Glucose Induces Anion Conductance and Cytosol-To-Membrane Transposition of ICln in INS-1E Rat Insulinoma Cells

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Key Words

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Abstract

The metabolic coupling of insulin secretion by pancreatic beta cells is mediated by membrane depolarization due to increased glucose-driven ATP production and closure of K_{ATP} channels. Alternative pathways may involve the activation of anion channels by cell swelling upon glucose uptake. In INS-1E insulinoma cells superfusion with an isotonic solution containing 20 mM glucose or a 30% hypotonic solution leads to the activation of a chloride conductance with biophysical and pharmacological properties of anion currents activated in many other cell types during regulatory volume decrease (RVD), i.e. outward rectification, inactivation at positive membrane

potentials and block by anion channel inhibitors like NPPB, DIDS, 4-hydroxytamoxifen and extracellular ATP. The current is not inhibited by tolbutamide and remains activated for at least 10 min when reducing the extracellular glucose concentration from 20 mM to 5 mM, but inactivates back to control levels when cells are exposed to a 20% hypertonic extracellular solution containing 20 mM glucose. This chloride current can likewise be induced by 20 mM 3-O-methylglucose, which is taken up but not metabolized by the cells, suggesting that cellular sugar uptake is involved in current activation. Fluorescence resonance energy transfer (FRET) experiments show that chloride current activation by 20 mM glucose and glucose-induced cell swelling are accompanied by a significant, transient redistribution of the membrane associated fraction of ICln, a multifunctional 'connector hub' protein involved in cell volume regulation and generation of RVD currents.

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Introduction

The metabolic regulation of insulin secretion is mainly determined by the plasma glucose concentration, but also by amino acids, triglycerols and free fatty acids. Glucose is taken up by pancreatic beta cells *via* the glucose transporter GLUT2 and metabolized in the process of glycolysis which results in an increased cellular ATP content. ATP binds to the sulfonylurea (SUR)-binding subunit of ATP-sensitive K⁺ channels (Kir 6.2/SUR1) which causes closure of these channels, membrane depolarization, opening of voltage-gated Ca²⁺ channels, Ca²⁺ influx and insulin secretion. Disturbances in this metabolic coupling lead to disease states like diabetes mellitus or hereditary hyperinsulinaemia [1-4]. A detailed knowledge of the molecular entities and signaling pathways involved in this coupling process is therefore inevitable for understanding these diseases and represents the basis for the development of new therapeutic strategies.

Besides this 'classical' stimulus secretion coupling (SSC) insulin release can be elicited or modified by additional mechanisms. These alternative mechanisms of SSC may be of particular importance in pathophysiological conditions, like in type 2 diabetes mellitus, where insulin secretion during hyperglycaemia cannot be satisfyingly explained by the closure of ATP-sensitive K⁺ channels. Of particular interest is the fact that glucose has been found to activate Cl⁻ currents in beta cells (ICl_{glucose}). Several lines of evidence indicate that the anion channels activated during regulatory volume decrease (RVD) after cell swelling (ICl_{swell} regulatory volume decrease channels/ currents, RVDC) play a pivotal role in the process of insulin secretion: (i) osmotic cell swelling induces Cl⁻ currents in insulin secreting cells (ICl_{swell}) [5-8]; (ii) glucose induces cell swelling in pancreatic beta cells [9]; (iii) glucose induces the activation of an anion conductance (ICl_{glucose}) with features resembling RVDC described in a great variety of cells [10-16]; (iv) cell swelling *per se* induces insulin release in clonal beta cells and isolated islets [9, 17-21]; (v) both ICl_{glucose} and the swelling induced insulin release have been shown to be inhibited by the Cl⁻ channel inhibitors 4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid (DIDS), 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB), 4-(2-butyl-6,7-dichloro-2-cyclopentyl-indan-1-on-5-yl)oxobutyric acid (DCPIB) and 4-hydroxytamoxifen [12, 13, 18, 22-26].

