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Glutamine potently stimulates glucagon-like peptide-1 secretion from GLUTag cells

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Abstract

Aims/hypothesis. Glucagon-like peptide-1 (GLP-1) and peptide YY (PYY) are secreted from enteroendocrine L cells in response to nutrient ingestion. As glutamine is an important metabolic fuel for the gut, the aim of this study was to investigate the effect of glutamine on the GLP-1-secreting cell line, GLUTag.
Methods. GLP-1 release was measured following incubation of GLUTag cells under a range of conditions. Single cells were studied by electrophysiology, calcium imaging and cytosolic ATP measurement using recombinant luciferase.
Results. Glutamine was a more potent GLP-1 secretagogue than glucose or other amino acids, increasing GLP-1 release 7.1±0.7-fold (n=19) at 10 mmol/l, with an estimated median effective concentration of between 0.1 and 1 mmol/l. Glutamine (10 mmol/l) induced a sodium-dependent inward current of 3.2±1.2 pA per cell (n=9), which triggered membrane depolarisation and an increase in intracellular calcium. Asparagine and alanine produced electrophysiological and calcium changes that were at least as large as those caused by glutamine, but they were less effective GLP-1 secretagogues, suggesting that glutamine also potentiates secretion downstream of the calcium signal. This was confirmed by measuring secretion in the presence of 30 mmol/l KCl + diazoxide, or in α-haemolysin-permeabilised cells. Glutamine increased cytosolic ATP, but was less effective than glucose.
Conclusions/interpretation. Glutamine acts as a trigger and potentiator of GLP-1 release, consistent with its role as the major metabolic fuel for the gut. The results suggest that nutritional agents like glutamine might have beneficial effects in diabetes and obesity.

Keywords Diabetes · Glucagon-like peptide-1 · GLUTag cells · Glutamine · Obesity

Introduction

Intestinal neuroendocrine L cells secrete glucagon-like peptides-1 and -2 (GLP-1, GLP-2) and peptide YY (PYY) in response to food ingestion [1, 2, 3, 4, 5, 6]. GLP-1 and PYY modulate glucose homeostasis and energy balance: GLP-1 by enhancing insulin release [7], and PYY by increasing satiety [8]. These peptides are therefore under investigation as candidates for the treatment of Type 2 diabetes and obesity. GLP-2 affects gut motility and is an intestinal trophic factor, with therapeutic potential for the treatment of intestinal disorders such as short bowel syndrome [9].

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Abbreviations: BCH, 2-amino-2-norbornanecarboxylic acid · CaM, calmodulin · DON, 6-diaza-5-oxo-L-norleucine · GDH, glutamate dehydrogenase · GLP-1, glucagon-like peptide-1 · GLP-2, glucagon-like peptide-2 · IBMX, 3-isobutyl-1-methylxanthine · K_{ATP} channel, ATP-sensitive potassium channel · MeAIB, α(methylamino)isobutyric acid · NMDG, *N*-methyl-D-glucamine · PKA, protein kinase A · PKC, protein kinase C · PYY, peptide YY

Developing strategies to enhance secretion from intestinal L cells could therefore provide alternative therapies for a range of conditions, including Type 2 diabetes and obesity.

Secretion from L cells in vivo occurs in response to various components of the diet, including carbohydrates, fats and proteins [10, 11, 12]. The mechanisms underlying the stimulation of secretion remain uncertain, although there is evidence for both direct L cell sensing of the luminal contents and indirect pathways involving neural/hormonal components [2, 13]. Using the murine GLP-1-secreting cell line, GLUTag, we have previously shown that GLP-1-secreting cells are electrically active and respond directly to increases in the glucose concentration by enhanced action potential firing and GLP-1 release [14, 15]. The mechanism partly resembles that found in the pancreatic beta cell, involving closure of ATP-sensitive potassium (K_{ATP}) channels [14], but in GLUTag cells an additional depolarising stimulus is provided by electrogenic coupled Na^+ and glucose entry via sodium-coupled glucose cotransporters [15].

Although protein has been found to stimulate GLP-1 release in a number of studies [10, 11, 16], the underlying mechanism has not been elucidated and some controversy remains about the effectiveness of protein on the L cell [17]. Whereas protein hydrolysate and peptones have been found to stimulate L cell secretion in several studies [18, 19, 20], amino acid mixtures are a less reliable stimulus [11, 12, 20]. Interestingly, however, the amino acid mixtures tested did not contain glutamine, which is believed to be a major energy source for the gut [21, 22]. Glutamine has a number of beneficial effects on the gut, not all of which can be accounted for by its metabolism alone [22], and it is used therapeutically as a nutritional supplement in conditions such as parenteral nutrition and cancer chemotherapy [23]. The question, therefore, arises as to whether L cells, like other intestinal cells, are sensitive to changes in the glutamine concentration.

In this paper, we have investigated the effect of glutamine on GLP-1-secreting cells using the cell line GLUTag [24]. This cell line was originally derived from a colonic tumour in a transgenic mouse expressing SV40 large T antigen under the control of the proglucagon promoter. It has been shown to respond to a range of physiological stimuli and is believed to be a good model cell line of GLP-1 secretion. We used a variety of techniques, including electrophysiology, intracellular calcium imaging, dynamic cytosolic ATP measurements and assays of GLP-1 release, to investigate the mechanisms underlying the effect of glutamine on GLP-1 secretion.

Materials and methods

Cell culture. GLUTag cells [24] were cultured in DMEM (5.5 mmol/l glucose), as described previously [14].

