUR1−/− islets is more effectively labeled than in the controls when glucose is at low levels of 5 mM, consistent with a higher rate of 13CO2 production and strongly suggests pyruvate cycling in SUR1−/− cells. One plausible reason for this activation of pyruvate cycling at low glucose is the high level of cytosolic Ca2+ of SUR1−/− islet cells (5–7). The molecular basis of such an hypothetical action of Ca2+ on pyruvate cycling remains elusive at this point because the potential participating enzymes and membrane transporters are so numerous and difficult to study. However, pyruvate carboxylase and the mitochondrial ME2 (malic enzyme isoform 2) are regulated enzymes and, thus, are primary candidates for the activation of the cycle in SUR1−/− beta cells. Pyruvate cycling is most likely a basic, glucose-independent process, and it can be extrapolated that it can take place as long as the pools of the participating intermediates of metabolism remain filled, for example in the presence of physiological levels of amino acids as used in the present experiments. The process would generate a
steady stream of acetyl-CoA to sustain a complete citric acid cycle when glutamate is a primary fuel (11, 13). It is then reasonable to hypothesize that pyruvate cycling of SUR1−/− cells is enhanced compared with controls even in the absence of glucose. If pyruvate cycling were to be an essential process participating in amino acid-stimulated insulin release, the higher responsiveness of SUR1−/− beta cells to amino acids could be explained, particularly when associated with elevated intracellular Ca2+. In line with this argument, glucose would not be an effective stimulus because it cannot increase pyruvate cycling above the basal rates, which are already maximal. The significance of the ME2 enzyme for pyruvate cycling when glutamine combined with leucine are the primary fuel was reported by Pongratz et al. (21) in studies with INS1 cells and rat islets.

The present data and interpretations regarding the role of the pyruvate cycle in fuel-stimulated mouse islets may seem to be irreconcilable with a large body of published material (9, 20, 21, 25, 26). First, one needs to deal with the repeated and independent claims that mouse pancreatic islets lack malic enzyme, an obligatory participant in any pyruvate cycling (25, 27, 28). The careful evaluation of the literature leads us to conclude that this claim is not strong. It appears that the analytical procedures suitable to determine the NADP-specific ME1 were not designed to detect the mitochondrial ME2 isomerase because the conditions were far from ideal. ME2, which operates with NAD and NADP but prefers the former cofactor, has an acidic pH optimum and low affinity for malate and NADP at the slightly alkaline pH that was used in the published reports (29, 30). Furthermore, the fact that ME2 is sigmoidal and activated by fumarate and inhibited by ATP, which could be employed to distinguish the various isoforms, was not explored (29, 30). In an attempt to address this controversial issue, we have applied reverse transcription-PCR and real-time PCR methods and have observed comparable levels of mouse ME1, -2, and -3 mRNA in control and SUR1−/− islets. Our results resemble those of Pongratz et al. (21) observed in INS1 cell and normal rat islets. We have discussed above a role of the pyruvate cycle, namely facilitation of citrate synthesis and, thus, of the operation of the complete citric acid cycle by providing acetyl-CoA when glutamate generated by glutaminase or transamination of amino acids is a predominant fuel, in agreement with views expressed in independent studies (31). It should also be remembered that malic enzymes are found in many different cell types studied so far including various tumor cells, the flight muscle of various insects, most impressively of the tsetse fly, and red muscle of certain fishes (29–35). ME2 seems to be an essential participant in the integration and regulation of energy and intermediary metabolism central to survival and function of these cells.

The concentrations of aspartate and glutamate in islets incubated with a 4.0 mM AAM but in the absence of glucose are about equal and contribute ~50% to the measured total amino acid pool of about 40 nmol/1000 islets (equivalent to about 20 mM). However, the response of the two amino acids to glucose is strikingly different; aspartate falls drastically, whereas glutamate is practically unchanged. The likely explanation for the fall of aspartate (the aspartate switch) is an equivalent decrease of oxaloacetate with little change of α-ketoglutarate and glutamate because the transaminase is a rapid equilibrium reaction. Oxaloacetate might fall because the citrate synthase and malate dehydrogenase reactions are activated as a result of increased provision of acetyl-CoA and NADH. The aspartate switch is comparable in SUR1−/− and control islets, and it, therefore, seems to have no direct bearing on glucose stimulation of insulin release (remember that the SUR1−/− beta cells show impaired glucose responsiveness). The present data provide little support for the hypothetical role of glutamate as metabolic coupling factor (36) since a correlation of glutamate levels and insulin secretion rates is entirely lacking over a wide range of conditions. These results and interpretations strengthen views expressed in an earlier report on studies of intermediary and energy metabolism with the insulin secreting beta-HC9 cell line (37). Simpson et al. (8), using different beta cell lines and isolated pig islets, observed decreased 13C labeling of aspartate when exposed to glucose and proposed that this anaplerotic process is essential for glucose-stimulated insulin secretion. But our data suggest that this process (here called aspartate switch) is not strongly associated with glucose-stimulated insulin secretion.

