Glutamine regulates expression of key transcription factor, signal transduction, metabolic gene, and protein expression in a clonal pancreatic β-cell line

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Abstract

We have investigated the effects of prolonged exposure (24 h) to the amino acid L-glutamine, on gene and protein expression using clonal BRIN-BD11 β-cells. Expression profiling of BRIN-BD11 cells was performed using oligonucleotide microarray analysis. Culture for 24 h with 10 mM L-glutamine compared with 1 mM resulted in substantial changes in gene expression with 148 genes upregulated more than 1·8-fold, and 18 downregulated more than 1·8-fold, including many genes involved in cellular signaling, metabolism, gene regulation, and the insulin-secretory response. Subsequent functional experiments confirmed that L-glutamine increased the activity of the Ca^{2+} regulated phosphatase calcineurin and the transcription factor Pdx1. Additionally, we demonstrated that β-cell-derived L-glutamate was released into the extracellular medium at high rates. As calcineurin is a regulator of the glutamate *N*-methyl-D-aspartate (NMDA) receptor activity, we investigated the action of NMDA on nutrient-induced insulin secretion, and demonstrated suppressed insulin release. These observations indicate important long-term effects of L-glutamine in regulating β-cell gene expression, signaling, and secretory function.

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Introduction

L-Glutamine circulates at the highest concentration of any amino acid (approximately 0·7 mmol/l in the human) and its metabolism is essential to the function of a large number of cells and tissues (reviewed in Newsholme *et al.* 2003). It has additionally been shown directly or indirectly to influence gene expression in specific target cells (Curi *et al.* 2005). It is readily metabolized in islets and β-cell lines, such as BRIN-BD11 (Malaisse *et al.* 1982*a*, 1982*b*, Rasschaert *et al.* 1996*a*, 1996*b*). Additionally, L-glutamate, which is derived intracellularly from L-glutamine, has been suggested to be a key messenger in the amplifying pathway of glucose-induced insulin release (Maechler & Wollheim 1999, Wollheim 2000) although others have questioned the insulinotropic action of L-glutamate (Bertrand *et al.* 2002). It may additionally enter the γ-glutamyl cycle to produce glutathione (Brennan *et al.* 2003), which can enhance islet cell viability and insulin secretion (Ammon *et al.* 1980, Avila *et al.* 2003, 2005). However, acutely L-glutamine alone weakly stimulates insulin secretion (approximately 0·7 increased to 1·0 ng/10^6 cells per 20 min in BRIN-BD11 cells, when it was increased from 0 to 10 mmol/l in the presence of 1·1 mmol/l glucose (McClenaghan *et al.* 1996)) and several papers question the proposed link between L-glutamate production and enhanced rates of exocytosis (MacDonald & Fahien 2000, Bertrand *et al.* 2002). The question, therefore, arises as to the functional role of L-glutamine in the β-cell.

L-Glutamine may be transported into the cell by a number of potential transport mechanisms (Prentki & Renold 1983, Brennan *et al.* 2003) and is rapidly converted into L-glutamate. Glutamate dehydrogenase (GDH) activity, as measured *in vitro*, is high in pancreatic β-cells (Sener *et al.* 2001) and in β-cell lines, such as BRIN-BD11 (Rasschaert *et al.* 1996*a*, 1996*b*). However, the activity of the enzyme is tightly regulated *in vivo* by negative and positive effectors, such as GTP, ADP, and leucine. In the presence of leucine or its non-metabolizable analog, BCH, L-glutamine potently enhances insulin secretion (Malaisse *et al.* 1982*b*, Gao *et al.* 1999). Leucine-activated GDH is considered to enhance L-glutamate oxidation and increase ATP production by providing the tricarboxylic acid (TCA) cycle with substrate (2-oxoglutarate), which will result in closure of plasma membrane K^+_{ATP} channels and stimulate insulin secretion. In support of this hypothesis, it is known that patients with rare mutations in the inhibitory GTP-binding allosteric site on GDH display elevated levels of insulin secretion, resulting in hypoglycemia (Stanley *et al.* 1998, 2000, MacMullen *et al.* 2001).

A recent study has shown that unregulated overexpression of GDH in MIN6 cells enabled glutamine alone to stimulate insulin secretion (Tanizawa *et al.* 2002). In contrast, glucose has been shown to inhibit the pathway of glutaminolysis

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(partial L-glutamine metabolism and oxidation) via inhibition of GDH (Gao *et al.* 1999).

We have previously presented ^{13}C NMR data, which provided novel evidence for substantial intracellular pancreatic β-cell metabolism of L-glutamine resulting in formation of L-glutamate, L-aspartate, and glutathione (Brennan *et al.* 2003). We proposed that L-glutamate production is important to the β-cell as an intermediate of the γ-glutamyl cycle, which regulates cellular glutathione concentration.