GLP-1 secretion. For secretion experiments cells were plated in 24-well culture plates and allowed to reach 60 to 80% confluency. On the day of the experiment, cells were washed twice with 500 µl nutrient-free KRB containing (mmol/l): 120 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 22 $NaHCO_3$, and 0.1 Diprotin A, bubbled with 95% O_2/5% CO_2 for 10 min and supplemented with 0.5% (w/v) BSA. Experiments were performed by incubating the cells with test reagents in 500 µl KRB for 2 h at 37 °C, 5% CO_2. In some experiments the KCl concentration was increased to 30 mmol/l (NaCl was reduced accordingly to 95 mmol/l) and 0.34 mmol/l diazoxide was added. IBMX, forskolin, KN93, H89, BIM-I and rapamycin were prepared as 1000× stocks in DMSO and the final DMSO concentration was adjusted to 0.25% for all conditions tested, including the control. Other test agents were directly dissolved in KRB and the pH was readjusted to 7.4 with NaOH or HCl. At the end of the incubation period, medium was collected and centrifuged to remove any floating cells. GLP-1 was assayed using an ELISA specific for GLP-1(7-36 amide) and GLP-1(7-37) (Linco GLP-1 active ELISA-kit; Biogenesis, Poole, UK). Secretion was normalised to the baseline release in the absence of nutrients measured in parallel on the same day. This baseline secretory rate was 28.5±2.6 fmol per dish in 2 h (n=37). To measure the time course of GLP-1 release, cells were incubated for different times in the presence or absence of glutamine, and the GLP-1 content of the medium was assayed as above.

Permeabilisation of cells. Cells were plated in Matrigel-coated 24-well culture plates (Matrigel supplied by BD Biosciences, Fahrenheit, Milton Keynes, UK), and allowed to reach 60 to 80% confluency. Secretion experiments in KRB with a range of different secretagogues revealed no difference in the responsiveness of cells cultured with or without Matrigel. On the day of the experiment, wells were washed twice with 500 µl Ca^{2+}-free buffer containing (mmol/l): 135 NaCl, 5 KCl, 2 $NaHCO_3$, 0.5 NaH_2PO_4, 0.5 $MgCl_2$, 0.4 EGTA, 10 HEPES (pH 7.4 with NaOH). Permeabilisation was achieved by incubating for 15 min at 37 °C with approximately 69 units of *Staphylococcus aureus* α-haemolysin in 100 µl lysis buffer containing (mmol/l): 107 KCl, 5 NaCl, 7 $MgSO_4$, 20 HEPES, 10.2 EGTA, 1.65 $CaCl_2$, 1 K_2ATP, 0.1% BSA (pH 7.0 with KOH; final $[K^+]$ ~140 mmol/l). In some control wells permeabilisation was assessed by subsequent incubation with 0.4% Eosin Y in lysis buffer for 5 min at 37 °C, which stained more than 90% of cells (compared with less than 5% of cells stained in wells not exposed to the toxin). The cells were subsequently washed with 250 µl lysis buffer, incubated for 15 min with 500 µl lysis buffer, and incubated for 10 min in 500 µl stimulation buffer (similar to lysis buffer but with 0 or 5 mmol/l ATP and 1.65 or 6.65 mmol/l $CaCl_2$) at 37 °C, 5% CO_2. The free Ca^{2+} concentrations of the lysis and stimulation buffer were estimated at 121 nmol/l (for 1.65 mmol/l $CaCl_2$) and 1.2 to 1.4 µmol/l (for 6.65 mmol/l $CaCl_2$), respectively, using BAD4-software [25]. DON, when included, was also present during the final 10 min of the pre-incubation. Experiments were performed by incubating the cells with test reagents in 500 µl stimulation buffer for 1 h at 37 °C, 5% CO_2. GLP-1 secretion was assayed by ELISA as above.

Electrophysiology. Cells were plated into 35-mm dishes 1 to 3 days prior to use. Experiments were performed on single cells and well-defined cells in small clusters. Microelectrodes

were pulled from borosilicate glass (GC150T; Harvard Apparatus, Edenbridge, UK) and the tips coated with refined yellow beeswax. Electrodes were fire-polished using a microforge (Narishige, London, UK) and had resistances of 2.5 to 3 MΩ when filled with pipette solution. Membrane potential and currents were recorded using an Axopatch 200B (Axon Instruments, Union City, Calif., USA) linked through a Digidata 1320A interface, and analysed using pCLAMP software (Axon Instruments). All electrophysiological recordings were made using the perforated patch configuration of the patch clamp setup at 22 to 24 °C. Membrane conductance was measured in voltage clamp experiments by applying a series of 1.2-s voltage ramps from −90 to −50 mV (holding potential −70 mV). The current voltage relation was linear in this voltage range, and the slope conductance was calculated from the average of more than ten voltage ramps.

Ca^{2+} measurements. Cells were plated on Matrigel-coated coverslips 1 to 3 days prior to use, and loaded with fura-2 by incubation in 1 μmol/l of the acetoxymethylester (Molecular Probes, Leiden, The Netherlands) for 30 min at 37 °C in bath solution containing 1 mmol/l glucose. Measurements were taken after mounting the coverslip in a perfusion chamber (Warner Instruments, Harvard Apparatus) on an inverted fluorescence microscope (Olympus IX71, Southall, UK) with a 40× oil immersion objective. Excitation at 340 and 380 nm was achieved using a combination of a 75-W Xenon arc lamp and a monochromator (Cairn Research, Faversham, UK) controlled by MetaFluor software (Universal Imaging, Cairn), and emission was recorded with a CCD camera (Orca ER; Hammamatsu, Cairn) using a dichroic mirror and a 510-nm long pass filter. Free cytoplasmic Ca^{2+} concentrations were estimated for individual cells from background-subtracted fluorescence using the equation of Grynkiewicz [26] assuming a K_d of 224 nmol/l. Minimal and maximal signals were recorded in the presence of 5 μmol/l ionomycin in 5 mmol/l EGTA/0 mmol/l Ca^{2+} and 5 mmol/l Ca^{2+}, respectively, at the end of the experiment.

