

A comparative study of amino acid consumption by rat islet cells and the clonal beta-cell line BRIN-BD11 – the functional significance of L-alanine

G Dixon¹, J Nolan², N McClenaghan³, P R Flatt³ and P Newsholme¹

¹Department of Biochemistry, Conway Institute of Biomolecular and Biomedical Research, University College Dublin, Belfield, Dublin 4, Ireland

²Department of Endocrinology, St James's Hospital, Dublin 8, Ireland

³School of Biomedical Sciences, University of Ulster, Coleraine, UK

(Requests for offprints should be addressed to P Newsholme; Email: philip.newsholme@ucd.ie)

Abstract

Evidence has been published that L-alanine may, under appropriate conditions, promote insulin secretion in normal rodent islets and various beta cell lines. Previous results utilising the clonal beta-cell line BRIN-BD11, demonstrated that alanine dramatically elevated insulin release by a mechanism requiring oxidative metabolism. We demonstrate in this paper that addition of L-alanine had an insulintropic effect in dispersed primary islet cells. Addition of D-glucose increased L-alanine consumption in both BRIN-BD11 cells and primary islet cells.

L-glutamine consumption in the BRIN-BD11 cell line and primary rat islets was also determined. The consumption rate was in line with that previously reported for cells of the immune system and other glutamine-utilising cells or tissues. However, L-alanine consumption was at least an order of magnitude higher than L-glutamine consumption. The metabolism of L-alanine in the beta-cell may result in stimulation of insulin secretion via generation of metabolic stimulus secretion coupling factors such as L-glutamate.

Journal of Endocrinology (2003) **179**, 447–454

Introduction

Amino acid metabolism, under appropriate conditions, is known to enhance insulin secretion from primary islet cells and beta-cell lines (Bratusch-Marrain *et al.* 1980, Charles *et al.* 1983, Yada 1994, Prentki 1996, Smith *et al.* 1997). L-alanine metabolism for example, is known to enhance insulin secretion alone or synergistically enhance glucose-stimulated insulin secretion from the clonal beta-cell line BRIN-BD11 (Brennan *et al.* 2002). Other investigators, however, have not demonstrated an insulintropic effect of L-alanine when added to rat islet cells (Sener & Malaisse 2002). L-glutamine while readily consumed by beta-cells does not stimulate insulin secretion in the absence of allosteric activation of glutamate dehydrogenase (GDH). L-leucine may enhance L-glutamine-stimulated insulin secretion by acting as a GDH allosteric activator (Sener & Malaisse 1980). In contrast to amino acids the key mechanism by which glucose enhances insulin secretion is known in considerable detail. Glucose enters the cell by facilitated diffusion, is converted by the glycolytic pathway to pyruvate and subsequently via pyruvate dehydrogenase (PDH) to acetyl CoA or via pyruvate carboxylase (PC) to oxaloacetate. Approximately equal amounts of pyruvate are utilised by the latter two enzymes (MacDonald 1993,

Khan *et al.* 1996, Schuit *et al.* 1997). After subsequent conversion to citrate the glucose carbon can be fully oxidised to CO₂ in the citrate (TCA) cycle (Wollheim 2000). This results in an increase in the ATP/ADP ratio, closure of ATP-sensitive K⁺ channels, membrane depolarisation, opening of voltage-activated Ca²⁺ channels, Ca²⁺ influx, a rise in [Ca²⁺]_i and activation of the exocytotic machinery (Wollheim 2000). The opening of Ca²⁺ channels is intermittent, oscillating with the membrane potential and therefore results in oscillations of [Ca²⁺]_i (Santos *et al.* 1991, Gilon & Henquin 1992) that in turn trigger oscillations of insulin secretion (Gilon *et al.* 1993).

To date, little comparative data exists on relative consumption rates of two key nutrients, L-glutamine and L-alanine, in islet cells and beta-cell lines. While transport and metabolism of D-glucose have been extensively studied, equivalent data for the insulintropic amino acids are generally not available. However, we have previously reported detailed aspects of L-alanine metabolism in the clonal cell line BRIN-BD11 (Brennan *et al.* 2002). While we determined routes of metabolism and quantitatively important end-products, we did not report rates of consumption of this amino acid. The present study has determined the relative consumption rates of L-alanine

and L-glutamine by primary islet cells and the clonal beta-cell line BRIN-BD11, further examined L-alanine consumption in the presence and absence of D-glucose and assessed the impact of the amino acid on insulin secretion. L-alanine has previously been reported to be oxidised in islet cells (Sener & Malaisse 2002) and BD11 cells (Brennan *et al.* 2002) while the first enzyme responsible for its metabolism, alanine aminotransferase, has a high activity in purified rat islet beta-cells (Sener *et al.* 2001). It is likely therefore that L-alanine metabolism is functionally significant in the beta-cell.

Our results suggest that L-alanine is consumed at extremely high rates by both the clonal beta-cell line and by rat islets. The functional significance of the high rates of L-alanine consumption is discussed.