Here, we report that chronic (24 h) exposure to L-glutamine weakly stimulated insulin secretion (by 30%), but induced significant changes in transcription factor, signal transduction, and metabolic enzyme gene expression. We have used 10 mM L-glutamine in the work reported in this paper, so as to saturate L-glutamine transport and allow comparison of our results with previously published studies, which utilized 10 mM L-glutamine to assess the effect on insulin secretion and β-cell function (Malaisse *et al.* 1982*b*, McClenaghan *et al.* 1996, Rasschaert *et al.* 1996*a*, 1996*b*, Brennan *et al.* 2003).

Materials and Methods

Culture of BRIN-BD11 pancreatic β-cells

Clonal insulin-secreting BRIN-BD11 cells were maintained in RPMI-1640 tissue culture medium (sigma) supplemented with 10% (v/v) fetal calf serum, 0·1% antibiotics (100 U/ml penicillin and 0·1 mg/ml streptomycin) and 11·1 mmol/l D-glucose (pH 7·4). The origin and characteristics of BRIN-BD11 cells are described elsewhere (McClenaghan *et al.* 1996). The cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air using a Forma Scientific incubator (Biosciences, Dublin, Ireland). The cells were cultured in 50–70 ml tissue culture medium in T175 sterile tissue culture flasks. Cells were subsequently seeded into six-well plates ($1·5 \times 10^6$ cells/well) and allowed to adhere overnight. Cells were then washed with PBS after which fresh medium, containing 11·1 mM D-glucose and varying concentrations of L-glutamine (0, 1, 2, and 10 mM) was added. After 24 h incubation, an aliquot of the media was removed and centrifuged at 200 ***g*** for 5 min and used for quantitation of metabolites (L-glutamate, NH_4^+, insulin, and urea).

Enzymatic determination of metabolites

Urea and NH_4^+ concentrations in the culture media were measured using an urease/GDH-based diagnostic kit (Fannin Healthcare, Dublin, Ireland). L-Glutamate concentration in the culture media was measured using a GDH-based diagnostic kit (Roche Diagnostics). Nitric oxide (NO) concentrations in the culture media were determined using the Griess Reagent System (Promega Medical Supply Co.). The rate of nitrite production was expressed as micromolar per milligram protein per 24 h.

Cellular glutamate determination

BRIN-BD11 cells were cultured as above in six-well plates in the presence of either 1 or 10 mM L-glutamine. After a 24 h incubation, cells were extracted with 6% perchloric acid and debris was removed from the wells using a cell scraper. After centrifugation, the supernatant was neutralized with KOH and the cellular L-glutamate concentration was quantified as described above. It is hydrolyzed to L-glutamate spontaneously at 37 °C at a rate of approximately 5%/24 h over a concentration range of 1–20 mM. This spontaneous rate of hydrolysis was taken into account when calculating rates of glutamate or NH_4^+ production.

Total glutathione assay

Total glutathione was quantified in a microtiter plate according to the technique of Baker *et al.* (1990) using the method originally described by Tietze (1969). Cell lysate or reduced glutathione standards (0–500 pmol of GSx/10 μl) was transferred into a microtiter plate and diluted with water. After addition of reaction mixture (0·1 M sodium phosphate buffer (pH 7·5) containing 1 mM EDTA, 0·3 mM dithionitrobenzoic acid, 0·4 mM NADPH, and 1 U/ml glutathione reductase), the increase in absorbance at 405 nm was detected at 15 s intervals over a range of 2·5 min using a microtiter plate reader. Glutathione contents were evaluated using a calibration curve.

Microarray analysis

BRIN-BD11 cells were cultured as above in six-well plates in the presence of either 1 mM (control) or 10 mM L-glutamine. After 24 h incubation, total RNA was isolated using RNeasy columns from control samples (1 mM L-glutamine) and high glutamine samples (10 mM L-glutamine cells) in triplicate. RNA samples were prepared for microarray analysis using a protocol provided by Affymetrix (High Wycombe, UK). In summary, first and second strand cDNA synthesis was performed using the SuperScript Choice system using a HPLC purified T7-oligo (dT) primer. The double stranded cDNA was cleaned using the GeneChip Sample Cleanup Module (Affymetrix). Biotin labeled cRNA was then prepared using the Enzo RNA transcript kit (Enzo Life Sciences Inc., Farmingdale, NY, USA). The biotin labeled cRNA was cleaned using the GeneChip Sample Cleanup Module and fragmented at 94 °C for 35 min, prior to hybridization with Affymetrix rat genome 230A arrays. The probe arrays were incubated for 16 h at 40 °C. Probe sets were visualized on the Affymetrix GeneArray 2500 scanner and quantified for intensity using Microarray Suite version 5.1. Further data analysis was performed using GeneSpring version 6.1. Statistical analysis is included in the GeneSpring software (Affymetrix), so that only reproducible levels in gene expression (between triplicate samples) are included in the resulting

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Supplementary data Table 1. To improve the robustness of the changes, genes were considered differentially expressed if they were present in 1 and 10 mM glutamine-treated cells. Some β-cell-specific genes that were upregulated at least 1·8-fold were chosen for further analysis (see below).

