Contrasting Effects of Glycerol and Urea Transport on Rat Pancreatic β -Cell Function

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Key Words

Islet • Pancreatic β -cell • Volume regulation • Anion channel • Aquaporin • Electrical activity • Insulin secretion

Abstract

Background/aims: Pancreatic β -cell function is influenced by changes in cell volume. Such volume changes depend on water permeability of the plasma membrane, conferred in part by aquaporins. Islet cells express aquaporin 7 (AQP7), which is permeable to urea and glycerol in addition to water. We therefore investigated the effects of glycerol and urea on rat pancreatic β -cell function. **Methods:** Electrical activity and whole-cell current were studied using the perforated patch technique. Cell volume was measured by video-imaging and insulin release by radioimmunoassay. Aquaporin 7 expression was studied by RT-PCR, Western blot and double fluorescent immunolabelling. **Results:** The isosmotic addition of glycerol and urea resulted in depolarization of the plasma membrane and electrical activity, accompanied by β -cell swelling, activation of the volume-regulated anion channel (VRAC) and insulin release. However, the effects of glycerol, in contrast to urea, persisted throughout exposure to the

osmolyte. Glycerol also caused β -cell activation when added hyperosmotically. A non-metabolizable glycerol analogue had comparable effects to urea on β -cells. The expression of AQP7 was demonstrated in rat β -cells. **Conclusion:** Glycerol and urea can activate β -cells via their rapid uptake across the β -cell plasma membrane, possibly via AQP7. This results in cell swelling, VRAC activation, electrical activity and insulin release. Glycerol appears to exert an additional effect, possibly related to its intracellular metabolism.

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Introduction

There is considerable evidence that the activity of pancreatic β -cells can be profoundly influenced by changes in cell volume. Indeed, several studies have demonstrated that exposure of insulin-secreting cells to hypotonic extracellular solutions causes cell swelling and depolarization of the cell membrane potential, leading to electrical activity and insulin release [1-7]. We have suggested that activation of the volume-regulated anion channel (VRAC) plays a central role in these responses

[8]. Briefly, VRAC activation in response to cell swelling results in Cl^- efflux thereby generating an inward (depolarizing) current, leading to activation of voltage-sensitive Ca^{2+} channels, Ca^{2+} entry and hence exocytosis.

In contrast to epithelial cells, pancreatic islet cells are not likely to be exposed to major changes in extracellular osmolarity. However, it is probable that the processes of transport and intracellular metabolism of substrates and metabolites in islet cells could result in alterations in intracellular osmolarity and hence cell volume. Indeed, a rise in glucose concentration has been shown to increase β -cell volume in a manner dependent upon metabolism of the sugar [9, 10]. Furthermore, there is increasing evidence that this response, coupled with VRAC activation, could play an important role in the regulation of β -cell electrical and thereby secretory activity of glucose [8].

Whether β -cell swelling results from exposure to hypotonic extracellular solutions or from a hypertonic intracellular medium, the participation of aquaporins in the plasma membrane is likely to be important in mediating water uptake necessary for cell swelling to occur. Aquaporins (AQPs) are a family of water channels consisting of 13 members, AQP0 to AQP12 [11]. AQPs are small hydrophobic integral membrane proteins of about 270 amino acids presenting six transmembrane domains in each monomer, as well as three extracellular and two intracellular loops [12]. Two repeating Asn-Pro-Ala (NPA) sequences, present in the first intracellular and third extracellular loop, represent the amino acid signature sequence motifs of AQPs [11]. Based on their permeability characteristics and their amino acid sequences, the members of the AQPs family can be divided in three groups: the aquaporins, the aquaglyceroporins, and the aquaporins containing unusual NPA motifs. The aquaporins (AQP0, AQP1, AQP2, AQP4, AQP5, AQP6, AQP8) are primarily permeable to water, whereas the aquaglyceroporins (AQP3, AQP7, AQP9, AQP10) also transport glycerol and small solutes [11, 13-17]. The aquaporins containing unusual NPA motifs appear to be more distantly related to the other mammalian aquaporins and aquaglyceroporins [11, 18]. While several AQPs are expressed in exocrine pancreas, only AQP7, an aquaglyceroporin, is expressed in mouse pancreatic β -cells [19]. AQP7 is permeable not only to water but also to certain small osmolytes, notably glycerol and to urea [20], and is thought to be involved in the transport of glycerol across the plasma membrane [13]. Accordingly, islets from AQP7^{-/-} knockout mice were reported to contain elevated islet cell glycerol and triglyceride content

[19]. Furthermore, islets from these mice also showed increased rates of basal and glucose-stimulated insulin release, reduced insulin content and reduced islet cell number and β -cell mass. From these findings, the authors suggested that AQP7 could be important in normal β -cell function, although no mechanistic explanations were suggested.

