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NKCC transport mediates the insulinotropic effects of taurine and other small neutral amino acids

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ABSTRACT

Aims: Despite its high concentration in pancreatic islets of Langerhans and broad range of antihyperglycemic effects, the route facilitating the import of dietary taurine into pancreatic β-cell and mechanisms underlying its insulinotropic activity are unclear. We therefore studied the impact of taurine on beta-cell function, alongside that of other small neutral amino acids, L-alanine and L-proline.
Main methods: Pharmacological profiling of insulin secretion was conducted using clonal BRIN BD11 β-cells, the impact of taurine on the metabolic fate of glucose carbons was assessed using NMR and the findings were verified by real-time imaging of Ca^{2+} dynamics in the cytosol of primary mouse and human islet beta-cells.
Key findings: In our hands, taurine, alanine and proline induced secretory responses that were dependent on the plasma membrane depolarisation, import of Ca^{2+}, homeostasis of K$^+$ and Na$^+$ as well as on cell glycolytic and oxidative metabolism. Taurine shifted the balance between the oxidation and anaplerosis towards the latter, in BRIN BD11 beta-cells. Furthermore, the amino acid signalling was significantly attenuated by inhibition of Na$^+$-K$^+$-Cl$^-$ symporter (NKCC).
Significance: These data suggest that taurine, like L-alanine and L-proline, acutely induces glucose-dependent insulin-secretory responses by modulating electrogenic Na$^+$ transport, with potential role of intracellular K$^+$ and Cl$^-$ in the signal transduction. The acute action delineated would be consistent with antidiabetic potential of dietary taurine supplementation.

1. Introduction

Alongside glucose, amino acids are the major physiological nutrient regulators of insulin secretion [1–3]. Different amino acids are believed to induce the release of insulin via different cellular mechanisms, either by directly carrying in a positive charge [4], by co-transport with Na$^+$ [5], by being metabolised with generation of ATP [6], or via a combination of such effects [7]. The diverse insulinotropic effects of amino acids represent one of the long-standing conundrums of diabetes research [8], the missing link between the metabolic and the exocytotic potency being the key highlight [1]. A critical step of the insulinotropic action, the transport of amino acids into pancreatic islet β-cells is accomplished by several empirically defined systems [9,10] enabling the import of a wide range of amino acids.

Small neutral amino acid L-alanine acutely potentiates insulin secretion [9], which is linked to its oxidation [7] as well as co-transport of Na$^+$ [11,12]. The Na$^+$-dependent transport system (A-system) is utilised by poorly **metabolisable** L-proline [10] and 2-aminoisobutyric

acid [5] that also exhibit acute insulinotropic effects [12]. At the same time, Na^{+}-independent Lat1 transporter, abundantly expressed in endocrine pancreas, mediates the transport of larger branched-chain amino acids [13], such as leucine or valine, in exchange for other amino acid species (L-system).

The sulphur-containing dietary amino acid taurine exerts a variety of actions ranging from osmoregulation to neurotransmission in many mammalian tissues [14–17]. Taurine is highly concentrated in pancreatic islets [18], prompting a causal link to its growing reputation of a potential insulinotropic [19,20] and antihyperglycaemic [21] agent. Despite the palpable elevation of the dietary profile of the amino acid, little is known about the transport of taurine into β-cells or its impact on β-cell biology. The molecular weight (125 Da) puts taurine in between the small and the large counterparts that are transported by the A- and L-systems, respectively. Despite a hint of Na^{+}-dependent transport in some systems [22], there is no compelling data to date that would unequivocally explain the insulinotropic effect of the amino acid [23].

Below, we profile the physiology and pharmacology of the insulinotropic effect of taurine into the β-cell, alongside two other neutral amino acids with more established transport parameters, namely L-alanine and L-proline. We utilise BRIN-BD11 β-cell line as a screening system and insulin secretion as a read-out to demonstrate the Na^{+}-dependence of the amino acid transport into the β-cell and its cellular metabolic and ionic actions that are subsequently verified in primary mouse and human pancreatic islets.

2. Materials and methods

2.1. Reagents

All chemicals were obtained from Sigma-Aldrich (Poole, Dorset UK), unless otherwise indicated, including taurine (Fluka, Buchs, Switzerland) and uniformly labelled ^{13}C D-glucose (D-[U-^{13}C]-glucose) (Goss Scientific, Great Baddow, Essex, UK).

2.2. Animals and islet isolation

C57Bl/6 J mice (Charles River) used throughout the study were kept in a conventional vivarium with a 12-hour-dark/12-hour-light cycle. The mice were allowed free access to food and water and were killed by cervical dislocation. All experiments were conducted in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986) and the Ulster University ethical guidelines. Pancreatic islets were isolated from non-fasted mice by injecting collagenase solution into the bile duct, with subsequent digestion of the connective and exocrine pancreatic tissue [24].

Human pancreatic islets were isolated from non-diabetic donors in the Oxford Diabetes Research & Wellness Foundation Human Islet Isolation Facility according to published protocols [25,26].

For imaging studies, mouse and human islets were cultured for 48 h in RPMI medium containing 11 and 5 mM glucose, respectively, supplemented with 10 % FBS, 100 IU/mL penicillin and 100 μg/mL streptomycin (all reagents from Life Technologies, Paisley, UK). Recombinant sensors GCaMP6f (Vector Biolabs, USA) or Perceval [27] were delivered via adenoviral vectors at 10[5] infectious units per islet, followed by 24-36 h culturing at 11 mM glucose (as above) to express the sensors.

2.3. Clonal beta cells

Clonal rat pancreatic BRIN-BD11 cells [28] were maintained in RPMI 1640 medium supplemented with 10 % (v/v) foetal calf serum, 100 IU/mL penicillin and 0.1 mg/mL streptomycin (all reagents were from Gibco, Paisley, UK), in a fully humidified atmosphere supplemented with 5 % CO_2, at 37 °C. The cells were washed with Hanks balanced saline solution (HBSS) before detachment from the culture flask by incubation with 0.025 % (w/v) trypsin containing 1 mM EDTA and

utilised immediately.

2.4. Hormone secretion

Monolayers of BRIN-BD11 cells were established following seeding at a density of 2×10^5 cells per well in 24-well (Falcon, New Jersey, USA) overnight, as above, after which the culture medium was replaced by 1 mL of extracellular solution EC1, containing, mM: 115 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.28 $CaCl_2$, 1.2 KH_2PO_4, 25 HEPES, 1 $NaHCO_3$, pH 7.4 with NaOH. The solution was supplemented with 0.01 % bovine serum albumin and 1.1 mM or 16.7 mM glucose, as indicated. Following a 40-min pre-incubation at 1.1 mM glucose, a 20 min incubation in 1 mL of the test solution, containing (ant)agonists and substrates, as indicated, was performed. Aliquots of the test buffer were subsequently removed, stored at −20 °C and **analyzed** using insulin radioimmunoassay [29]. To model the removal of extracellular Ca^{2+}, $CaCl_2$ was omitted from the EC1 solution above and 0.5 mM EGTA was supplemented instead. Na^{+}-free solution, EC2, contained, mM: 250 sucrose, 4.9 KCl, 1.28 $CaCl_2$, 1.2 $MgCl_2$, 1.2 KH_2PO_4, 25 HEPES, 1 $KHCO_3$, pH 7.4 with KOH.