Real-time PCR

We selected seven genes that had shown more than 1·8-fold change in expression for further study by reverse transcriptase (RT)-PCR in a separate experiment. BRIN-BD11 cells were cultured as above in six-well plates in the presence of either 1 or 10 mM L-glutamine. After 24 h incubation, total RNA from three biological replicates of each treatment was isolated using the QIAGEN RNeasy Mini Kit (Qiagen Ltd., West Sussex, UK). Target mRNA was quantified using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems Foster City, CA, USA). Primers and probes (Table 3) were designed using ABI PRISM Primer Express software v.1.5 (Applied Biosystems). Primers were purchased from Sigma-Genosys and probes from Applied Biosystems. The probes were labeled with a 5′-reporter dye (FAM, 6-carboxyfluorescein) and a 3′-quencher dye (TAMRA, 6-carboxytetramethylrhodamine). RT-PCR were carried out in a microamp optical 96-well plate in a total volume of 25 µl/well, consisting of TaqMan 1-step RT-PCR master mix Reagent kit (Applied Biosystems), 20 ng total RNA, 0·25 U/µl Multiscribe, and concentrations of primers and probes ranging from 100 to 500 nmol/l. Reverse transcription was performed for 30 min at 48 °C, AmpliTaq gold activation for 10 min at 95 °C, followed by 40 PCR cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Reactions were carried out in triplicate and data were analyzed by the ABI PRISM 7700 Sequence Detection System software (Applied Biosystems) using a standard curve to quantify the mRNA. Standard curves were produced for each set of primers and probes using 5–80 ng total RNA per reaction. Results were normalized on the basis of 18S rRNA quantification. The genes analyzed by real time PCR were: calcineurin (catalytic subunit, *Pppcb3* and regulatory subunit, *Ppp3r1*), pancreatic and duodenal homeobox gene 1 (*PDX1*), acetyl CoA carboxylase (*Acac*), potassium inwardly rectifying channel (*Kcnj6*), potassium channel (*Kcnk2*) and glutamate receptor, ionotropic (*Grin1a*).

Western blot analysis

Twenty micrograms protein extracts were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Pierce, Rockfort, IL, USA). Samples were subsequently subjected to 6% SDS-PAGE and electrophoretically transferred onto a nitrocellulose sheet. The sheet was blocked in 5% milk protein and incubated with polyclonal anti acetyl CoA carboxylase (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). The blots were washed and probed with horseradish peroxidase and visualized with Supersignal West Pico chemiluminescent substrate (Pierce).

Calcineurin enzymatic activity assay

Calcineurin activity assays were performed on BRIN-BD11 cell lysates using a colorimetric calcineurin activity assay kit as described in the manufacturer's protocol (Calbiochem, kit no. 2007007; Merk). Reactions were terminated after 30 min, and absorption was read on an u.v. spectroscope at 660 nm. The activity was corrected for differences in protein concentration between samples.

Electrophoretic mobility shift assay (EMSA)

BRIN-BD11 cells were cultured as above in six-well plates in the presence of either 1 or 10 mM L-glutamine. After 24 h incubation, nuclear protein extract was prepared using NE-PER reagent. A LightShift Chemiluminescent EMSA Kit (Pierce) was used to detect DNA–protein interactions. A double-stranded oligonucleotide corresponding to the FAR-FLAT element of the rat insulin 1 promoter was biotin end-labeled and incubated with a nuclear extract. This reaction was then subjected to gel electrophoresis on a native polyacrylamide gel and transferred to a nylon membrane. The biotin end-labeled DNA was detected using the streptavidin–horseradish peroxidase conjugate and the chemiluminescent substrate.

Insulin secretion (in the presence of glutamate receptor agonists and antagonists)

BRIN-BD11 cells were cultured in 24-well plates for 24 h in RPMI-1640 medium containing 11·1 mM D-glucose. After 40 min pre-incubation in Krebs ringer bicarbonate buffer (KRB; NaCl 115 mM, KCL 4·7 mM, $CaCl_2.6H_2O$ 1·28 mM, KH_2PO_4 1·2 mM, $MgSO_4.7H_2O$ 1·2 mM, $NaHCO_3$, 10 mM, BSA 0·1%, pH 7·4) with 1·1 mM glucose at 37 °C, the BRIN-BD11 cells were incubated in KRB containing 16·7 mM D-glucose in the presence or absence of 10 mM L-alanine for 20 min in order to stimulate the first phase of insulin secretion. The combination of glucose and L-alanine is required to stimulate robust and reproducible levels of insulin secretion (Brennan *et al.* 2002, Dixon *et al.* 2003). Various glutamate receptor agonists (AP4, L-CCG1, (S)-3, 5-dihydroxy-phenylglycine (DHPG), and NMDA; Sigma) were included in this 20-min incubation period, after which, an aliquot was removed from each well and analyzed for insulin content using the Mercodia Ultrasensitive Rat Insulin ELISA kit (Uppsala, Sweden).