In the present study, we have investigated the effects of glycerol and urea on several aspects of rat pancreatic β -cell function and also detected the expression of AQP7 in these cells. Our findings indicate that glycerol and urea are highly permeable across β -cell plasma membrane. Since aquaglyceroporins represent the only known membrane pathway that can explain high glycerol permeability [17], it is possible that this uptake is mediated by AQP7. The rapid entry of glycerol and urea leads to intracellular hypertonicity, cell swelling and VRAC activation. This results in depolarization of the plasma membrane potential, electrical activity and insulin release. The effects of glycerol on β -cell function were more sustained than those of urea and therefore probably involve an additional mechanism, possibly related to its intracellular metabolism.

Materials and Methods

Islet cell preparation

Pancreatic islets were isolated from Sprague-Dawley rats (300-350g; either sex) by collagenase digestion (Worthington type 4, Cambridge Biosciences, Cambridge, UK). Islets were dispersed into single cells by brief exposure to a Ca^{2+} -free medium consisting of 130 mM NaCl, 5 mM KCl, 2 mM MgSO_4 , 4 mM glucose, 1 mM EGTA, 1% (w/v) bovine serum albumin and 25 mM Hepes-NaOH (pH 7.4), centrifuged at 100g for 5 min and re-suspended in Hepes-buffered Minimal Essential Medium (MEM; Gibco, Paisley, Scotland) containing 5% (v/v) bovine serum albumin and gentamycin (50 $\mu\text{g ml}^{-1}$). Cells were then plated onto 30 mm diameter polystyrene dishes and cultured for 2-12 days in humidified air at 37°C. β -cells were identified by their size (larger than non β -cells), typical granular appearance and polarized membrane potential (~ -60 mV) in the presence of a low glucose concentration. Cells identified by such criteria correspond to insulin-stained cells [21]. The standard incubation medium used for islet cell preparation and incubations consisted of 130 mM NaCl, 5 mM KCl, 1 mM MgSO_4 , 1 mM NaH_2PO_4 , 1.2 mM CaCl_2 , 25 mM Hepes-NaOH (pH 7.4) and 5 mM glucose. For isosmotic substitution experiments, the basal medium contained 50 mM mannitol substituted for 25 mM NaCl. The addition of urea, glycerol or 1,3 propanediol to the medium was then substituted for an equivalent amount of mannitol. In other hyperosmotic addition experiments, these osmolytes were added to the incubation medium with no substitution.

Electrophysiology

Cells were preincubated for at least 10 min in the basal medium containing 50 mM mannitol substituted for 25 mM NaCl (this had no significant impact on the resting membrane potential), then superfused at a rate of approximately 2 ml min⁻¹ with incubation medium. Membrane potential was recorded from single β -cells using the perforated patch technique with a List EPC-7 amplifier (List, Darmstadt, Germany). The pipette solution consisted of 130 mM KCl, 5 mM NaCl, 1 mM MgCl₂ and 25 mM Hepes-NaOH (pH 7.2) and 50 μ g/ml gramicidin D. Activity of the VRAC was recorded at the whole-cell level, again using the perforated patch configuration. The pipette solution was designed to eliminate all cationic conductances and contained 40 mM CsCl, 40 mM Cs₂SO₄, 1 mM MgCl₂, 25 mM HEPES-CsOH (pH 7.2) and 240 μ g/ml amphotericin B. Current was recorded at a holding potential of -65 mV. Under such conditions, VRAC activation is manifest as a characteristic noisy inward current [22, 23]. Urea, glycerol, 1,3 propanediol and all other chemicals were obtained from the Sigma Chemical Co. (Poole, UK).

Cell volume measurements

Changes in β -cell volume were measured by a video-imaging technique as described previously [9, 24]. Cell volume is expressed as relative cell volume (i.e. normalized with respect to the volume observed for the initial reading which is given as 1.00). All measurements were carried out 'blind' in order to avoid bias.

Insulin secretion

The effects of urea and glycerol on insulin release were studied using groups of 5 intact islets incubated in 1 ml of medium. Following a 30 min incubation, samples of supernatant were removed and the insulin content measured by radioimmunoassay. Secretion experiments were carried out at 37°C.