Materials and Methods

Culture of BRIN-BD11 cells

Clonal insulin-secreting BRIN-BD11 cells were maintained in RPMI-1640 tissue culture medium with 10% (v/v) foetal calf serum (FCS), 0.1% antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) and 11.1 mmol/l D-glucose, pH 7.4. The cells were maintained at 37 °C in a humidified atmosphere of 5% CO₂ and 95% air using a Forma Scientific incubator (Marietta, OH, USA). The cells were cultured in 50–70 ml of tissue culture medium in T175 sterile tissue culture flasks.

Culture of rat islets

Female Wistar, 8–10 weeks old, were obtained from the Biomedical Facility, University College Dublin. Islet cells were obtained by collagenase digestion of the pancreas as previously described (Appels *et al.* 1989). The islets were kept in culture for 1–3 days in RPMI-1640 medium supplemented with 10% FCS, 0.1% antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) and 11.1 mmol/l D-glucose, pH 7.4. Islets were subsequently hand picked and placed into individual wells of a 24-well plate.

Insulin secretion

For evaluation of insulin secretion, BRIN-BD11 cells or islets were seeded into 24-well multiplates at 1.0×10^5 cells/well; islets were seeded at 30 islets/well. After overnight culture the medium was removed gently and cells washed twice with 1 ml Krebs Ringer bicarbonate buffer (KRB; 115 mM NaCl, 4.7 mM KCl, 1.28 mM CaCl₂, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄·7H₂O, 10 mM NaHCO₃, 5 g/l BSA, pH 7.4). After the final wash 1 ml KRB was added containing 1.1 mM D-glucose. After a 40 min preincubation at 37 °C, buffer was removed and the cell monolayers were incubated in the

same buffer containing either 1.1 mM D-glucose alone or supplemented with 10 mM L-alanine. After 20 min incubation at 37 °C, an aliquot (900 µl) of buffer was removed from each well and centrifuged at 500 g for 5 min at 4 °C. The supernatant was stored at –20 °C for subsequent measurement of insulin by Mercodia Ultrasensitive rat insulin ELISA (Uppsala, Sweden), according to the manufacturer's instructions. Cell density and insulin content were not significantly different for any of the incubation conditions.

Alanine consumption determination

BRIN-BD11 cells were seeded into T25 sterile tissue flasks (4×10^6 cells per flask; final volume 5 ml) and islets were seeded into 24-well plates (30 islets/well; final volume 500 µl). The cells were incubated for 24 h after which time the medium was removed and the cells were preincubated at 37 °C in KRB supplemented with 1.1 mmol/l glucose for 40 min. The cells were subsequently incubated in 1.1 mmol/l D-glucose with either 0.5, 1, 5 or 10 mmol/l L-alanine for 60 min or 16.7 mmol/l D-glucose with 0.5, 1, 5 or 10 mmol/l L-alanine for 60 min (BRIN-BD11 cell incubation volume 5 ml; islet cell incubation volume 500 µl). The L-alanine concentration was determined before and after the 60 min incubation period by the oxidation of L-alanine to pyruvate and ammonia in the presence of NAD and alanine dehydrogenase. The increase in absorbance at 339 nm due to the formation of NADH is a measure of the amount of L-alanine present. The consumption of L-alanine after a 20 min incubation period in KRB supplemented with 1.1 mmol/l D-glucose and 10 mM L-alanine by BRIN-BD11 cells (2×10^7 /T175 flask; final incubation volume 10 ml) and islets (150 islets/well; final incubation volume 500 µl) were also determined. The consumption of L-alanine (10 mmol/l) in RPMI-1640-supplemented medium by BRIN-BD11 cells (4×10^6 /T25 flask; final volume 5 ml) over 24 h was also determined.

Glutamine consumption determination

For evaluation of L-glutamine consumption, BRIN-BD11 cells were seeded into T25 sterile tissue flasks (4×10^6 cells/flask; final incubation volume 5 ml) and islets were seeded into 24-well plates (30 islets/well; final incubation volume 500 µl). The glutamine concentration was determined at 0 h and then again after a 24 h incubation period by its hydrolysis to glutamate and ammonium ions in a reaction catalysed by asparaginase. The ammonium ions were then reacted with α-ketoglutarate in the presence of NADH to form glutamate, NAD⁺ and water. The concentration of glutamine in the sample was quantified indirectly by measuring the decrease in absorbance at 340 nm due to the conversion of NADH to NAD⁺. The

amount consumed over 24 h was converted to absolute amounts and expressed as $\mu\text{mol}/\text{mg}$ protein/h.

ATP and glutamate determinations

BRIN-BD11 cells were seeded into T175 sterile tissue flasks (2×10^7 cells per flask) and islets were seeded into 24-well plates (150 islets/well). After a 48 h incubation period the medium was removed and the cells were preincubated at 37°C in KRB supplemented with 1.1 mmol/l glucose for 40 min. The cells were subsequently incubated in 1.1 mmol/l D-glucose with or without 10 mmol/l L-alanine for 20 min (BRIN-BD11 cells final incubation volume 10 ml , islets final incubation volume $500 \mu\text{l}$). Medium was removed and cells were immediately frozen by brief exposure to liquid nitrogen. Cells were extracted with 6% PCA and debris was removed from the flask using a cell scraper. After centrifugation, the supernatant was neutralised with KOH. The ATP concentration in the supernatant was determined as described by Trautschold *et al.* (1985). Cellular glutamate concentration was quantified using a glutamate dehydrogenase-based assay kit supplied by Roche Diagnostics (Lewes, Sussex, UK).

