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GLUCOSE PROMOTION OF GABA METABOLISM CONTRIBUTES TO THE STIMULATION OF INSULIN SECRETION IN β -CELLS

Short Title: Stimulation of islet insulin secretion by an increased GABA metabolism

Keywords: GABA, glucose, insulin, pancreatic islets, GAD, GABA transaminase

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¹Abbreviations: GABA (γ -amino butyric acid); SSA (succinic acid semialdehyde); KIC (oxo-4-methylpentanoate or α -ketoisocaproate); GAD (glutamic acid decarboxylase); GABA-T (GABA transaminase); SSA-R (semialdehyde succinic acid reductase); SSA-dh (semialdehyde succinic acid dehydrogenase); KRBH (Krebs-Ringer bicarbonate HEPES buffer; 2-OG-dh (2-oxoglutarate dehydrogenase); Succ-dh (succinic acid dehydrogenase); MAD (malonic acid dimethyl ester); K_{ATP} channel (ATP sensitive K⁺ channel)

SYNOPSIS

We have recently demonstrated that branched-chain α -ketoacid stimulation of insulin secretion is dependent on islet GABA metabolism: GABA transamination to semialdehyde succinic acid is increased by 2-oxoglutarate, generated in α -ketoacid transamination to its corresponding α -amino acid. This work aimed at investigating whether glucose also promotes islet GABA metabolism and whether the latter contributes to the stimulation of insulin secretion. Glucose (20 mM) decreased both the content and release of islet GABA. Gabaculine (1 mM), a GABA transaminase inhibitor, partially suppressed the secretory response of rat perfused islets to 20 mM glucose at different L-glutamine concentrations (0, 1 and 10 mM) as well as sugar-induced decrease of islet GABA. The drug also reduced islet ATP content and the ATP/ADP ratio at 20 mM glucose. Exogenous semialdehyde succinic acid dose-dependently increased islet GABA content by reversal of GABA transamination and induced biphasic insulin secretion in the absence of glucose. It depolarized isolated β -cells and triggered action potential firing, accompanied by a reduction of membrane currents through ATP-sensitive K^+ channels. Gene expression and enzyme activity of GABA transaminase were several-fold higher than those of 2-oxoglutarate dehydrogenase in islet homogenates. We conclude that, at high glucose concentrations, there is an increased diversion of glucose metabolism from the citric acid cycle into the "GABA shunt". Semialdehyde succinic acid is a permeable "GABA-shunt" metabolite that increases ATP and the ATP/ADP ratio, depolarizes β -cells, and stimulates insulin secretion. In summary, an increased islet GABA metabolism may trigger insulin secretion.

INTRODUCTION

Pancreatic β -cells contain high amounts of GABA [1-2] and express glutamic acid decarboxylase (GAD, isoforms 65 and 67), which catalyzes its synthesis [3-6]. GABA localizes to the rough endoplasmic reticulum, mitochondria and synaptic-like microvesicles (SLMV) [7, 8]. Recent studies also suggest that GABA is taken up by the insulin-containing secretory granules [8, 9]. GABA uptake into secretory vesicles is mediated by the vesicular GABA transporter VGAT and dependent on a proton gradient generated by the vesicular H^+ -ATPase [10, 11]. GABA is subsequently released from β -cells by regulated exocytosis [9] and a poorly characterized but quantitatively important tonic release by a non-vesicular mechanism [9, 12].

GABA released from β -cells has been shown to exert a paracrine inhibitory effect on glucagon secretion from neighbouring α -cells [13-15] as well as an autocrine suppressor effect on insulin secretion [16]. The presence of GABA_B-receptors in β -cells [16, 17] and of GABA_A-receptors in α -cells [13, 15] supports the putative autocrine/paracrine role of locally secreted GABA in islets.

Although an intracellular function of GABA in β -cells has also been suggested [18], this area is less well explored. The presence of all enzymatic activities required for the function of the "GABA-shunt" has been demonstrated in islet homogenates [3-6, 19-22]. GABA is produced by the decarboxylation of Glu (by glutamic acid decarboxylase, GAD) and it enters the "GABA-shunt" after its transamination with 2-oxoglutarate (GABA transaminase, GABA-T) to succinic acid semialdehyde (SSA) and Glu. SSA may be oxidized to succinic acid (NAD⁺-dependent SSA dehydrogenase, SSA-dh) and enter the citric acid cycle. Alternatively, SSA might be reduced to γ -aminobutyric acid (NADP⁺-dependent SSA reductase, SSA-R) and exit the "GABA-shunt" (see the

scheme in reference 25). GABA has a high metabolic turnover rate in islets [20] determined by the balance between synthesis, catabolism, and release. Acutely, glucose stimulates GABA release from β -cells over-expressing GABA_A-receptors [22]. By contrast, in long-term experiments (1-24 h) high glucose promotes islet GABA catabolism and decreases its content, resulting in a lower rate of release [21]. It has been shown that increasing the extracellular L-glutamine concentration increases both islet GABA content and GABA release [19-22]. While a decreased GABA content has been associated with an increased insulin secretion [21], overexpression of GAD65 in transgenic mice or INS-1 cells results in impaired insulin release [23, 24] but it was not determined whether intracellular GABA levels were correspondingly modified.