2.5. High-throughput imaging in cell lines

Dynamics of membrane potential and $[Ca^{2+}]_{cyt}$ in the populations of BRIN-BD11 cells was recorded using a FLIPR Membrane Potential Assay Kit and a FLIPR Calcium Assay Kit (Molecular Devices, Sunnyvale, CA, USA). BRIN-BD11 cells were seeded in 96 well microplates (black walls, clear bottom, Costar, Roskilde, Denmark) at 1×10^5 cells/well and allowed to attach for 18 **h** at 37 °C. The attached cells were preincubated in with 100 μl of EC1 containing 5.6 mM glucose and loaded with 100 μl of the respective FLIPR dye that was reconstituted using assay buffer for 60 min. Higher levels of basal glucose (5.6 vs 1.1 mM) were deemed to maximise the impact of the sugar on $[Ca^{2+}]_{cyt}$, as the latter is sequestered into the ER or extracellular compartment via active transport. Changes in signal intensity were detected in a FlexStation 3 scanning fluorimeter equipped with a fluid transfer workstation (Molecular Devices, USA). The dyes were excited at 485 nm, with the emission collected at 525 nm. The acquisition was performed for 10 min, in 2.5 s intervals. Test solutions were introduced at the rate of 78 μL/s.

2.6. NMR spectroscopy

BRIN-BD11 cells were cultured as above but in six T175 flasks per condition, which was repeated at least three times on independent cultures. The cells were washed with phosphate-buffered saline (PBS), preincubated for 20 min in EC1 solution supplemented with 1.1 mM glucose and subsequently incubated for 2 h in the presence of 16.7 mM [U—^{13}C]-glucose supplemented with(out) 10 mM taurine. The metabolites were extracted, for which the cells were washed with ice-cold PBS, lysed by perchloric acid (6 %) and scraped off the culture flasks. The extracts were pooled, centrifuged at 200 ×g at 4 °C for 5 min, with the resulting supernatant neutralised with KOH (5 M, 1 M and 0.1 M) and the pellets were soaked overnight in 0.1 M NaOH. The neutralised supernatant was centrifuged as above, with the supernatant treated with Chelex-100 resin and subsequently lyophilised. The lyophilised cell extracts, dissolved in 3 mL of 100 mM K_2HPO_4 buffer (pH 7.0) were centrifuged as above, with the supernatant removed and 10 % D_2O supplemented for subsequent NMR experiments.

The ^{13}C NMR experiments used an insert containing 5 % v/v dioxane in water as an external signal intensity reference. Proton decoupled ^{13}C spectra were acquired on a Bruker DRX 500 spectrometer using a 10 mm broadband probe at 25 °C with 32 K data points using 90° pulses, 260 ppm spectral width, 2.5 s relaxation delay and 12,000–24,000 scans. Chemical shifts were referenced to tetramethylsilane at 0 ppm.

NMR data was processed using Bruker WINNMR software, utilising exponential multiplications with 2 Hz line broadening. The assignments of the intermediate metabolites were made by comparison with

chemical shift tables in the literature [30]. The amount of ^{13}C in each resonance was evaluated by integration of the extract peaks and the corresponding peaks in the standard sample relative to the dioxane signal, with the corrections for the natural abundance signal. The enrichment percentage was calculated using the total glutamate in the extracts measured using a YSI amino acid analyser. The fluxes reported were calculated using the glutamate C2 and C4 isotopomers. The ratio between flux through PC and PDH was calculated as ([2,3-^{13}C2] + [1,2,3-^{13}C3])/[4,5-^{13}C2] + [3,4,5-^{13}C3] [31]. The fraction of acetyl-CoA labelled from [U—^{13}C]glucose was derived from the C4 peak as: (3,4,5-^{13}C) × C4/C3 [32]. 3D rendering (Fig. 3E) was performed using MolView (**https://molview.org**).

2.7. Real-time imaging of $[Ca^{2+}]_{cyt}$ and $[ATP/ADP]_{cyt}$ in islets

Time-lapse imaging of $[Ca^{2+}]_{cyt}$ and $[ATP/ADP]_{cyt}$ in isolated mouse and human islets was performed on an upright Zeiss Axioskop FS2 200 microscope equipped with Zeiss 510-META laser confocal scanning system. Islets expressing Ca^{2+} sensor GCaMP6f or ATP/ADP sensor Perceval were immobilized inside the open-air imaging chamber [33]. Bath solution EC3 containing, mM: 140 NaCl, 4.6 KCl, 2.6 CaCl2, 1.2 MgCl2, 1 NaH2PO4, 5 NaHCO3, 10 HEPES (pH 7.4, with NaOH) and (ant)agonists, as indicated, was perifused continuously at a rate of 60 μL/min. The sensor was excited at 488 nm and emission was collected at 530 nm, using 512 × 512 frame scanning mode with the pixel dwell time of 6 μs (image frequency: 0.25 Hz). α- and β-cells were differentiated by their response to 10 μM adrenaline [24]. Images were acquired using ZenBlack (Carl Zeiss).

2.8. Data analysis

Image sequences were analyzed (registration, background subtraction, ROI intensity vs time analysis, F/F$_0$ calculation) using open-source FIJI software (**http://fiji.sc/Fiji**). The numerical data was analyzed using IgorPro package (Wavemetrics). To calculate partial areas under the curve (pAUC), the recordings were split into 30s intervals, and area under the curve was computed for each individual interval, using the trapezoidal integration [34]. Numbers of measurements/cells are specified in Figure legends; the experiments on human islets were performed on islets isolated from 3 donors. Statistical analysis was performed using R [35]. Data are presented as the mean values ± S.E.M. Mann-Whitney *U* test or Wilcoxon's paired test were used to compute the significance of difference between independent and dependent samples, respectively. Multiple comparisons within one experiment were performed using Kruskal-Wallis test with Nemenyi post-hoc analysis (independent samples) or Friedman test with Nemenyi post-hoc analysis (dependent samples).

3. Results

3.1. Amino acids induce insulin secretion at basal and high glucose

Assayed at basal glucose, as little as 1 mM of alanine, taurine or proline induced a small but significant insulin release from BRIN BD11 clonal β-cells (Fig. 1A). The effect was further potentiated by applying higher (10 mM) concentrations of the amino acids (Fig. 1A) and using suprathreshold levels of glucose (16.7 mM) (Fig. 1A). Of note, the effect of the amino acid concentration was significantly higher in the case of alanine, whereas the elevation of glucose had identical effects for each of the tested amino acids (Fig. 1A).

3.2. The potentiation of insulin secretion by amino acids depends on Ca^{2+} influx

The perturbation of plasma membrane excitability or Ca^{2+} influx using K$_{ATP}$ channel opener diazoxide (Fig. 1B), L-type Ca^{2+} channel

inhibitor verapamil (Fig. 1C) or chelator of extracellular Ca^{2+} EGTA (Fig. 1D) reduced secretion of insulin in response to 16.7 mM glucose, in BRIN BD11 cells (first pair of bars in Fig. 1B–D). Taurine, alanine or proline triggered insulin secretion in the presence of diazoxide or verapamil (Fig. 1B, C) but not EGTA (Fig. 1D), albeit to a significantly reduced extent. Of note, in the presence of the three inhibitors of β-cell Ca^{2+} dynamics, the impact of 10 mM alanine on the secretion was significantly higher than that of equimolar taurine or proline (Fig. 1B–D).

Both plasma membrane potential (Fig. 1E) and Ca^{2+} influx (Fig. 1F) quantified in BRIN BD11 cells in a high-throughput modality, were elevated upon the switch between sub- (5.6 mM) and suprathreshold (16.7 mM) glucose. The addition of taurine, alanine or proline significantly depolarised the cell at 5.6 mM glucose (Fig. 1E). Likewise, added at basal glucose (5.6 mM), the amino acids significantly elevated cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) (Fig. 1F). The impact of taurine on the membrane potential was, however, significantly higher than that of alanine or proline, at low glucose (Fig. 1E). It was furthermore significantly potentiated by high (16.7 mM) glucose, likely reflecting a more prolonged timecourse of taurine effect.