Protein determination

Protein determination was by the Bradford method (Bradford 1976). For BRIN-BD11 the conversion factor for cell number to mg protein was 8.9×10^6 cells/mg protein and for islets this was 2.7×10^3 cells/mg protein.

Statistical analysis and protein determination

Results are expressed as means \pm s.d. Analysis was performed by Student's *t*-test. $P < 0.05$ was considered to be statistically significant.

Results

Nutrient consumption in BRIN-BD11 beta cells and islets

In the present study the consumption of L-glutamine over 24 h (from a standard incubation concentration of 2 mmol/l in RPMI-1640) and L-alanine (from an expected insulinotropic incubation concentration of 10 mmol/l) over 20 mins by BRIN-BD11 cells or islets were determined. The consumption of L-alanine was substantially greater than L-glutamine for both BRIN-BD11 cells and rat islets, therefore requiring different incubation periods to optimise the respective assays so as to obtain reproducible results. The cellular utilisation of L-glutamine adjusted to a rate describing consumption over 1 h by islets was substantial ($0.288 \pm 0.036 \mu\text{mol}/\text{mg}$

Table 1 Consumption rates of L-glutamine and L-alanine by BRIN-BD11 beta cell and rat islets. Results are expressed as means \pm s.d. for 5–6 independent experiments

	BRIN-BD11 cells ($\mu\text{mol}/\text{mg}$ protein)	Islets ($\mu\text{mol}/\text{mg}$ protein)
L-Glutamine (2 mmol/l) Consumption/h	0.219 ± 0.024	$0.288 \pm 0.036^*$
L-Alanine (10 mmol/l) Consumption/20 min	2.01 ± 0.37	7.79 ± 2.79^s

Statistically significant differences in nutrient consumption rates (Student's *t*-test) between BRIN-BD11 cells and islets are indicated by $^sP < 0.001$, $^*P < 0.05$. D-glucose concentration was 11.1 mM in the RPMI 1640 medium used for the L-glutamine determinations, whereas D-glucose was 1.1 mM in the incubation medium used for the L-alanine determinations.

protein/h) and significantly higher ($P < 0.05$) than that by the clonal BRIN-BD11 cells ($0.219 \pm 0.024 \mu\text{mol}/\text{mg}$ protein/h) (Table 1).

A striking finding was that L-alanine was consumed at extremely high rates by both BRIN-BD11 cells ($2.01 \pm 0.37 \mu\text{mol}/\text{mg}$ protein/20 mins) and rat islets ($7.79 \pm 2.79 \mu\text{mol}/\text{mg}$ protein/20 mins) (Table 1). In BRIN-BD11 cells L-alanine (initial incubation concentration of 10 mM) was consumed at a rate at least an order of magnitude greater than that of L-glutamine (initial incubation concentration of 2 mM). In islets the rate of L-alanine consumption was also at least an order of magnitude greater than that of L-glutamine. However preculture of BRIN-BD11 cells for 24 h in RPMI medium supplemented with 10 mM L-alanine substantially reduced L-alanine consumption measured over a subsequent 20 min acute incubation period (results not shown).

Effect of glucose on L-alanine consumption

L-alanine consumption increased on addition of D-glucose for both BRIN-BD11 beta-cells (Fig. 1) and islets (Fig. 2). To gain further insight into the L-alanine consumption rates under these conditions, kinetic experiments were performed which required the use of L-alanine at several different concentrations including 0.5 mM , therefore the incubation period was extended to 1 h to ensure reproducibility of results in the L-alanine consumption assay. The V_{max} for L-alanine consumption by BRIN-BD11 cells increased 60% on addition of 16.7 mM glucose as determined by intercept on the $1/V$ axis (Fig. 1).

There was a 26% increase in the V_{max} for L-alanine consumption in rat islets following a similar increment in glucose concentration (Fig. 2). Apparent K_m for L-alanine consumption was calculated to be 4.5 mM in the presence of 1.1 mM glucose and 4.9 mM in the presence of 16.7 mM glucose for BRIN-BD11 cells. In rat islets the apparent K_m for L-alanine consumption was 2.8 mM in

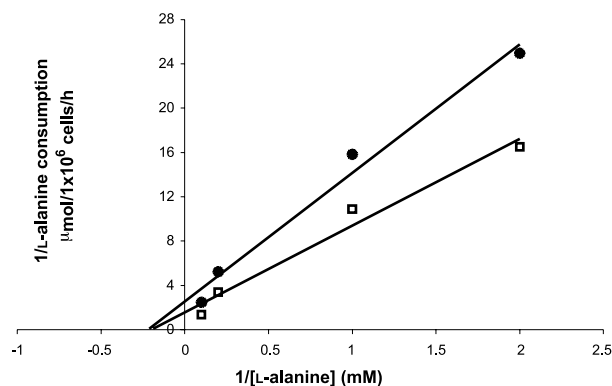


Figure 1 Lineweaver–Burk plot of the effect of D-glucose on the consumption of L-alanine by BRIN-BD11 beta cells. Rates of L-alanine consumption by BRIN-BD11 cells were measured after 60 min at various concentrations of L-alanine (0.5, 1, 5 or 10 mM) in the presence of 1.1 mM ● or 16.7 mM D-glucose □. Results are presented as mean for six separate experiments.

the presence of 1.1 mM glucose and 3.0 mM in the presence of 16.7 mM glucose. These results suggest that glucose increases L-alanine consumption in both BRIN-BD11 cells and rat islets possibly by increasing transport rates rather than the apparent K_m for the amino acid.