Solutions and chemicals. The bath solution contained (mmol/l): 5.6 KCl, 138 NaCl, 4.2 $NaHCO_3$, 1.2 NaH_2PO_4, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES (pH 7.4). Amino acids and drugs were added to the bath solution as indicated. The Na^+-free bath solution contained (mmol/l): 148.4 NMDG, 4.4 $KHCO_3$, 1.2 KH_2PO_4, 2.6 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES (pH 7.4 with HCl). The perforated patch pipette solution contained (mmol/l): 76 K_2SO_4, 10 KCl, 10 NaCl, 55 sucrose, 10 HEPES, 1 $MgCl_2$ (pH 7.2), to which amphotericin B was added to a final concentration of 200 μg/ml. Drugs and chemicals were purchased from Sigma-Aldrich (Poole, UK) unless otherwise stated. H89, KN93, BIM-1, rapamycin and ionomycin were purchased from Calbiochem (Merck Eurolab, Lutterworth, UK) and DON from Bachem (Merseyside, UK).

Measurement of cytosolic free ATP concentration. Cells plated on glass coverslips were infected with adenoviruses encoding humanised firefly luciferase (Promega, Southampton, UK) under cytomegalovirus promoter control [27]. Changes in cytosolic ATP were measured 24 h after infection. Single-cell photon counting was performed using an Olympus IX-70 microscope fitted with a ×10 air objective, 0.4 numerical aperture, and a Photek ICCD218 intensified camera (Photek, Lewes, UK) as previously described [27, 28]. Cells were cultured in 0 mmol/l glucose and then stimulated with 10 mmol/l glutamine or 1 mmol/l glucose.

Statistical analysis. Results are presented as means ± SE. Statistical significance was tested in the first case by ANOVA, and subsequently by one-sample or unpaired Student's *t* tests (as indicated). A *p* value of less than 0.05 was considered statistically significant.

RT-PCR. As previously described [14], RT-PCR was performed using the following specific primers, which were designed using sequence information obtained from http://www.ensembl.org/mus_musculus/ (from 5′ to 3′): SLC38A1/ATA1 sense TCGGGAGAGTAGGAGGAGTC, antisense GCTCCACACTCATGCAGGG; SLC38A2/ATA2 sense ACAGACTTTCATCCAGGTACTAC, antisense TTCACAGTTTCGTTGGCCACC; SLC38A3/SN1 sense CCAACACTGTGGAGAGGGC, antisense CCTCTGCCACCACCATGTG; SLC38A4/ATA3 sense ACGCGGATGAGCATCACCC, antisense TGTTGTGATCCAGAGCAGGC; SLC38A5/SN2 sense CTGCCCACCACCCGTAACC, antisense TTCAGCCTCCACTACTGTGTC; SLC7A6/y^+LAT2 sense GTGTCAGGGACACACTGAGC, antisense GACATCCTGAATGTTCAGCACTG; SLC7A7/y^+LAT1 sense TTGGCCAGGGAGCCACAGC, antisense CTGGAAGCAGCCACGATGG; SLC6A14/$ATB^{o,+}$ sense TCTGACCTACACCAATGGCG, antisense CACTGGGAAGTTGTCCTGGC; SLC1A7/mASCT2 sense TGCTGACGCGCTGGGTCC, antisense CCGCTGGATACAGGATTGCG. The predicted sizes (bp) of fragments were: (ATA1) 611, (ATA2) 583, (SN1) 715, (ATA3) 576, (SN2) 624, (y^+LAT2) 251, (y^+LAT1) 382, ($ATB^{o,+}$) 440, (mASCT2) 448. All primer pairs span intron–exon borders to distinguish amplification from genomic DNA. No bands were amplified when H_2O was used instead of cDNA. Identity of amplified bands was confirmed by sequencing.

Results

To investigate whether glutamine stimulates GLP-1 release from GLUTag cells, we cultured cells in 10 mmol/l glutamine or 10 mmol/l glucose for 2 hours. As reported previously [14, 15], glucose stimulated GLP-1 secretion, which increased approximately three-fold. Glutamine was a more potent stimulus, enhancing secretion approximately seven-fold at 10 mmol/l (Fig. 1a), with an estimated median effective concentration of between 0.1 and 1 mmol/l (Fig. 1b). A time course study showed that 10 mmol/l glutamine enhanced GLP-1 release within minutes of its application, with a subsequent slow increase in the rate of secretion over 2 hours (Fig. 1c). A range of other amino acids (each at 10 mmol/l) also stimulated GLP-1 release (Fig. 1a), but none were as effective as glutamine (*t* test vs glutamine $p<0.05$ for all agents tested).

Electrophysiological response to glutamine. Glutamine (10 mmol/l) potently triggered action potentials in GLUTag cells recorded in the perforated patch configuration. While asparagine stimulated the firing of action potentials as strongly as glutamine, glutamate was much less effective (Fig. 2a, b). To investigate the underlying mechanism, we applied a series of voltage ramps in the absence and presence of amino acids. Glutamine shifted the current voltage relationship downwards, corresponding to an increase in the in-

ward current at −70 mV of 3.2 pA/cell (Fig. 2c, d), with no significant effect on the slope conductance. A current of similar magnitude was induced by asparagine (10 mmol/l; Fig. 2d). Substitution of extracellular Na^+ with $NMDG^+$ abolished the response to glutamine (Fig. 2d). These data are consistent with the idea that glutamine and asparagine trigger action potentials by electrogenic amino acid uptake coupled to Na^+ influx.