Glutamine has emerged as a quantitatively very important fuel stimulant of insulin release as most convincingly demonstrated by studies designed to elucidate the molecular basis of hyperinsulinism syndromes in infants caused by mutations of the SUR1 and the glutamate dehydrogenase genes (38, 39). Pancreatic beta cells of SUR1−/− mice and mice overexpressing an activated mutant of glutamate dehydrogenase are markedly hyper-responsive to glutamine stimulation (6, 13). The pancreatic beta cell defect in glutamate dehydrogenase linked hyper-insulinism has been fully elucidated; hypersecretion of insulin is caused by pathologically enhanced glutaminolysis and concomitant ATP generation because mutant glutamate dehydrogenases escape the physiological inhibitory control by the phosphate-potential (13). The hyper-responsiveness to amino acids (primarily glutamine) of SUR1-linked hyperinsulinemia is less well understood. The difficulty arises from the fact that beta cells lacking the SUR1 protein are hyper-responsive to glutamine and also to a physiologial AAM but are largely refractory to glucose stimulation (6). Two explanations are being considered, but the question remains unsettled; 1) metabolism of glucose is impaired, whereas the metabolism of amino acids is enhanced (perhaps as a result of the persistent elevation of cytosolic calcium), which could result in enhanced stimulus secretion coupling via the phosphate-potential in one but not in the other condition; 2) the AAM or glutamine trigger insulin release directly by hypothetical novel mechanisms, e.g. by activation of protein kinase pathways, to mention just one of the countless possibilities (6). The observation of a reduced respiratory response to high glucose, the block of the GABA shunt, and the glucose refractoriness of pyruvate cycling in SUR1−/− islets favor the former explanation. It had also been proposed in a previous publication that glutamine might participate as metabolic coupling factor in glucose-stimulated insulin release, but earlier attempts had failed to demonstrate that glutamine synthesis is enhanced when beta cells are exposed to high glucose, as would be required if such a mechanism were to operate (6). The present study clearly shows that high glucose does indeed
Pyruvate carbon enters the citric acid cycle through pyruvate carboxylase (with succinate as end product. Aspartate and glutamate are suitable readouts for oxaloacetate and followed by ATP-dependent amination, respectively. GAD is the entry point for glutamate into the GABA shunt equilibrium with malate via MEs. Glucose carbon is channeled to glutamate and glutamine via transamination (citrate synthase, and reactions shown here, there are only four irreversible steps: pyruvate dehydrogenase, pyruvate carboxylase, generation of acetyl-CoA from islet lipid stores cannot be ignored but is not shown here. Note that of all

FIGURE 7. Network of glucose and amino acid metabolism. The GABA shunt is shown in red, pyruvate cycling is in purple, and alanine-pyruvate transamination is in blue. Pyruvate arising from glycolysis is in equilibrium with alanine via alanine aminotransferase (ALT). Alanine is, therefore, a suitable readout for pyruvate labeling. Pyruvate carbon enters the citric acid cycle through pyruvate carboxylase (PC) or with pyruvate dehydrogenase (PDH) via oxaloacetate (OAA) or acetyl-CoA, the building blocks of citrate. Pyruvate is probably also in equilibrium with malate via MEs. Glucose carbon is channeled to glutamate and glutamine via transamination followed by ATP-dependent amination, respectively. GAD is the entry point for glutamate into the GABA shunt with succinate as end product. Aspartate and glutamate are suitable readouts for oxaloacetate and α-ketoglutarate (KG) labeling due to rapid equilibrium reactions catalyzed by the corresponding transaminases. The generation of acetyl-CoA from islet lipid stores cannot be ignored but is not shown here. Note that of all reactions shown here, there are only four irreversible steps: pyruvate dehydrogenase, pyruvate carboxylase, citrate synthase, and α-ketoglutarate dehydrogenase. GABA-T, GABA transaminase; TCA, tricarboxylic acid cycle; PDG, phosphate-dependent glutaminase; AST, aspartate aminotransferase; SSA, succinic semialdehyde; SSADH, succinic semialdehyde dehydrogenase.

results of the following model experiments supported this contention; glyburide (at saturating levels of 0.3 μM) reduced GAD protein and mRNA in control islets by about 30%, and nifedipine at 20 μM partially reversed the mRNA loss in SUR1−/− islets. It is possible that the observed impairment of the GABA shunt is the primary cause or a contributor to the glucose refractoriness of the SUR1−/− beta cells because a main pathway of glucose metabolism is interrupted by the SUR1 gene deletion. The lack of effect of vigabatrin on glucose-induced insulin release of control islets could be interpreted as speaking against this explanation. However, additional pharmacological studies are required before reaching such a conclusion. The GABA shunt could be important in glucose sensing because it could bypass α-ketoglutarate dehydrogenase, a strongly controlled step, which might restrict flux through the citric acid cycle. The shunt would generate NADH as a result of glutamate (produced by transamination of α-ketoglutarate) being converted to succinate as an intermediate of the citric acid cycle (however, at the cost of 1 GTP or ATP depending on the isoenzyme). A metabolic role of the GABA shunt had been suggested by results showing that phenylpyruvate played a permissive role for stimulation of insulin secretion by mM substrate levels of

augment glutamine synthesis about 2-fold, an effect paralleled by a moderate rise of glutamine levels. Enhanced glutamine synthesis is most likely the result of an increased provision of ATP for the corresponding synthetase reaction and of decreased Pi, which would inhibit glutaminase. Glutamine has, therefore, emerged as a plausible indicator of the cell phosphate-potential and remains on the large list of candidate metabolic coupling factors until proven otherwise.