Chronic (24 h) insulin secretion

BRIN-BD11 cells were seeded in 24-well plates (1×10^5 cells/well) in RPMI-1640 medium containing 11·1 mM D-glucose in the presence of either 1 or 10 mM L-glutamine.

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After 24 h incubation, an aliquot of the media was removed and centrifuged at 200 ***g*** for 5 min and analyzed for insulin content using the Mercodia Ultrasensitive Rat Insulin ELISA kit.

Protein determination

Cellular protein was determined using a bianchoninic acid protein assay kit (Pierce; kit no. 23225), which utilizes a modification of the biuret reaction.

Statistical analysis

The results are presented as means ± S.D. Groups of data were compared using a Student's unpaired *t*-test or ANOVA where appropriate. Differences were considered significant at a *P* value $<0{\cdot}05$.

Results

L-Glutamate, NH_4^+, urea and nitrite production in BRIN-BD11 β-cells

No previous study has as yet reported the release and extracellular production rates of L-glutamate from pancreatic β-cells. Incubation of BRIN-BD11 cells in the presence of 10 mM L-glutamine significantly ($P<0{\cdot}05$) increased the extracellular glutamate concentration from 1·26 μmol/10^6 cells per 24 h (0 mM L-glutamine) to 2·59 μmol/10^6 cells per 24 h (Table 1). Extracellular NH_4^+ production dramatically increased on addition of extracellular L-glutamine, resulting in an up to eightfold ($P<0{\cdot}02$) increase over basal (0 mM glutamine) production rates (Table 1). However, addition of L-glutamine to the culture medium did not alter urea production rates over 24 h. Nitrite concentrations in the culture medium were not significantly altered by addition of extracellular L-glutamine (Table 1). The intracellular glutamate concentration was also significantly increased on raising the extracellular glutamine concentration from 1 to 10 mM (15·2–20·9 nmol/10^6 cells per 24 h), as was the intracellular glutathione concentration (5·1–6·6 nmol/10^6 per 24 h; Table 2).

Effect of chronic exposure to L-glutamine on BRIN-BD11 cell insulin secretion

We have previously reported that acute addition (20 min) of L-glutamine alone weakly stimulated insulin secretion (McClenaghan *et al.* 1996). In this study, the 24 h secretion rate of insulin by BRIN-BD11 β-cells exposed to L-glutamine was determined. Increasing the extracellular L-glutamine concentration from 1 to 10 mM resulted in a 30% increase in the insulin secretion rate ($P<0{\cdot}05$; Table 1).

Microarray analysis

Analysis performed using the Affymetrix rat genome 230A microarray revealed that the expression of a total of 148 genes were increased 1·8-fold or greater after 24 h culture of BRIN-BD11 β-cells in the presence of 10 mM L-glutamine compared with culture in the presence of 1 mM L-glutamine. Additionally, 18 genes were downregulated by 1·8-fold or more by 10 mM L-glutamine. These genes are grouped according to molecular function in the Supplementary data Table 1. The largest group of genes up/downregulated by L-glutamine was functionally classified as signal transduction. This group could further be subdivided into genes involved in phosphorylation/dephosphorylation, G-protein coupling and Ca^{2+} binding. Both the regulatory and catalytic subunits of the calcium-binding protein, calcineurin were upregulated approximately 6- and 1·9-fold respectively. Interestingly, the homeodomain transcription factor *PDX1* was upregulated more than fivefold by L-glutamine. *PDX1* plays a key role in pancreatic development and β-cell function. Genes altered by L-glutamine addition were grouped as follows: signal transduction (16%), growth/gene regulation (16%), metabolism (10%), structural (7%) channels/receptors (6%), apoptosis/inflammatory response (4%), transport (2%), miscellaneous (13%), and ESTs (26%).