Effect of L-alanine on insulin secretion

In a previous study L-alanine was shown to be the most potent amino acid insulin secretagogue in the BRIN-BD11 cell line (McClenaghan *et al.* 1996). In the present study the basal rate of insulin secretion in BRIN-BD11 cells at 1.1 mM glucose was 1.32 ng/ 1×10^6 cells/20 min and addition of 10 mM L-alanine

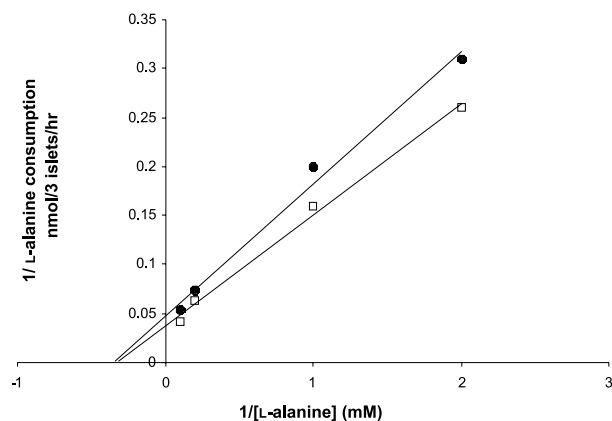


Figure 2 Lineweaver–Burk plot of the effect of D-glucose on the consumption of L-alanine by islets. Rates of L-alanine consumption by islets were measured after 60 min at various concentrations of L-alanine (0.5, 1, 5 or 10 mM) in the presence of 1.1 mM ● or 16.7 mM D-glucose □. Results are presented as mean for six separate experiments.

produced a significant ($P < 0.001$) 3-fold increase (Fig. 3). There was also a significant ($P < 0.05$) positive effect on insulin secretion from dispersed rat islet cells (treated with trypsin to produce single cells) when treated with 10 mM L-alanine in the presence of 1.1 mM glucose (Fig. 3).

Effect of L-alanine on cellular ATP and glutamate concentration

Addition of 10 mM L-alanine significantly reduced the ATP content of BRIN-BD11 cells ($P < 0.05$) and that of rat islet cells ($P < 0.05$), determined after 20 min of incubation (Table 2). However, addition of 10 mM L-alanine significantly increased ($P < 0.001$) the intracellular BRIN-BD11 cellular glutamate concentration by 2.3-fold and the rat islet glutamate concentration by 1.8-fold after 20 min ($P < 0.01$, Table 2).

Discussion

Glucose is consumed at high rates by primary rat islet cells and BRIN-BD11 cells *in vitro* (Brennan *et al.* 2002). Glucose metabolism is closely correlated with insulin secretion in the pancreatic beta-cell (Henquin 2000). L-glutamine is known to be an important nutrient fuel for a number of rapidly dividing cells (e.g. enterocytes, tumour cells, fibroblasts, reticulocytes; Lund 1980, Krebs 1981) and also resting lymphocytes and thymocytes, which have the potential for rapid cell division (Ardawi & Newsholme 1983, Brand *et al.* 1984). L-glutamine is also an essential nutrient for cells such as macrophages and neutrophils, which, while they do not have a high proliferative potential, require high rates of protein synthesis and secretion (Newsholme 2001). In this study, we report that both rat islets and BRIN-BD11 cells consumed L-glutamine at high rates (Table 1). Islets may have a high rate of protein turnover even under basal conditions, which would require L-glutamine for purine and pyrimidine synthesis, subsequent mRNA production and in addition, protein synthesis. Remarkably L-alanine was consumed at significantly higher rates than glutamine in both BRIN-BD11 cells and rat islets (Table 1). Addition of 16.7 mM glucose significantly enhanced L-alanine consumption in both BRIN-BD11 beta-cells ($P < 0.01$) and primary islet cells ($P < 0.05$), suggesting a critical role for L-alanine in beta-cell function.

