Conversion into GABA (γ-aminobutyric acid) may reduce the capacity of L-glutamine as an insulin secretagogue

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We have carried out a detailed examination of L-glutamine metabolism in rat islets in order to elucidate the paradoxical failure of L-glutamine to stimulate insulin secretion. L-Glutamine was converted by isolated islets into GABA (γ-aminobutyric acid), L-aspartate and L-glutamate. Saturation of the intracellular concentrations of all of these amino acids occurred at approx. 10 mmol/l L-glutamine, and their half-maximal values were attained at progressively increasing concentrations of L-glutamine (0.3 mmol/l for GABA; 0.5 and 1.0 mmol/l for Asp and Glu respectively). GABA accumulation accounted for most of the $^{14}CO_2$ produced at various L-[U-^{14}C]glutamine concentrations. Potentiation by L-glutamine of L-leucine-induced insulin secretion in perifused islets was suppressed by malonic acid dimethyl ester, was accompanied by a significant decrease in islet GABA accumulation, and was not modified in the presence of GABA receptor antagonists [50 μmol/l saclofen or 10 μmol/l (+)-bicuculline]. L-Leucine activated islet glutamate dehydrogenase activity, but had no effect on either glutamate decarboxylase or GABA transaminase activity, in islet homogenates. We conclude that (i) L-glutamine is metabolized preferentially to GABA and L-aspartate, which accumulate in islets, thus preventing its complete oxidation in the Krebs cycle, which accounts for its failure to stimulate insulin secretion; (ii) potentiation by L-glutamine of L-leucine-induced insulin secretion involves increased metabolism of L-glutamate and GABA via the Krebs cycle (glutamate dehydrogenase activation) and the GABA shunt (2-oxoglutarate availability for GABA transaminase) respectively, and (iii) islet release of GABA does not seem to play an important role in the modulation of the islet secretory response to the combination of L-leucine and L-glutamine.

Key words: γ-aminobutyric acid (GABA), glutamate dehydrogenase (GDH), glutamic acid decarboxylase (GAD), insulin secretion, rat islet.

INTRODUCTION

Fundamental to the initiation of insulin secretion by glucose is the metabolism of the sugar by the β-cell. There is also a close correlation between the ability of many other metabolites to be oxidized by the β-cell and to stimulate the release of insulin. Paradoxically, however, L-glutamine does not stimulate insulin secretion by itself [1], and neither does it potentiate the secretory response to glucose [2], even though it is metabolized significantly by rat islets at a similar rate as glucose, judged by the production of $^{14}CO_2$ from L-[U-^{14}C]glutamine [2]. The failure of L-glutamine to stimulate secretion has been ascribed to its marked sparing action on the oxidation of endogenous nutrients, possibly lipids [2]. Cytoplasmic alkalinization due to the intracellular accumulation of NH_4^+, resulting from L-glutamine deamination, has also been considered an additional factor contributing to the lack of the secretagogue capacity of the amino acid, which may partially be antagonized by increasing the partial pressure of CO_2 of the incubation medium [3].

L-Leucine and its non-metabolizable analogue BCH (2-aminobicyclo[2.2.1]heptane-2-carboxylic acid) each stimulate a predominantly monophasic release of insulin, which becomes biphasic in the presence of L-glutamine [4,5]. This secretory synergism between L-leucine (or BCH) and L-glutamine was attributed initially to the allosteric activation of islet GDH (glutamate dehydrogenase) by L-leucine (or BCH) [1], resulting in increased conversion of L-glutamine-derived L-glutamate into 2-oxoglutarate (α-ketoglutarate) and its subsequent oxidation in the Krebs cycle [6]. However, a closer examination of their metabolic interactions has led to the conclusion that L-leucine induces a net decrease in the rate of 2-oxoglutarate production: stimulation of the oxidative deamination of L-glutamate to 2-oxoglutarate was overcome by its increased transamination (with leucine, alanine and aspartate), resulting in a net decrease in the rate of oxidative metabolism of 2-oxoglutarate [7]. In view of this negative metabolic interaction, the secretory synergism between the two amino acids was ascribed to their marked sparing action on the rate of oxidation of endogenous fatty acids [7].

It has been proposed that L-glutamate may be synthesized from 2-oxoglutarate by the reverse reaction catalysed by GDH, and plays a role as a second messenger (independently of ATP-dependent closure of K^+ channels) in the metabolism–secretion coupling mechanism of β-cells stimulated by glucose [8]. At variance with this hypothesis, L-glutamine increases the intracellular concentration of L-glutamate [2,7,9–11] after being deaminated by the mitochondrial enzyme glutaminase [12], without stimulating insulin secretion. Moreover, it is debatable whether glucose increases the intracellular content of L-glutamate [8,11] or not [10,13]. It is vital to this debate to know whether GDH predominantly catalyses *in vivo* the net oxidative deamination of glutamate, or if it is also capable of carrying out net synthesis of glutamate by the reverse reaction, depending on the relative concentrations of the reactants (2-oxoglutarate and glutamate).

Abbreviations used: BCH, 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid; dmGlu, dmAsp and dmOG, dimethyl esters of Glu, Asp and 2-oxoglutarate respectively; GABA, γ-aminobutyric acid; GAD, glutamic acid decarboxylase; GDH, glutamate dehydrogenase; KRBH, Krebs–Ringer solution buffered with 0.5 mM $NaHCO_3$ and 20 mM Hepes and supplemented with 0.5 % (w/v) BSA; MAD, malonic acid dimethyl ester; Tau, taurine.

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It has also been shown previously that L-glutamine is converted in islets into GABA (γ-aminobutyric acid) [2,14], and β-cell GAD (glutamic acid decarboxylase; GAD-65 isoform) has been recognized as one of the more prevalent autoantigens in Type I diabetes [15,16]. However, very little is known about the possible role of GABA in the mechanism of stimulation of glucose-induced insulin secretion. Interestingly, transgenic mice overexpressing the *GAD-65* gene and GABA develop glucose intolerance without any apparent sign of insulitis or β-cell loss [17]; similarly, INS-1 cells or islets overexpressing GAD-65 show a diminished response to glucose [18].

Thus L-glutamine can be converted in islets not only into L-glutamate, which might behave as a positive regulator of insulin secretion, but also into GABA, which may inhibit secretion. The paradoxical failure of L-glutamine to stimulate insulin secretion may arise from this pattern of metabolism. In order to investigate this hypothesis in the present study, we carried out a detailed examination of the metabolism of L-glutamine by rat islets. Conversion of L-glutamine into several amino acids [Asp, Glu, Ser, Gln, His, Gly, Thr, Arg, Tau (taurine), Ala, Tyr and GABA] was investigated over a wide range of amide concentrations (0.1 to 10.0 mmol/l) using a highly sensitive and specific HPLC method. The rate of L-glutamine metabolism ($^{14}CO_2$ production from L-[U-^{14}C]glutamine) and its capacity to stimulate secretion, as well as their modification by L-leucine, were also studied concomitantly. The data obtained suggest an alternative mechanism that might explain the apparent dissociation between L-glutamine metabolism and insulin secretion, and how it is affected by L-leucine.

EXPERIMENTAL

Materials

Collagenase P was obtained from Roche Diagnostics S.L. (Barcelona, Spain). BSA and most organic compounds were obtained from Sigma-Aldrich Química S.A. (Madrid, Spain). Saclofen and (+)-bicuculline were from Tocris (Biogen Científica S.L., Madrid, Spain). Rat insulin was from Linco Research, Inc. (St. Charles, MO, U.S.A.). Na^{125}I was obtained from Amersham Iberica S.A. (Madrid, Spain); D-[U-^{14}C]glutamine and NaH$^{14}CO_3$ were from New England Nuclear (Itisa Biomedica, Madrid, Spain) or American Radiolabeled Chemicals (Itisa Biomedica S.A., Madrid, Spain). Inorganic compounds and organic solvents were obtained from Merck Farma y Química S.A. (Madrid, Spain).