Effect of glutamine and glucose on intracellular free ATP concentration. We reported previously that K_{ATP} channel closure contributes to the electrical response to glucose in GLUTag cells. The lack of change in the slope conductance upon addition of glutamine, however, does not support the idea that K_{ATP} channel closure also underlies the response to glutamine. We examined this idea further by measuring dynamic changes in the ATP concentration using recombinant luciferase and bioluminescence imaging. Addition of 10 mmol/l glutamine in the presence of 0 mmol/l glucose led to a 10±4% increase in apparent free cytosolic ATP (Fig. 3a). Subsequent addition of 1 mmol/l glucose led to a substantially greater (23±5%) further increase in ATP (Fig. 3a). When the order of addition was reversed (Fig. 3b), 1 mmol/l glucose prompted a 57±9% increase in cytosolic ATP, whereas 10 mmol/l glutamine caused an 18±9% increase. Thus, glutamine was less effective than glucose at raising cytosolic ATP when added as the first or second substrate. The

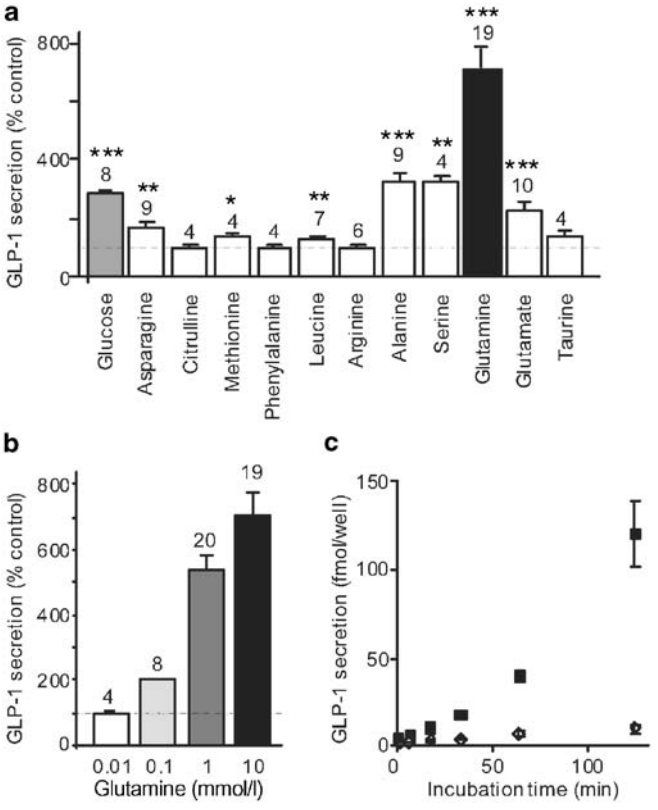

Fig. 1. Secretory responses of GLUTag cells to glutamine. **a.** GLP-1 secretion from GLUTag cells cultured with 10 mmol/l glucose or the amino acid indicated. The number of wells is indicated above each bar. Statistical significance was assessed relative to secretion in the absence of nutrients using Student's one-sample *t* test: ***p<0.001, **p<0.01, *p<0.05. Where bars are not marked with asterisks, results were not significant. **b.** Concentration dependence of glutamine-induced secretion. Secretion in **a** and **b** was normalised to baseline secretion in the absence of nutrients, measured in parallel on the same day. In **a** and **b** the 10 mmol/l glutamine data is the same. **c.** Time course of GLP-1 release in the absence (open diamonds) or presence (closed squares) of 10 mmol/l glutamine. n=4 per point

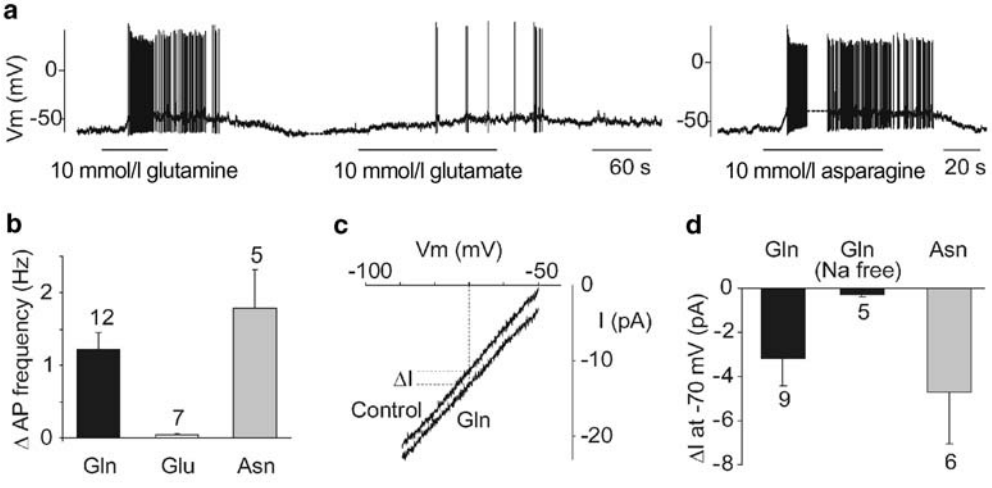

Fig. 2. Electrophysiological responses of GLUTag cells to glutamine. **a.** Action potentials triggered by glutamine, glutamate and asparagine (10 mmol/l), as indicated. **b.** Mean change (Δ) in action potential (AP) frequency in 10 mmol/l glutamine (Gln), glutamate (Glu) and asparagine (Asn). The numbers of cells analysed are indicated above the bars. **c.** Current (I)–voltage (Vm) relationship in the presence and absence of 10 mmol/l glutamine. The lines represent the means of more than ten voltage ramps in a single GLUTag cell. ΔI represents the incremental current induced at −70 mV. **d.** Mean ΔI induced by 10 mmol/l glutamine (Gln) in the presence or absence (Na free) of extracellular sodium, or 10 mmol/l asparagine (Asn). The number of cells is given below each bar