RT-PCR

Total RNA from rat tissues was extracted using the AURUMTM total RNA fatty acid and tissue kit (BioRad, Hercules, CA, USA). The purified total RNA was quantified on a Nanodrop spectrophotometer (NanoDrop Technologies, Inc, Wilmington, DE, USA). Quality of the total RNA was verified on an Experion Automated Electrophoresis System (BioRad, Hercules, CA, USA). The total RNA was reverse transcribed using the Revert AidTM first strand cDNA synthesis kit (Fermentas, St. Leon-Rot, Germany), starting from 1 μ g total RNA in a final 20 μ l reaction volume. The sense rat AQP7 primer used was 5'-ACC ACT ATG CAG GTG GAG AAC T-3' (nt 629-650) and the antisense primer 5'-TGA AGT GCT GGA CTG TTCAAC T-3' (nt 788-809) (from accession n° NM_019157, amplicon: 181 bp). Control β -actin cDNA sense primer was 5'-TGA CGG GGT CAC CCA CAC TGT GCC CGT C-3' (nt 539-566) and the antisense 5'-CTA GAA GCA TTA GCG GTG GAC GAT GGA GG-3' (nt 1171-1199) (from accession n° BC002409, amplicon: 661 bp). All PCR reactions were performed in a total volume of 20 μ l containing 1 μ l of cDNA, 0.5 U GoTaq DNA polymerase (Promega, Madison, WI, USA), 0.2 mM dNTP,

0.5 μ M of each primer, and 4 μ l GoTaq Green 5x buffer using My iCycler Thermal cycler (Bio-Rad Laboratories, Hercules, USA). Thermocycling conditions were 94° C for 1.5 min followed by 35 cycles of 30 sec at 95° C, 30 sec at 60° C and 1 min at 72° C. To further exclude amplification from potential contaminating genomic DNA (despite an on column DNase treatment included during the RNA purification procedure), primers were designed in separate exons flanking an intron. The PCR amplification products were submitted to electrophoresis on a 1.2% agarose gel in TAE buffer: 40 mM Tris, 40 mM Acetate, 1 mM EDTA in the presence of 0.5 μ g/ml of ethidium bromide. Gels were visualized by UV transillumination using a GelDoc apparatus (Bio-Rad Laboratories, Hercules, USA).

Western blot analysis

Total proteins were prepared from rat pancreatic islet cells and visceral adipose tissue by homogenization of cells in lysis buffer (50 mM HEPES, 150 mM NaCl, 10 mM EDTA, 10 mM tetrasodium pyrophosphate, 1.5% CHAPS, and a protease inhibitor cocktail (Complete EDTA-free; Roche Diagnostics, Mannheim, Germany), 1 tablet per 25 ml of buffer), followed by centrifugation of the homogenate at 17,000 g and finally collecting the supernatant. Total proteins were used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE). 5% (v/v) of β -mercaptoethanol was added to the sample buffer. The samples were analyzed by SDS-PAGE using 12% polyacrylamide gels. Proteins were transferred to polyvinylidene difluoride membranes and immunolabeled using an affinity-purified rabbit antibody to AQP7 at a 1:500 dilution (Alomone, Jerusalem, Israël). The bound antibodies were detected by using an anti-rabbit secondary antibody at a dilution 1:5000 (GE Healthcare, Little Chalfont, United Kingdom) and an ECL chemiluminescence method (GE Healthcare, Little Chalfont, United Kingdom).

Double fluorescence immunolabelling

Several sections of rat pancreas, embedded in paraffin (4 μ m thickness), were deparaffined, rehydrated and then incubated in a solution containing 1% H₂O₂ for 30 min, 10% horse serum for 60 min. A 15 min incubation in avidin and biotin solutions followed (Vectra Laboratories, Geneva, Switzerland). Primary antibodies were incubated overnight as follows: rabbit polyclonal affinity purified anti-AQP7, dilution 1:100 (Alomone, Jerusalem, Israel); mouse monoclonal affinity-purified anti-insulin, dilution 1:100 (Abcam, Cambridge, United Kingdom); mouse monoclonal affinity-purified anti-glucagon, dilution 1:200 (Sigma-Aldrich, Saint Louis, USA); mouse monoclonal affinity-purified anti-somatostatin, dilution 1:25 (GeneTex, Sydney, Australia). Bound antibodies were revealed by incubating the tissue slices with anti-rabbit biotin antibodies at a dilution of 1:200 in PBS containing 5% horse serum (GE Healthcare, Little Chalfont, United Kingdom) for 120 min. This was followed by incubation with streptavidin-Cyanin2 (dilution 1:300 in PBS containing 5% horse serum (GE Healthcare, Little Chalfont, United Kingdom)) and anti-mouse-Cyanin3 (dilution 1:300 in PBS containing 5% horse serum (GE Healthcare, Little Chalfont, United Kingdom)) for 120 min. Slides were mounted using Pro-Long Gold antifade reagent (Invitrogen, Eugene,

Fig. 1. Effects of isosmotic addition of urea, glycerol and 1,3 propanediol (1,3 PD) on membrane potential in isolated rat β -cells. In all cases, these substances were substituted for an equivalent amount of mannitol. 5 mM glucose was present throughout. The gap * in B represents a period of approximately 10 min following withdrawal of glycerol. Traces A and B are typical of 8-10 similar recordings.