Methods

Islets were isolated from the pancreas of male Wistar albino rats (body weight 250 g) by collagenase digestion. Insulin secretion was studied using perifused or incubated islets. Two groups, each of 40 rat islets, were perifused in parallel at a flow rate of 0.5 ml/min with KRBH [Krebs–Ringer solution buffered with 0.5 mM $NaHCO_3$ and 20 mM Hepes and supplemented with 0.5 % (w/v) BSA], and heated at 37 °C. The perifusion pattern was similar in all experiments. After a pre-perifusion period of 45 min under basal conditions (in the absence of nutrients or at non-stimulatory concentrations of substrates), the perifusion medium was switched to one containing the test substances and maintained for the next 30 min. Finally, the medium was changed back to pre-perifusion conditions, which were maintained for 25 min. The perifusate was collected at 1 min intervals during the final 60–70 min of perifusion and its insulin concentration measured radioimmunologically. Alternatively, three batches each of ten islets were incubated in 1 ml of KRBH at 37 °C for 60 min and the concentration of insulin released into the incubation medium was measured radioimmunologically. Pig insulin was radio-iodinated with Na^{125}I [19] and rat insulin was used as a standard in the RIA of insulin. Anti-insulin serum was kindly provided by Dr Janove Sehlin (Department of Medical Cell Biology, University of Umeå, Sweden).

L-Glutamine oxidation was measured as the production of $^{14}CO_2$ from L-[U-^{14}C]glutamine [20] and was corrected according to the recovery of NaH$^{14}CO_3$, measured in triplicate during each experiment. One or two groups of islets were taken from each preparation for the fluorimetric determination of DNA [21].

Islet α-amino acids were separated by reverse-phase HPLC after pre-column derivatization with *o*-phthaldialdehyde [22] and quantified by fluorescence detection. Islets (groups of 20) were incubated for 60 min at 37 °C in 100 μl of the same buffer as that used in perifusions. Incubations were stopped by placing the tubes containing the islets on ice. The medium was aspirated and the islets were washed twice with 100 μl of cold PBS. Finally, 100 μl of 35 % (w/v) 5-sulphosalicylic acid was added in order to extract islet amino acids. Final extracts were kept at − 80 °C until their amino acid content was determined. Twelve amino acids were regularly identified and separated in the extracts, and were measured with a sensitivity close to 1 pmol (Asp, Glu, Ser, Gln, His, Gly, Thr, Arg, Tau, Ala, Tyr and GABA). The amount of protein was measured in the extracts using the method of Lowry [22a], and BSA was used as the standard.

GAD activity was measured in islet homogenates using a previously established method, modified slightly from [23] Isolated islets were homogenized by sonication (four times, five strokes at 50 % of the cycle and the minimum potency of a Branson sonifier 450) in 50 mM phosphate buffer (pH 7.2) containing 1 % Triton X-114, protease inhibitors (0.5 mM PMSF and 6.25 μg/ml each of leupeptin and aprotinin) and 2 mM EDTA. The islet homogenate was kept on ice for 30 min with occasional shaking. The reaction was performed at 37 °C in the same phosphate buffer as used for homogenization, after the addition of (final concentrations) 16 mmol/l L-glutamate, 0.75 mmol/l pyridoxal 5-phosphate and 40 μmol/l 3-amino-2,3-dihydrobenzoic acid (gabaculine; a GABA transaminase inhibitor). The reaction was stopped by the addition of 5-sulphosalicylic acid (3.5 %, w/v, final concentration). GABA was derivatized with *o*-phthaldialdehyde, separated by reverse-phase HPLC, and quantified by fluorescence detection.

GDH activity was measured in islet homogenates as the formation (amination) or disappearance (deamination) of NADH at 340 nm, following closely a previously established method [24]. Islets were sonicated in the buffer described above for the study of GAD activity, but in the absence of Triton. The reaction was performed at 30 °C in the same homogenization buffer, supplemented with 5 μmol/l rotenone, 2 mmol/l aminooxiacetate, and 0.5 % BSA. The final concentrations of substrates were 25 mmol/l L-glutamate and 1 mmol/l NAD^+ (deamination reaction) or 10 mmol/l 2-oxoglutarate, 0.1 mmol/l NADH and 100 mmol/l NH_4Cl (amination reaction).

GABA transaminase activity in islet homogenates was measured as the formation of GABA from saturating concentrations (20 mmol/l) of L-glutamate and succinic acid semialdehyde. The reaction was performed at 37 °C in the same homogenization buffer used in the study of GDH activity, and was stopped by the addition of 5-sulphosalicylic acid (3.5 %, w/v, final concentration). The amounts of GABA formed were measured by fluorescence detection after pre-column derivatization with *o*-phthaldialdehyde and HPLC separation.

The total ATP content of islets was measured using the luciferin/luciferase system [25]. Islets (groups of 25) were incubated at

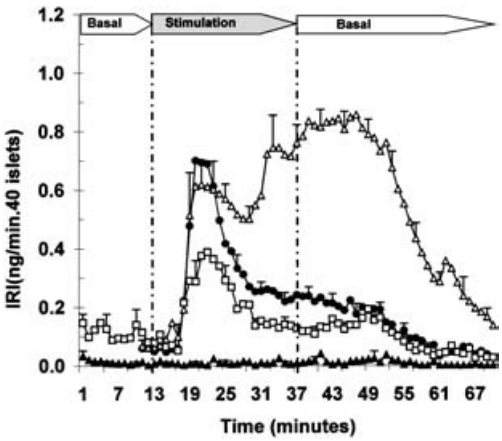

Figure 1 Effects of L-leucine, alone and in combination with L-glutamine, on insulin secretion by rat perifused islets

Groups of 40 islets each, pre-perifused without substrates for 45 min, were then stimulated for 30 min (between vertical broken lines) with 10 mmol/l L-glutamine (▲) or with 10 mmol/l L-leucine, alone (●, $n = 13$) or together with 0.5 mmol/l (□, $n = 5$) or 10 mmol/l (△, $n = 41$) L-glutamine. Pre-perifusion conditions were then re-established during the last 25 min. Values are means ± S.E.M. IRI, immuno-reactive insulin.

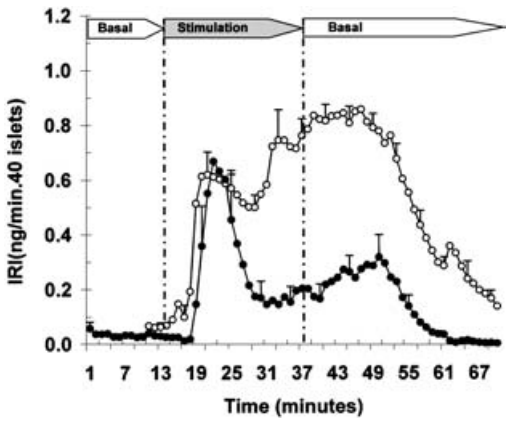

Figure 2 Effects of 10 mmol/l MAD on the insulin secretory response of rat perifused islets to 10 mmol/l L-leucine plus 10 mmol/l L-glutamine

Groups of 40 islets each, pre-perifused without substrates for 45 min, were then stimulated for 30 min (between vertical broken lines) with 10 mmol/l L-leucine plus 10 mmol/l L-glutamine, in the absence (○, $n = 41$) or presence (●, $n = 6$) of 10 mmol/l MAD. Pre-perifusion conditions were then re-established during the last 25 min. Values are means ± S.E.M. IRI, immuno-reactive insulin.

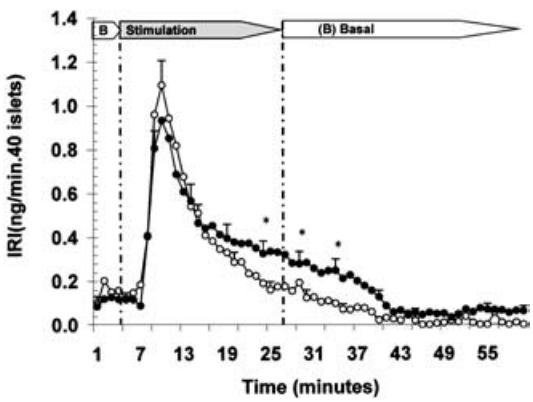

Figure 3 Insulin secretory response of rat perifused islets to depolarization by 30 mmol/l KCl, and the effect of 10 mmol/l L-glutamine

Groups of 40 islets each, pre-perifused with 3 mmol/l glucose alone for 45 min, were then stimulated for 30 min (between vertical broken lines) with 30 mmol/l KCl plus 3 mmol/l glucose, in the absence (○, $n = 8$) or presence (●, $n = 13$) of 10 mmol/l L-glutamine. Pre-perifusion conditions (3 mmol/l glucose) were then re-established during the last 25 min. Values are means ± S.E.M. $^*P < 0.05$ compared with the corresponding points of time in the absence of L-glutamine. IRI, immuno-reactive insulin.