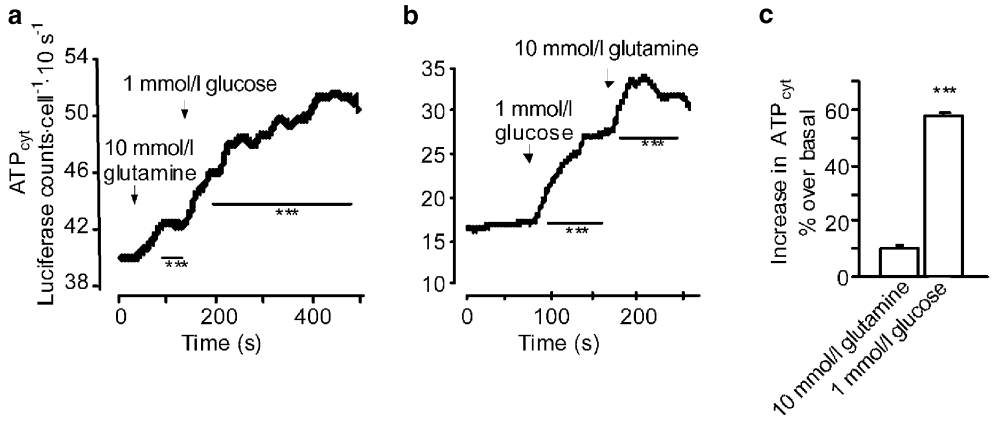

Fig. 3. Metabolic response of GLUTag cells to glutamine and glucose. **a, b.** Cytosolic-free ATP (ATP_{cyt}) was measured in cells cultured in KRB containing 0 mmol/l glucose, following the addition of 10 mmol/l glutamine and then 1 mmol/l glucose (**a**), or with the additions in reverse order (**b**). **c.** Mean change (%) in cytosolic ATP resulting when glutamine or glucose were added first, as determined in the experiments shown in **a** and **b**. Data are the means ± SE of three individual experiments, with analysis of a minimum of 15 cells per experiment. ***p<0.001 for the effects of indicated additions vs basal light output (**a, b**) or vs the effect of glutamine (**c**)

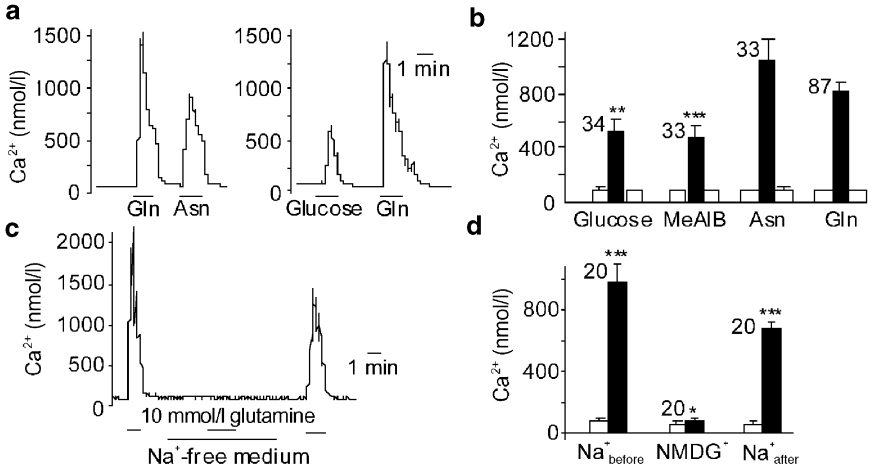

Fig. 4. Intracellular Ca^{2+} responses of GLUTag cells to glutamine. **a.** Cytoplasmic Ca^{2+} concentration in response to 10 mmol/l glutamine (Gln), asparagine (Asn) or glucose, as indicated. **b.** Mean $[Ca^{2+}]_i$ measured during a 30-s period before, during and after application of 10 mmol/l glucose, MeAIB, asparagine or glutamine. Where filled bars are not marked with asterisks, results were not significant. **p<0.01, ***p<0.001 vs glutamine. Open bars, control; filled bars, secretagogue. **c.** $[Ca^{2+}]_i$ changes in response to 10 mmol/l glutamine in the presence and absence (Na^+-free medium) of extracellular sodium. **d.** Mean $[Ca^{2+}]_i$ measured over 30 s, as in **c**, in the absence and presence of glutamine. The response was measured initially in the presence of Na^+ (Na^+_{before}), then during substitution of extracellular Na^+ with $NMDG^+$ ($NMDG^+$), and finally in the presence of Na^+ again (Na^+_{after}). Open bars, control; filled bars, glutamine. *p<0.05, ***p<0.001 vs mean of $[Ca^{2+}]_i$ measured before and after application of the amino acid. The number of analysed cells is given above each bar

response to 1 mmol/l glucose was approximately five-fold greater than the response to 10 mmol/l glutamine (Fig. 3c) when only the effects of the initial nutrient addition were compared.

Intracellular calcium transients. In other neuroendocrine cell types such as pancreatic beta cells, the link between electrical activity and exocytosis is provided by calcium, which enters the cells through L-type voltage-gated Ca^{2+} channels [29]. We therefore investigated whether amino acids and glucose trigger a rise in intracellular Ca^{2+} in GLUTag cells. Cells were loaded by incubation with fura2-AM, and the fluorescence ratio at 340 and 380 nm was followed during application of test agents. Glutamine (10 mmol/l) triggered a reversible increase in $[Ca^{2+}]_i$ (Fig. 4a). In contrast, glucose triggered a smaller response and asparagine a comparable response to glutamine (Fig. 4a, b). The response to 10 mmol/l alanine was much stronger than that to glutamine, and was too large to measure accurately due to saturation of the fura-2. Substitution of extracellular Na^+ by $NMDG^+$ abolished the glutamine-induced rise in $[Ca^{2+}]_i$ (Fig. 4c, d).

Expression of sodium-dependent glutamine transporters. The sodium dependency of the glutamine-induced inward current and calcium transient, taken