Fig. 2. A: Lack of effect of 1,3 propanediol (PD) on a β -cell despite subsequent depolarisation by glycerol. B: Inhibition of glycerol-induced depolarisation by VRAC blocker 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB; 50 μ M). C: Irreversible depolarisation of β -cells caused by the aquaporin blockers silver sulphadiazine (AgS) and mercuric chloride.

USA). As negative controls, tissue sections were incubated either with secondary antibody alone, or with anti-AQP antibody pre-adsorbed with a 100-fold excess of the immunizing peptide. Images were taken using an Axiocam MRB fluorescent microscope using a 40x objective.

Statistical analysis

Where appropriate, data are expressed as mean $\pm$ SEM, statistical significance being ascribed using Student's paired or unpaired t-test.

Results

Effects of isoosmotic urea, glycerol, and 1,3 propanediol on membrane potential in isolated rat β -cells

As shown in Fig. 1A, the isosmotic addition of urea (50 mM by substituting for mannitol) caused a depolarization in 6/8 cells resulting in electrical activity.

This effect presumably reflects the entry of urea into the cell resulting in cell swelling and VRAC activation. This effect of urea was transient in nature, the cells eventually repolarized in the continued presence of urea, possibly reflecting a process of regulatory volume decrease (RVD). In contrast, the isosmotic addition of glycerol caused a marked depolarization in all 10 cells tested such that a brief period of electrical activity was followed by a prolonged 'silent' depolarization (Fig. 1B). This silent depolarization was sustained throughout the period of exposure to glycerol although the cells gradually repolarized following the replacement of glycerol by mannitol. We next investigated the effect of 1,3 propanediol, a non-metabolisable analogue of glycerol which also permeates through aquaglyceroporin AQP7, on β -cell membrane potential. In 6/10 cells, isosmotic addition of 50 mM 1,3 propanediol caused a modest, transient depolarization, in some cells resulting in electrical

activity (e.g. Fig. 1C). However, the remainder of the cells tested showed little or no response to 1,3 propanediol, despite a marked response to the subsequent application of glycerol (Fig. 2A). These findings suggest that the effects of glycerol on β -cell membrane potential involved not only its transport into the cell but an additional component, presumably its metabolism.

Effects of VRAC and AQP inhibitors on the depolarizing action of glycerol in isolated rat β -cells

The depolarizing action of glycerol was reversibly inhibited by the VRAC inhibitor blocker 5-nitro-2-(3-phenylpropylamino) benzoic acid (NPPB; Fig. 2B). Similarly, urea failed to depolarize the cells in the presence of this inhibitor (not shown). Although NPPB is not a specific inhibitor of VRAC, these findings are consistent with the idea that depolarization in response to glycerol and urea is the result, at least in part, of β -cell swelling and VRAC activation. Attempts were made to investigate the role of aquaporins in the β -cell responses to urea and glycerol by using purported aquaporin blockers. However, as shown in Fig. 2C, two such inhibitors, silver sulfadiazine (AgS) and mercuric chloride (HgCl_2), caused a rapid and essentially irreversible depolarization of the cell membrane potential. Thus, these aquaporin blockers are clearly unsuitable for the study of the role of aquaporins in β -cell electrical activity. Indeed, the gross appearance of the cells following exposure to AgS and HgCl_2 strongly suggested a major cytotoxic action of these substances.

Effects of isosmotic urea, glycerol and 1,3 propanediol on whole-cell current in β -cells

The effects of isosmotic urea, glycerol and 1,3 propanediol on whole-cell current in β -cells are shown in Fig. 3. Both urea and glycerol evoked a noisy inward current in cells voltage-clamped at -65 mV, amounting to a maximal net increase in inward current of -13.8 ± 1.9 (n=10) and -19.3 ± 6.0 (n=6) pA respectively. The addition of 1,3 propanediol led to a somewhat smaller increase in inward current (-7.9 ± 1.4 pA; n=6), consistent with the relatively modest effect on β -cell membrane potential noted earlier. The characteristics of this current closely resembled those of the VRAC current evoked under similar conditions by increased glucose concentrations [22] and in response to cell swelling [2], and were inhibited in the presence of NBBP (not shown), consistent with the suggestion that osmolyte (and hence water) entry into the cells causes cell swelling and thus VRAC activation. Consequently, the effects on β -cell volume of isosmotic

Fig. 3. Effects of isosmotic addition of urea, glycerol and 1,3 propanediol on whole-cell current in rat pancreatic β -cells. Cells were voltage-clamped at -65 mV under perforated patch conditions. In each case, addition of these osmolytes was made by substituting for an equivalent amount of mannitol. 5 mM glucose was present throughout.

addition of urea, glycerol and 1,3 propanediol were investigated.