37 °C for 60 min in 25 μl of KRBH containing the selected substrates. The incubation was stopped by plunging the tubes containing the islets plus medium into acetone chilled with solid CO_2, then 20 μl of 1.35 mol/l perchloric acid was added and the tubes were spun briefly in a Microfuge to permit rapid thawing and mixing of their contents. The perchloric acid was neutralized and precipitated with 15 μl of 0.1 mol/l Tris plus 2.8 mol/l $KHCO_3$. Supernatant aliquots (10 μl) of the neutralized extract of samples and ATP standard solutions (1–50 μmol/l) were then added to a luciferin/luciferase mixture, and the emitted light was measured in a bioluminometer. The intensity of the emitted light was linearly related to the concentration of ATP. Luciferase (1 mg/ml) and D-luciferin (0.1 mmol/l) were dissolved in a buffer containing 50 mmol/l Hepes, 10 mmol/l $MgCl_2$ and 0.1 % (w/v) fatty acid-poor BSA, and adjusted to pH 7.6. The luciferin/luciferase mixture was prepared by adding 10 μl of the luciferase solution to 400 μl of 0.1 mmol/l luciferin.

All experimental data are presented as mean values ± S.E.M., and the numbers of separate experiments are given in parentheses. Statistical comparisons were performed using non-paired, two-tailed Student's *t* tests.

RESULTS

Insulin secretion

As shown in Figure 1, 10 mmol/l L-glutamine did not stimulate insulin secretion, whereas 10 mmol/l L-leucine induced a predominantly monophasic release of insulin from rat perifused islets. The perifusion of both amino acids together, each at 10 mmol/l, converted the monophasic response to L-leucine into a clearly biphasic stimulation, with a specific and significant increase in the second phase of secretion. However, L-glutamine did not potentiate the second phase of L-leucine-induced insulin secretion when it was perifused at 0.5 mmol/l (Figure 1). The potentiation by 10 mmol/l L-glutamine of the secretory response to L-leucine was strongly suppressed by 10 mmol/l MAD (malonic acid dimethyl ester), a membrane-permeable analogue of malonic acid which is a known competitive inhibitor of the Krebs cycle enzyme succinic acid dehydrogenase [26] (Figure 2). BCH (10 mmol/l), a non-metabolizable analogue of L-leucine, triggered a much lower insulin response than L-leucine in the presence of 10 mmol/l L-glutamine (results not shown).

We then investigated whether the potentiation of insulin release by L-glutamine was specific for L-leucine-induced release. Under continuous perifusion with 3 mmol/l glucose to provide basal production of ATP, plasma membrane depolarization with 30 mmol/l K^+ induced a transient (monophasic) release of insulin which was qualitatively and quantitatively similar to that stimulated by 10 mmol/l L-leucine alone (Figure 3). The addition of 10 mmol/l L-glutamine did not alter the overall secretory response induced by K^+ depolarization, but the secretory rate was significantly higher at some points during the second phase of secretion (Figure 3). These experiments were repeated with incubated islets in the presence of 250 μM diazoxide to keep the K^+

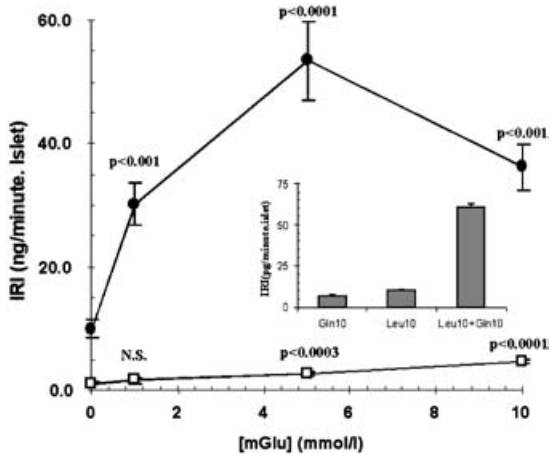

Figure 4 Concentration dependence of the stimulation of insulin secretion by dmGlu alone and in combination with 10 mmol/l L-leucine

Three batches each of ten isolated islets were incubated at 37 °C in 1 ml of KRBH containing various concentrations of L-glutamate alone (dmGlu, □) or in the presence of 10 mmol/l L-leucine (●). The concentration of insulin released into the incubation medium was measured radioimmunologically. The inset shows, for comparison, the potentiation of the secretory response to 10 mmol/l L-leucine (Leu10) by 10 mmol/l L-glutamine (Gln10) in incubated islets. Values are means ± S.E.M. ($n = 6$). IRI, immuno-reactive insulin.

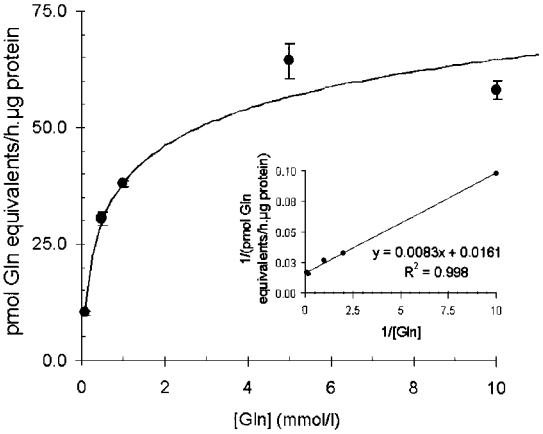

Figure 5 Concentration dependence of the rate of L-glutamine oxidation by isolated rat islets

Glutamine oxidation was measured as the rate of $^{14}CO_2$ production from various concentrations of L-[U-^{14}C]glutamine (5 to 500 Ci/mol): 0.1 ($n = 5$), 0.5 ($n = 21$), 1.0 ($n = 5$), 5.0 ($n = 5$), and 10.0 mmol/l ($n = 17$). Two groups, each of 20 islets, were incubated at 37 °C for 120 min. The inset shows the double-reciprocal plot used to calculate the ED_{50} of L-glutamine (0.56 mmol/l). Values are means ± S.E.M.

channels open, and in this case glutamine addition induced a slight but significant enhancement of the secretory response (10.9 ± 0.95 compared with 7.91 ± 1.0 ng/60 min per islet; $n = 10$; $P < 0.05$).

dmGlu (the dimethyl ester of L-glutamate) showed a poor capacity for stimulating insulin secretion by incubated islets by itself, but it potentiated the response to 10 mmol/l L-leucine in a concentration-dependent manner (Figure 4). At 5 mmol/l, it potentiated the secretory effect of 10 mmol/l L-leucine to a similar extent as 10 mmol/l L-glutamine (see inset of Figure 4 for comparison), whereas at a higher concentration (10 mmol/l) it induced a decrease. In contrast, dmOG (the dimethyl ester of 2-oxoglutarate) stimulated the insulin secretory response of incubated islets at 5 mmol/l (24.7 ± 2.0 compared with 2.7 ± 0.2 pmol/min per islet; $n = 6$; $P < 0.0001$). The combination of 10 mmol/l L-leucine with 5 mmol/l dmOG induced a secretory response (31.8 ± 2.3 pmol/min per islet; $n = 6$) that was approximately equivalent to the summation of the separate effects of L-leucine (14.0 ± 1.2 pmol/min per islet; $n = 6$) and dmOG (24.7 ± 2.0 pmol/min per islet; $n = 6$). The methyl ester derivatives of 2-oxoacids, like those of pyruvate and 2-oxoglutarate, seem to be more unstable in solution than other methyl esters used previously, such as succinic acid dimethyl ester or the methyl esters of amino acids. At high concentrations, they may acidify the pH of the medium due to their spontaneous hydrolysis and thus alter the secretory response.

The stimulation of insulin secretion induced by depolarization with 30 mmol/l K^+ in the presence of 0.25 mmol/l diazoxide and 5 mmol/l dmOG (34.1 ± 3.0 pmol/min per islet, compared with a baseline value of 2.7 ± 0.2 pmol/min per islet; $n = 6$; $P < 0.0001$) was approximately equal to the summation of the separate responses to each stimulus (K^+ depolarization, 9.2 ± 0.9 pmol/min per islet; dmOG stimulation, 24.7 ± 2.0 pmol/min per islet; $n = 6$).This suggests that islet 2-oxoglutarate metabolism is not sensitive to increases in cytosolic Ca^{2+}. Neither 10 mmol/l dmAsp (the dimethyl ester of aspartate) nor L-aspartate, L-glutamate or GABA stimulated the rate of insulin secretion by incubated islets.