We could recently demonstrate that oxo-4-methylpentanoic acid (KIC) promotes islet cells GABA metabolism whose inhibition by gabaculin suppresses insulin secretion [20, 25]: oxo-4-methylpentanoic acid (KIC) transamination to L-leucine at the expenses of Glu generates 2-oxoglutarate that may be used by GABA-transaminase (GABA-T) to initiate "GABA-shunt" metabolism. As glucose metabolism in the citric acid cycle can also generate 2-oxoglutarate, the latter might also be used by GABA-T to promote "GABA-shunt" metabolism. Therefore, we have investigated how glucose affects intracellular GABA metabolism and whether this contributes to the stimulation of insulin secretion.

EXPERIMENTAL

Materials

Collagenase P and free-FFA bovine serum albumin were obtained from Roche Diagnostics S.L. (Barcelona, Spain). Gabaculine, bovine serum albumin and most of the substances, enzymes and coenzymes were obtained from Sigma-Aldrich Química S.A. (Madrid, Spain). Rat insulin was from Linco Research, Inc. (St. Charles, Missouri, U.S.A.). Na¹²⁵I was obtained from PerkinElmer España, S.L. (Madrid, Spain). Inorganic compounds and organic solvents were obtained from VWR International Eurolab S.L.(Spain).

Islet insulin secretion

Islets were isolated from the pancreas of male Wistar-albino rats (250-275 g BW) by collagenase digestion. Insulin secretion was studied in perifused islets. Four groups, each of 40 rat islets, were perifused in parallel and at a flow rate of 0.5 ml/min with Krebs-Ringer, buffered with 0.5 mM NaHCO₃ and 20 mM HEPES, supplemented with 0.5 % FFA-free BSA (KRBH), and heated at 37°C. The perifusion pattern was similar in all the experiments. After a pre-perifusion period of 45 minutes under basal conditions (in the absence of nutrients or at 5 mM glucose), the perifusion medium was switched to one containing the test substances and maintained for the next 30 minutes. Finally, the medium was changed back to pre-perifusion conditions where it was maintained for the last 25 minutes. The perfusate was collected at 1 minute intervals during the last 60-70 minutes of perifusion and its insulin concentration radioimmunologically measured. Pig insulin was radio-iodinated with Na¹²⁵I [26] and rat insulin was used as a standard in the radioimmunoassay of insulin. Insulin antiserum was kindly provided by Dr. Janove Sehlin from the Department of Medical Cell Biology at the University of Umeå (Sweden).

Islet amino acid measurement

Islet amino acids were separated by reverse-phase HPLC after precolumn derivatization with o-phthalaldehyde [27] and quantified by fluorescence detection. The content and release of the measured islet amino acids (Asp, Glu, Ser, Gln, His, Gly, Thr, Arg, Tau, Ala, Tyr, and GABA) were studied following previously published methods [19,

20]. Three groups, each of 20 to 30 islets, were pre-incubated for 1 hour at 37°C with different Gln concentrations. After discarding the medium and washing the islets twice with saline, they were further incubated for a second hour at 0 or 20 mM glucose. In the cases in which amino acid release was not studied, the islets were only incubated for 1 hour. The incubations were stopped by placing the islets on ice. Islets were washed twice with PBS and their amino acids extracted with 35% (w/v) 5-sulfosalicylic acid. Aliquots of the incubation medium were also saved for measurements of amino acid release. The amount of protein was measured in the islet extracts with the method of Lowry [28] and BSA was used as the standard.

Islet adenine nucleotides measurement

Total islet ATP content was measured with the luciferin-luciferase system [18]. Islets (groups of 25) were incubated at 37 °C for 60 minutes in 25 µl of KRBH+BSA. The incubation was stopped on acetone chilled with dry ice and 20 µl of 1.35 M perchloric (PCA) were added. PCA was neutralized and precipitated with 15 µl of 0.1 M Tris + 2.8 M KHCO₃. Supernatant aliquots and ATP standard solutions (1-50 µM) were then added to a luciferin-luciferase mixture and the emitted light was measured in a bioluminometer. Luciferase (1 mg/ml) and D-luciferin (0.1 mM) were dissolved in a buffer containing 50 mM HEPES, 10 mM MgCl₂, and 0.1 % (w/v) fatty acid-free BSA, and adjusted to pH 7.6. The luciferin-luciferase mixture was prepared by adding 10µl of the luciferase solution to 400 µl of 0.1 mM luciferin. ADP was converted to ATP by pyruvate kinase (10 IU/ml) in the presence of 1 mM phosphoenolpyruvate.

RNA extraction and retrotranscription

Total RNA was harvested from four rats' livers (< 20 mg) and from each group of islets isolated from six rats using the RNeasy[®] Mini, RNA isolation kit (Qiagen, Izasa S.A., Madrid, Spain) and total RNA from cerebellum (< 100 mg) was harvested from three rats using RNeasy Lipid Tissue Mini Kit (Qiagen, Izasa S.A., Madrid, Spain), according to manufacturer's protocol. Total RNA was eluted from the RNeasy[®] Mini columns with 30 µl of RNase-free water. RNA retrotranscription was performed with the Quantitect[®] Reverse Transcription Kit (Qiagen, Izasa S.A., Madrid, Spain), as per manufacturer's instructions.