Effects of isosmotic addition of urea, glycerol and 1,3 propanediol on β -cell volume

As shown in Figure 4A, application of 50 mM urea increased relative cell volume (RCV) from 1.0 to a peak value at 6 min. of 1.115 ± 0.017 (n=6), reflecting urea uptake into the cells. Cell swelling was followed by a gradual regulatory volume decrease (RVD) to a relative value at 12 min. of 1.049 ± 0.027 ($P < 0.005$ by paired t-test), presumably due to VRAC activation. A similar degree of cell swelling was provoked by the isosmotic addition of 50 mM glycerol (Fig. 4B). However, in this case no subsequent RVD was observed, the 6 and 12 min. values being 1.090 ± 0.026 and 1.064 ± 0.017 respectively (n=6; $P > 0.1$). This lack of significant RVD could be due to the intracellular accumulation of glycerol metabolites and hence the maintenance of a hypertonic intracellular medium. Consistent with this suggestion, the isosmotic addition of non-metabolisable 1,3 propanediol

Fig. 4. Effects of isosmotic addition of urea, glycerol and 1,3 propanediol (1,3 PD) on relative cell volume (RCV) in rat pancreatic β -cells. Addition of these osmolytes was made by substituting for an equivalent amount of mannitol. Each point represents the mean $\pm$ SEM from 4-6 experiments.

caused cell swelling to 1.108 ± 0.036 followed by an RVD to 0.989 ± 0.012 ($P < 0.05$; Fig. 4C), thus resembling the response to urea.

Effects of isosmotic addition of urea, glycerol and 1,3 propanediol on insulin release from intact pancreatic islets

The effects of isosmotic addition of urea, glycerol and 1,3 propanediol on insulin release is shown in Fig. 5. While it is clear that all of the above osmolytes stimulated insulin release, the extent of the secretory effect was not entirely consistent with the corresponding pattern of electrical activity. For example, urea and glycerol stimulated insulin release to a similar extent, but glycerol provoked a more marked and sustained effect on

Fig. 5. The effects of isosmotic addition of urea, glycerol (glycerol) and 1,3 propanediol (1,3 PD; all 50 mM, substituted for mannitol) on insulin release from intact pancreatic islets. Each point represents the mean $\pm$ SEM of 6 determination. * $P < 0.05$; ** $P < 0.01$ compared to controls.

membrane potential. However, it should be borne in mind that, in contrast to the electrophysiological experiments, the secretory measurements involved the use of intact islets in static 'batch' type incubations. It is also possible that the above osmolytes could influence insulin release by mechanisms independent of electrical activity, including distal sites of the secretory pathway.

Effects of hypertonic addition of urea and glycerol on membrane potential in rat pancreatic β -cells

The effects of isosmotic addition of urea on β -cell function described above are consistent with uptake of urea, possibly via an aquaporin, accompanied by water uptake leading to cell swelling, VRAC activation, depolarization and electrical activity. As noted above, an additional component, possibly involving metabolism, appears to contribute to the actions of glycerol. Consistent with this possibility, glycerol was found to depolarize β -cells and evoke electrical activity even when added hyperosmotically (i.e. with no substitution for mannitol; Fig. 6B). The hyperosmotic addition of urea had no such effect but rather, in 5/13 cells, caused a modest, transient hyperpolarization of the membrane potential (Fig. 6A), presumably reflecting cell shrinkage followed by urea entry and volume recovery. In such cases, the subsequent withdrawal of urea was followed by a depolarization, possibly due to cell swelling on exposure to the relatively hypotonic solution. It should be stressed that the

Fig. 6. Effects of hyperosmotic addition of urea and glycerol on membrane potential in rat pancreatic β -cells. These osmolytes were added to the bath medium with no substitution for mannitol. The gap * in trace B represents a period of approximately 15 min. Each trace is typical of 4-9 similar recordings.

hyperosmotic addition of urea or glycerol shrank the cell as expected, albeit very transiently (data not shown), and this was quickly followed by volume recovery due to rapid entry of glycerol or urea. The effect of hyperosmotic glycerol was concentration-dependent, lower concentrations tending to require longer exposure to depolarize the cells (Fig. 7).