We investigated whether increased release of GABA might play a role in modulation of the insulin secretory response of isolated islets to the combination of L-glutamine and L-leucine (via suppression of somatostatin release from δ-cells and/or feedback inhibition of β-cells). The secretory response of perifused islets to these two α-amino acids was not modified by the presence of either 10 μmol/l (+)-bicuculline (a $GABA_A$ receptor antagonist; 28.9 ± 3.1 compared with 28.6 ± 4.1 ng/30 min per 40 islets; $n = 6$) or 50 μmol/l saclofen [a $GABA_B$ receptor antagonist; 21.1 ± 4.0 ($n = 10$) compared with 21.6 ± 2.5 ($n = 9$) ng/30 min per 40 islets]. Addition of 100 μmol/l GABA did not modify the islet secretory response to 10 mmol/l L-leucine [10.3 ± 2.6 ($n = 10$) compared with 15.3 ± 3.0 ($n = 8$) ng/30 min per 40 islets].

Oxidation of L-[U-^{14}C]glutamine

$^{14}CO_2$ production from L-[U-^{14}C]glutamine showed a hyperbolic relationship with the medium concentration of the α-amino acid in the range 0.1 to 10.0 mmol/l (Figure 5). It was characterized by an ED_{50} value close to 0.5 mmol/l L-glutamine, as calculated from a double-reciprocal plot (see inset of Figure 5). The rate of $^{14}CO_2$ production from 10 mmol/l L-[U-^{14}C]glutamine was not modified significantly by 10 mmol/l L-leucine or by 2 μg/ml oligomycin, while a combination of the two induced a slight increase (Table 1). In contrast, at 0.5 mmol/l L-[U-^{14}C]glutamine the production of $^{14}CO_2$ was significantly decreased by 10 mmol/l L-leucine, irrespective of the presence of 2 μg/ml oligomycin, which had no effect by itself (Table 1). The succinic acid dehydrogenase inhibitor MAD (10 mmol/l) [26] did not modify the islet production of $^{14}CO_2$ from either 10 or 0.5 mmol/l L-[U-^{14}C]glutamine (Table 1). Allylglycine, a precursor of a competitive inhibitor of GAD [27], significantly suppressed the rate of $^{14}CO_2$ production from both 0.5 mmol/l (−34 %) and 10 mmol/l (−25 %) L-[U-^{14}C]glutamine (Table 1).

Islet ATP levels

The total ATP content of islets incubated for 1 h in the absence of substrates was significantly increased by 3 mmol/l glucose

Table 1 L-Glutamine oxidation: effects of L-leucine and different types of inhibitors

L-Glutamine oxidation was measured as the rate of $^{14}CO_2$ production from L-[U-^{14}C]glutamine (5 or 100 Ci/mol). Two groups, each of 20 islets, were incubated at 37 °C for 120 min. Values are means ± S.E.M. for the numbers of experiments given in parentheses. N.S., not significant.

	$^{14}CO_2$ production (pmol/h per μg of protein)			
Addition	0.5 mmol/l L-Glutamine	*P*	10.0 mmol/l L-Glutamine	*P*
Control	30.41 ± 1.57 (21)	–	53.79 ± 4.30 (13)	–
10 mmol/l L-Leucine	19.87 ± 1.36 (8)	< 0.001	55.97 ± 3.36 (8)	N.S.
2 μg/ml Oligomycin	27.49 ± 2.36 (8)	N.S.	47.54 ± 4.53 (6)	N.S.
10 mmol/l L-Leucine + 2 μg/ml oligomycin	16.10 ± 1.09 (4)	< 0.001	68.55 ± 4.54 (4)	< 0.05
10 mmol/l MAD	24.10 ± 1.53 (4)	N.S.	58.12 ± 4.76 (4)	N.S.
10 mmol/l Allylglycine	20.23 ± 1.15 (12)	< 0.001	40.41 ± 2.53 (4)	< 0.02
10 mmol/l MAD + 10 mmol/l allylglycine	27.02 ± 1.72 (8)	N.S.	39.31 ± 2.37 (4)	< 0.01

[3.52 ± 0.2 ($n = 8$) compared with 2.66 ± 0.14 ($n = 22$) pmol/islet; $P < 0.003$], but the ATP/ADP ratio was not [1.17 ± 0.10 ($n = 8$) and 1.00 ± 0.06 ($n = 22$) respectively]. At 20 mmol/l, glucose increased still further the total amount of islet ATP [4.82 ± 0.23 ($n = 14$) compared with 3.52 ± 0.2 ($n = 8$) pmol/islet; $P < 0.001$] and the ATP/ADP ratio [1.88 ± 0.13 ($n = 14$) and 1.17 ± 0.10 ($n = 8$) respectively; $P < 0.001$]. Glutamine (10 mmol/l) increased islet ATP [3.6 ± 0.25 ($n = 10$) compared with 2.66 ± 0.14 ($n = 22$) pmol/islet; $P < 0.002$] and the ATP/ADP ratio [1.38 ± 0.14 ($n = 10$) and 1.00 ± 0.06 ($n = 22$) respectively; $P < 0.02$] above the levels recorded in the absence of substrates, and to values similar to those induced by 3 mmol/l glucose. L-Leucine alone increased islet ATP levels (4.63 ± 0.38 compared with 3.52 ± 0.2 pmol/islet; $n = 8$; $P < 0.022$) and the ATP/ADP ratio (1.74 ± 0.21 and 1.17 ± 0.10 respectively; $n = 8$; $P < 0.028$) above the values obtained at 3 mmol/l glucose, and close to those induced by 20 mmol/l glucose. The combination of L-leucine (10 mmol/l) with L-glutamine (10 mmol/l) maintained ATP levels (4.94 ± 0.47 pmol/islet, $n = 7$) and the ATP/ADP ratio (1.8 ± 0.28, $n = 7$) at values very close to those obtained with either 20 mmol/l glucose or 10 mmol/l L-leucine.

Conversion of L-glutamine into other amino acids

Extracellular L-glutamine was converted by isolated islets into GABA, L-aspartate and L-glutamate in a concentration-dependent manner (Figure 6). No significant variations were observed for any of the other amino acids measured. The intracellular concentrations of GABA, L-aspartate and L-glutamate followed a hyperbolic relationship with L-glutamine concentration. Half-maximal values for GABA, L-aspartate, and L-glutamate were attained at 0.3, 0.5, and 1.0 mmol/l L-glutamine respectively, as calculated from the corresponding double-reciprocal plots. Near-saturation of GABA, L-aspartate and L-glutamate was obtained at approx. 10 mmol/l L-glutamine. The islet (intracellular) content of L-glutamine itself increased almost linearly with its extracellular concentration (Figure 6). In a separate series of experiments, the uptake of L-leucine in the presence of 10 mmol/l L-glutamine was studied using a different gradient elution profile for the HPLC separation of islet amino acids. The basal content of Leu is very low compared with the most abundant amino acids in islets (Tau, Glu, Asp and GABA), and it was increased by more than 7-fold by 10 mmol/l extracellular L-leucine (1.8 ± 0.3 compared with 13.3 ± 2.0 pmol/μg of protein; $n = 6$; $P < 0.0002$). This increased content was not modified by the simultaneous presence of 10 mmol/l Gln (15.9 ± 1.7 pmol/μg of protein; $n = 6$).