3.3. The potentiation of insulin secretion by amino acids relies on glycolytic metabolism

We probed potential mechanisms linking the exposure to extracellular amino acids to the membrane excitability and Ca^{2+} flux, the prerequisites of the potentiation of insulin secretion (Fig. 1). The K$_{ATP}$ channel-dependent pathway, utilised by many fuels, includes the upregulation of the energy metabolism, with the putative agonist working as a substrate or a regulator for intracellular exergonic processes. Thus, a membrane-permeable glycolytic metabolite glyceraldehyde (10 mM) induced insulin secretion by BRIN BD11 cells at basal (1.1 mM) glucose (Fig. 2A). The effect of glyceraldehyde was comparable to that of proline (10 mM), albeit the combination of the two secretagogues failed to enhance the secretory output any further (Fig. 2A). In contrast, taurine or alanine displayed additive stimulatory effect on insulin secretion, to that of glyceraldehyde (Fig. 2A).

An inhibitor of the key glycolytic enzyme glucokinase, mannoheptulose (15 mM) significantly reduced glucose-induced insulin secretion from BRIN BD11 cells (Fig. 2B). It furthermore significantly attenuated the impact of each of the three amino acids on insulin secretion, both at high (16.7 mM) and low (1.1 mM) glucose (Fig. 2B), in agreement with our earlier reports of the hexokinase activity in BRIN BD 11 cells [36]. Notably, in the presence of taurine but not alanine or proline, mannoheptulose was unable to fully reverse glucose-induced insulin secretion (Fig. 2B, inset).

3.4. The impact of oxidative metabolism of the amino acid-induced insulin secretion

Pancreatic islet β-cells bear a number of metabolic adjustments enabling a tight coupling between metabolic fuels and oxidative metabolism [37–39], which is translated into a rapid sustained elevation of cytosolic ATP levels [40,41]. We investigated the role of oxidative metabolism in the amino acid-induced insulin secretion using a battery of pharmacological tools perturbing mitochondrial metabolism. Inhibitors of cytochrome C oxidise sodium azide (Fig. 2C) and sodium cyanide (Fig. 2D) as well as the inhibitor of mitochondrial F$_O$F1 ATP synthase oligomycin (Fig. 2E) abolished insulin secretion induced by high glucose (16.7 mM). The three inhibitors significantly reduced the stimulatory effect of taurine, alanine and proline on insulin secretion, albeit they failed to cancel the effect fully (Fig. 2C–E). Notably, we were able to observe an additive effect of high glucose on the amino acid-induced secretion, in the presence of each of the inhibitors, something that was not detectable in the absence of the amino acid stimulus (Fig. 2C–E). The effect of glucose was fully cancelled upon inhibition of
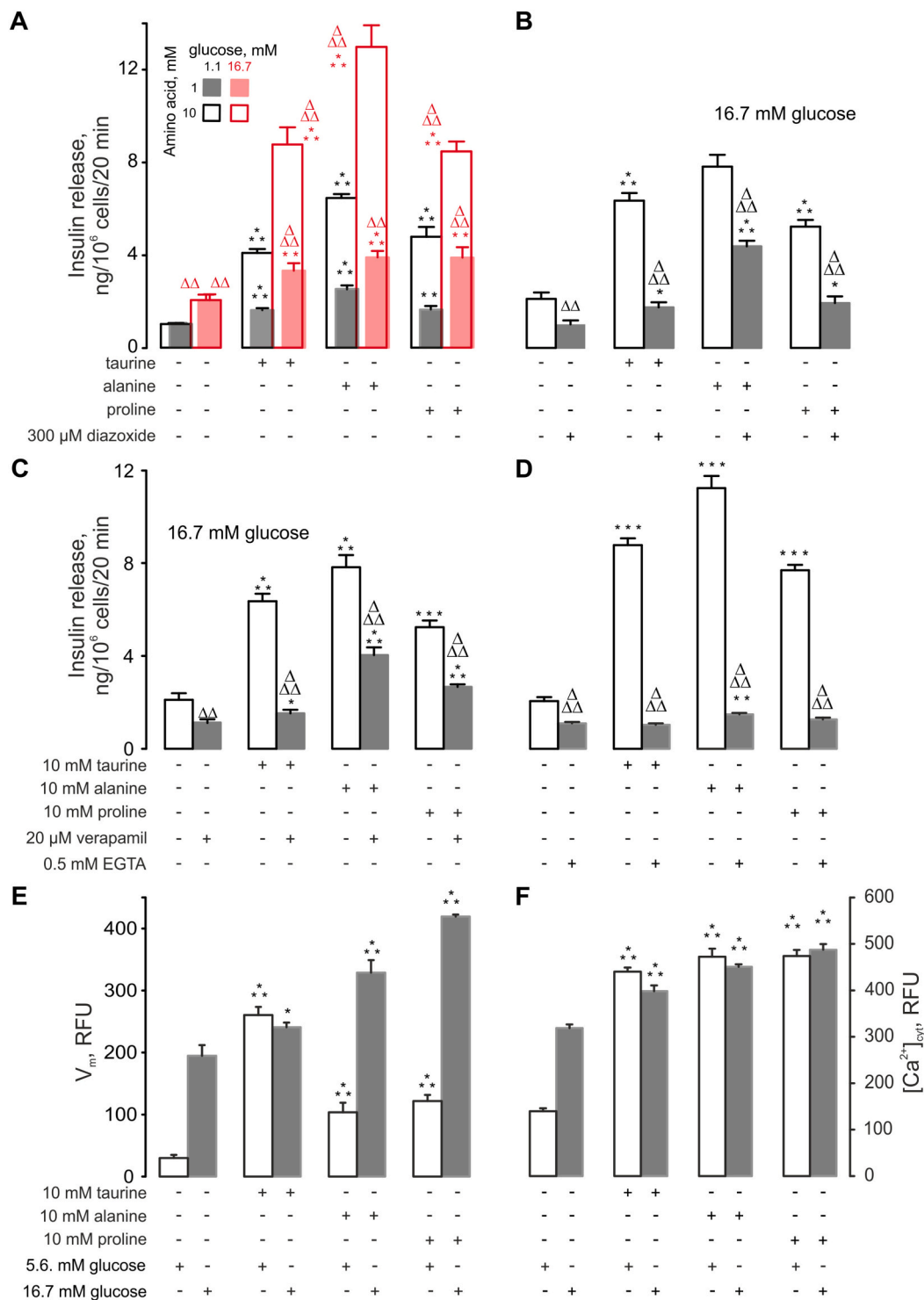
Fig. 1. Taurine, alanine and proline enhance glucose-induced insulin secretion by increasing $[Ca^{2+}]_{cyt}$.

A: Effects of acute (20 min) exposure to 1 mM or 10 mM of taurine, alanine or proline, as indicated, on insulin release from BRIN-BD11 cells at 1.1 mM (black) or 16.7 mM (red) glucose ($n = 6$). **$P < 0.01$, ***$P < 0.001$ vs control (absence of the amino acid). $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ vs corresponding effects at 1.1 mM glucose.

B: Effects of acute exposure (20 min) to diazoxide on 10 mM amino acid-induced insulin release from BRIN-BD 11 cells at 16.7 mM glucose ($n = 6$). *$P < 0.05$, ***$P < 0.001$ vs control (absence of the amino acid). $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ vs corresponding effect in the absence of diazoxide.