A number of amino acids promote or synergistically enhance insulin release from pancreatic beta-cells (Fajans *et al.* 1967, McClenaghan *et al.* 1996). The mechanisms by which amino acids enhance insulin secretion are varied. The cationically charged amino acid, L-arginine, does so by direct depolarisation of the plasma membrane at neutral pH but only in the presence of glucose. Other amino acids, which are co-transported with Na^+ , can also depolarise the cell membrane as a consequence of Na^+

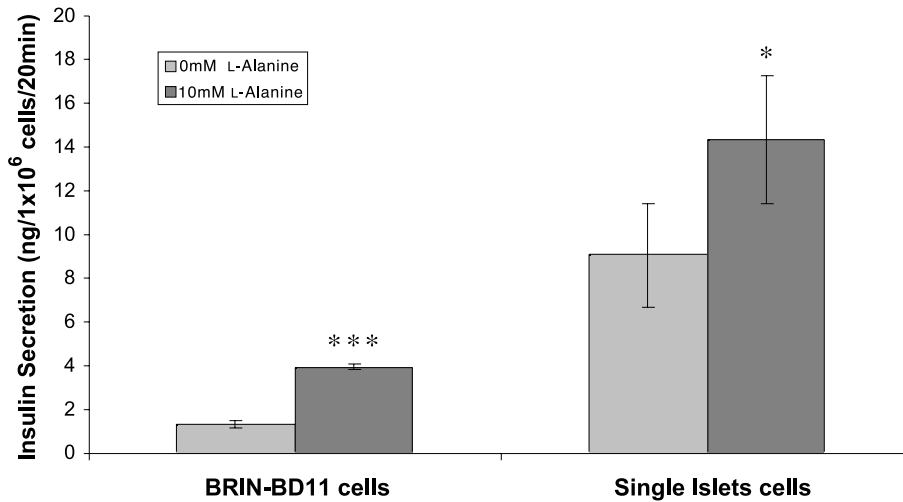


Figure 3 Effect of 10 mM L-alanine on insulin secretion from BRIN-BD11 beta cells and single islet cells in the presence of 1.1 mM D-glucose. Results are expressed as means \pm S.D. for four independent experiments. *** $P < 0.001$ compared with insulin secretion in the absence of 10 mM L-alanine. * $P < 0.05$ compared with insulin secretion in the absence of 10 mM L-alanine.

transport and thus induce insulin secretion by activating voltage-dependent calcium channels. Metabolism of amino acids, resulting in partial oxidation e.g. L-alanine (Brennan *et al.* 2002), may initially increase the cellular content of ATP, leading to closure of the ATP-sensitive K^+ channel, depolarisation of the plasma membrane, activation of voltage-activated Ca^{2+} channel, Ca^{2+} influx and insulin exocytosis. However it is possible to additionally stimulate insulin secretion via allosteric effects on regulatory proteins such as GDH (as described for leucine) (Malaisse-Lagae *et al.* 1982). After 20 min incubation in the presence of 10 mM L-alanine there was a small but significant reduction in ATP content of BRIN-BD11 cells and rat islets (Table 2). This may have been due to activation of the Na^+/K^+ ATPase which will be required to remove the Na^+ co-transported with L-alanine into the beta-cell.

A recent study has definitively demonstrated the importance of the oxidative metabolism of L-alanine to insulin secretion (Brennan *et al.* 2002). The respiratory

poison, oligomycin, dramatically decreased alanine-stimulated insulin secretion from BRIN-BD11 beta-cells. These investigators additionally provided ^{13}C -NMR-based evidence for substantial oxidative L-alanine metabolism in the BRIN-BD11 cells. While metabolism of L-alanine was required for stimulation of insulin secretion, L-alanine also synergistically enhanced glucose metabolism and thus insulin secretion in BRIN-BD11 cells. Despite the many previous reports of the insulinotropic effect of L-alanine, there have been relatively few studies on utilisation rates of L-alanine by beta-cells (Hellman *et al.* 1971, Prentki & Renold 1983). In the study by Hellman *et al.* (1971) the uptake of L-[U- ^{14}C] alanine from obese-hyperglycaemic mice was found to be concentration (1.0–25.0 mM) and time (10–120 min) dependent. We have demonstrated in this paper, increased L-alanine consumption in the presence of glucose by both BRIN-BD11 cells and islets. In BRIN-BR11 cells, increasing the glucose concentration from 1.1 to 16.7 mM in the presence of various L-alanine concentrations increased the

Table 2 Intracellular concentrations of L-glutamate and ATP in BRIN-BD11 cells and islets after a 20 min incubation in the presence or absence of 10 mM L-alanine

Cell type	[D-glucose] (mM)	[L-alanine] (mM)	Glutamate (nmol/mg protein)	ATP (nmol/mg protein)
BRIN-BD-11	1.1	0	63.87 \pm 10.42	18.90 \pm 1.71
BRIN-BD11	1.1	10	148.90 \pm 20.30 [§]	15.53 \pm 1.74*
ISLETS	1.1	0	215.66 \pm 30.37	52.96 \pm 4.53
ISLETS	1.1	10	393.65 \pm 68.09 [#]	44.89 \pm 4.34*

Values are means \pm S.D. for four independent experiments.

[§] $P < 0.001$, [#] $P < 0.01$, * $P < 0.05$ compared with same cell type without L-alanine.