L-Leucine (10 mmol/l) decreased significantly the synthesis of GABA from either 0.5 mmol/l (−52 %) or 10 mmol/l (−38 %)

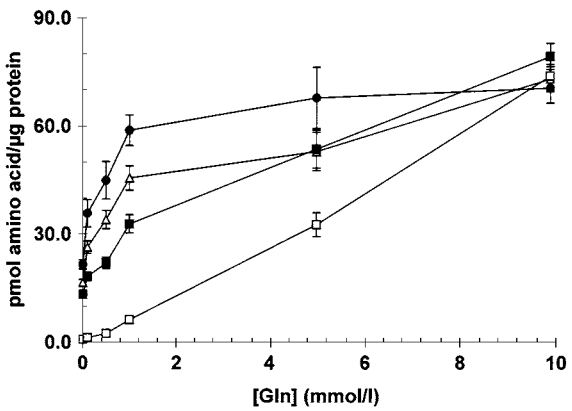

Figure 6 Concentration dependence of the conversion of L-glutamine into other amino acids by isolated rat islets

Two groups, each of 20 islets, were incubated with different L-glutamine concentrations at 37 °C for 60 min. They were then washed with saline twice and extracted with 35 % (w/v) 5-sulphosalicylic acid. Twelve amino acids were separated and quantified by HPLC after derivatization with *o*-phthaldialdehyde. Significant variations were found only in three of the four most abundant endogenous amino acids (△, Asp; ■, Glu; ●, GABA) and L-glutamine (□). The concentration of Tau, the most abundant amino acid, was unaffected by the different L-glutamine concentrations tested (results not shown). Values are means ± S.E.M. of seven different experiments.

L-glutamine (Table 2). BCH (10 mmol/l) decreased significantly the islet content of L-aspartate (−26 %; $P < 0.01$), L-glutamate (−26 %; $P < 0.01$), GABA (−56 %; $P < 0.001$) and L-glutamine (−35 %; $P < 0.002$) at 10 mmol/l extracellular L-glutamine. Either L-leucine and BCH, alone at 10 mmol/l, prevented the decline in the islet content of L-aspartate, L-glutamate and L-glutamine produced by the incubation of freshly isolated islets for 1 h (Table 2). At 10 mmol/l, allylglycine, a precursor of a competitive inhibitor of GAD [27], decreased significantly the islet synthesis of GABA from either 0.5 or 10 mmol/l L-glutamine, but did not modify the intracellular level of any of the other amino acids generated from the amide. Islet depolarization with 30 mmol/l KCl did not affect the conversion of 10 mmol/l L-glutamine into any of the amino acids investigated (results not shown).

Methyl ester derivatives as permeable precursors of amino acids and 2-oxoglutarate

dmGlu was converted by isolated islets into GABA, L-aspartate and L-glutamate in a concentration-dependent manner (0.25, 1.0,

Table 2 Modification by L-leucine, its non-metabolizable analogue BCH and the GAD inhibitor allylglycine (AllGly) of the islet content of L-glutamine-derived amino acids

Two to three groups, each of 20 islets, were incubated with L-glutamine and the indicated additives for 60 min at 37 °C. They were washed with saline twice and extracted with 35 % (w/v) 5-sulphosalicylic acid. Twelve amino acids were separated and quantified by HPLC after their derivatization with *o*-phthaldialdehyde, but only the amino acids whose content varied with the concentration of L-glutamine are presented. Values are means ± S.E.M. for the numbers of experiments indicated.

		Content (pmol of amino acid/μg of protein)							
Additions	*n*	Asp	*P*	Glu	*P*	Gln	*P*	GABA	*P*
None	19	16.5 ± 0.8	–	13.2 ± 0.6	–	0.8 ± 0.2	–	21.6 ± 1.3	–
10 mmol/l L-Leucine	6	30.0 ± 2.0	< 0.001	20.4 ± 2.4	< 0.01	2.6 ± 0.7	< 0.02	21.3 ± 1.7	N.S.
10 mmol/l BCH	6	22.0 ± 1.2	< 0.01	24.1 ± 2.0	< 0.001	2.0 ± 1.0	N.S.	21.3 ± 2.0	N.S.
0.5 mmol/l L-Glutamine	16	40.2 ± 1.8	–	27.5 ± 1.8	–	4.1 ± 0.4	–	45.8 ± 2.8	–
+ 10 mmol/l L-leucine	6	35.5 ± 2.3	N.S.	22.7 ± 1.4	< 0.05	3.3 ± 0.4	N.S.	22.2 ± 1.7	< 0.001
+ 10 mmol/l AllGly	6	42.3 ± 2.0	N.S.	28.3 ± 2.2	N.S.	2.0 ± 0.2	< 0.001	32.2 ± 2.3	< 0.02
10 mmol/l L-Glutamine	6	73.1 ± 3.5	–	79.3 ± 3.6	–	73.9 ± 3.3	–	70.6 ± 4.2	–
+ 10 mmol/l L-leucine	6	79.9 ± 4.4	N.S.	77.2 ± 5.6	N.S.	55.6 ± 3.5	< 0.01	43.5 ± 3.4	< 0.001
+ 10 mmol/l BCH	6	54.5 ± 3.3	< 0.01	59.0 ± 5.0	< 0.01	48.4 ± 4.6	< 0.002	31.0 ± 2.4	< 0.001
+ 10 mmol/l AllGly	7	83.5 ± 4.5	N.S.	84.4 ± 4.3	N.S.	58.6 ± 6.9	N.S.	41.1 ± 4.3	< 0.001

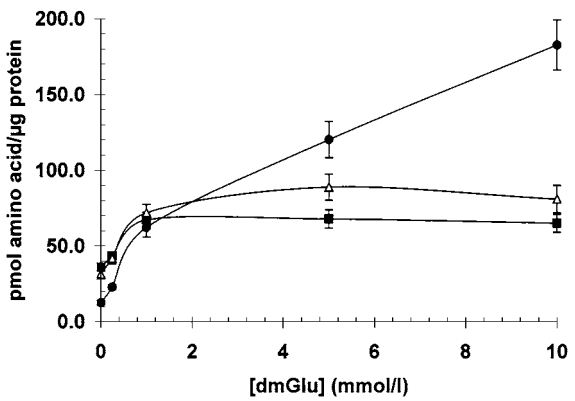

Figure 7 Concentration dependence of the conversion of dmGlu into other amino acids by isolated rat islets

Two groups, each of 20 islets, were incubated with various dmGlu concentrations at 37 °C for 60 min. Other experimental details were the same as those described in the legend of Figure 6 and the Experimental section. dmGlu was converted mainly into Glu (●), GABA (△) and Asp (■). The intracellular amounts of Gln synthesized are given in the text. Values are means ± S.E.M. of seven different experiments. The increases in Glu and Asp were statistically significant at 0.25 mmol/l dmGlu ($P < 0.001$ and $P < 0.05$ respectively).

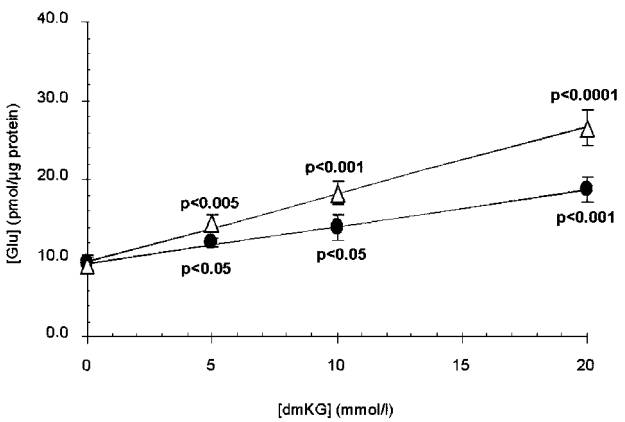

Figure 8 Concentration dependence of the conversion of dmOG into L-glutamate by isolated rat islets, and effects of L-leucine

Two groups, each of 20 islets, were incubated with different dmOG (dmKG) concentrations at 37 °C for 60 min in the absence (●) or presence (△) of 10 mmol/l L-leucine. Other experimental details were the same as those described in the legend of Figure 6 and the Experimental section. Values are means ± S.E.M. of six different experiments. *P* values above or below each symbol represent the degree of statistical significance compared with the corresponding control in the absence of dmOG.