C, D: Effects of acute (20 min) exposure to verapamil (C) or depletion of extracellular Ca^{2+} by 0.5 mM EDTA (D) on insulin release from BRIN-BD11 cells induced by 16.7 mM glucose and 10 mM amino acids, as indicated ($n = 6$). *$P < 0.05$, ***$P < 0.001$ vs control (absence of the amino acid). $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ vs corresponding effect in the absence of verapamil (C) or EDTA (D).

E, F: Effects of acute (10 min) exposure to 10 mM taurine, alanine and proline on membrane potential (E) or $[Ca^{2+}]_{cyt}$ (F) of BRIN-BD11 cells exposed to 5.6 or 16.7 mM glucose, as indicated ($n = 12$ for each panel). *$P < 0.05$, ***$P < 0.001$ vs control (absence of the amino acid).
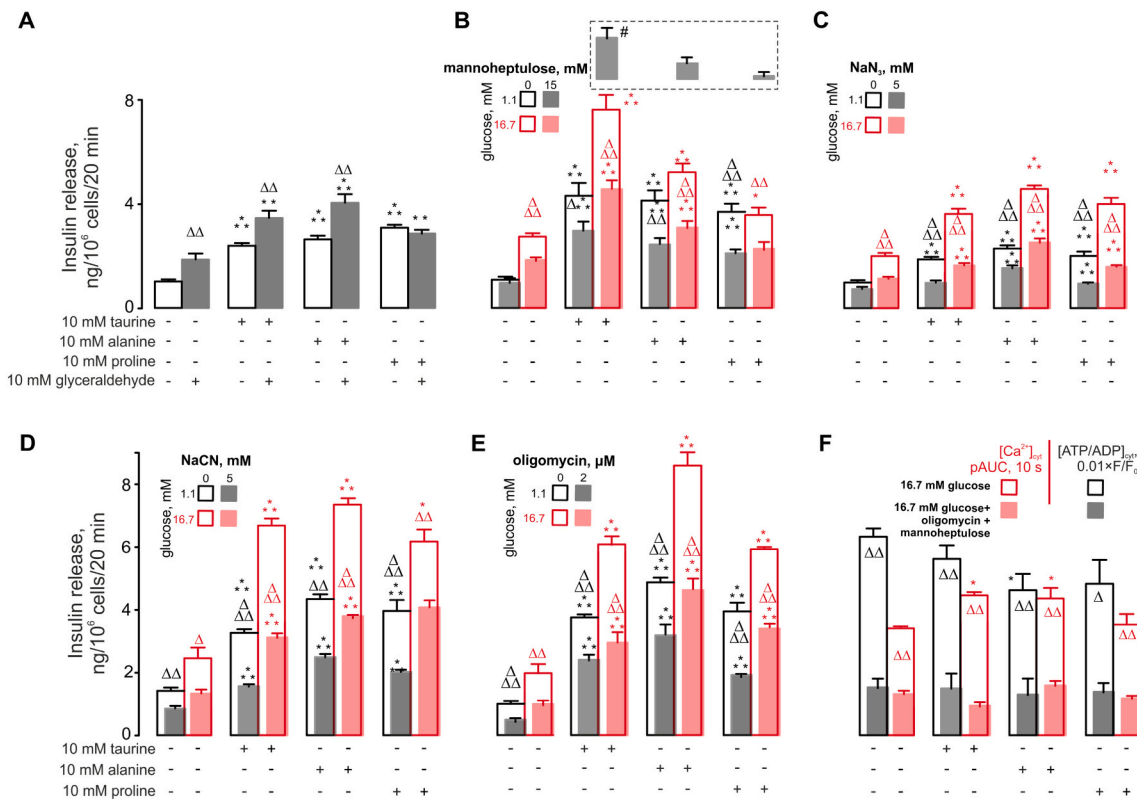
Fig. 2. Potentiation of insulin secretion by taurine, alanine and proline relies on oxidative metabolism.

A: Effects of acute (20 min) exposure to glyceraldehyde on insulin release from BRIN-BD 11 cells induced by 10 mM amino acids (as indicated), at 1.1 mM glucose ($n = 6$). **P < 0.01, ***P < 0.001 vs control (absence of the amino acid). $\Delta\Delta$P < 0.01 vs corresponding effect in the absence of glyceraldehyde (control).

B–E: Effects of acute (20 min) exposure to mannoheptulose (B), sodium azide (C), sodium cyanide (D) or oligomycin (E) on insulin release from BRIN-BD 11 cells induced by on 10 mM amino acids (as indicated) at 1.1 mM or 16.7 mM glucose (each dataset, n = 6). *P < 0.05, ***P < 0.001 vs control (absence of the amino acid). ΔP < 0.05, $\Delta\Delta$P < 0.01, $\Delta\Delta\Delta$P < 0.001 vs respective effect in the absence of mannoheptulose (B), sodium azide (C), sodium cyanide (D) or oligomycin (E).

F: Effects of acute (20 min) co-exposure to mannoheptulose and oligomycin on $[Ca^{2+}]_{cyt}$ and $[ATP/ADP]_{cyt}$ dynamics in mouse pancreatic islet cells induced by on 10 mM amino acids (as indicated) at 16.7 mM glucose (each dataset, $n = 50$ cells). *P < 0.05, ***P < 0.001 vs control (absence of the amino acid). ΔP < 0.05, $\Delta\Delta$P < 0.01, $\Delta\Delta\Delta$P < 0.001 vs respective effect in the absence of mannoheptulose and oligomycin.

both glycolytic and oxidative metabolism, which we probed at the level of $[Ca^{2+}]_{cyt}$ and $[ATP/ADP]_{cyt}$ dynamics in primary cells from mouse pancreatic islets (Fig. 2F).

We probed the metabolic fate and impact of taurine in extracts of BRIN-BD11 cells that were pre-loaded with 16.7 mM [U—^{13}C]glucose with(out) taurine (10 mM) and subsequently examined using NMR. The metabolites produced from [U—^{13}C]glucose included glutamate (labelled at positions C2, C3 and C4), lactate (C2 and C3), alanine (C2 and C3) and acetate (C2) (Fig. 3A). Two singlet peaks corresponding to unlabelled taurine appeared, corresponding to taurine N-CH2 and taurine S-CH2, indicating significant transport of taurine into the cells.

The amount of amino acid labelled at each position, calculated for glutamate, lactate and alanine, displayed no significant changes in response to taurine (Fig. 3B). Taurine, however, caused a significant (20 ± 5 %) increase in the ratio of flux through pyruvate carboxylase (PC) to that through pyruvate dehydrogenase (PDH) (Fig. 3C). The labelling of acetyl-CoA, that is exclusively accomplished via conversion of glycolytically-derived pyruvate through the PDH complex, was significantly lowered in cells exposed to taurine where the fraction of acetyl-CoA labelled with ^{13}C was reduced from 73.3 to 66.7 % ($p < 0.05$ vs control). Thus, whereas the observations (Fig. 2C–E) indicate low oxidation of intracellular taurine, the NMR data suggests that the latter impacts the balance between anaplerotic (PC) and oxidative (PDH) fluxes in mitochondria.