Real-time PCR analysis of glutamine-regulated genes

Selected targets, as described below, were confirmed using real time PCR. The sequences of the primer sets used in real

Table 1 Metabolite production and insulin secretion in BRIN-BD11 cells after a 24 h incubation in the presence of various concentrations of L-glutamine

Glutamine conc. (mM)	**Glutamate production** (extracellular)	**NH_4^+ production**	**Urea production**	**Insulin secretion**	**Nitrite production**
0	1·26±0·43	0·63±0·21	–	–	–
1	1·62±0·29	1·74±0·37	0·814±0·19	233·84±24·87	0·15±0·04
2	1·95±0·28	3·01±0·34*	0·801±0·23	–	–
10	2·59±0·43*	5·29±0·42*	0·775±0·25	304·66±21·41*	0·07±0·02

Values are expressed as μmol/10^6 cells per 24 h (except for insulin secretion which is expressed in ng/10^6 cells per 24 h) ± S.D. for five to six independent experiments. Metabolite concentrations and insulin secretion was determined as described in Materials and Methods. Statistically significant differences in production (ANOVA) are indicated by *$P<0{\cdot}05$ compared with consumption or production or secretion in the presence of 1 mM L-glutamine.

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Table 2 Cellular L-glutamate and total glutathione levels in BRIN-BD11 cells after 24 h incubation in the presence of 1 or 10 mM extracellular L-glutamine

	Cellular glutamate	**Total glutathione**
Glutamine conc. (mM)		
1	15·28±1·98	5·09±0·62
10	20·89±3·21*	6·61±0·56*

Values are expressed as nmol/10^6 cells per 24 h±S.D. for five to six independent experiments. Cellular glutamate and total glutathione were determined as described in Materials and Methods. *$P<0·05$ compared with intracellular levels after incubation in the presence of 1 mM glutamine.

time PCR analysis are described in Table 3. The levels of the regulatory and catalytic subunits of calcineurin, the transcription factor PDX1, the metabolic enzyme acetyl CoA carboxylase, the KIR subunit of the K^+_{ATP} channel, and the ionotropic L-glutamate receptor Grinl1a were chosen for independent verification as they are involved in signal transduction, insulin gene expression, metabolism, and regulation of insulin secretion in pancreatic β-cells. As shown in Fig. 1, the upregulation of the latter genes determined by microarray analysis was confirmed using real time PCR. Overall, there was very good agreement between increases in gene expression determined by the two methods.

Western blot analysis of acetyl coA carboxylase expression and calcineurin activity measurement in the presence of 1 or 10 mM L-glutamine

Western blot analysis of acetyl CoA carboxylase expression clearly demonstrated that increasing the extracellular L-glutamine concentration from 1 to 10 mM over 24 h resulted in an increase of approximately twofold in acetyl CoA carboxylase protein expression (Fig. 2A). Following real-time PCR confirmation that L-glutamine increased the mRNA expression of both the catalytic and regulatory subunits of calcineurin, the calcineurin-specific phosphatase activity in BRIN-BD11 β-cells was determined. Calcineurin activity increased almost twofold ($P<0·05$), when L-glutamine was increased from 1 to 10 mM over 24 h (Fig. 2B).

Electrophoretic mobility shift assay

Following real time PCR confirmation of significant glutamine-dependent upregulation of PDX1 mRNA, the specific DNA:protein interactions were investigated further via EMSA. Increasing the L-glutamine concentration from 1 to 10 mM resulted in increased binding of the PDX1 protein to its specific binding site, the FAR-FLAT element of the rat insulin 1 promoter (Fig. 3).

Effects of NMDA glutamate receptor agonists and specific metabotropic glutamate receptor (mGluR) agonists on nutrient-induced insulin secretion

RINm5F pancreatic β-cells (parental cell line of the BRIN-BD11) have been shown to express NMDA receptors and mGluRs (Molnar *et al.* 1995, Brice *et al.* 2002). In a previous study, L-alanine was shown to be a potent amino acid insulin secretagog in the BRIN-BD11 cell line (McClenaghan *et al.* 1996). In this study, alanine-induced insulin secretion in the presence of various glutamate receptor agonists was determined. One hundred micromolars of NMDA significantly reduced L-alanine-induced insulin secretion ($P<0·05$) in the presence of either 1·1 or 16·7 mM D-glucose (Fig. 4). The group III specific mGluR agonist L-AP4 also significantly ($P<0·05$) reduced L-alanine-induced insulin secretion in the presence of either 1·1 or 16·7 mM D-glucose (Fig. 4). However, Groups I and II specific mGluR agonists DHPG and L-CCG1 respectively had no effect on insulin secretion under any of the conditions tested (Fig. 4).