5.0 and 10 mmol/l). The synthesis of GABA and L-aspartate reached apparent saturation at 1 mmol/l dmGlu, whereas islet L-glutamate increased linearly with dmGlu concentrations above 1 mmol/l (Figure 7). dmGlu was also converted into L-glutamine at a very low rate, as compared with the formation of L-aspartate, L-glutamate or GABA: islet L-glutamine increased linearly with dmGlu concentration, and the increments were statistically significant at 5 mmol/l (2.5 ± 0.3 compared with 1.1 ± 0.2 pmol/μg of protein; $n = 7$; $P < 0.002$) and 10 mmol/l (3.2 ± 0.5 pmol/μg of protein; $n = 7$; $P < 0.002$).

dmAsp, tested at 10 mmol/l, resulted in a 6.5-fold increase in L-aspartate (193.0 ± 20.3 compared with 29.7 ± 2.2 pmol/μg of protein; $n = 6$; $P < 0.0001$); the islet content of L-glutamate remained constant, and that of GABA decreased slightly (61.7 ± 4.9 compared with 80.3 ± 5.5 pmol/μg of protein; $n = 6$; $P < 0.05$). The islet concentrations of Glu, Asp and GABA were not increased significantly by incubation of islets with 10 mmol/l Glu, Asp or GABA respectively (results not shown).

dmOG increased the islet content of Glu linearly in a concentration-dependent manner (Figure 8). This effect was augmented further by the simultaneous presence of 10 mmol/l L-leucine, reaching statistical significance at 20 mmol/l dmOG (26.51 ± 2.2 compared with 18.7 ± 1.5 pmol/μg of protein; $n = 6$; $P < 0.016$) (Figure 8). dmOG exerted a dual effect on islet GABA content: it was increased at the lowest dmOG concentration used (5 mmol/l) (28.1 ± 2.1 compared with 20.9 ± 1.9 pmol/μg of protein; $n = 6$; $P < 0.03$), but was decreased by concentrations of 10 mmol/l (non-significant) and 20 mmol/l (17.1 ± 1.8 pmol/μg of protein; $n = 6$; $P < 0.03$) (Figure 9). In the presence of 10 mmol/l L-leucine, dmOG increased islet GABA content at 5 and 10 mmol/l, but this increment was almost completely suppressed at 20 mmol/l

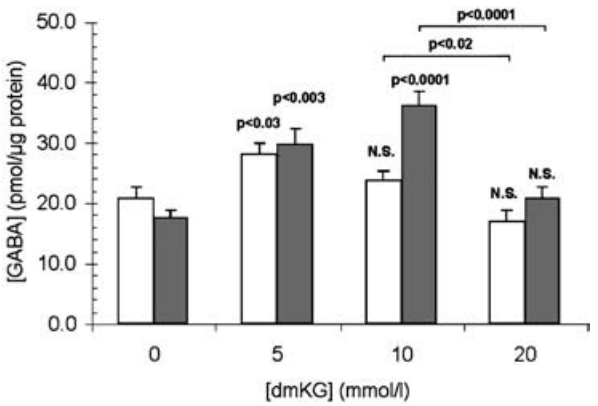

Figure 9 Conversion of dmOG into GABA by isolated rat islets, and effects of L-leucine

Two groups, each of 20 islets, were incubated with different dmOG (dmKG) concentrations at 37 °C for 60 min in the absence (empty bars) or presence (solid bars) of 10 mmol/l L-leucine. Other experimental details were the same as those described in the legend of Figure 6 and the Experimental section. Values are means ± S.E.M. of six different experiments. Each value was compared with the corresponding control in the absence of dmOG, and *P* values are shown (N.S., not significant).

Table 3 GAD and GDH activities in islet homogenates at saturating concentrations of their respective substrates

GAD activity was measured as the accumulation of GABA, and GDH activity as the formation (amination) or disappearance (deamination) of NADH, recorded at 340 nm. Experimental details are given in the Experimental section. Values are means ± S.E.M. for the numbers of experiments given in parentheses. BCH, an L-leucine analogue, is an allosteric effector of GDH; allylglycine is a precursor of a competitive inhibitor of GAD; 3-mercaptopropionic acid is a known GAD inhibitor. N.S., not significant.

Additives	GAD activity (pmol of GABA/15 min per islet)	*P*
None	12.55 ± 0.57 (15)	–
10 mmol/l L-leucine	15.60 ± 0.90 (9)	< 0.01
10 mmol/l α-Oxoisocaproic acid	11.07 ± 0.70 (9)	N.S.
10 mmol/l Allylglycine	12.51 ± 0.96 (6)	N.S.
1 mmol/l 3-mercaptopropionic acid	1.14 ± 0.23 (6)	< 0.001
10 mmol/l BCH	16.35 ± 0.97 (7)	< 0.002

	GDH activity (nmol/min per μg of protein)		
Reaction	Control	+ 10 mmol/l L-Leucine	*P*
Amination (A)	62.88 ± 4.90 (8)	110.12 ± 9.42 (8)	< 0.001
Deamination (D)	8.01 ± 1.07 (8)	11.06 ± 1.07 (8)	< 0.05
Ratio (A/D)	8.16 ± 0.53 (8)	10.10 ± 0.62 (8)	< 0.05

(Figure 9). No significant variations in the islet content of Asp were observed.

GAD activity of islet homogenates

Under saturation conditions of L-glutamate and its coenzyme, pyridoxal 5-phosphate, the activity of GAD measured in islet homogenates was linear for at least 30 min at 37 °C. A shorter time of 15 min was used to test routinely the effects of different substances on enzyme activity. As shown in Table 3, 10 mmol/l L-leucine (or its non-metabolizable analogue BCH) enhanced enzyme activity slightly but significantly, whereas its deaminated product, 2-oxoisocaproic acid, had no effect. The enzyme activity was not affected by allylglycine, but it was very strongly suppressed (> 90 %) by 1 mmol/l 3-mercaptopropionic acid, a known competitive inhibitor. Allylglycine is known to be effective only *in vivo*, because it needs to be converted into an active metabolite, possibly 2-oxo-4-pentenoic acid [27].

GABA transaminase activity of islet homogenates

GABA transaminase activity, measured at saturating concentrations of L-glutamate and semialdehyde succinic acid, was of the same order of magnitude as the recorded GAD activity. It was strongly suppressed by its known inhibitor gabaculine at 40 μM [2.1 ± 0.3 ($n = 5$) compared with 19.7 ± 1.1 pmol/15 min per islet ($n = 14$); $P < 0.0005$], but was not affected by 10 mmol/l L-aspartate (16.7 ± 1.7 pmol/15 min per islet; $n = 4$) or L-leucine (19.8 ± 1.8 pmol/15 min per islet, $n = 14$).

GDH activity

In islet homogenates, the rate of 2-oxoglutarate amination was 8-fold higher than the rate of oxidative deamination of L-glutamate under conditions of saturation with the corresponding substrates and in the absence of allosteric effectors (Table 3). L-Leucine (10 mmol/l) increased the rate of amination (+75 %) to a greater extent than that of deamination (+38 %), so that the amination/deamination ratio was increased significantly (Table 3).

DISCUSSION

The dose–response study of the conversion of L-glutamine into other amino acids showed that the amide is converted preferentially into GABA, followed by its conversion into L-aspartate and L-glutamate, according to their respective ED_{50} values obtained from the double-reciprocal plots of amino acid content against extracellular L-glutamine concentration. Islet L-glutamine levels increased almost linearly in relation to its extracellular concentration, and all four amino acids (GABA, Asp, Glu and Gln) reached similar levels at the highest amide concentration (10 mmol/l). This probably means that transport of L-glutamine (and not its deamination to L-glutamate) is limiting its conversion into other amino acids at low concentrations, and suggests that islet GAD has a high activity and affinity for L-glutamate [23]. It has been shown previously that GABA is synthesized in islets from L-glutamine, but no detailed dose–response relationship was investigated [2,7,14].

Islet accumulation of GABA (after subtracting the basal value obtained in the absence of exogenous substrates) accounted for most of the $^{14}CO_2$ produced from L-[U-^{14}C]glutamine at each of the extracellular L-glutamine concentrations studied. The lack of effect of oligomycin (a respiration inhibitor) and MAD (a plasma membrane-permeable form of malonic acid, and a demonstrated inhibitor of the Krebs cycle in islets) [26] on the rate of $^{14}CO_2$ production from L-[U-^{14}C]glutamine argues against the possibility that $^{14}CO_2$ was released mainly in the Krebs cycle. The concomitant accumulation of islet Asp together with GABA is probably the result of oxaloacetate transamination with glutamate. Through the partial reactions of the 'GABA shunt' (GABA transaminase and semialdehyde succinic acid dehydrogenase), GABA might be metabolized into succinic acid, which would then be converted into oxaloacetate following the partial reactions of the Krebs cycle (succinic dehydrogenase and malic acid dehydrogenase). This pathway skips Krebs

cycle decarboxylations and does not contribute to the production of $^{14}CO_2$ from L-[U-^{14}C]glutamine.