AQP7 expression in rat pancreatic islet cells

As noted above, AQP7 expression has been reported in mouse pancreatic islet cells [19]. We therefore next examined whether expression of this aquaporin could be detected in rat islet cells. RT-PCR was performed using β -actin primers or rat AQP7 specific primers on cDNA obtained from purified total RNA isolated from rat visceral adipose tissue (positive control), total pancreas and rat pancreatic islet cells. For each tissue, the amplification reaction yielded single amplicons of the expected molecular weight corresponding to actin (660 bp) and AQP-7 (181 bp) (Fig. 8A). Western blot analysis with anti-AQP7 antibody revealed a band of approximately 37 kDa in total proteins from visceral adipose tissue, used as a positive control, and from rat pancreatic islets (Fig. 8B). On sections from rat pancreas, AQP7 expression colocalized with insulin positive rat pancreatic β -cells by double fluorescent immunolabelling (Fig. 8C,1). AQP7 expression was not detected in rat pancreatic α -cells as its labeling did not colocalize with that of glucagon (Fig. 8C,2), but was detected in some D-cells as its labeling colocalized with some somatostatin positive cells (Fig. 8C,3).

Discussion

It has been previously shown that the isosmotic addition of permeant molecules, namely urea and 3-O-methyl glucose, to pancreatic islets stimulates the release of insulin [25], an effect presumably related to an ensuing increase in β -cell volume. Since this effect persisted in the absence of Ca^{2+} , the authors suggested that it was the result of cell swelling per se, rather than any ionic movements involved in cell volume regulation. However, the present study clearly demonstrates that the isosmotic

Fig. 7. Effects of hyperosmotic addition of glycerol on membrane potential in rat pancreatic β -cells. Glycerol was added to the bath medium with no substitution for mannitol. 5 mM glucose was present throughout. The gap * in trace C represents a period of approximately 12 min. Each trace is typical of 3-6 similar recordings.

Fig. 8. AQP7 expression in rat pancreatic islet cells. A: Gel Electrophoresis of the RT-PCR reactions: upper panel showing amplicons using AQP7 primers on mRNA extracted from visceral adipose tissue (TAV), isolated pancreatic islets (Islets) and total pancreas (Pancreas). Ladder: 100 bp DNA ladder; NTC: non-target control. Lower panel: results obtained for the amplification on the same tissue cDNA preparations using control β -actin primers. B: Western blot analysis performed using total proteins from rat submandibular gland (15 μ g), used as negative control, visceral adipose tissue (15 μ g), used as positive control, and rat pancreatic islets (20 μ g) and anti-AQP7 antibodies. MW: molecular weight markers. C: Double fluorescent immunolabelling of AQP7 protein expression in rat pancreatic β -cells. 1: AQP7 labelling (in green) overlaps with that of insulin (in red); 2: AQP7 labelling does not overlap with that of glucagon (in red); 3: AQP7 labelling (in green) overlaps with that of somatostatin (in red) (indicated by arrows); 4: negative control performed in the absence of primary antibodies. Bar represents 100 μ m.

addition of urea to rat pancreatic β -cells causes cell swelling, activation of the VRAC, depolarization, electrical activity and insulin release. VRAC activation by isosmotic cell swelling is consistent with, and could explain the previously reported stimulation of taurine efflux from INS-1 cells by urea under such conditions [26]. All the above effects are clearly reminiscent of the effects of hypo-osmotic cell swelling on these parameters described earlier, and suggest that ionic effects are indeed implicated in swelling-induced β -cell activation. Such ionic effects of cell swelling probably explain the finding that swelling-induced insulin release from BRIN-BD11 cells was sensitive to removal of extracellular Ca^{2+} [1]. This does not of course preclude the possibility of additional, possibly distal effects of cell swelling on the exocytotic pathway.

The present study also demonstrates that glycerol, like urea, is able to cross the β -cell plasma membrane causing cell swelling, VRAC activation, electrical activity and insulin release. However, in contrast to urea, these effects persisted throughout exposure to the osmolyte.

Furthermore, glycerol was found to activate β -cells even when added hyperosmotically. These findings strongly suggest that β -cell activation by glycerol is not solely the result of its transport into the cell but involves an additional action. One possibility is that glycerol metabolism could contribute to its stimulatory effects. Consistent with this suggestion, the non-metabolisable glycerol analogue 1,3 propanediol exerted a very modest, transient effect on β -cell function. In fact, the stimulatory effects of this compound were invariably less pronounced than equivalent concentration of urea, despite a comparable degree of cell swelling induced by both agents. Though the reason for this is as yet unclear, it is unlikely due to slower 1,3 propanediol uptake since cell swelling occurs at similar rates with both urea and 1,3 propanediol. Moreover, it is possible that urea and 1,3 propanediol exerts effects on β -cell function in addition to volume changes.