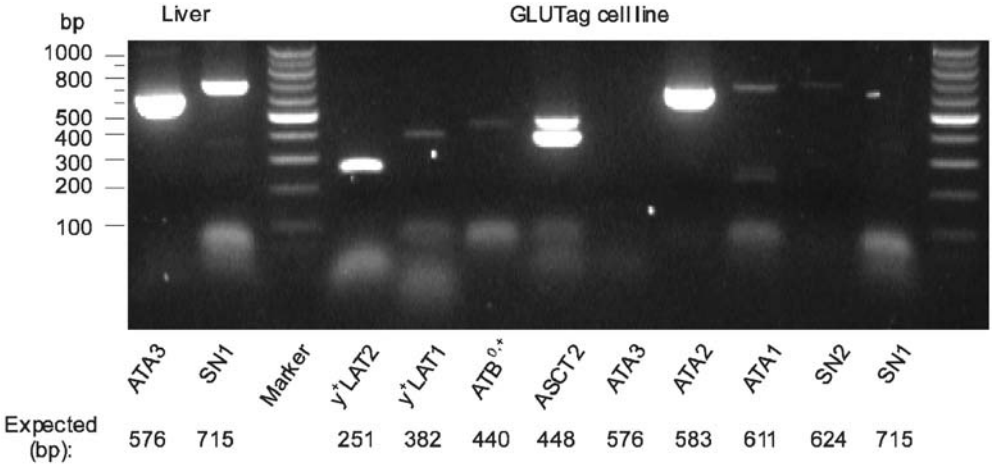

Fig. 5. Sodium-dependent glutamine transporter expression in the GLUTag cell line (RT-PCR). Positive controls (using mouse liver cDNA) for SN1 and ATA3, which were not detected in GLUTag cells, are shown on the left. Predicted band sizes are indicated. The non-specific lower band in the ASCT2 lane was also amplified with control cDNA from small intestine, and originates from a genomic sequence on murine chromosome 14

Table 1. Effect of candidate inhibitors on basal and stimulated GLP-1 release

Inhibitor	% change in GLP-1 release relative to secretion triggered by:			
	Baseline conditions	1 mmol/l glutamine	10 mmol/l glucose	Forskolin + IBMX
BCH (10 mmol/l)	4.8±17.1 (3) NS	–5.1±7.8 (4) NS	–	–
MeAIB (10 mmol/l)	19.5±5.4 (8) **	–26.9±5.2 (4) *	–	–
DON (20 µmol/l)	–	4.7±14.2 (4) NS	–	–
DON (1 mmol/l)	–3.7±2.2 (4) NS	–76.6±2.7 (7) ***	43.8±10.6 (4) *	1.7±11.5 (4) NS
KN93 (10 µmol/l)	–	–63.5±2.7 (9) ***	–92 (2)	–
BIM-1 (250 nmol/l)	–	0.0±5.3 (6) NS	–	–
H89 (1 µmol/l)	–	–4.3±5.2 (6) NS	–	–
Rapamycin (100 ng/ml)	–	–7.4±3.1 (4) NS	–	–

Data are presented as means ± SEM (n). Significance was evaluated using Student's one-sample t test, compared with zero (no change). NS, not significant; *$p<0.05$; **$p<0.01$; ***$p<0.001$. Negative values represent inhibition of secretion

together with the similarity of the responses induced by asparagine, suggest a possible involvement of Na-coupled amino acid transporters. We therefore used RT-PCR to identify which Na-coupled glutamine transporters [30] are expressed in GLUTag cells. The mRNA for several sodium-coupled glutamine transporters was detected (Fig. 5). The strongest bands were for ATA-2 (system A: an electrogenic, Na^+-coupled neutral amino acid transporter), ASCT-2 (a non-electrogenic Na^+-coupled neutral amino acid transporter) and y^+LAT2 (an Na^+-coupled amino acid exchanger). Weaker bands were detected for SN2, ATA-1, ATB^{0+}, and y^+LAT1. One of the characteristics of system A is its ability to transport the amino acid analogue MeAIB. Consistent with the idea that system A contributes to electrogenic glutamine uptake in GLUTag cells, MeAIB (10 mmol/l) induced calcium transients in GLUTag cells that were smaller than those triggered by glutamine (Fig. 4a), triggered a small but significant increase in GLP-1 secretion, and reduced the secretory response to 1 mmol/l glutamine by approximately 25% (Table 1).

Additional effects of glutamine. The results of the electrophysiological and calcium imaging experiments suggest that the efficacy of glutamine as a GLP-1 secretagogue, compared with that of other amino acids such as asparagine and alanine, cannot be explained

by differences in the magnitude of the depolarising current or calcium transient. An alternative possibility, therefore, is that glutamine in some way potentiates the secretory response, analogous to the effect of glucose on pancreatic beta cells [31]. To investigate this possibility, we compared the effects of glutamine, glucose and alanine on GLP-1 secretion in the presence of 30 mmol/l KCl + diazoxide (340 µmol/l). Under these conditions the potassium current through open K_{ATP} channels produces a clamped membrane depolarisation that prevents small currents, such as those arising from electrogenic transporter activity, from significantly affecting the membrane potential. 30 mmol/l KCl + diazoxide was itself a potent GLP-1 trigger, increasing secretion approximately six-fold. Glutamine doubled the secretory rate, whereas glucose and alanine induced significantly smaller effects (Fig. 6; $p<0.001$ vs glutamine for each). This suggests that glutamine enhances stages of the secretory pathway downstream of membrane depolarisation.

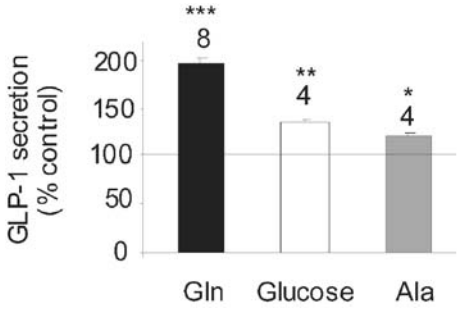

Fig. 6. Effects of glutamine independent of the plasma membrane potential. GLP-1 secretion measured in 30 mmol/l KCl/340 µmol/l diazoxide and 10 mmol/l of glutamine (Gln), glucose or alanine (Ala) as indicated. Secretion was normalised to that measured in the absence of nutrient on the same day (mean 301±45 fmol·well^{-1}·2 h^{-1}; n=8). Statistical significance was assessed relative to secretion in the absence of nutrients using Student's one-sample t test: ***$p<0.001$, **$p<0.01$, *$p<0.05$