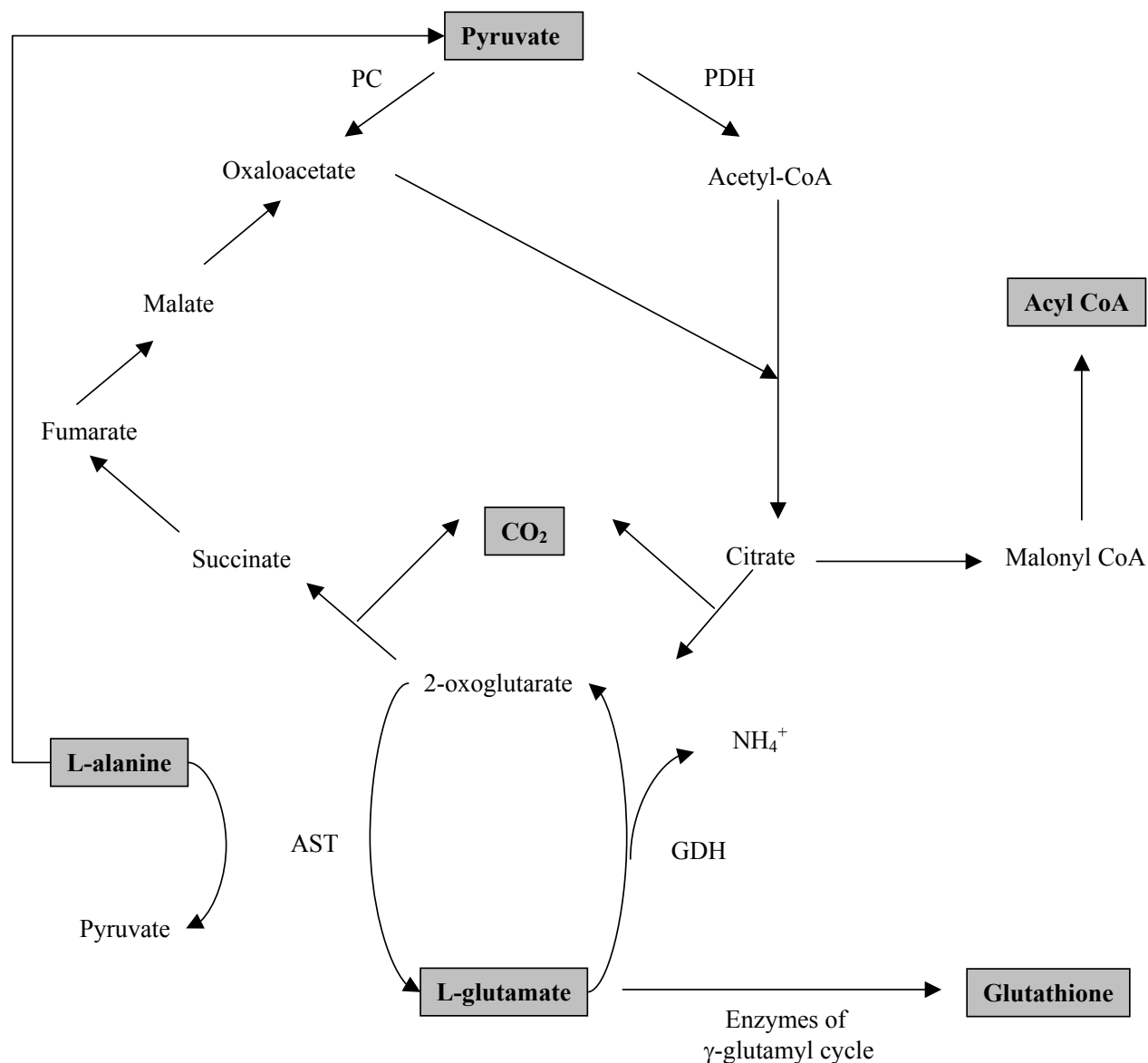
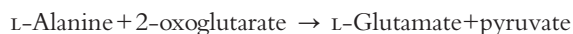


Figure 4 L-alanine metabolism in the beta cell. ALT, alanine aminotransferase; GDH, glutamate dehydrogenase; PC, pyruvate carboxylase; PDH, pyruvate dehydrogenase.

V_{\max} for L-alanine consumption by 60% from 3.51 to 5.62 $\mu\text{mol}/\text{mg}$ protein/h (Fig. 1). However the increased consumption of L-alanine cannot be correlated with an increase in alanine oxidation as demonstrated using ¹³C-NMR in BRIN-BD11 cells (Brennan *et al.* 2002). It is possible L-alanine may be converted to metabolic stimulus-secretion coupling factors which do not require oxidation. Increased L-alanine consumption may increase the intracellular concentration of specific stimulus-secretion metabolic coupling factors via enhanced mitochondrial metabolism due to an elevation in intracellular Ca^{2+} . The Ca^{2+} dependence of a number of

mitochondrial metabolic steps connected with TCA cycle activity (McCormack & Denton 1980, Civelek *et al.* 1996) would support such a hypothesis. Thus insulin secretion would be promoted. The most likely metabolic coupling factor would be L-glutamate, generated by the alanine aminotransferase reaction:



The pyruvate produced may subsequently be converted to oxaloacetate by PC and then enzymes of the TCA cycle to 2-oxoglutarate to provide substrate for the reaction

shown above (see Fig. 4 for further details). Thus, a significant increase in the cellular content of L-glutamate, originating from L-alanine, can occur as reported here after a 20 min incubation (Table 2) and as previously described after a 1 h incubation in the presence of L-alanine (Brennan *et al.* 2002). Glutamate has previously been shown to act as a metabolic stimulus secretion-coupling factor in glucose-stimulated insulin secretion in islet and beta-cell lines (Maechler & Wollheim 1999, Rubi *et al.* 2001, Hoy *et al.* 2002, Broca *et al.* 2003). While evidence for a direct stimulatory role of L-glutamate on insulin secretion is controversial (MacDonald & Fahien 2000, Bertrand *et al.* 2002) we have recently reported that L-glutamate can be converted to glutathione in the beta-cell via reactions of the gamma glutamyl cycle, indirectly regulating insulin secretion (Brennan *et al.* 2003).