Real-time quantitative PCR

Succinic dehydrogenase, GABA transaminase, 2-oxoglutarate dehydrogenase (E2 subunit), semialdehyde succinic reductase (AKR7A2) and glutamic acid decarboxylase (GAD65) genes expression were evaluated using TaqMan[®] Gene Expression Assays (20X) (Applied Biosystems, Applera Hispania S.A., Madrid, Spain). Semialdehyde succinic dehydrogenase gene expression was evaluated using Custom Gene Expression Assay (20X) (Applied Biosystems, Applera Hispania S.A., Madrid, Spain) using the extreme 3' end of the genetic sequence (L34821). Two internal controls were used: 18S for tissue-comparison and GAPDH for intra-islet comparison (TaqMan[®] Gene Expression Assays (20X), Applied Biosystems, Applera Hispania S.A., Madrid, Spain). FastStart TaqMan[®] Probe Master (Rox) (Roche Diagnostics S.L.) supplemented with Rox (Roche Diagnostics S.L.) to 820 nM was used as master mix. Amplification was performed in a 7300 Real Time PCR System (Applied Biosystems, Applera Hispania S.A.). Gene expression is represented as 2^{-ΔΔCt} values and the statistical comparisons made between mean 2^{-ΔCt} values of 3 to 6 experiments.

Enzymes' activities

Isolated islets were homogenized by sonication (four times, ten strokes at 10-20 % of the cycle and the minimum potency of a Branson sonifier 450) in a buffer containing 10 mM KH₂PO₄, 0.5 mM EDTA, leupeptin (60 µM), aprotinin (0.019 TIU/ml), PMSF (2 mM in DMSO), β-mercaptoethanol (10 mM), and Triton-X100 (0.5 %, vol/vol) (pH=

7.2). The islet homogenate was then centrifuged at 4°C and 141,500 g for 30 minutes in a 100.Ti rotor of the ultracentrifuge model Optima™-L-100 XP (Beckman Coulter España S.A.).

2-Oxoglutarate dehydrogenase (2-OG-dh) activity in islets' homogenates was studied as the amount of NADH produced at saturating concentrations of 2-oxoglutarate (10 mM) and NAD⁺ (2.5 mM) and measured fluorometrically in a multiwell plate (Costar assay plate no. 3915, Corning Incorporated, NY, U.S.A.) recorder (Varioskan model, Bioanalysis Labsystems S.A., Barcelona, Spain). The enzyme reaction was performed in a 50 mM KH₂PO₄ buffer (pH=7.2) supplemented with 4 µM rotenone, 0.5 mM coenzyme A, 0.3 mM thiamine pyrophosphate, 2.5 mM dithiotreitol, 2 mM GTP, 0.8 mM CaCl₂, 1 mM MgCl, 5 mg/ml BSA and the substrates.

GABA transaminase (GABA-T) activity in islets' homogenates was studied as the amount of NADH produced at saturating concentrations of succinic acid semialdehyde (SSA, 10 mM), Glu (5mM) and NAD⁺ (2.5 mM) in the presence of exogenous 2-oxoglutarate dehydrogenase (0.18 IU/ml) and measured as described above. The enzyme reaction was performed in the same buffer used for 2-OG-dh (pH= 7.2). In parallel aliquots of islet homogenates, the activity of SSA dehydrogenase (SSA-dh) was measured in the absence of exogenous 2-OG-dh and this activity was subtracted from the overall GABA transaminase reaction. All enzyme activities were calculated as the slopes of the linear increase of NADH fluorescence expressed as the amount of formed product / min x mg protein by reference to a nucleotide standard curve. The amount of protein was measured in the islet extracts with the method of Lowry [21] and BSA was used as the standard.

Electrophysiology

Patch-clamp experiments performed in the perforated-patch whole-cell configuration, using an EPC9 patch-clamp amplifier and Pulse software (HEKA Electronics, Lambrecht, Germany). The extracellular solution consisted of (in mM) 140 NaCl, 3.6 KCl, 0.5 MgSO₄, 1.5 CaCl₂, 10 HEPES, 0.5 NaH₂PO₄, 5 NaHCO₃ (pH adjusted to 7.4 with NaOH) and 1 glucose. The pipette solution contained (in mM) 76 K₂SO₄, 10 KCl, 10 NaCl, 1 MgCl₂ and 5 HEPES (pH 7.35 with KOH). Electrical contact was established by inclusion of amphotericin B (0.24 mg/ml) in the pipette solution. β-cells were identified based on the inactivation properties of voltage-gated Na⁺-currents. All measurements were carried out at 32-33°C.

RESULTS

Islet amino acids

We examined the effects of extracellular Gln on the islet content of Asp, Gln, Glu and GABA. Rat islets were first incubated in the presence of increasing concentrations of Gln during one hour, followed by incubation in the absence of Gln for another hour at either 0 or 20 mM glucose. The amounts of amino acids within the islets were determined at the end of the second hour. Gln induced a concentration-dependent increase in the islet content of Glu (Fig. 1A), Asp (Fig. 1B) and GABA (Fig. 1C), but not Gln (results not shown). Glucose (20 mM) reduced the islet content of Glu in the absence of Gln (Fig. 1A) but did not modify the content of GABA under these conditions (Fig. 1C). GABA content was reduced by glucose by ~40% in islets preloaded with either 0.5 (p<0.01) or 10 mM Gln (p<0.001; Fig. 1C). The amounts of the amino acids measured in the extracellular medium at the end of the second hour provide an estimate of the overall rate of release. Glucose (20 mM) decreased the release of

Asp, Glu, and GABA by 14-75% (1A-C, right). It also reduced Gln release from islets pre-exposed to 10 mM Gln during the first hour (result not shown). Fig. 1D summarizes the correlation between GABA content measured at the 0, 0.5 and 10 mM Gln and the amount of GABA released. It can be seen that release is linearly related to the content of the amino acid ($r = 0.95 \pm 0.15$, $n = 6$; $p = 0.01$).