3.5. The effect of amino acids on insulin secretion depends on extracellular K^+ and Na^+

The monovalent cation homeostasis plays a principal role in the β-cell energy balance, with Na^+/K^+ ATPase consuming >15 % of intracellular ATP [42]. In line with the key role of oxidative metabolism in mediating the stimulatory effect of small neutral amino acids on β-cell secretory output, the latter has been historically linked to Na^+ transport [10]. We therefore tested, using BRIN BD11 cells, whether the perturbation of K^+ or Na^+ affects the insulinotropic potency of taurine, alanine or proline. The direct membrane depolarisation with 30 mM KCl, applied at 16.7 mM glucose, had an additive effect to that of each of the amino acids (Fig. 4A). In line with this observation, the omission of K^+ from the extracellular solution significantly attenuated insulin secretion induced by each of the three amino acids at low (1.1 mM) or high (16.7 mM) glucose (Fig. 4B). Interestingly, the omission of K^+ from the extracellular solution had a more significant effect when applied for 40 min prior to the experiment, at 16.7 mM glucose, as the insulinotropic effect of each of the amino acids was completely cancelled (Fig. 4C).

The sensitivity of the amino acid-induced insulin secretion to prolonged omission of extracellular K^+ suggests a mediatory role of Na^+/K^+ ATP-ase in the amino acid transport. To our surprise, the acute inhibition of the enzyme with ouabain (1 mM, $IC_{50} > 100$ mM [43]) dramatically enhanced the insulinotropic effect of each of the amino acids, both at low (1.1 mM) and high (16.7 mM) glucose (Fig. 5A). Notably, the enhancement by 10 mM taurine, at low glucose, ranked highest among the three amino acids (Fig. 5A).
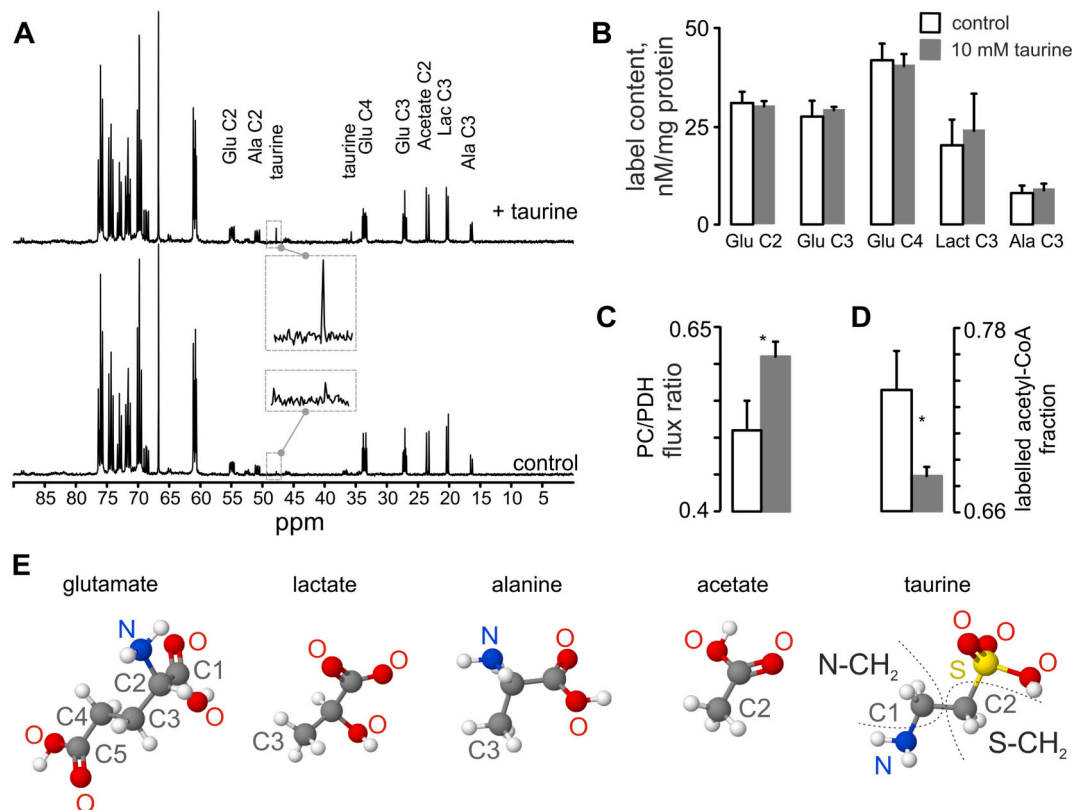
Fig. 3. Taurine alters the balance between anaplerosis and oxidative metabolism in BRIN BD11 cells.

A: NMR spectra of the extract from BRIN BD11 cells exposed or not to 10 mM taurine, as indicated. Peaks corresponding to the carbons included by glutamate ('Glu'), alanine ('Ala'), lactate ('Lac'), taurine, or acetate, deriving from the labelled [U-^{13}C]glucose are indicated. Inset: magnified taurine peak.

B: ^{13}C Label distribution in glucose metabolites following 2 h incubation in the presence of [U-^{13}C]glucose under control conditions and in the presence of 10 mM taurine.

C, D: The ratio of flux through pyruvate carboxylase (PC) and pyruvate dehydrogenase (PDH) and the fraction acetyl-CoA labelled from [U—^{13}C]glucose. *$P < 0.05$ vs control (absence of taurine).

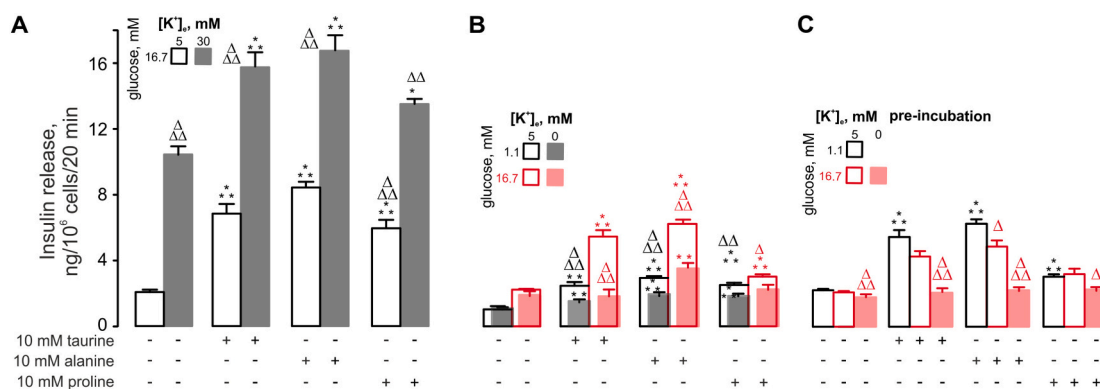E: Structural schemes of metabolites and groups presented or quantified in panels A, B.

Fig. 4. Taurine, alanine and proline rely on K$^+$ transport to enhance insulin secretion.

A: Effects of acute (20 min) exposure to high K$^+$ (30 mM KCl) on insulin release from BRIN-BD 11 cells induced by on 10 mM amino acids (as indicated) at 1.1 mM or 16.7 mM glucose (each dataset, $n = 6$). *P < 0.05, ***P < 0.001 vs control (absence of the amino acid). $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ vs corresponding effect in the absence of KCl.

B, C: Effects of acute (20 min, A) or long term (3 h, B) removal of extracellular K$^+$ on insulin release induced by 10 mM amino acids (as indicated), at 1.1 mM or 16.7 mM glucose ($n = 6$). **P < 0.01, ***P < 0.001 vs control (absence of the amino acid). $\Delta P < 0.05$, $\Delta\Delta P < 0.01$, $\Delta\Delta\Delta P < 0.001$ vs respective condition in the presence of extracellular K$^+$ (A) and presence of K$^+$ and 1.1 mM glucose in the extracellular medium.