The cellular mechanisms leading to an activation of RVDC and/or ICl_{glucose} and the molecular identity of these

channels are still elusive. One protein crucially involved in the activation of RVDC is the multifunctional 'connector hub' protein ICl_h [27]. This protein is highly conserved and ubiquitously expressed in all cell types investigated so far [15] and - while knock-out of ICl_h is lethal - knock-down of the protein has been found to impair the activation of RVD and RVDC. Conversely, overexpression of ICl_h enhances RVDC during hypotonic challenge. ICl_h is water soluble and resides primarily in the cytosol where it forms heteromultimeric complexes with several proteins. It is involved in the processes of RNA-splicing by interaction with Sm proteins and the arginine methyltransferase PRMT5. In addition ICl_h interacts with cytoskeletal proteins like β -actin, erythrocyte protein 4.1 or the non-muscle isoform of the alkali myosin light chain [15]. A fraction of the protein is associated with the cell membrane and the ability of ICl_h to spontaneously insert into artificial lipid bilayers has been shown [28]. An interaction with the membrane protein integrin $\alpha_{\text{IIb}}\beta_3$ has been reported [29]. NMR solution structure analysis of ICl_{h159}, a truncated form of ICl_h, showed that ICl_h belongs to the pleckstrin homology (PH) domain superfold family of proteins [27]. PH domains are highly conserved in regulatory proteins involved in intracellular signaling and are frequently utilized to tether host proteins to the cell membrane. Using the FRET technique we have recently demonstrated that hypotonic cell swelling leads to a cytosol-to-membrane shift and a cellular redistribution of ICl_h [30].

The aim of this study was to further investigate the mechanisms of ICl_{glucose} activation and to test, whether glucose-induced cell swelling - similar to hypotonicity-induced cell swelling - results in an altered interaction of ICl_h with the cell membrane. We show that glucose and the non metabolizable analogue 3-O-methylglucose induce swelling of INS-1E cells and induction of Cl⁻ currents (ICl_{glucose}) which resemble Cl⁻ currents activated by hypotonic cell swelling (ICl_{swell}, RVDC). Cell swelling and current activation are accompanied by a significant redistribution of membrane-associated ICl_h.

Materials and Methods

Salts, Chemicals, Drugs

All salts, chemicals, and drugs (*p.a.* grade) were purchased from Sigma, Germany. Cell culture reagents were from Invitrogen, Germany. Optical filters were from AHF Analysentechnik, Germany.

Plasmids and fusion proteins

Cloning procedures, plasmid preparation and DNA sequencing were performed according to standard protocols [31] and procedures according to the manufacturers' protocols. A plasmid coding for a fusion protein of ICln attached to the C-terminus of ECFP (CFP-ICln) was prepared by cloning the ORF coding for the canine (MDCK) ICln in frame into the pECFP-C1 vector (Clontech) using the XhoI and BamHI restriction sites. For cell membrane labeling with fluorescent proteins (YFP-Mem) the pEYFPMem vector (Clontech) was used. All constructs were sequenced using an automated sequencer (Gene ReadIR 4200, LiCor).

Culture of INS-1E cells

Cells were grown in culture dishes in RPMI 1640 medium containing 11 mM D-glucose supplemented with 10% fetal calf serum, 100 µg/ml penicillin and 100 units/ml streptomycin at 37°C, 5% CO₂ and 95% air. Subcultures were routinely established every second to third day by trypsin/EDTA treatment. For electrophysiological recordings and cell volume measurements cells were seeded onto poly-D-lysine-coated glass coverslips and used for experiments after 24–48 h at 40–70% confluency. Unless otherwise stated cells were starved for 12–24 hours in glucose free RPMI 1640 medium and preincubated for 1 hour in 5 mM glucose medium immediately before the experiments. For transfection with the appropriate plasmids, subconfluent cells were incubated for 6 h with 3 µg of plasmid DNA and 7 µl of LipofectAMINE 2000 (Invitrogen). For double transfection with CFP-ICln and YFP-Mem a ratio of 2:1 yielded the best results. Thereafter the cells were subcultured and grown on uncoated glass cover slips for 24–48 h. The transfection efficiency was approximately 15–20%.

Cell volume measurements

Relative volume of INS-1E cells was measured at room temperature by digital imaging using a Zeiss IM35 inverted microscope (40x objective lens). Images of cells were taken at one minute intervals with a Moticam 1000 digital camera controlled by the Motic Images Plus 2.0 software (Motic, China) under control conditions (isotonic extracellular solution in the absence or presence of 5 mM D-glucose) and after switching to a 30% hypotonic extracellular solution or to an isotonic solution containing 20 mM D-glucose or 20 mM 3-O-methylglucose. The surface area of individual cells was determined using the ImageJ software (NIH, USA; <http://rsb.info.nih.gov/ij/>) and cell volume was calculated assuming cells to be spherical. Values are expressed as relative volume at a given point of time (V) with respect to the volume measured under control conditions (V₀).