As islet GABA content was decreased by 20 mM glucose without any concomitant increase of amine release, it was assumed that an increased GABA metabolism might have occurred. D-Mannoheptulose, a specific competitive glucokinase inhibitor [29], was used to confirm if the observed decrease of islet GABA content was dependent on glucose metabolism. 10 mM D-mannoheptulose increased the islet GABA content at 20 mM glucose by more than 70% whereas neither 10 mM 2-deoxyglucose (non-competitive inhibitor of low-K_m-hexokinases), nor 10 mM malonic acid dimethylester (MAD, a succinic acid dehydrogenase inhibitor) had any effect (online Fig.1). Islet Asp was similarly increased by 10 mM D-mannoheptulose, unaffected by 10 mM 2-deoxy-D-glucose but decreased by 10 mM malonic acid dimethylester (online Fig.1). Measurement of the amino acid contents at the end of perfusion experiments (shown in Figure 4A) revealed that 1 mM gabaculine, a specific GABA-T inhibitor [30], more than doubled islet GABA content at 20 mM glucose (104.8 ± 12.6 , $n = 6$ vs. 51.6 ± 10.1 pmol/ μ g islet protein, $n = 6$; $p < 0.009$) and decreased Asp (17.9 ± 2.0 , $n = 5$ vs. 29.9 ± 3.9 pmol/ μ g islet protein, $n = 5$; $p < 0.03$). Allylglycine, a precursor of a competitive inhibitor of glutamic acid decarboxylase (GAD) (31), suppressed basal levels of islet GABA (in the absence of any substrate) at a concentration of 10 mM (31.2 ± 2.8 , $n = 4$ vs. 49.1 ± 2.8 pmol/ μ g islet protein, $n = 4$; $p < 0.004$) or 20 mM (31.1 ± 1.8 , $n = 4$ vs. 49.1 ± 2.8 pmol/ μ g islet protein, $n = 4$; $p < 0.002$). Semialdehyde succinic acid (SSA) is an intermediary metabolite in the "GABA shunt" pathway and originates in the transamination of GABA by a specific transaminase. As transamination is a reversible process, we checked whether SSA was membrane permeable and then able to increase the intracellular GABA concentration of islet cells. Islet GABA content was concentration-dependently increased by SSA (2 mM: 64.5 ± 10.5 , $n = 4$ vs. 49.1 ± 2.8 pmol/ μ g islet protein, $n = 4$, N.S.; 5 mM: 104.0 ± 11.7 , $n = 4$ vs. 49.1 ± 2.8 pmol/ μ g islet protein, $n = 4$; $p < 0.004$; 10 mM: 108.8 ± 6.0 , $n = 4$ vs. 49.1 ± 2.8 pmol/ μ g islet protein, $n = 4$; $p < 0.0001$). No significant change was observed in any of the other α -amino acids measured.

Islet ATP

As expected, 20 mM glucose increased islet ATP content (4.61 ± 0.08 , $n = 17$ vs. 2.82 ± 0.14 pmol/ islet, $n = 18$; $p < 0.0001$) as well as the ATP/ADP ratio (2.14 ± 0.16 , $n = 17$ vs. 1.19 ± 0.06 , $n = 18$; $p < 0.0001$) by 60-80 % in comparison with 3 mM glucose. As shown in Figure 2, both islet ATP content and ATP/ADP ratio measured in the presence of 20 mM glucose were significantly decreased by incubation with 1 mM gabaculine (without previous pre-treatment) irrespective of the L-glutamine concentration (0, 1, or 10 mM). Oxo-4-methylpentanoic acid (KIC, 10 mM), a well known nutrient insulin secretagogue, increased islet ATP content (4.4 ± 0.1 , $n = 3$ vs. 2.8 ± 0.1 pmol/ islet, $n = 18$; $p < 0.0003$) and the ATP/ADP ratio (1.9 ± 0.3 , $n = 2$ vs. 1.2 ± 0.06 , $n = 18$; $p < 0.003$) in the presence of 3 mM glucose. 1 mM Gabaculine decreased the elevation of islet ATP content (2.6 ± 0.3 , $n = 3$ vs. 4.4 ± 0.1 pmol/ islet, $n = 3$; $p < 0.005$) and ATP/ADP ratio (1.0 ± 0.1 , $n = 3$ vs. 1.9 ± 0.3 , $n = 2$; $p < 0.04$) induced by 10 mM KIC. 10 mM SSA also increased islet ATP content both in the absence (+ 93.5 %) and presence (+ 45.0 %) of 3 mM glucose (Fig. 2) and elevated the corresponding ATP/ADP ratios by 84 and 26 %, respectively.