Discussion

Amino acids may exert chronic influences on cell function via a number of mechanisms (Haussinger 1996, Averous *et al.* 2003, Curi *et al.* 2005). In the work reported here, L-glutamine increased the 24 h insulin secretion rate of BRIN-BD11 β-cell line by 30%. This was associated with an upregulation of 148 genes of at least 1·8-fold and similarly, downregulation of 18 genes. Notably, BRIN-BD11 cells (in common with all transformed cell lines) required exposure to L-glutamine at a minimum concentration of 1 mM, to avoid

Table 3 Sequences of primer sets used in real time PCR analysis

	Genbank accession no.	**Forward primer** (5′–3′)	**Reverse primer** (5′–3′)
Gene name			
Acac	NM_022193	CGGCTGTGGAAATTGCG	GAGGCGGATGGGAATCG
Pdx1	NM_022852	TTCCCGTGGATGAAATCCA	TCCGGTTCTGCTGCGTATG
Ppp3r1	NM_017309	TGTCAAAGGCCGATAAGGAACAG	TTGAGGTTCGCTTTCCGTATCT
Ppp3cb	NM_017042	CCCACAGGGATGTTGCCTAGT	AACTGTGGCACTTTGCAAGGT
Kcnk2	NM_172041	CTACAAGCCCGTTGTGTGGTT	GCTCAGAACAGCCGCAAAGT
Kcnj6	NM_013192	AATGGTGGAAGCCACAGGAA	CCGGTAACCCCACAGGCTCT
Grinl1a	NM_183402	TTACAGCAGCACGCCTTCTTC	GAGCCAGCGACTCTTCTATGGA

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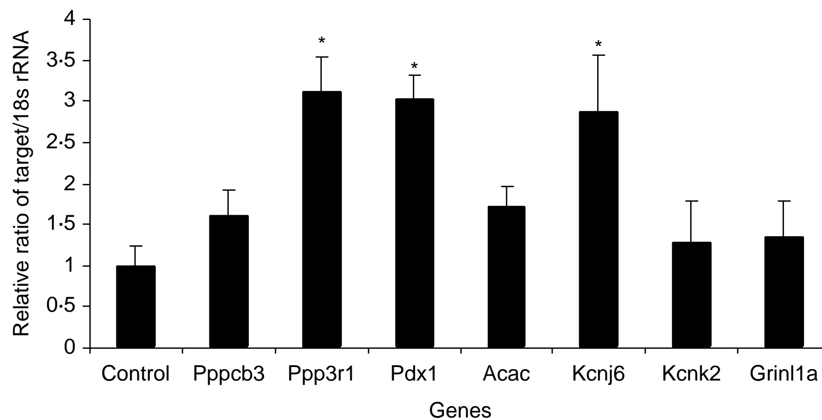

Figure 1 Real-time PCR analysis of L-glutamine-affected genes. Confirmation by real time-PCR of seven genes that had shown more than 1·8-fold 10 mM L-glutamine-induced change in expression by microarray analysis. BRIN-BD11 cells were cultured in the presence of either 1 or 10 mM L-glutamine. After 24 h incubation, total RNA from three biological replicates of each treatment was isolated using the QIAGEN RNeasy Mini Kit. Target mRNA was quantified using an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) as described in Materials and Methods. The data are presented as a percentage of the respective 1 mM L-glutamine control which received an arbitrary value of 1 in each experiment. Values are means ± S.D., $*P<0·05$.

significant loss of viability during a chronic period of incubation.

Detailed microarray analysis revealed that the largest functional clusters of genes changed by 24 h exposure to L-glutamine were those involved in signal transduction, gene regulation, and metabolism (see Supplementary data Table 1). Not all the genes described in the Supplementary data Table 1 would be expected to be expressed in the β-cell (especially those listed in the 'Miscellaneous' or 'Signaling – Other' sections) but those selected for further analysis by real time PCR, Western blot, or activity measurement are normally expressed in β-cells. L-Glutamine is rapidly metabolized via glutaminase to L-glutamate and NH_4^+ (Brennan *et al.* 2003). However, the production rate of urea was not altered by L-glutamine, in contrast to the production rate of NH_4^+. Urea production from pancreatic β-cells in culture has been previously reported by others (Webb *et al.* 2000, 2001) but the significance of this finding is not known. It may be related to the regulation of NO production (as inducible nitric oxide synthase will compete with arginase for available L-arginine, as described for the mouse macrophage and human monocyte (Murphy & Newsholme 1998). We propose that the β-cell glutamate is converted to 2-oxoglutarate on activation of GDH, and then to malate via reactions of the TCA cycle.