The results of this study add weight to the hypothesis that L-alanine is an important beta-cell fuel *in vitro* and *in vivo*. *In vivo* L-alanine may be produced from L-glutamine metabolism (primary fuel for intestinal epithelia – News-holme *et al.* 2003) and secreted by the intestinal epithelial cells. It may reach 0.1–1.0 mM concentrations in the hepatic vein and may thus be important for regulation of beta cell function by nutrients.

Acknowledgements

This work was generously supported by a Health Research Board of Ireland Research Project Grant. We thank Mr. Paul Rooney, Department of Biochemistry, University College Dublin, for technical assistance.

References

- Appels B, Burkart V, Kantwerk-Funke G, Funda J, Kolb-Bachofen V & Kolb H 1989 Spontaneous cytotoxicity of macrophages against cultured pancreatic islet cells. *Journal of Immunology* **142** 3803–3808.
- Ardawi MS & Newsholme EA 1983 Glutamine metabolism in lymphocytes of the rat. *Biochemical Journal* **212** 835–842.
- Bertrand G, Ishiyama N, Nenquin M, Ravier MA & Henquin J-C 2002 The elevation of glutamate content and the amplification of insulin secretion in glucose-stimulated pancreatic islets are not causally related. *Journal of Biological Chemistry* **277** 32883–32891.
- Bradford MM 1976 A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein-dye binding. *Analytical Biochemistry* **72** 248–254.
- Brand K, Williams JF & Weidemann MJ 1984 Glucose and glutamine metabolism in rat thymocytes. *Biochemical Journal* **221** 471–475.
- Bratusch-Marrain P, Ferenci P & Waldhausl W 1980 Leucine assimilation in patients with diabetes mellitus. *Acta Endocrinologica* **93** 461–465.
- Brennan L, Shine A, Hewage C, Malthouse JPG, Brindle KM, McClenaghan N, Flatt PR & Newsholme P 2002 A nuclear magnetic resonance-based demonstration of L-alanine metabolism and L-alanine-enhanced glucose metabolism in a clonal pancreatic β -cell line. *Diabetes* **51** 1714–1721.
- Brennan L, Corless M, Hewage C, Malthouse JPG, McClenaghan NH, Flatt PR & Newsholme P 2003 ^{13}C NMR analysis reveals a link between L-glutamine metabolism, D-glucose metabolism and γ -glutamyl cycle activity in a clonal pancreatic β -cell line. *Diabetologia*. **46** 1503–1512.
- Broca C, Brennan L, Petit P, Newsholme P & Maechler P 2003 Mitochondria-derived glutamate at the interplay between branched-chain amino acid and glucose-induced insulin secretion. *FEBS Letters* **545** 167–172.
- Charles S & Henquin JC 1983 Distinct effects of various amino acids on $^{45}\text{Ca}^{2+}$ fluxes in rat pancreatic islets. *Biochemical Journal* **214** 899–917.
- Civelek VN, Deeney JT, Shalosky NJ, Tornheim K, Hansford RG, Prentki M & Corkey BE 1996 Regulation of pancreatic β -cell mitochondrial metabolism: influence of Ca^{2+} , substrate and ADP. *Biochemical Journal* **318** 615–621.
- Fajans SS, Floyd JC Jr, Knopf RF, Guntsche EM, Rull JA, Thiffault CA & Conn JW 1967 A difference in mechanisms by which leucine and other amino acids induce insulin release. *Journal of Clinical Endocrinology and Metabolism* **27** 1600–1606.
- Gilon P & Henquin JC 1992 Influence of membrane potential changes on cytoplasmic Ca^{2+} concentration in an electrically excitable cell, the insulin-secreting pancreatic β -cell. *Journal of Biological Chemistry* **267** 20713–20720.
- Gilon P, Shepherd RM & Henquin JC 1993 Oscillations of secretion driven by oscillations of cytoplasmic Ca^{2+} as evidenced in single pancreatic islets. *Journal of Biological Chemistry* **268** 22265–22268.
- Hellman B, Sehlin J & Taljedal IB 1971 Uptake of alanine, arginine and leucine by mammalian pancreatic β cells. *Endocrinology* **89** 1432–1439.
- Henquin JC 2000 Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes* **49** 1751–1760.
- Hoy M, Maechler P, Efanov AM, Wollheim CB, Berggren P-O & Gromada J 2002 Increase in cellular glutamate levels stimulates exocytosis in pancreatic beta cells. *FEBS Letters* **531** 199–203.
- Khan A, Ling ZC & Landau BR 1996 Quantifying the carboxylation of pyruvate in pancreatic islets. *Journal of Biological Chemistry* **271** 2539–2542.
- Krebs HA 1981 In *Glutamine: Metabolism Enzymology and Regulation*, pp 319–329. Eds J Mora & R Palacios. New York: Academic Press.
- Lund P 1980 Glutamine metabolism in the rat. *FEBS Letters* **117** K86–K92.
- McClenaghan NH, Barnett CR, O'Harte FPM & Flatt PR 1996 Mechanisms of amino acid-induced insulin secretion from the glucose-responsive BRIN-BD11 pancreatic β -cell line. *Journal of Endocrinology* **15** 349–357.
- McCormack JG & Denton RM 1980 Role of calcium ions in the regulation of intramitochondrial metabolism. Properties of the Ca^{2+} -sensitive dehydrogenases within intact uncoupled mitochondria from the white and brown adipose tissue of the rat. *Biochemical Journal* **190** 95–105.
- MacDonald MJ 1993 Glucose enters mitochondrial metabolism via both carboxylation and decarboxylation of pyruvate in pancreatic islets. *Metabolism* **42** 1229–1231.
- MacDonald MJ & Fahien LA 2000 Glutamate is not a messenger in insulin secretion. *Journal of Biological Chemistry* **275** 34025–34027.
- Maechler P & Wollheim CB 1999 Mitochondrial glutamate acts as a messenger in glucose-induced insulin exocytosis. *Nature* **402** 595–596.
- Malaisse-Lagae F, Sener A, Garcia-Morales P, Valverde I & Malaisse WJ 1982 The stimulus–secretion coupling of amino acid-induced insulin release. Influence of a nonmetabolized analog of leucine on the metabolism of glutamine in pancreatic islets. *Journal of Biological Chemistry* **257** 3754–3758.