respectively. Islet pre-treatment with 1 mM gabaculin for 45 minutes (as in the perfusion experiments shown in Figure 6B), decreased the ATP content of islets incubated with 10 mM SSA and the GABA-T inhibitor during the 30 following minutes (2.7 ± 0.2 , $n=3$ vs. 3.3 ± 0.08 pmol/ islet, $n=3$; $p < 0.047$). MAD suppressed the elevation of islet ATP levels by 10 mM SSA at 20 (4.1 ± 0.05 , $n=3$ vs. 4.6 ± 0.1 pmol/ islet, $n=7$; $p < 0.035$) and 40 mM (3.2 ± 0.2 , $n=5$ vs. 4.6 ± 0.1 pmol/ islet, $n=7$; $p < 0.00003$). MAD was more effective reducing islet ATP content at 20 mM glucose: a significant decrease was already obtained at 10 mM (data not shown). This is probably due to a stronger competition between MAD and the succinic acid generated by SSA as compared with 20 mM glucose. 2 mM Fluoroacetate, an aconitase inhibitor, also suppressed the elevation of islet ATP content by 10 mM SSA (3.1 ± 0.1 , $n=4$ vs. 4.6 ± 0.1 pmol/ islet, $n=7$; $p < 0.00003$), suggesting that the generated succinic acid may be converted into acetyl-CoA (via the malate / pyruvate cycle) and further metabolized in the citric acid cycle.

Islet perfusion experiments

Perfused rat islets responded with a biphasic release of insulin after stimulation with 20 mM glucose (Figure 3A). Gabaculine (1 mM), when included during the pre-perfusion at 5 mM glucose for 45 minutes as well as the subsequent stimulation by 20 mM glucose, significantly reduced both the first phase (13.3 ± 2.0 , $n=5$ vs. 25.5 ± 4.0 ng /40 islets x 10 min, $n=5$; $p < 0.025$) and the second phase (initial 20 minutes, 65.1 ± 6.5 , $n=5$ vs. 95.3 ± 10.4 ng /40 islets x 20 min, $n=5$; $p < 0.031$) of the response to glucose elevation (Figure 3A).

Gln is the most abundant α -amino acid in the blood and the main precursor for the synthesis of GABA. GABA reaches a close to maximal intracellular content at physiological Gln concentrations (0.5 to 1.0 mM) (Fig. 1) [19]. Gln does not stimulate insulin secretion, nor does it potentiate the secretory response to glucose, as deduced from the statistical comparisons among perfusions in Figure 3. However, it is conceivable that in its presence the contribution of "GABA-shunt" metabolism to total islet metabolism would be higher when the islets were stimulated by glucose. At 1 mM Gln, islets treated with only 0.25 mM gabaculine during pre-perfusion for 45 minutes showed a trend to decreased second phase insulin secretion (93.0 ± 10.2 , $n=6$ vs. 124.6 ± 10.7 ng /40 islets x 20 min, $n=7$; $p = 0.057$) when subsequently stimulated with 20 mM glucose in the presence of the inhibitor: as indicated in Figure 3B, there were statistically significant differences between the secretory rate of control and treated islets along several time points of the second phase of secretion. At a supraphysiological Gln concentration of 10 mM, a similar treatment with 1 mM gabaculin induced significant decreases of both the first (22.0 ± 3.2 , $n=6$ vs. 31.2 ± 2.1 ng /40 islets x 10 min, $n=6$; $p < 0.037$) and second phase of insulin release (50.0 ± 6.2 , $n=5$ vs. 84.0 ± 5.3 ng /40 islets x 20 min, $n=6$; $p < 0.002$) (Fig. 3C).

Pre-perfusion of islets with 5 mM glucose and 20 mM allylglycine, a GAD inhibitor precursor, during the 45 minutes preceding the stimulation with 20 mM glucose, suppressed the total amount of insulin released during the stimulation period by 35% (62.8 ± 12.9 , $n=4$ vs. 97.0 ± 8.0 ng /40 islets x 30 min, $n=6$; $p < 0.05$) (online Fig. 2). No significant effect of 20 mM allylglycine was recorded during islet stimulation with 7 mM glucose (data not shown).

Islet depolarization with high KCl (70 mM) induced a monophasic insulin secretion in islets perfused with 5 mM glucose (data not shown). It also stimulates GABA release and produces a secondary decrease of the amine content: Under this condition, the

insulin secretory response to oxo-4-methylpentanoic acid (KIC) is strongly suppressed [25]. The stimulation of insulin secretion by 20 mM glucose was also decreased in the presence of 70 mM KCl, although not as intensively as the response to KIC, and affected mainly the second phase of release (58.3 ± 9.1 , $n=5$ vs. 89.0 ± 6.4 ng /40 islets x 20 min, $n=12$; $p<0.03$) (Figure 4A).

Similarly, 70 mM KCl suppressed the second phase of the islet secretory response to 20 mM glucose in presence of 10 mM Gln (49.4 ± 11.8 , $n=6$ vs. 94.5 ± 14.7 ng /40 islets x 20 min, $n=4$; $p<0.05$) (Figure 4B). Islet pre-exposure to 5 mM glucose plus 70 mM KCl for 45 minutes induced a stronger suppression of both the first (7.3 ± 1.2 , $n=5$ vs. 23.6 ± 4.5 ng /40 islets x 10 min, $n=6$; $p<0.01$) and the second phase (40.8 ± 6.7 , $n=5$ vs. 80.6 ± 13.8 ng /40 islets x 20 min, $n=6$; $p<0.04$) of the insulin response to 20 mM glucose (Figure 4C). Insulin content of islets pre-incubated for 1 hour at 5 mM glucose plus 70 mM KCl and a second hour at 20mM glucose was the same as in the controls, incubated at 5 mM glucose during the first hour and at 20 mM glucose during the second hour (643.3 ± 59.9 , $n=6$ vs. 597.0 ± 42.9 ng insulin/islet, $n=6$; N.S.). Addition of 10 mM L-glutamine together with 20 mM glucose did not reverse KCl-induced inhibition (data not shown).