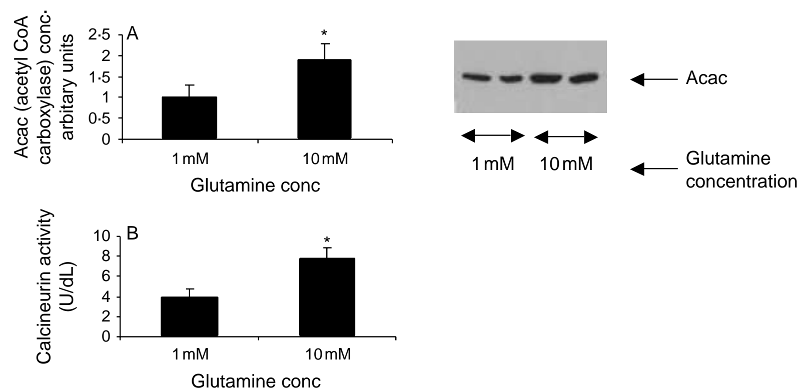

Figure 2 (A) Western blot analysis confirming L-glutamine induced upregulation of acetyl CoA carboxylase protein was performed as described in Materials and Methods. (B) Measurement of calcineurin activity in BRIN-BD11 cells after a 24 h incubation in the presence of 1 or 10 mM L-glutamine. Calcineurin activity assays were performed on BRIN-BD11 cell lysates using a colorimetric calcineurin activity assay kit, as described in the manufacturer's protocol (Calbiochem).

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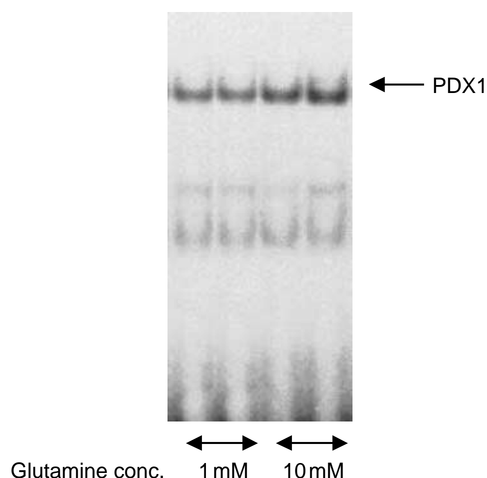

Figure 3 An electrophoretic mobility shift assay was used to confirm an increase in PDX1 transcription factor-binding activity after incubation in 10 mM L-glutamine compared to 1 mM L-glutamine, as described in Materials and Methods.

Subsequently, malate may be converted to pyruvate and then citrate (Newsholme *et al.* 2003). Citrate may then leave the mitochondrial matrix and be converted to acetyl-CoA and oxaloacetate in the cytosol. Acetyl-CoA can then be converted to malonyl CoA by acetyl-CoA carboxylase (which we report here to be upregulated by L-glutamine at the mRNA and protein level) and so enter the pathway of fatty acid synthesis. Fatty acid synthesis appears to be essential for optimal stimulation of insulin secretion in the β-cell (Haber *et al.* 2003) due to generation of lipid-derived signaling molecules. Long chain acyl-CoAs, glutamate, glutathione, or reactive oxygen species could activate a number of kinases, such as JNK, p38 MAPK, or protein kinase C (Yaney & Corkey 2003). Glutamate may additionally enter the γ-glutamyl cycle as previously described (Brennan *et al.* 2003) and so contribute to β-cell glutathione production and antioxidant defenses. Thus, the mechanisms by which L-glutamine metabolism influences β-cell signal transduction and gene expression are probably related to generation of key amino acid stimulus secretion coupling factors (L-glutamate), generation of lipid metabolites (long chain acyl-CoAs), and by ensuring adequate levels of cellular glutathione, which may be important for optimizing mitochondrial function, as previously described (Brennan *et al.* 2003).

One important gene significantly upregulated by L-glutamine was *pdx1*, a factor essential for pancreatic β-cell differentiation and function. Studies of rat islets following adenoviral transfection with a dominant-negative *pdx1* gene demonstrated reduced glucose-stimulated insulin secretion, decreased islet ATP content, and reduced expression of a number of mitochondrial genes required for ATP generation (Gauthier *et al.* 2004). The significant glutamine-dependent upregulation of PDX1 mRNA and increased PDX-1 transcription binding assessed by EMSA observed in this study is a novel finding and deserves further evaluation. This finding may have relevance for the optimal *in vitro* differentiation of pancreas-derived and possibly embryonic stem cells towards β-cell phenotype.