Electrophysiological recordings

For the assessment of Cl⁻ currents in INS-1E cells experimental conditions were chosen as described earlier [30]. Whole-cell currents were recorded in the ruptured- or perforated patch clamp configuration at room temperature using an EPC-10 amplifier and data were acquired and analysed with Pulse/FitMaster software (HEKA, Germany). Currents are given as

normalized currents (pA/pF). The intracellular solution for perforated patch clamp recordings contained (in mM): 100 CsCl, 5 MgCl₂, 11 EGTA, 10 HEPES FA, 65 raffinose, pH 7.2 adjusted with CsOH and 120 µg/ml amphotericin B; 305 mosm/l (measured by freezing point depression). Recordings were started when access resistances were <20 MΩ. Cell capacitances were 8–15 pF. Since channels formed by amphotericin B are permeable for monovalent anions and cations this pipette solution allows to establish symmetrical intra- and extracellular Cl⁻ concentrations and to minimize contaminating K⁺ currents due to blockage by Cs⁺. For ruptured whole-cell experiments 2 mM MgATP was added to the intracellular solution. The control isotonic extracellular solution contained (in mM): 100 NaCl, 2.5 CaCl₂, 2.5 MgCl₂, 10 HEPES FA, 80 mannitol, pH 7.2; 305 mosm/l. Omission of mannitol yielded a 30% hypotonic extracellular solution used for activation of RVDC (221 mosm/l). The control isotonic extracellular solution for studying glucose-activated Cl⁻ channels/currents was (in mM): 100 NaCl, 2.5 CaCl₂, 2.5 MgCl₂, 10 HEPES FA, 5 D-glucose, 75 mannitol, pH 7.2; 305 mosm/l. For current activation studies the glucose concentration was increased from 0 or 5 mM to 20 mM. In separate series of experiments 20 mM 3-O-methylglucose was added to this solution instead of 5 mM glucose. Isotonicity was adjusted at the expense of mannitol. The 20% hypertonic extracellular solution containing glucose was composed of (in mM): 100 NaCl, 2.5 CaCl₂, 2.5 MgCl₂, 10 HEPES FA, 20 D-glucose, 115 mannitol, pH 7.2; 360 mosm/l. Extracellular solution exchange was performed using a gravity flow perfusion system. Timecourses of current activation were studied by repetitively applying voltage ramps from -100 mV to +100 mV from a holding potential of 0 mV separated by 10 s intervals. Inward and outward currents were measured at -100 mV and +100 mV, respectively. Current-voltage relations, current rectification and inactivation at positive holding potentials was assessed by applying 500 ms voltage steps from -100 mV to +100 mV (20 mV step increments) from a holding potential of 0 mV. Peak and steady state currents were measured 10–20 ms and 480–490 ms after the onset of voltage pulses, respectively. As a measure for time-dependent current inactivation the ratio between the steady state (ss)- and peak current at +100 mV was calculated (I_{ss}/I_{peak}) with values <1.0 indicating current inactivation.

Fluorescence Resonance Energy Transfer (FRET) measurements

FRET measurements were performed as described previously [30]. ECFP served as a donor and EYFP as an acceptor in all FRET experiments. Cells were cotransfected with CFP-ICln and YFP-Mem. Transfected cells were superfused with a solution composed of (in mM) 130 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.4 and 20 sucrose and allowed to adapt for at least 20 min at room temperature (20–23°C). For glucose stimulation sucrose was replaced by 20 mM D-glucose. Visualization of ECFP- and/or EYFP-expressing cells and FRET detection was performed on an Olympus IX70 inverted microscope equipped with a monochromator (Polychrome 4, TILL Photonics) and a cooled charge-coupled device camera (TILL Imago SVGA) controlled by TILLvislon software (version

4.0). Experiments were performed by changing three separate Olympus BX cubes equipped with the appropriate filter combinations for ECFP (436/20, 455 DCLP, 480/40), EYFP (510/20, 530 DCLP, 560/40), and FRET (436/20, 455 DCLP, 560/40) measurements as described earlier [30]. All images were adjusted to pixel-by-pixel alignment and corrected for background, which was then clamped to zero. Corrected FRET images (NFRET) were calculated from the sensitized emission of EYFP. Each of the images was corrected for ECFP cross-talk and EYFP cross-excitation, and this is referred to as netFRET = $I_{\text{FRET-bg}} - (I_{\text{CFP-bg}} \cdot k_1) - (I_{\text{YFP-bg}} \cdot k_2)$, where $I_{\text{CFP-bg}}$ and $I_{\text{YFP-bg}}$ are the background-corrected pixel gray values in the windows for FRET (ex. 436, em. 560 nm), ECFP (ex. 436, em. 480 nm), and EYFP (ex. 515, em. 560 nm), and k_1 and k_2 denote the relative contribution to the fluorescence intensity in the FRET window by ECFP cross-talk (k_1 ; 0.3) and EYFP cross-excitation (k_2 ; 0.04). The obtained netFRET values were normalized for protein expression levels and are referred to as NFRET = netFRET $\cdot 100 / (I_{\text{CFP-bg}} \cdot I_{\text{YFP-bg}})^{1/2}$. The integrated fluorescence density values of the images from whole cells were analyzed using TILLvision and Microsoft Excel software. Images were taken under control conditions and 2, 5, 10 and 20 min after starting glucose stimulation.