- Newsholme P 2001 Why is L-glutamine metabolism important to cells of the immune system in health, postinjury, surgery or infection? *Journal of Nutrition* **131** 2515–2522.
- Newsholme P, Procopio J, Ramos Lima MM, Pithon-Curi TC & Curi R 2003 Glutamine and glutamate: their central role in cell metabolism and function. *Cell Biochemistry and Function* **21** 1–9.
- Prentki M 1996 New insights into pancreatic beta-cell metabolic signalling in insulin secretion. *European Journal of Endocrinology* **134** 272–286.
- Prentki M & Renold AE 1983 Neutral amino acid transport in isolated rat pancreatic islets. *Journal of Biological Chemistry* **258** 14239–14244.
- Rubi B, Ishihara H, Hegardt FG, Wollheim CB & Maechler P 2001 GAD65-mediated glutamate decarboxylation reduces glucose-stimulated insulin secretion in pancreatic beta cells. *Journal of Biological Chemistry* **276** 36391–36396.
- Santos RM, Rosario LM, Nadal A, Garcia-Sancho J, Soria B & Valdeolmillos M 1991 Widespread synchronous $[Ca^{2+}]_i$ oscillations due to bursting electrical activity in single pancreatic islets. *Pflügers Archiv* **418** 417–422.
- Schuit F, De Vos A, Farfari S, Moens K, Pipeleers D, Brun T & Prentki M 1997 Metabolic fate of glucose in purified islet cells: glucose-regulated anaplerosis in beta cells. *Journal of Biological Chemistry* **272** 18572–18579.
- Sener A & Malaisse WJ 1980 L-leucine and a nonmetabolized analogue activate pancreatic islet glutamate dehydrogenase. *Nature* **288** 187–189.
- Sener A & Malaisse WJ 2002 The stimulus-secretion coupling of amino acid-induced insulin release. Insulinotropic action of L-alanine. *Biochimica et Biophysica Acta* **1573** 100–104.
- Sener A, Mercan D & Malaisse WJ 2001 Enzymatic activities in two populations of purified rat islet beta-cells. *International Journal of Molecular Medicine* **8** 285–289.
- Smith PA, Shakura H, Coles B, Gummerson N, Proks P & Ashcroft FM 1997 Electrogenic arginine transport mediates stimulus-secretion coupling in mouse pancreatic β -cells. *Journal of Physiology* **499** 625–635.
- Trautschold I, Lamprecht W & Schweitzer G 1985 ATP:UV-method with hexokinase and glucose-6-phosphate dehydrogenase. In *Methods of Enzymatic Analysis*, edn 3, vol VII, pp 346–357. Eds HU Bergmeyer. Germany: VCH.
- Wollheim CB 2000 Beta-cell mitochondria in the regulation of insulin secretion: a new culprit in type II diabetes. *Diabetologia* **43** 265–277.
- Yada T 1994 Action mechanisms of amino acids in pancreatic β -cells. In *Frontiers of Insulin Secretion and Pancreatic β -Cell Research*, pp 129–135. Eds PR Flatt & S Lenzen. London: Smith-Gordon.

Received 6 August 2003

Accepted 1 September 2003

Made available online as an

Accepted Preprint 9 September 2003