Thus, our data strongly indicate that glycerol metabolism could contribute to its effects on β -cell function. Glycerol metabolism by pancreatic islet cells is

subject to some disagreement. Indeed, an initial study of glycerol kinase activity in rat islet homogenates revealed appreciable activity of the enzyme, amounting to approximately 35% of that found in liver homogenates, whilst intact islets also showed efficient oxidation of glycerol [27]. A subsequent report found negligible levels of glycerol kinase in rat islets virally transfected with the β -galactosidase gene [28, 29]. However, recently Matsumura and colleagues [19] have reported again detectable levels of glycerol kinase activity in normal mouse islets.

The concentrations of glycerol and urea used in the present study were obviously supraphysiological. However, this study was designed to investigate the effects of these permeant osmolytes on β -cell volume and activation, rather than to reflect possible effects of circulating glycerol or urea on β -cell function. Such high concentrations of osmolytes were used in order to generate an osmotic gradient comparable to that produced by the removal of 50 mM NaCl from the extracellular medium, a manoeuvre previously shown to induce cell swelling and VRAC activation [1]. The finding that such isosmotic addition of urea or glycerol causes rapid β -cell swelling implies that the cell membrane is highly

permeable to these osmolytes. The finding that AQP7 is expressed in rat as well as mouse β -cells raises the possibility that this aquaporin provides a major pathway for glycerol, and possibly urea uptake. Indeed, aquaglyceroporins are the only known glycerol carrier in mammalian cells. However, we were not able in the present study to investigate the specific role of AQP7 in the β -cell response to urea and glycerol owing to the lack of a suitable AQP blocker. A number of sulphhydryl reagents, including silver and mercury salts, have been used to study osmolyte transport via aquaporins [30, 31], but these compounds appeared to cause major irreversible cytotoxic effects on β -cells. Thus, the exact role of AQP7 in the β -cell responses to urea, glycerol and related osmolytes awaits the identification of new selective and non-toxic AQP blockers or further studies using transgenic animals lacking the AQP7 gene.

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References

- 1 Beauwens R, Best L, Markadieu N, Crutzen R, Louchami K, Brown P, Yates A, Malaisse WJ, Sener S: Stimulus-secretion coupling of hypotonicity-induced insulin release in BRIN-BD11 cells. *Endocrine* 2006;30:353-364.
- 2 Best L, Miley HE, Yates AP: Activation of an anion conductance and B-cell depolarization during hypotonically-induced insulin release. *Exp Physiol* 1996;81:927-933.
- 3 Blackard WG, Kikuchi M, Rabinovitch A, Renold AE: An effect of hyposmolarity on insulin release in vitro. *Am J Physiol* 1975;228:706-713.
- 4 Drews G, Zempel G, Krippeit-Drews P, Britsch S, Busch GL, Kaba NK, Lang F: Ion channels involved in insulin release are activated by osmotic swelling of pancreatic B-cells. *Biochim Biophys Acta* 1998;1370:8-16.
- 5 Jakab M, Grundbichler M, Benicky J, Ravasio A, Chwatal S, Schmidt S, Strbak V, Furst J, Paulmichl M, Ritter M: Glucose induces anion conductance and cytosol-to-membrane transposition of I_{Cl} in INS-1E rat insulinoma cells. *Cell Physiol Biochem* 2006;18:21-34.
- 6 Kinard TA, Goforth PB, Tao Q, Abood ME, Teague J, Satin LS: Chloride channels regulate HIT cell volume but cannot fully account for swelling-induced insulin secretion. *Diabetes* 2001;50:992-1003.
- 7 Marström A, Lund PE, Hellman B: Regulatory volume decrease of pancreatic beta-cells involving activation of tetraethylammonium-sensitive K^+ conductance. *Mol Cell Biochem* 1990;96:35-41.
- 8 Best L, McLaughlin J: Nutrients as regulators of endocrine and neuroendocrine secretion; in: Taylor P, Winderickx J, (eds): *Topics in Current Genetics*. Springer-Verlag, 2004;7:79-111.
- 9 Miley HE, Sheader EA, Brown PD, Best L: Glucose-induced swelling in rat pancreatic beta-cells. *J Physiol* 1997;504:191-198.
- 10 Semino MC, Gagliardino AM, Bianchi C, Rebolledo OR, Gagliardino JJ: Early changes in rat pancreatic B cell size induced by glucose. *Acta Anat* 1990;138:293-296.
- 11 Agre P: Aquaporin water channels (Nobel Lecture). *Angew Chem Int Ed Engl* 2004;43:4278-4290.
- 12 Preston GM, Agre P: Isolation of the cDNA for erythrocyte integral membrane protein of 28 kilodaltons: member of an ancient channel family. *Proc Natl Acad Sci USA* 1991;88:11110-11114.