10 mM SSA elicited biphasic insulin secretion in the presence of a basal (5 mM) glucose concentration that was similar in magnitude to that triggered by 20 mM glucose (Figure 5A). The combination of 5 or 10 mM SSA together with 20 mM glucose did not modify the magnitude, nor the pattern, of the insulin response induced by glucose alone (Figure 5B). In the absence of glucose, 10 mM SSA induced a much lower first (10.8 ± 1.6 , $n=12$ vs. 19.2 ± 2.7 ng /40 islets x 10 min, $n=6$; $p<0.01$) and second phase of insulin secretion (48.4 ± 3.7 , $n=12$ vs. 64.0 ± 5.6 ng /40 islets x 20 min, $n=6$; $p<0.03$) than in presence of 5 mM glucose (compare Figures 5A and 6A). Biphasic insulin secretion induced by 10 mM SSA was unaffected by the presence of Gln (Figure 6C). Pre-perifusion with 1 mM gabaculine for 45 minutes suppressed the second phase of insulin secretion stimulated by 10 mM SSA both in the absence (32.6 ± 2.5 , $n=6$ vs. 50.9 ± 5.6 ng /40 islets x 10 min, $n=6$; $p<0.01$) and presence of 10 mM L-glutamine (30.6 ± 6.1 , $n=5$ vs. 61.1 ± 8.7 ng /40 islets x 10 min, $n=5$; $p<0.02$) (Figures 6B and 6C, respectively). β -Cell depolarization with 70 mM KCl did not modify the secretory response to 10 mM SSA alone in perifused islets (online Fig. 4).

SSA effects on β -cell membrane potential

Addition of SSA (10 mM, at 1 mM extracellular glucose) to the extracellular medium depolarized isolated β -cells and induced action potential firing (Fig. 7A and B). This was accompanied by a reduction of membrane currents through ATP-sensitive K^+ channels (K_{ATP} channels; Fig. 7C). The ability of SSA to depolarize β -cells and inhibit K_{ATP} channels was preserved in cells that had been incubated with gabaculine (1 mM) in the culture medium for 1 h prior to the experiments (data not shown). Increasing the extracellular glucose concentration from 1 to 7, or 20 mM, depolarized β -cells in a concentration-dependent manner. This effect of glucose was unaltered when cells had been pre-incubated with gabaculine (1 mM, 1-2 h) prior to the experiments (data not shown).

Islet expression profile of the genes of some citric acid cycle and "GABA-shunt" enzymes and their activities

At the mRNA level, 2-OG-dh was the least expressed among the six genes studied, calculated as percentage of D-glyceraldehyde dehydrogenase expression which was used as the reference, housekeeping gene (online Fig. 3). Very similar results were obtained using 18S rRNA as the reference gene (data not shown). The messages for GAD65 and GABA-T were 6-fold more abundant than that of 2-OG-dh. The “GABA-shunt” enzymes catalyzing SSA oxidation (SSA-dh) and reduction (SSA-R) were expressed at slightly higher levels than 2-OG-dh. The citric acid cycle enzyme accepting the final product of the “GABA-shunt”, Succ-dh, was expressed 3-fold more intensively than the 2-OG-dh gene. This expression profile is similar to that of rat cerebellum but very different from liver tissue which does not express GAD65 (data not shown). A comparative study of 2-OG-dh and GABA-T activities in islet homogenates showed that the former was several fold lower than the latter (2.5 ± 0.1 , $n=4$ vs. 7.0 ± 1.6 nmol NADH/min. x mg. protein, $n=4$; $p<0.03$).

DISCUSSION

High glucose (20 mM) significantly diminished the islet GABA content at a physiological L-glutamine concentration (0.5 mM) without modifying the content of any other α -amino acid and it decreased not only GABA but also Glu and Asp release. Overall analysis of the data at the Gln (0, 0.5 and 10.0 mM) and glucose concentrations (0 and 20 mM) used showed a positive correlation between islet GABA release and content, suggesting that the free (exchangeable) fraction of GABA is proportional to the content. A study of separate correlations revealed that the slope was steeper at 0 than 20 mM glucose (data not shown), indicating a higher usage and less availability of free GABA at high glucose. The stable islet content of Asp and Glu between 0 and 20 mM glucose probably reflects metabolic changes secondary to an increased succinic acid production in the “GABA shunt” and hence of oxaloacetate which is then transaminated to Asp. The Glu content may be maintained by increased citric acid cycle provision of 2-oxoglutarate at 20 mM glucose. In fact, malonic acid dimethyl ester, a permeable succinic acid dehydrogenase inhibitor [32], decreased islet Asp content without modifying that of GABA or Glu. Moreover, gabaculine inhibition of GABA metabolism significantly decreased Asp content.