Pancreatic β-cells express functional voltage-gated Na$^+$ channels [44] that however do not have a proven role in the electrical excitability of these glucose-sensing cells. Inhibition of the voltage-gated Na$^+$ conductance with tetrodotoxin (TTX, 5 μM) had no effect on the ability of the three amino acids to induce insulin secretion, either at 1.1 or at 16.7 mM glucose (Fig. 5B). At the same time, the omission of Na$^+$ from the extracellular solution fully cancelled the effects of each of the amino acids on insulin secretion (Fig. 5C). The inhibition of Na+/K$^+$ ATPase
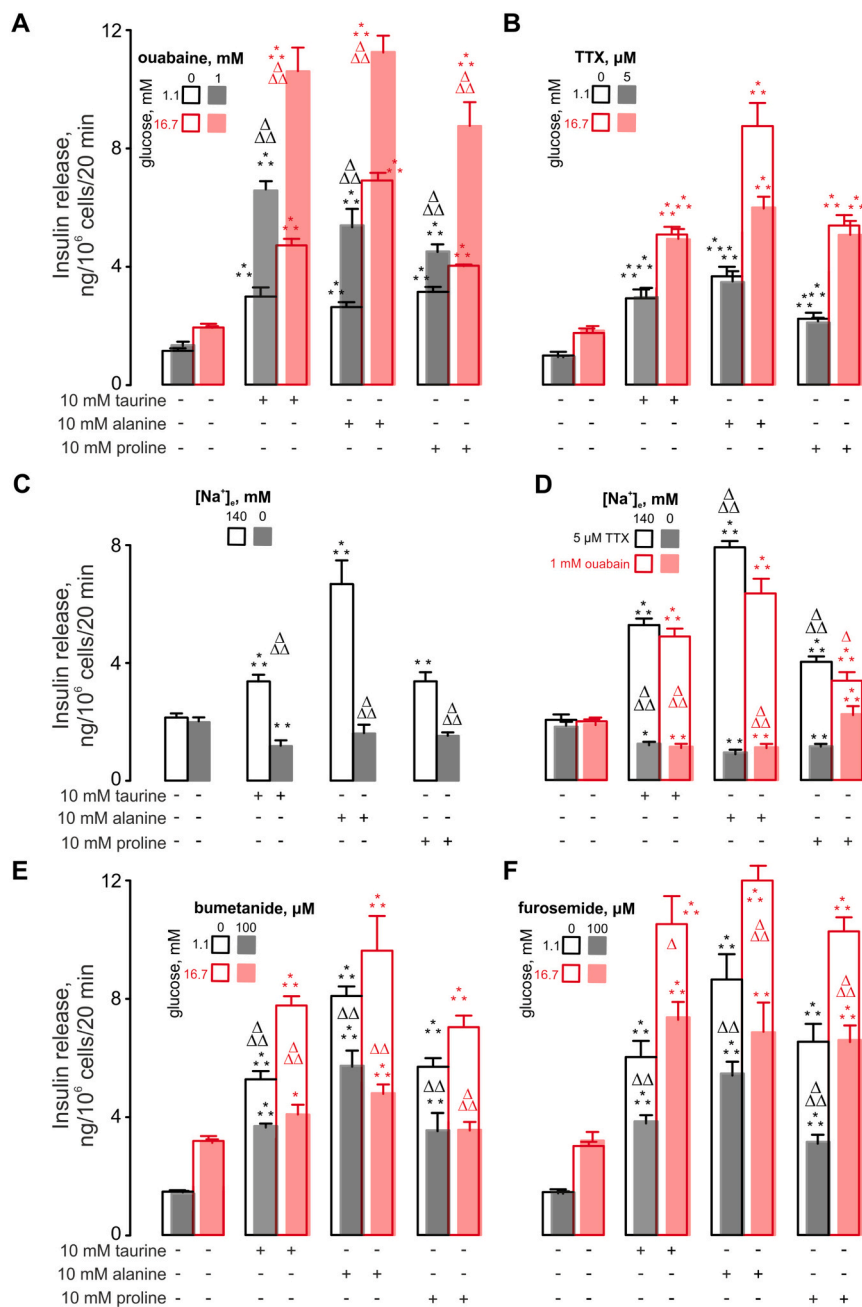
Fig. 5. Na+-K+-Cl− symporter mediates the transport of taurine, alanine and proline into β-cells.

A, B: Effects of acute (20 min) exposure of Na-K+ exchanger blocker ouabain (A) and Nav channel blocker tetrodotoxin (TTX, B) and on insulin release by BRIN-BD 11 cells induced by 10 mM amino acids (as indicated), at 1.1 mM or 16.7 mM glucose ($n = 6$). ***$P < 0.001$ vs control (absence of the amino acid). ΔΔΔ$P < 0.001$ vs respective condition in the absence of TTX (*A*) or ouabain (*B*).

C, D: Effects of substitution on extracellular Na+ by sucrose in the absence (C) or presence of TTX and ouabain (D) on insulin release by BRIN-BD 11 cells induced by 10 mM amino acids (as indicated), at 1.1 mM or 16.7 mM glucose ($n = 6$). **$P < 0.01$, ***$P < 0.001$ vs control (absence of the amino acid). Δ$P < 0.05$, ΔΔΔ$P < 0.001$ vs respective condition in the presence of extracellular Na+.

E, F: Effects of acute (20 min) exposure of Na+-K+-Cl− symporter blockers bumetanide (E) and furosemide (F) on insulin release by BRIN-BD 11 cells induced by 10 mM amino acids (as indicated), at 1.1 mM or 16.7 mM glucose ($n = 6$). **$P < 0.01$, ***$P < 0.001$ vs control (absence of the amino acid). Δ$P < 0.05$, ΔΔΔ$P < 0.001$ vs respective condition in the absence of bumetanide (E) or furosemide (F).

(with ouabain) or voltage-gated Na+ channel (with TTX) produced no significant effect on the insulinotropic properties of the taurine, alanine or proline, upon the removal of extracellular Na+ (Fig. 5D).

Interestingly, the expression and the activity of Na+-K+-2Cl− symporter (NKCC) was reported in β- but not α-cells from rodent islets [45]. The inhibition of NKCC either with bumetanide (100 μM, IC50 = 2 μM [46]) or furosemide (100 μM, IC50 = 9 μM [46]) had no effect on glucose-induced insulin secretion in BRIN BD11 cells (Fig. 5E, F) but significantly reduced the secretory output in response to 10 mM taurine, alanine or proline (Fig. 5E, F).

3.6. Loop diuretics inhibit [Ca2+]cyt dynamics induced by amino acids in islet β-cells

We verified the involvement of NKCC in mediation of the amino acid signal, by imaging [Ca2+]cyt dynamics in response to taurine, alanine