- 13 Hara-Chikuma M, Verkman AS: Physiological roles of glycerol-transporting aquaporins: the aquaglyceroporins. *Cell Mol Life Sci* 2006;63:1386-1392.
- 14 King LS, Kozono D, Agre P: From structure to disease: the evolving tale of aquaporin biology. *Nat Rev Mol Cell Biol* 2004;5:697-698.
- 15 Krane CM, Goldstein DL: Comparative functional analysis of aquaporins/glyceroporins in mammals and anurans. *Mamm Genome* 2007;18:452-462.
- 16 Takata K, Matsuzaki T, Tajika Y: Aquaporins: water channel proteins of the cell membrane. *Prog Histochem Cytochem* 2004;39:1-83.
- 17 Rojek A, Praetorius J, Frokiaer J, Nielsen S, Fenton RA: A current view of the mammalian aquaglyceroporins. *Ann Rev Physiol* 2008;70:301-327.
- 18 Ishibashi K: Aquaporin subfamily with unusual NPA boxes. *Biochim Biophys Acta* 2006;1758:989-993.
- 19 Matsumura K, Chang B H-J, Fujimiya M, Chen W, Kulkarni RN, Eguchi Y, Kimura H, Kojima H, Chan L: Aquaporin 7 is a β -cell protein and regulator of intraislet glycerol content and glycerol kinase activity, β -cell mass, and insulin production and secretion. *Mol Cell Biol* 2007;27:6026-6037.
- 20 Ishibashi K, Kuwahara M, Gu Y, Kageyama Y, Tohsaka A, Suzuki F, Marumo F, Sasaki S: Cloning and functional expression of a new water channel abundantly expressed in the testis permeable to water, glycerol, and urea. *J Biol Chem* 1997;272:20782-20786.
- 21 Majid A, Speake T, Best L, Brown PD: Expression of the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter in α and β cells isolated from the rat pancreas. *Pflugers Arch* 2001;442:570-576.
- 22 Best L: Glucose-sensitive conductances in rat pancreatic beta-cells: contribution to electrical activity. *Biochim Biophys Acta* 2000;1468:311-319.
- 23 Best L: Glucose-induced electrical activity in rat pancreatic β -cells: dependence on intracellular chloride concentration. *J Physiol* 2005;568:137-144.
- 24 Best L, Sheader EA, Brown PD: A volume-activated anion conductance in insulin-secreting cells. *Pflugers Arch* 1996;431:363-370.
- 25 Lund PE, Berts A, Hellman B: Stimulation of insulin release by isosmolar addition of permeant molecules. *Mol Cell Biochem* 1992;109:77-81.
- 26 Grant ACG, Thomson J, Zammit VA, Shennan DB: Volume-sensitive amino acid efflux from a pancreatic β -cell line. *Mol Cell Endocr* 2000;162:203-210.
- 27 Yilmaz MT, Sener A, Malaisse WJ: Glycerol phosphorylation and oxidation in pancreatic islets. *Mol Cell Endocr* 1987;52:251-256.
- 28 Noel RJ, Antinozzi PA, McGarry JD, Newgard CB: Engineering of glycerol-stimulated insulin secretion in islet beta cells. *J Biol Chem* 1997;272:18621-18627.
- 29 Skelly RH, Wicksteed B, Antinozzi PA, Rhodes CJ: Glycerol-stimulated proinsulin biosynthesis in isolated pancreatic rat islets via adenoviral-induced expression of glycerol kinase is mediated via mitochondrial metabolism. *Diabetes* 2001;50:1791-1798.
- 30 Edashige K, Tanaka M, Ichimaru N, Ota S, Yazawa K-I, Higashino Y, Sakamoto M, Yamaji Y, Kuwano T, Valdez DM Jr, Kleinhans FW, Kasai M: Channel-dependent permeation of water and glycerol in mouse morulae. *Biol Reprod* 2006;74:625-632.
- 31 Yang B, Kim JK, Verkman AS: Comparative efficacy of HgCl_2 with candidate aquaporin-1 inhibitors DMSO, gold, TEA^+ and acetazolamide. *FEBS Lett* 2006;580:6679-6684.