It can be reasonably argued that, given that glucose decreases GABA release, the suppression of its content can only be attributable to its increased metabolism via the “GABA shunt”. This effect was dependent on glucose metabolism in the β -cells as inhibition of the latter by D-mannoheptulose blocked the reduction of islet GABA content. This confirms the previously described ability of D-mannoheptulose to suppress glucose-induced decrease of islet GABA content and release in cultured human and rat β -cells at 2 mM L-glutamine [33, 21]. Several findings suggest that glucose promotion of islet GABA metabolism relies on citric acid supply of 2-oxoglutarate that transaminates with GABA to produce Glu and SSA in the first reaction of the “GABA shunt”: 1. The GABA transaminase inhibitors gabaculine (this work) and γ -vinyl-GABA in [21] suppressed glucose-induced reduction of GABA content. 2. Addition of a permeable form of 2-oxoglutarate (its dimethyl ester) increased islet Glu but decreased islet GABA production above a certain concentration [19]. 3. KIC suppresses more strongly than glucose (even in the absence of L-glutamine) islet GABA content in a transamination dependent mechanism: Inhibition of either GABA-T with gabaculine or the branched-chain α -keto acid transaminase (generating L-leucine

and 2-oxoglutarate) with 4-methyl valeric acid, blocked GABA suppression in both cases as well as insulin secretion [20, 25].

In support of the involvement of islet GABA metabolism in the stimulation of insulin secretion, islet pre-treatment with 1 mM gabaculine reduced (by 30 to almost 50%) both phases of the insulin secretory response to 20 mM glucose in the absence and presence of L-glutamine. Additionally, gabaculine diminished the glucose-induced elevation of islet ATP content and ATP/ADP ratio. Moreover, pre-exposure of islets to allylglycine, a GAD inhibitor precursor, also decreased the subsequent secretory response to 20 mM glucose. These data may seem at odds with the failure of gabaculine to interfere with glucose-induced membrane depolarization of isolated β -cells. However, the ATP content of islets incubated with 20 mM glucose plus 1 mM gabaculine was higher than the content of islets incubated at 3 mM glucose (3.47 ± 0.12 , $n=5$, vs. 2.82 ± 0.14 pmol/islet, $n=18$; $p<0.03$). It has previously been shown that the amplifying pathway of glucose stimulation has a higher K_m for glucose than the triggering pathway [34]. It is thus possible that the smaller increase in ATP attained with gabaculine is sufficient to close K_{ATP} channels but not to fully support the amplifying pathway of glucose-induced insulin secretion.

Our data confirm a previous report showing that γ -vinyl-GABA (another GABA-T inhibitor) tends to decrease glucose-induced secretion and significantly (by 40 %) reduces 3-phenylpyruvate stimulated insulin secretion in the perfused rat pancreas [24]. The authors concluded their results were compatible with the hypothesis that GABA metabolism via the GABA shunt might have a role in insulin secretion. At variance with this and our own interpretation, the authors of a recent report [21] suggested that γ -vinyl-GABA decreases glucose-stimulated insulin secretion of 24 hours cultured rat β -cells independently of their modification of GABA metabolism, as the effect was observed only at an inhibitor concentration (>7 mM) well above the concentration range (0.3 and 1.6 mM) required to increase cellular GABA content. However, in our freshly isolated rat islet preparation, an inhibition of insulin secretion was observed at 0.25 and 1.0 mM gabaculine after 45 to 60 minutes of pre-exposure to the drug (see Figure 6). Furthermore, we have previously demonstrated that the simultaneous perfusion of 10 mM KIC together with 0.25 mM gabaculine (without previous exposure to the inhibitor) significantly reduced the magnitude of the biphasic response to the α -keto acid and elevated the islet GABA content (19). The discrepancy between Wang et al. and our own results might be attributable to the different preparations used (24 hours cultured rat β -cells versus the perfused pancreas and freshly isolated rat islets). A role for GABA metabolism in GSIS is further supported by the inhibitory effect of 70 mM KCl as this treatment is known to deplete intracellular GABA and to strongly reduce KIC-induced insulin secretion [25]. However, the mechanism of GABA release from β -cells must be further explored before an unambiguous interpretation of the data can be made.

The stimulation of insulin secretion by SSA, together with its effects on islet adenine nucleotides, constitutes strong evidence in support of the "GABA-shunt" as an alternative or complementary pathway of glucose metabolism. This is corroborated by the ability of SSA to depolarize β -cells through a reduction of the ATP-sensitive K^+ -current. SSA is converted to GABA by reversion of GABA-T catalyzed reaction that also generates 2-oxoglutarate and it is probably also converted to succinic acid by SSAdh. Both succinic acid and 2-oxoglutarate can be metabolized in the citric acid cycle and

contribute to ATP generation. In support of this interpretation, GABA-T inhibition by gabaculine partially suppressed the second phase of the secretory response to SSA. Even though the inhibitor failed to modify SSA-induced membrane depolarization of isolated β -cells, succinic acid production might contribute to ATP generation during the second phase of release. SSA metabolism by the same mitochondrial pathways as glucose explains why the two secretagogues did not show any synergism.