and proline, in primary islet β-cells. At low glucose (1.1 mM), mouse or human islet β-cells displayed stable low [Ca2+]cyt, whereas the addition of 10 mM taurine induced a rapid reversible increase in the level of the ion (Fig. 6A). Alanine and proline, likewise, reversibly elevated [Ca2+]cyt (Fig. 6B), in mouse and human islets. The effect of the amino acids on [Ca2+]cyt was significantly inhibited by furosemide (100 μM) or bumetanide (100 μM) (Fig. 6B), in line with the effect of these two loop diuretics on amino acid-induced insulin secretion in the BRIN BD11 cell line (Fig. 5E, F). Despite the similarity of the magnitude and pharmacological profile of the effects, the per cell correlation between the three amino acids was fairly modest ($r = 0.41$ (0.44), $r = 0.51$ (0.34), $r = 0.43$ (0.32) for taurine vs alanine, taurine vs proline and alanine vs proline in human (mouse) islet β-cells, $p < 0.05$) (Fig. 6C, D).
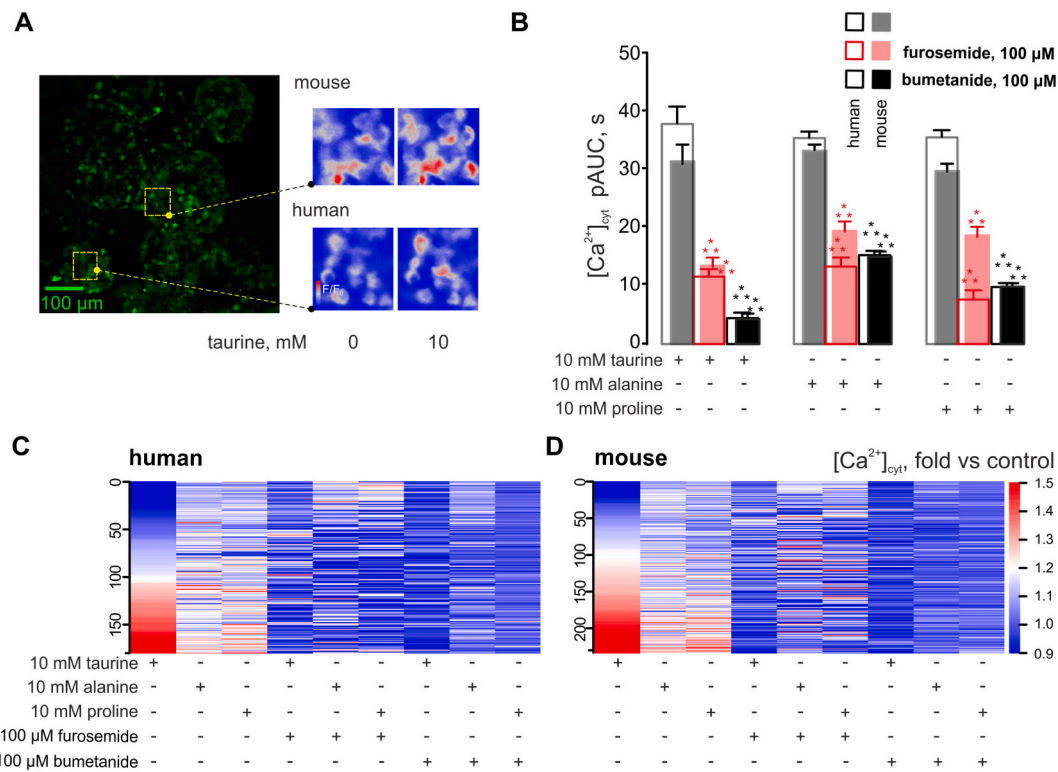
Fig. 6. Inhibition of NKCC attenuates the $[Ca^{2+}]_{cyt}$ dynamics induced by taurine, alanine and proline in mouse and human islets of Langerhans.
A: Representative confocal images of mouse (top) and human (bottom) islets of Langerhans expressing GCaMP6f Ca^{2+} reporter. Inset: GCaMP6f fluorescence in β-cells, before and after the addition of taurine (10 mM), pseudocolour.
B: Quantification of the effect of acute addition of taurine, alanine or proline (10 mM) on $[Ca^{2+}]_{cyt}$ dynamics in human and mouse islet β-cells with(out) the loop diuretics bumetanide or furosemide, recorded at 1.1 mM glucose. ***$P < 0.001$ compared with control.
C, D: Individual cell data representing the effect of acute addition of taurine, alanine or proline (10 mM) on $[Ca^{2+}]_{cyt}$ dynamics in human (C, $n = 181$ cells from 3 separate preparations) and mouse (D, $n = 235$ cells from 3 separate preparations) islet β-cells in the presence of the inhibitors indicated. The cell profiles were ranked by to the response to the addition of taurine (10 mM).

4. Discussion

Several mechanisms with different degrees of electrogenicity have been implicated in the transport of neutral amino acids into the fuel-sensing pancreatic islet β-cells [10,47]. Whereas L-alanine and L-proline were demonstrated to be predominantly co-transported with Na^+, thereby depolarising the plasma membrane and evoking Ca2+ influx [48], L-leucine seemingly utilises the non-electrogenic system L amino acid transporter 1 and triggers insulin release predominantly through its metabolism [13] or by impacting on the metabolism of other fuels such as glutamate [49]. We now for the first time demonstrate that the transport system in the β-cell for taurine, bears strong similarities to that of alanine and proline. In our hands, the import of the three amino acids into clonal, mouse and human pancreatic β-cells, and therefore their insulin-releasing actions, relied on the symport of Na^+ via NKCC (Fig. 7).

4.1. The electrogenic mode of amino acid transport into β-cells

We demonstrate that the insulinotropic effects of taurine and the other small neutral amino acids alanine and proline are mediated by the depolarisation of plasma membrane (Fig. 1B, F) and initiation of Ca^{2+} dynamics (Fig. 1C, E) in the cytosol of β-cells. The latter finding has been verified in primary β-cells within mouse and human pancreatic islets (Fig. 6). The dependence of the insulinotropic effect of the three amino acids on Ca^{2+} is well in line with their ability to induce insulin release alone, at substimulatory glucose levels (Fig. 1A), as the elevation of $[Ca^{2+}]_{cyt}$ has a proven indispensable role in triggering insulin secretion [50,51].

The resting membrane potential of excitable cells is governed by high

K^+ conductance and the function of Na^+/K^+ ATP-ase that maintains the gradient of the two cations across the plasma membrane. Enhancing or attenuating the control over the resting membrane potential by omitting K^+ from the extracellular solution (Fig. 3B, C) or inhibiting Na^+/K^+ ATP-ase with ouabain (Fig. 5A) led to abolition or enhancement of the effects of taurine and the other two amino acids, respectively, confirming the pivotal role of the plasma membrane excitability in mediating the amino acid signal in β-cells.

4.2. The role and the identity of Na^+ conductance

These depolarising effects in β-cells are linked to the increases of Ca^{2+} or Na^+ conductances, that are low under the resting conditions [52]. With this in mind, voltage-gated Na^+ channels of mouse, human or clonal [53] β-cells represent a puzzling component of the secretory machinery, as they tend to open at extremely low membrane potentials, unreachable under physiological conditions [44,54]. In line with this, inhibition of $Na_V1.7$ and $Na_V1.3$ channels with TTX had no effect on the insulinotropic potency of taurine, alanine or proline (Fig. 5B).

Given the small size of the resting Na^+ conductance, omission of Na^+ in extracellular solution (Fig. 5C) is unlikely to produce any acute effect on the plasma membrane potential in β-cells; the backward induction would therefore indicate the role of Na^+ conductance in mediating the amino acid signal, which is abolished upon the depletion of extracellular Na^+ (Fig. 5C). This observation therefore extends earlier findings of the indispensable role of extracellular Na^+ in triggering of membrane depolarisation and Ca^{2+} dynamics by L-alanine (MW = 89 Da) [55], adding two larger neutral amino acids, taurine (MW = 125) and proline (MW = 115) to the cohort of species handled by system A amino acid
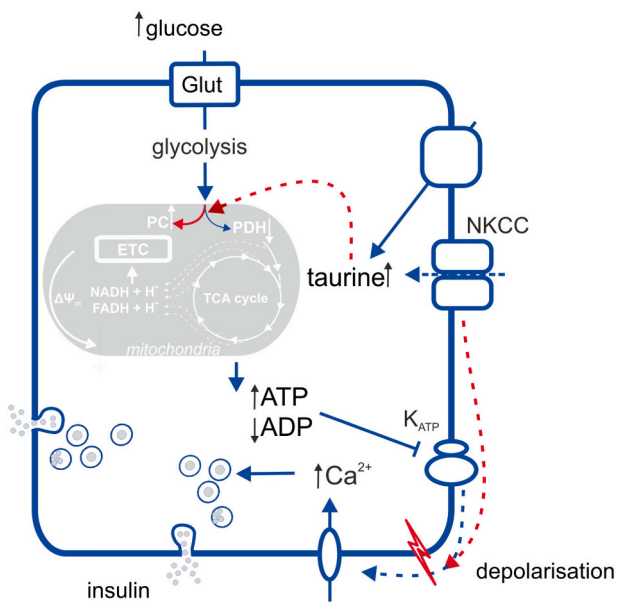
Fig. 7. Schematic of the proposed mechanism of the insulinotropic effect of taurine.