The failure of L-glutamine to maintain the same levels of islet ATP as glucose similarly suggests that $^{14}CO_2$ production from L-[U-^{14}C]glutamine is poorly coupled to ATP production. The finding that allylglycine, a precursor of a competitive inhibitor of GAD [27], partially decreased L-glutamine oxidation as well as its conversion into GABA at two amide concentrations (0.5 and 10.0 mmol/l) also strongly suggests that most of the L-glutamine-derived L-glutamate is predominantly decarboxylated to GABA instead of being oxidized in the Krebs cycle. GABA may accumulate in synaptic-like microvesicles [28] within β-cells and be released via spontaneous exocytosis [29]. This interpretation provides an alternative explanation for the paradoxical dissociation between metabolism and secretion in islets incubated with L-glutamine. It has been accepted for more than two decades that L-glutamine is not a secretagogue because it exerts a strong sparing action on the oxidation of endogenous substrates [2]. It could not be demonstrated previously that $^{14}CO_2$ production from L-[U-^{14}C]glutamine was accounted for by L-glutamate decarboxylation to GABA, but it was investigated only at one amide concentration, and GABA was measured by ion-exchange radiochromatography; as far as we know, no direct, chemical measurement was performed [2]. In a more recent work, GABA synthesis from L-glutamine was measured using an enzymic method, but hardly any difference was found between two amide concentrations as different as 0.25 and 2 mmol/l [14].

Potentiation of L-leucine-induced insulin secretion by L-glutamine transformed the predominantly monophasic release triggered by the latter into a biphasic and sustained release pattern, thus confirming previous reports [4,5]. This potentiating effect of L-glutamine was almost totally abolished by MAD, a permeable form of the succinic acid dehydrogenase inhibitor malonic acid [26]. This supports the idea that increased metabolism of L-glutamine in the Krebs cycle mediates the potentiation of secretion [6]. Moreover, the present results do not favour the view [7] that L-leucine diverts the metabolism of L-glutamine-derived 2-oxoglutarate out of the Krebs cycle and towards transamination reactions with Asp, Ala or Leu itself: the combination of 10 mmol/l L-glutamine and 10 mmol/l L-leucine did not decrease the islet content of Asp, nor those of Leu or Ala, whose basal concentrations are severalfold lower than those of the four most abundant islet amino acids (Tau, Glu, Asp and GABA) [30]. Moreover, the fact that 10 mmol/l dmAsp only gave rise to free Asp suggests that the glutamate–oxaloacetate transaminase equilibrium *in vivo* is displaced in favour of Asp accumulation and not Glu formation. In contrast, L-leucine drastically reduced the islet content of GABA at two L-glutamine concentrations (0.5 and 10 mmol/l). This cannot be attributed to L-leucine-induced inhibition of islet GAD activity, which showed slight stimulation, but it might be due to increased GABA metabolism through the reactions of the GABA shunt, initiated at the GABA transaminase step and leading to succinic acid [28]. A direct effect of L-leucine on GABA transaminase activity has been disproved experimentally. However, L-leucine might facilitate GABA transamination by increasing the availability of 2-oxoglutarate through the allosteric activation of GDH: (1) addition of a membrane-permeable form of 2-oxoglutarate (dmOG) stimulated insulin secretion, increased the islet content of Glu and decreased that of GABA above a critical concentration; and (2) L-leucine activated GDH activity in islet homogenates, as has been substantiated previously [1,24,31,32]. It is also demonstrated in the present work that L-leucine significantly increased the conversion of 2-oxoglutarate (added as its dimethyl ester) into L-glutamate in incubated islets without decreasing the Asp concentration. This seems to contradict the view [33,34] that mitochondrial energization with glucose (or dmOG) inhibits GDH activity through an increased GTP concentration and/or a decreased ADP concentration in the matrix. Although L-leucine favoured 2-oxoglutarate amination over L-glutamate oxidative deamination, net conversion of Glu into 2-oxoglutarate might be possible at sufficiently elevated levels of Glu. This is supported by the fact that a permeable form of Glu (dmGlu) greatly potentiated L-leucine-induced stimulation of insulin secretion depending on its intracellular conversion into free Glu. Similar to L-glutamine, dmGlu showed very poor secretagogue capacity by itself, and was also converted mainly into GABA, Asp and Glu, but poorly into Gln. This similarity does not support the idea that β-cell alkalinization is the main reason for the lack of stimulation of insulin secretion by Gln [3]. In fact it has been shown recently that Gln has no effect on β-cell cytosolic pH [11].

It is well known that a specific isoform of GAD, GAD-65, is very frequently implicated in the autoimmune reaction that β-cells suffer in cases of Type I diabetes [15,16], but less is known about the possible functional role of the enzyme product, GABA. It is generally assumed that GABA, synthesized in and released by β-cells, might act as a paracrine regulator of glucagon- and somatostatin-secreting cells within the islets [28,35]. However, it has not yet been possible to demonstrate that glucose or any other physiological stimulus increases the release of GABA by islets [14,30]. On the other hand, an autocrine inhibitory effect of the released GABA on insulin secretion cannot be discounted, since the presence of $GABA_B$ receptors (types R1 and R2) has been demonstrated in a β-cell line and in human islets [36]. However, addition of GABA did not modify the secretory response to L-leucine, and nor was the response to L-leucine plus L-glutamine affected by the presence of type A (bicuculline) or type B (saclofen) GABA receptor antagonists. We therefore conclude that GABA does not seem to exert a primarily extracellular effect. In contrast, increased expression of a GAD-65 transgene and GABA in pancreatic β-cells induces impaired glucose tolerance and diabetes in the transgenic mice, with no sign of insulitis or β-cell loss [17]. Therefore it remains to be investigated whether GABA has any regulatory role in the mechanism of stimulation of insulin secretion within β-cells. On the one hand, its metabolism through the GABA shunt (to succinic acid) and the Krebs cycle might contribute to generate ATP for the closure of ATP-sensitive K^+ channels. The activities of GAD and GABA transaminase measured in islet homogenates suggest that the metabolic turnover of GABA in islets may be relatively high. This is also supported by the marked accumulation of GABA observed in the culture medium of rat islets relative to their content [37]. On the other hand, the excessive intracellular accumulation of GABA might generate negative signals for secretion, as suggested by the effects of GAD overexpression [17,18]. L-Leucine, besides opening a mitochondrial gate for L-glutamate oxidation (GDH activation), might indirectly fuel the Krebs cycle, promoting GABA metabolism and in that way contributing to decreasing a possible negative influence of intracellular GABA accumulation on insulin secretion.

Contrary to what might be predicted from the proposed direct messenger role (independent of the ATP-dependent closure of K^+ channels) of L-glutamate in the metabolism–secretion coupling mechanism of β-cells [8], it is shown here that L-glutamine and dmGlu greatly increased the islet content of L-glutamate, but induced a poor secretory response. The predominant conversion of L-glutamine and dmGlu into GABA, L-aspartate and L-glutamate, rather than being completely oxidized in the Krebs cycle, probably precludes closure of the ATP-dependent K^+ channels and the opening of the voltage-dependent, L-type Ca^{2+} channels.

However, membrane depolarization with 30 mmol/l KCl in the presence of 3 mmol/l glucose did not allow strong stimulation of insulin secretion by 10 mmol/l L-glutamine, thus confirming the results of a recent report [11]. We therefore conclude that an increased islet content of L-glutamate is necessary, but not sufficient, to allow its net conversion into 2-oxoglutarate and its further metabolism in the Krebs cycle or the GABA shunt. This, and the subsequent stimulation of insulin secretion, requires activation of GDH by L-leucine.