Regulation and Significance of the GABA Shunt of Pancreatic Beta Cells—The present study demonstrates directly that glucose is converted into GABA and that raising the glucose from 5 to 25 mM increases flux through the GABA shunt in isolated pancreatic islets, remarkably without significant changes of cellular GABA levels. Based on the histochemical observations of Sorenson (16) and the biochemical results of Pipeleers and coworkers (40, 41) with sorted islet cells, one can safely assume that the observations pertain specifically to beta cells. The apparent rates of interconversion are comparable with the rates of glucose conversion to alanine, aspartate, and glutamate, suggesting that the pathway is relatively active and plays a prominent role in intermediary metabolism of pancreatic beta cells during glucose stimulation, in agreement with independent
Metabolic Integration and Insulin Secretion

GABA, which apparently involved generation of intracellular succinic semialdehyde and succinate paralleled by production of phenylalanine by transamination (43, 44). It is noteworthy that inhibition of the GABA shunt in SUR1−/− islets did not interfere with the suppression of glucagon secretion observed at low glucose, which suggests that under the conditions of this experiment GABA plays no paracrine role as mediator of glucocose inhibition of glucagon secretion.

A Minimal Model of Metabolic Coupling in Pancreatic Beta Cells—The present study offers new insights into the nature and central role of intermediary metabolism of pancreatic beta cells in fuel stimulated insulin release. It also illustrates the enormous difficulties that have to be faced as the metabolic data are interpreted because of the complex network character of the metabolic pathways that are involved and because of the fact that most enzymatic steps of these pathways are at equilibrium or near equilibrium, which results when islet cells are fuel-stimulated in countless interconnected changes of metabolites and cofactors (45). Any such changes should not be taken as evidence that the metabolites and cofactors in question serve as “metabolic coupling factors” in the true sense of the term. For example, numerous indicators of the phosphate- and the redox-potential change concomitantly, as dictated by the equilibrium constants of the participating reactions, and should be classified as “metabolic indicators” rather than metabolic coupling factors unless there is a clear molecular basis demonstrating otherwise. We have chosen to focus our attention on the metabolism of amino acids because their study promised to open a broad window for observing the metabolic processes associated with stimulation of insulin release by glucose. We have used the SUR1 knock-out mouse as an experimental model because of its unique fuel sensing characteristics. The results of the study clearly demonstrate that amino acids, in particular alanine, aspartate, glutamate, glutamine, and GABA, can serve as powerful indicators of the metabolic response of normal and genetically modified pancreatic beta cells to a glucose stimulus.

What is the physiological significance of this complex metabolic network of fuel sensing pancreatic beta cells? We hypothesize that it serves primarily the role of a central integrator system for the confluent input about fuel availability from numerous specific pathways which participate in the catabolism of glucose, amino acids, fatty acids, and ketone bodies with the citric acid cycle, electron transport, and oxidative phosphorylation at the core and closely linked to several shuttles and shunts, as for instance the glycerol-phosphate and the malate/aspartate hydrogen shuttles, pyruvate cycling, and the GABA shunt. The existence of a central metabolic integrator system in the pancreatic beta cell would assure that the levels of the three known metabolic coupling factors ATP, ADP, and AMP are finely tuned to provide an accurate minute by minute measure of the fuel availability in the blood ready for transmission to the cell membrane constituents that control insulin release (most importantly the K+ and Ca2+ channels) and the AMP kinase system. Four interdependent second messengers (Ca2+, cAMP, diacylglycerol, and inositol triphosphate) also participate in this basic process of stimulus secretion coupling when fuels rise above threshold levels, as was amply documented by earlier investiga-

tions. Second messengers are, however, more prominently involved in mediating the action of neuroendocrine modifiers of fuel-stimulated insulin release (e.g. acetylcholine and GLP-1) with surprisingly little manifestation of altered beta cell intermediary or energy metabolism because the energy cost of exocytosis is relatively low.

The molecular characterization of monogenic diseases of glucose homeostasis caused by mutations of glucokinase, glutamate dehydrogenase (GHD), short-chain 3-hydroxy acyl-CoA dehydrogenase, SUR1, and KIR6.2 has been guided by this minimal concept of metabolic coupling and has been of great heuristic value as illustrated again by the present study using the SUR1 knock-out mouse model of one of these human syndromes. Guided by the tenets of the minimal model (as illustrated in Fig. 7) it has therefore been possible to provide near complete accounts of the molecular basis of the corresponding clinical phenotypes and to use the results of the basic research to design rational and effective strategies for treatment.

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