The major metabolites of glutamine in the gut are glutamate, proline, citrulline, ornithine and ammonium [21, 22]. To investigate whether metabolism of glutamine via these pathways is required for the potentiation of GLP-1 release, we measured secretion in the presence of α-haemolysin and fixed concentrations of ATP and free calcium. The advantages of this experimental design are that entry of the test agent occurs through membrane pores generated by the toxin and is not limited by the presence of appropriate transporters [32]. Only the secretory pathway downstream of the calcium signal is therefore tested. Glutamine (10 mmol/l) stimulated GLP-1 release 1.5- to 2.3-fold in the presence of elevated concentrations of ATP (5 mmol/l) and free Ca^{2+} (~1 µmol/l; Fig. 7). No glutamine-triggered secretion was observed at basal free Ca^{2+} concentrations (~100 nmol/l), and hormone release was severely impaired by removal of ATP from the high-Ca^{2+} medium (Fig. 7a). Under stimulatory

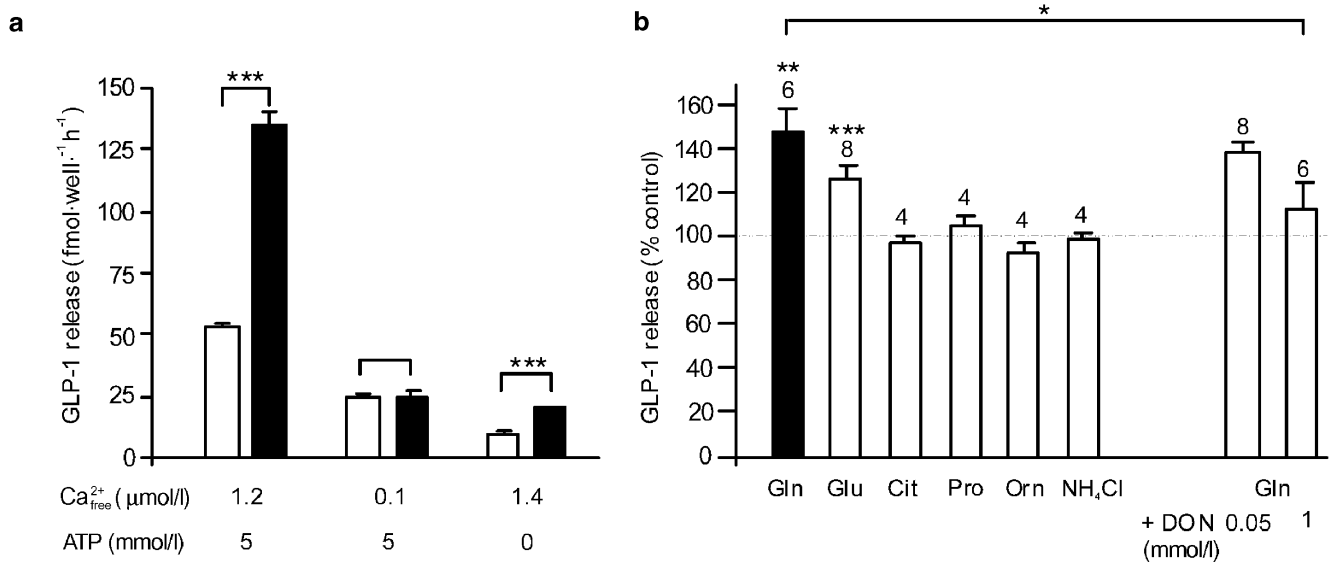

Fig. 7. Effects of glutamine and its metabolites in permeabilised cells. **a.** GLP-1 secretion from GLUTag cells permeabilised with α-haemolysin and subsequently cultured with 10 mmol/l glutamine at concentrations of ATP and free Ca^{2+} (estimated using BAD4-software) as indicated. Open bars, no glutamine; closed bars, 10 mmol/l glutamine. n=4 for each condition. ***$p<0.001$ vs no glutamine. Where bars are not marked with asterisks, results were not significant. **b.** GLP-1 secretion from permeabilised GLUTag cells in the presence of 5 mmol/l ATP, ~1.2 µmol/l free Ca^{2+} and 10 mmol/l glutamine (Gln), glutamate (Glu), citrulline (Cit), proline (Pro), ornithine (Orn) or 1 mmol/l ammonium chloride as indicated, or with 10 mmol/l glutamine in the presence of DON (50 µmol/l or 1 mmol/l). Secretion was normalised to the baseline rate measured on the same day in the absence of nutrients (mean 162±52 fmol·well^{-1}·h^{-1}; n=6). The number of wells is indicated above the bars. *$p<0.05$, **$p<0.01$, ***$p<0.001$ vs baseline or, in the case of DON, vs 10 mmol/l glutamine. Where bars are not marked with asterisks, results were not significant

conditions (high free Ca^{2+}, 5 mmol/l ATP), glutamine was more effective than any of the metabolites tested (Fig. 7b).

We further investigated whether conversion to glutamate is required for the action of glutamine, by application of the glutaminase inhibitor DON in non-permeabilised and α-haemolysin-permeabilised cells. In non-permeabilised cells, 20 μmol/l DON did not significantly affect the secretory response to 1 mmol/l glutamine, but 1 mmol/l DON reduced the response by approximately 75% (Table 1). The inhibitory action of 1 mmol/l DON was specific to glutamine-triggered GLP-1 release, as it impaired neither basal secretion nor secretion triggered by 10 mmol/l glucose or forskolin plus IBMX (10 μmol/l each). In α-haemolysin-permeabilised cells, the response to 10 mmol/l glutamine was also inhibited by high, but not low, concentrations of DON (Fig. 7b). While low concentrations of DON block glutaminase in pancreatic beta cells [33], higher concentrations also block a range of other glutamine-utilising pathways [34]. In support of the lack of importance of carbon entry into the citric acid cycle via glutamate dehydrogenase (GDH), the response to 1 mmol/l glutamine in non-permeabilised cells was not significantly affected by the allosteric GDH activator BCH (10 mmol/l; Table 1).