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REFERENCES

1 Sener, A. and Malaisse, W. J. (1980) L-Leucine and a nonmetabolized analogue activate pancreatic islet glutamate dehydrogenase. Nature (London) **288**, 187–189
2 Malaisse, W. J., Sener, A., Carpinelli, A. R., Anjaneyulu, K., Lebrun, P., Herchuelz, A. and Christophe, J. (1980) The stimulus-secretion coupling of glucose-induced insulin release XLVI. Physiological role of L-glutamine as a fuel for pancreatic islets. Mol. Cell. Endocrinol. **20**, 171–189
3 Sener, A. and Malaisse, W. J. (1980) Stimulation of insulin release by L-glutamine. Mol. Cell. Biochem. **33**, 157–159
4 Somers, G., Carpinelli, A. R., Devis, G., Sener, A. and Malaisse, W. J. (1982) Stimulus-secretion coupling of amino acid-induced insulin release VII. The B-cell memory for L-glutamine. Metab. Clin. Exp. **31**, 229–237
5 Panten, U., Zielmann, S., Lange, J., Zünkler, B.-J. and Lenzen, S. (1984) Regulation of insulin secretion by energy metabolism in pancreatic B-cell mitochondria. Studies with a non-metabolizable analogue. Biochem. J. **219**, 189–196
6 Malaisse, W. J., Sener, A. and Malaisse-Lagae, F. (1981) Insulin release: reconciliation of the receptor and metabolic hypothesis. Nutrient receptors in islet cells. Mol. Cell. Biochem. **37**, 157–165
7 Malaisse, W. J., Sener, A., Malaisse-Lagae, F., Welsh, M., Matthews, D. E., Bier, D. M. and Hellerström, C (1982) The stimulus-secretion coupling of amino acid-induced insulin release. Metabolic response of pancreatic islets to L-glutamine and L-leucine. J. Biol. Chem. **257**, 8731–8737
8 Maechler, P. and Wollheim, C. B. (1999) Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. Nature (London) **402**, 685–689
9 Sener, A., Malaisse-Lagae, F. and Malaisse, W. J. (1982) The stimulus-secretion coupling of glucose-induced insulin release. Environmental influences on L-glutamine oxidation in pancreatic islets. Biochem. J. **202**, 309–316
10 MacDonald, M. J. and Fahien, L. A. (2000) Glutamate is not a messenger in insulin secretion. J. Biol. Chem. **275**, 34025–34027
11 Bertrand, G., Ishiyama, N., Nenquin, M., Ravier, M. A. and Henquin, J. C. (2002) The elevation of glutamate content and the amplification of insulin secretion in glucose-stimulated pancreatic islets are not causally related. J. Biol. Chem. **277**, 32883–32891
12 Michalik, M., Nelson, J. and Erecinska, M. (1992) Glutamate production in islets of Langerhans: properties of phosphate-activated glutaminase. Metab. Clin. Exp. **41**, 1319–1326
13 Danielsson, A., Hellman, B. and Idahl, L.-Å. (1970) Levels of α-ketoglutarate and glutamate in stimulated pancreatic β-cells. Horm. Metab. Res. **2**, 28–31
14 Smismans, A., Schuit, F. and Pipeleers, D. (1997) Nutrient regulation of gamma-aminobutyric acid release from islet beta cells. Diabetologia **40**, 1411–1415
15 Degli Esposti, M. and Mackay, I. R. (1997) The GABA network and the pathogenesis of IDDM. Diabetologia **40**, 352–356
16 Pozzilli, P. and Leslie, R. D. G. (1997) Hyperexpression of GAD in islets may be relevant but is it sufficient to induce autoimmune insulitis? Diabetologia **40**, 357–361
17 Shi, Y., Kanaani, J., Menard-Rose, V., Ma, Y. H., Chang, P. Y., Hanahan, D., Tobin, A., Grodsky, G. and Baekkeskov, S. (2000) Increased expression of GAD65 and GABA in pancreatic β-cells impairs first-phase insulin secretion. Am. J. Physiol. Endocrinol. Metab. **279**, E684–E694
18 Rubí, B., Ishihara, H., Hegardt, F. G., Wollheim, C. B. and Maechler, P. (2001) GAD65-mediated glutamate decarboxylation reduces glucose-stimulated insulin secretion in pancreatic beta cells. J. Biol. Chem. **276**, 36391–36396
19 Hunter, W. M. and Greenwood, F. C. (1962) Preparation of iodine-131 labeled human growth hormone of high specific activity. Nature (London) **194**, 495–496
20 Ashcroft, S. J. H., Weerasinghe, C. C., Bassett, J. M. and Randle, P. J. (1972) The pentose cycle and insulin release in mouse pancreatic islets. Biochem. J. **126**, 525–532
21 Vytasek, R. (1982) A sensitive assay for the determination of DNA. Anal. Biochem. **120**, 243–248
22 Jones, B. N., Paabo, S. and Stein, S. (1981) Amino acid analysis and enzymatic sequence determination of peptides by an improved o-phthalaldialdehyde precolumn labeling procedure. J. Liquid Chromatogr. **4**, 565–586
22a Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**, 265–275
23 Michalik, M., Nelson, J. and Erecinska, M. (1993) GABA production in rat islets of Langerhans. Diabetes **42**, 1506–1513
24 Bryla, J., Michalik, M., Nelson, J. and Erecinska, M. (1994) Regulation of the glutamate dehydrogenase activity in rat islets of Langerhans and its consequence on insulin release. Metab. Clin. Exp. **43**, 1187–1195
25 Ashcroft, S. J. H., Chatra, L., Weerashinge, C. and Randle, P. J. (1973) Interrelationship of islet metabolism, adenosine triphosphate content and insulin release. Biochem. J. **132**, 223–231
26 Mukala-Nsengu, A., Fernández-Pascual, S., Martín, F., Martín-del-Río, R. and Tamarit-Rodriguez, J. (2004) Similar effects of succinic acid dimethyl ester and glucose on islet calcium oscillations and insulin release. Biochem. Pharmacol. **67**, 981–988
27 Reingold, D. F. and Orlowski, M. (1979) Inhibition of brain glutamate decarboxylase by 2-keto-4-pentenoic acid, a metabolite of allylglycine. J. Neurochem. **32**, 907–913
28 Sörenson, R. L., Garry, D. G. and Brelje, T. C. (1991) Structural and functional considerations of GABA in islets of Langerhans, β-Cells and nerves. Diabetes **40**, 1365–1374
29 Thomas-Reetz, A. C. and deCamilli, P. (1994) A role for synaptic vesicles in non-neuronal cells: clues from pancreatic β cells and from chromaffin cells. FASEB J. **8**, 209–216
30 Bustamante, J., Lobo, M. V. T., Alonso, F. J., Mukala-Nsengu-Tshibangu, A., Giné, E., Solís, J. M., Tamarit-Rodriguez, J. and Martín del Río, R. (2001) An osmotic-sensitive taurine pool is localized in glucagon and somatostatin containing cells in rat pancreatic islets. Am. J. Physiol. Endocrinol. Metab. **281**, E1275–E1285
31 Gylfe, E. (1976) Comparison of the effects of leucine, non-metabolizable leucine analogues and other insulin secretagogues on the activity of glutamate dehydrogenase. Acta Diabetol. Lat. **13**, 20–24
32 Fahien, L. A., MacDonald, M. J., Kmiotek, E. H., Mertz, R. J. and Fahien, C. M. (1988) Regulation of insulin release by factors that also modify glutamate dehydrogenase. J. Biol. Chem. **263**, 13610–13614
33 Gao, Z.-Y., Li, G., Najafi, H., Wolf, B. A. and Matschinsky, F. M. (1999) Glucose regulation of glutaminolysis and its role in insulin secretion. Diabetes **47**, 1535–1542
34 Li, C., Najafi, H., Daikhin, Y., Nissim, I. B., Collins, H. W., Yudkoff, M., Matschinsky, F. M. and Stanley, C. A. (2003) Regulation of leucine-stimulated insulin secretion and glutamine metabolism in isolated rat islets. J. Biol. Chem. **278**, 2853–2858
35 Rorsman, P., Berggren, P. O., Bokvist, K., Ericson, H., Möhler, H., Östenson, C. G. and Smith, P. A. (1989) Glucose-inhibition of glucagon secretion involves activation of $GABA_A$-receptor chloride channels. Nature (London) **341**, 233–236
36 Brice, N. L., Varadi, A., Ashcroft, S. J. H. and Molnar, E. (2002) Metabotropic glutamate and $GABA_B$ receptors contribute to the modulation of glucose-stimulated insulin secretion in pancreatic beta cells. Diabetologia **45**, 242–252
37 Winnock, F., Ling, Z., de Proft, R., Dejonghe, S., Schuit, F., Gorus, F. and Pipeleers, D. (2002) Correlation between GABA release from rat islet β cells and their metabolic state. Am. J. Physiol. Endocrinol. Metab. **282**, E937–E942

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