Glucose metabolism elevates cytosolic ATP/ADP ratio, which inhibits K_{ATP} channels thereby depolarising the membrane. NKCC function that facilitates taurine entry into the β-cell, depolarises the membrane per se and/or enhances the glucose-induced depolarisation. At the same time, regulatory effect of taurine results in an enhanced flux through pyruvate carboxylase thereby enhancing the anaplerosis.

transport in β-cells.

The inhibition of NKCC, that symports $1Na^+$ and $1 K^+$ alongside 2 Cl^- into the cell with loop diuretics resulted in a significant attenuation of the effects induced by the amino acids in clonal and primary β-cells (Figs. 5E, F; 6). Although this finding unequivocally indicates a key role of NKCC in the amino acid signalling, we should note that the effects of the taurine, alanine and proline were not completely abolished by high concentration (100 μM) of bumetanide or furosemide that exhibit IC_{50} values for NKCC of 2.5 μM and 8 μM, respectively. There exists no evidence of the direct transport of amino acids by NKCC. At the same time, the import of Na^+ by NKCC would shunt the flux of the amino acid – Na^+ symporter (hypothetically Slc6a6/TauT) that was demonstrated to mediate taurine influx in other systems [56]. The complex interplay between the activities of NKCC and TauT may however require consideration of the other two transported ionic species, Cl^- and K^+. Had the transport via NKCC been driven by the reversal potentials of K^+ and Cl^-, the transporter would be operating in the reverse direction thereby clearing the β-cell cytosol off the elevated $[Cl^-]$ and reducing the gradient of the chemical potential that TauT works against when co-importing Cl^-.

4.3. Does oxidation of taurine and other neutral amino acids underlie their insulinotropic effect?

Added at basal glucose, taurine and the two other neutral amino acids tested in this study acutely induced secretion of insulin in clonal β-cells and altered Ca^{2+} dynamics in primary mouse and human β-cells (Figs. 1A, 6). The pharmacological profiles of the insulinotropic effect clearly indicate the involvement of Na^+ transport via NKCC and plasma membrane depolarisation. Although the system A Na^+-dependent transport into the β-cell has been advocated for alanine [12,55,57] and proline [58], the former amino acid has been viewed as an important oxidative substrate [7,59], with a marked saturation of the insulinotropic effect upon prolonged exposure [59]. The similarity of insulinotropic profiles for alanine and proline may prompt the involvement on

the oxidative metabolism in proline signalling in β-cells, presumably though the conversion into glutamate [60] and further oxidation by glutamate dehydrogenase [61]. At the same time, as the insulinotropic effect of taurine is not associated with oxidation of the amino acid [23], our data rather argues against the importance of direct oxidation of the three amino acids for their insulinotropic effect.

4.4. Can neutral amino acids regulate fuel metabolism in β-cells?

The major physiological stimulator of insulin secretion, glucose is metabolised in the β-cell cytosol, tightly linked to pyruvate entering the mitochondria. The latter is processed by pyruvate dehydrogenase and pyruvate carboxylase to fuel and maintain oxidative metabolism, which has the elevated levels of cytosolic ATP as an output. Subsequent specific inhibition of ATP-sensitive K^+ (K_{ATP}) channels by the phosphonucleotide [62], a step dysfunctional in some forms of diabetes [63], links the metabolic stimulus to the membrane excitability and triggers the influx of Ca^{2+} into the cytosol that drives the hormone exocytosis. The site of interaction between the metabolism of taurine and glucose in β-cells is however unclear. Speculatively, the anaerobic degradation of taurine via the production of alanine and 2-sulfoacetaldehyde by taurine-pyruvate aminotransferase [64], may deplete intracellular levels of pyruvate and hence acetyl-CoA (Fig. 3D). The feasibility of this machinery in mammalian system is however a matter of further research.

In our hands, the potentiating effect of taurine and alanine on glucose-induced insulin secretion exceeded the arithmetic sum of the effects of the two stimuli added alone (Fig. 1A), indicating potential regulatory role for the two amino acids in fuel metabolism. The NMR data suggested that taurine may regulate the balance between the oxidative metabolism and anaplerosis in β-cells, an observation that, however, cannot be extrapolated to other small amino acids. Whereas taurine could reduce intracellular pyruvate, alanine is a proven pyruvate precursor, in BRIN BD11 cells [65].

Given its systemic abundance, taurine bears a potentially very impactful ability to ameliorate various aspects of diabetes, such as the elevated platelet aggregation in diabetes [66]. However, the clinical link between taurine signalling and insulin secretion or diabetes pathophysiology overall remains unclear [47]. Recent studies have proposed also that dietary taurine may be of utility in the treatment of diabetes, exerting stimulatory actions on islet cell turnover as well stimulating beta cell function [23]. Such data suggest possible new horizons for this amino acid and the present study is important in so far as it demonstrates that taurine has potent glucose-dependent stimulatory effects on insulin secretion. We show that taurine principally exerts its actions on beta cells by virtue of its being co-transported with Na^+, where Na^+ influx causes membrane **depolarisation**, resulting in an increase in intracellular Ca^{2+} and insulin secretion. However, of course, the present data do not exclude the possibility of additional intracellular β-cell metabolic actions of taurine, which warrant further investigation.

These actions are highlighted by mechanistic discrepancies between the effects of taurine, alanine and proline. For instance, the stronger impact of taurine on plasma membrane potential (Fig. 1E) may reflect the ability of the amino acid to inhibit K_{ATP} channels reported earlier in several systems [16,67]. Secondly, the partial reversal of the inhibitory effect of mannoheptulose on glucose-induced insulin secretion by taurine but not by alanine or proline (Fig. 2B, inset) highlights an exclusive impact of taurine on glucose metabolism, in line with the metabolomics data (Fig. 3).

5. Conclusion

In contrast to carbohydrate fuels, the full spectrum of insulinotropic effects of amino acids could be viewed as a conundrum as many key molecular players are yet to be elucidated. The physiological impact of the amino acid signalling also remains a matter for future research. Whilst from a clinical standpoint, 10 mM systemic levels of taurine and

other neutral amino acids are deemed unlikely, small concentrations can have a critical role under the condition of partial inhibition of K_{ATP} channels, at moderate glycaemia. Thus, dietary intake of taurine or production of systemic alanine via Cahill cycle are likely to represent special cases of physiological regulation of insulin output by small neutral amino acids.

CRediT authorship contribution statement

JT: Methodology and investigation, data curation, visualisation. LB: Methodology and investigation, data curation, visualisation, reviewing, editing. RCM: Conceptualization, supervision, methodology. PRVJ: Conceptualization, methodology. PRF, NHM: Conceptualization, supervision, writing, reviewing, editing. AIT: Data curation, investigation, visualisation, conceptualization, writing, reviewing, editing.

Declaration of competing interest

All authors declare no conflicts of interest.

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