The gene expression profile, as well as the study of the corresponding enzymatic activities, showed that GABA-T is almost three-fold more abundant than 2-OG-dh in islet homogenates. Both enzymes have similar K_m values for their common substrate 2-oxoglutarate. Therefore, it seems reasonable to assume that 2-oxoglutarate generated in the citric acid cycle is preferentially metabolized in the "GABA-shunt" to succinic acid rather than being decarboxylated in the cycle by 2-OG-dh. Moreover, 2-OG-dh is subject to regulation by the NADH / NAD⁺ and ATP/ADP ratios (35) and has long been considered as a rate limiting step in the citric acid cycle (36). Inhibition of 2-OG-dh by an increased ATP/ADP ratio at high glucose concentrations might increase the availability of 2-oxoglutarate for GABA transaminase which would then bypass the citric acid cycle bottleneck via the "GABA shunt" to succinic acid. Therefore, the metabolic flow of glucose in the "GABA shunt" might increase with its concentration. This might explain the partial dependence of glucose-induced insulin secretion on "GABA-shunt" metabolism as previously suggested [24].

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LEGENDS TO FIGURES

Figure 1. Effect of 20 mM glucose on islet Glu (A), Asp (B) and GABA (C) content (isl) and release (med) after pre-incubation with the indicated Gln concentrations. (D) Relationship between GABA content and GABA release.

Three groups, each of 30 islets, were pre-incubated for 1 hour at 37°C with different Gln concentrations (0, 0.5, or 10 mM, as indicated in each graph). After discarding the medium and washing the islets twice with saline, they were further incubated for a second hour in the absence of substrates (G0) or in the presence of 20 mM glucose (G20). At the end of incubation, the medium (med) and islets (isl) were separated for the measurement of their respective content of amino acids. The symbols represent mean values $\pm$ SE of 7-8 different experiments. Each value was statistically compared with its corresponding control obtained in the absence of Gln during the pre-incubation phase (* $p < 0.025$, † $p < 0.01$, ‡ $p < 0.001$). Comparisons between 0 and 20 mM glucose are indicated by brackets.

Figure 2. Effect of 0, 3 and 20 mM glucose (G0, G3 and G20, respectively) and 10 mM semialdehyde succinic acid (SSA) on islet ATP levels and the ATP/ADP ratio. Modification of the adenine nucleotide profile at 20 mM glucose by 1 mM gabaculine (GABA-T inhibitor).

Groups each of 25 islets were incubated for 1 hour at the conditions indicated in the figure. Incubations were stopped by freezing islets and medium and adenine nucleotides extracted with perchloric acid. After neutralization, ATP and ADP contents were determined with the luciferin/luciferase system, as described in detail in the Methods section.

Figure 3. Effect of gabaculine (GABA-T inhibitor) on the secretory response of perfused rat islets to 20 mM glucose in the absence (A) and presence of 1 (B) and 10 mM Gln (C).

Groups of 40 islets each, pre-perfused with 5 mM glucose ($\pm$ 1 mM gabaculine) for 45 min, were stimulated for 30 min (between vertical broken lines) with 20 mM glucose and the indicated additives. Pre-perfusion conditions were then re-established during the last 25 min. Symbols represent means $\pm$ S.E.M.

Figure 4. Modification of the insulin secretory response of rat perfused islets to 20 mM glucose by the concomitant depolarization with 70 mM KCl in the absence (A) and presence of 10 mM Gln (B) or by a previous 45 minutes exposure to 70 mM KCl (C).

Groups of 40 islets each were pre-perfused with 5 mM glucose alone for 45 min (A and B) or together with 70 mM KCl (C). They were then stimulated for 30 min (between vertical broken lines) with 20 mM glucose alone (C) or together with 70 mM KCl (A and B). Pre-perfusion conditions were then re-established during the last 25 min. Symbols represent means $\pm$ S.E.M.

Figure 5. Insulinotropic capacity of semialdehyde succinic acid (SSA) in the presence of 5 (G5) and 20 mM glucose (G20).

Groups of 40 islets each, pre-perfused with G5 alone for 45 min were then stimulated for 30 min (between vertical broken lines) with G20 alone or 5 mM glucose plus 10 mM SSA (A). In (B) secretion was stimulated with G20 together with 5 and 10 mM SSA. Pre-perfusion conditions were then re-established during the last 25 min. Symbols represent means $\pm$ S.E.M.

Figure 6. Effect of Gln and gabaculine (GABA-T inhibitor) on the stimulation of insulin secretion of perfused rat islets by 10 mM SSA alone.

Groups of 40 islets each, pre-perfused without any substrate for 45 min were then stimulated for 30 min (between vertical broken lines) with 10 mM SSA alone and together with 10 mM Gln (A) or 1 mM gabaculine (B). In (C), insulin secretion was stimulated by 10 mM SSA plus 10 mM Gln in the absence or presence of 1 mM

gabaculine. Pre-perifusion conditions were then re-established during the last 25 min. Symbols represent means $\pm$ S.E.M.

Figure 7. Modification of β -cell membrane potential by SSA

A) Recording of β -cell membrane potential at 1 mM extracellular glucose. SSA (10 mM) was applied as indicated by the bar. B) Average membrane potential of β -cells at 1 mM glucose before (control) and after addition of 10 mM SSA (n=5; *p<0.01). C) β -cell K_{ATP} current (measured by 10 mV de- and hyper-polarizing pulses from -70 mV) before and after application of 10 mM SSA (n=4; *p<0.01).

FIGURES

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