To investigate whether specific protein kinases were necessary for the response to glutamine, we applied a range of kinase inhibitors (Table 1). The PKA inhibitor H-89 did not significantly affect glutamine-triggered GLP-1 release, suggesting that the response does not involve raised cAMP levels. This was further supported by the finding that the additional enhancement of secretion by glutamine was maintained in the presence of 10 μmol/l forskolin plus 10 μmol/l IBMX (4.5-fold increase by 10 mmol/l glutamine vs 3.0-fold increase by 25 mmol/l glucose; from a baseline secretory rate of 139±12 fmol·well^{-1}·2 h^{-1} in forskolin/IBMX alone). The PKC inhibitor BIM-1 also had no effect on glutamine-induced secretion. Inhibition of CaM kinase by KN-93 reduced the response to glutamine by approximately 60%, but the effect was not specific to glutamine, as KN-93 similarly impaired the secretory responses to glucose and alanine (data not shown). We also tested whether the intracellular amino acid target mTOR (mammalian target of rapamycin) played a role [35], by addition of 100 ng/ml rapamycin. This did not, however, significantly impair the response to glutamine.

Discussion

Our results show that glutamine is a potent stimulus of GLP-1 secretion from GLUTag cells. The median effective concentration was approximately 0.5 mmol/l, close to the normal plasma glutamine concentration [36], suggesting that physiological changes in glutamine following a meal could modulate GLP-1 release. Glutamine acted as both an initiator and a potentiator of secretion. The initiating stimulus triggered membrane depolarisation and action potential generation. However, the markedly potent effect of glutamine on secretion, compared with other amino acids or glucose, appears to be attributable to its ability to potentiate later stages of the secretory pathway. The ability of glutamine to stimulate GLP-1 secretion within 5 minutes of its application suggests that it acts by enhancing the release of preformed secretory vesicles.

Triggering pathway. Glutamine triggered GLP-1 release from GLUTag cells by generating a depolarising current of approximately 3 pA per cell. As the typical membrane resistance of GLUTag cells was 2 to 5 GΩ, this would be sufficient to depolarise the cells by approximately 5 to 15 mV, thereby initiating action potential firing and calcium entry. Both the glutamine-induced inward current and the calcium transient were mimicked using 10 mmol/l asparagine and abolished by extracellular sodium removal. Taken together with the ability of MeAIB to induce calcium transients and GLP-1 release, and the results of the RT-PCR, these findings suggest that electrogenic glutamine entry via ATA-2 contributes to the depolarising action of these amino acids. The 10 mmol/l MeAIB also inhibited GLP-1 secretion in response to 1 mmol/l glutamine, albeit only partially. This might be explained by the competitive nature of this inhibitor and also by the fact that GLUTag cells express a number of other glutamine-carrying transporters. ASCT-2 and y^+LAT2, for example, gave strong RT-PCR bands and might therefore play a role in total glutamine uptake, although both of these transporters catalyse electroneutral amino acid exchange [30, 37] and would not, therefore, directly contribute to membrane depolarisation. The increase in intracellular Ca^{2+} from approximately 100 nmol/l to approximately 600 to 1000 nmol/l, triggered by glucose, glutamine and asparagine, is similar to that which triggers exocytosis in other secretory cells.

Amplifying pathway. The efficacy of glutamine in stimulating GLP-1 release cannot be accounted for by its depolarising action only, as asparagine and alanine were less effective as secretagogues even though they induced currents and calcium transients that were, if anything, larger than those produced by glutamine. The idea that glutamine has an independent effect on secretion was supported by further experiments carried out under conditions that eliminate the effects of small depolarising currents. Measuring secretion in the presence of diazoxide and a depolarising concentration of KCl (30 mmol/l) is routinely used to demonstrate an amplifying effect of glucose on insulin release [31]. Under these conditions, glutamine still en-

hanced GLP-1 release more effectively than glucose or alanine.

To investigate whether glutamine metabolites were responsible for the amplifying action of glutamine, we measured GLP-1 secretion in cells permeabilised by α-haemolysin. In permeabilised cells with fixed stimulatory concentrations of free Ca^{2+} and ATP, glutamine remained a potent trigger of secretion. This action was severely impaired by removal of ATP or reduction of the free Ca^{2+} concentration to basal levels. The principal gastrointestinal metabolites of glutamine (glutamate, proline, ornithine, citrulline and ammonium) [21] were less effective or inactive as secretagogues, suggesting that metabolism via these pathways is not responsible for the amplifying action of glutamine. This is supported by the finding that low concentrations of DON did not markedly impair the response. The metabolic generation of ATP is also unlikely to be directly implicated, as ATP levels were elevated less by glutamine than by glucose. Furthermore, BCH, which activates glutamate dehydrogenase and enhances carbon flux from glutamate into the citric acid cycle, did not enhance GLP-1 secretion in the presence of glutamine. The results suggest that the potentiating effect of glutamine is mediated either by the amino acid itself or by the product of a different metabolic pathway. Similar, but not identical, conclusions have been reached in studies investigating a role for glutamine in insulin secretion from pancreatic beta cells [33, 38].

Although PKA and PKC have been previously shown to play a role in GLP-1 secretion from GLUTag cells [39], inhibition of these protein kinases did not affect glutamine-stimulated secretion. Application of the CaM kinase inhibitor KN93 impaired the action of glutamine, glucose and alanine, suggesting that it acts at a common step in the secretory pathway. However, previous results show that the related CaM kinase inhibitor KN62 also blocks L-type Ca-channels [40].

Physiological significance. Glutamine is the major metabolic fuel of the gut and has been shown to have beneficial effects on gut integrity. It is therefore perhaps not surprising that it has such a profound effect on GLP-1 release. If the concentration dependence of GLP-1 release in vivo mirrors that observed in GLUTag cells, the glutamine content of a meal could provide a significant stimulus to the L cells. Indeed, it has been shown that glutamine significantly increased PYY release after small bowel resection in rats [41]. Studies are now required to test whether glutamine stimulates or potentiates GLP-1 release in vivo, and whether glutamine supplementation could be used as a nutritional therapy to enhance GLP-1 and PYY concentrations in diabetic and obese subjects. A further interesting question is whether enhanced GLP-2 concentrations, which might occur following glutamine supplementation, contribute to the positive effect of glutamine on gut function.

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