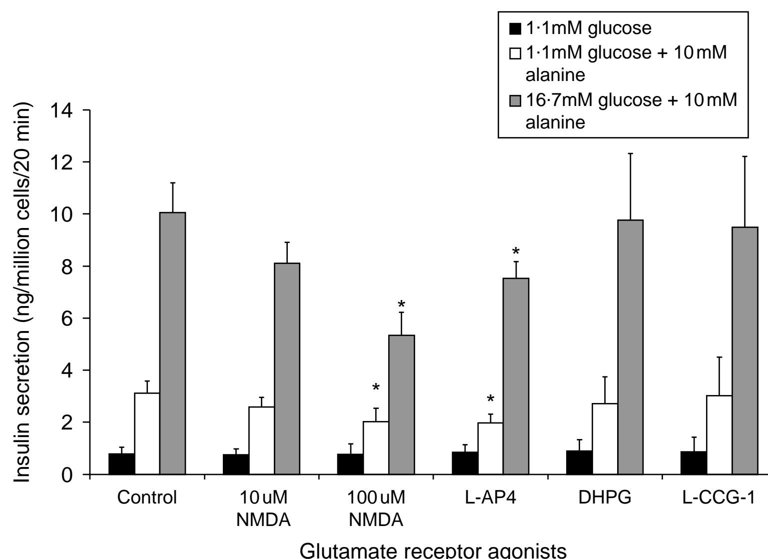

Figure 4 Basal, L-alanine, or glucose plus L-alanine-induced insulin secretion in the presence of specific metabotropic glutamate receptor (mGluR) agonists (L-AP4, DHPG, and L-CCG1) or NMDA. Effects of glutamate receptor agonists were determined as described in Materials and Methods. Values are means ± S.D. Statistically significant differences (ANOVA) are indicated by $^*P<0{\cdot}05$ compared to appropriate control (absence of receptor agonist).

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L-Glutamine strongly upregulated both the calcineurin catalytic and regulatory subunit mRNA expression in BRIN-BD11 cells. Calcineurin, or protein phosphatase 2B, is a calcium-binding protein that has been shown to contribute to the mechanism of somatostatin-induced inhibition of exocytosis in mouse pancreatic β-cells (Renstrom *et al.* 1996). In addition, it is now appreciated that the cAMP response element-binding protein (CREB) transcription factor regulates specific pro-survival genes in the β-cell. CREB translocation to the nucleus is regulated by specific Ca^{2+}-dependent dephosphorylation of TORC (transducers of regulated CREB) by calcineurin (Schuit *et al.* 2005). Indeed, a recent report has shown that the addition of 5 mM L-glutamine to the isolation medium applied through the main pancreatic duct, improved yield, survival, and function of human islets that were destined for transplantation (Avila *et al.* 2005). Calcineurin has also been reported to modulate NMDA receptor activity (Lieberman & Mody 1994). NMDA-specific glutamate receptors are expressed in pancreatic β-cells (Molnar *et al.* 1995) but their function has not been clearly defined. In the present study, upregulation of the calcineurin gene resulted in increased calcineurin-specific phosphatase activity, which *in vivo* will lead to NMDA receptor inactivation. NMDA receptors normally stimulate Na^{+} and Ca^{2+} influx (Dingledine *et al.* 1999), which would be expected to depolarize the cell membrane and promote insulin secretion. However, chronic receptor stimulation by L-glutamate (when released at high levels) may desensitize the β-cell to subsequent nutrient-stimulated insulin secretion. Calcineurin may reduce NMDA receptor-mediated desensitization.

We have uniquely reported here that L-glutamate is released from the BRIN-BD11 β-cell into the extracellular medium. The quantity of glutamate released was large – indeed, based on the reported increase of extracellular and intracellular glutamate on increasing extracellular glutamine from 1 to 10 mM (approximately 1 μmol/10^6 cells per 24 h reported in Table 1, compared with approximately 5 nmol/10^6 cells per 24 h reported in Table 2 respectively), then glutamate was released into the extracellular medium at a rate almost 200-fold above the intracellular production rate. While we appreciate that the acute rate of glutamate release from the β-cell (i.e. <1 h) is probably low, chronic glutamate release may cause glutamate receptor activation and desensitization of the cell to further insulinotropic signals, representing a novel autocrine mechanism for regulation of β-cell function. Exogenously applied NMDA or the metabotropic glutamate receptor agonist L-AP4 significantly reduced acute L-alanine and glucose-induced insulin secretion. Although the present study has utilized acute periods of stimulation of insulin secretion to determine the effects of NMDA or L-AP4, we appreciate that chronic regulation of insulin secretion may be influenced in a different manner by glutamate receptor-dependent mechanisms. Interestingly, glutamine metabolism in the β-cell may therefore play multiple roles: (i) production of intracellular glutamate and glutathione, which acutely stimulate insulin secretion (Brennan *et al.* 2003), (ii) stimulation of acyl-CoA production, which would be important for the amplification of insulin secretion, (iii) chronic release of glutamate from the cell therefore inhibiting insulin secretion via interaction with ionotropic and metabotropic glutamate receptors, and (iv) upregulation of calcineurin expression and activity, which may impact on pro-survival gene expression and NMDA receptor-dependent ion fluxes.

In conclusion, we have demonstrated multiple roles for L-glutamine in the acute and chronic regulation of β-cell function. However, it should be noted that, like many other published studies, high concentrations of L-glutamine were used *in vitro*. Such an approach results in optimal glutamine transport into target cells and metabolism thus facilitating production of signaling molecules, which at lower concentrations, might not be detectable.

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Supplementary material

Supplementary data table 1 associated with this article can be found in the online version of Journal of Endocrinology at http://joe.endocrinology-journals.org/content/vol190/issue3.

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