Statistics

Group data are presented as mean $\pm$ SEM. Statistical comparisons were made using the paired or unpaired t-test, were applicable. A *p*-value <0.05 was taken to be statistically significant.

Results

Cell volume (CV) measurements

Increasing the glucose concentration from 5 mM to 20 mM in the isotonic extracellular solution causes a significant increase of the relative CV to a peak value of 1.09 ± 0.01 ($n=21$; $p<0.01$) within 5 min (fig. 1). The CV remains increased for further 15 min with no significant RVD being evident. Cell swelling can be only observed in cells adapted to a substimulatory glucose concentration (5 mM) as described in the Methods section. Under the same conditions cells directly transferred from the normal culture medium containing 11 mM glucose to the experiment do not swell, but show a slow reduction of their relative CV to 0.93 ± 0.03 ($n=12$; not significant) within 20 min. A similar reduction of the CV to 0.93 ± 0.02 ($n=14$; $p=0.04$) can be observed when cells were kept in an isotonic glucose-free extracellular solution for 20 min.

Exposure to a 30% hypotonic solution in the absence of glucose leads to a fast and significant increase of the relative CV to a peak value of 1.19 ± 0.01 ($n=14$; $p<0.01$) within 5 min followed by an RVD to 1.11 ± 0.02 ($n=14$;

$p<0.01$ compared to the peak value) over the next 15 min.

Electrophysiological measurements

A) Ruptured whole-cell patch clamp measurements. Under isotonic conditions, symmetrical Cl⁻ concentrations in the intra- and extracellular solutions and 2 mM ATP in the pipette solution, a basal outwardly rectifying Cl⁻ current (ICl_{basal}) of 14.32 ± 0.84 pA/pF ($n=23$) at +100 mV and -4.11 ± 0.62 pA/pF ($n=24$) at -100 mV is evident (fig. 2a and b). ICl_{basal} is significantly higher in cells cultured in the presence of glucose containing medium (11 mM) compared to cells starved for 12-24 hours ($p<0.01$ at +100 mV). The reversal potential (E_{rev}) of the basal currents under isotonic conditions is 4.12 ± 0.87 mV ($n=40$), i.e. close to the expected E_{rev} of 0 mV. To unmask a possible contribution of a Na⁺ and/or Cs⁺ conductance, NaCl was replaced by choline-Cl in the isotonic extracellular solution. Under these conditions E_{rev} of the basal currents is significantly shifted to -2.40 ± 0.88 mV ($n=40$; $p<0.01$), which suggests the contribution of a small background Cs⁺ conductance to the measured currents. Osmotic cell swelling induced by omission of 80 mM mannitol from the extracellular solution (30% hypotonicity) causes a significant increase of the outwardly rectifying Cl⁻ current (ICl_{swell}) within 10 min to a peak current of 29.29 ± 7.09 pA/pF ($n=13$; $p<0.01$) at +100 mV and -6.61 ± 1.02 pA/pF ($n=14$; $p=0.03$) at -100 mV and time-dependent inactivation at potentials $>+40$ mV (fig. 2a and b). The current inactivation parameter $I_{\text{ss}}/I_{\text{peak}}$ calculated from voltage steps to +100 mV is 0.90 ± 0.06 ($n=12$). In the continued presence of hypotonic conditions the Cl⁻ channel blocker NPPB (100 μ M) significantly reduces both the inward- and outward current (I/I_{max}) to 0.52 ± 0.09 and 0.29 ± 0.11 ($n=9$) of the hypotonicity-activated current, respectively (fig. 2c).

B) Perforated whole-cell patch clamp measurements. In order to avoid depletion of intracellular constituents by dialysis with the pipette solution and to allow physiological intracellular Ca²⁺ concentrations, all further patch clamp experiments were performed using the perforated patch technique.

Under isotonic conditions in the absence of glucose ICl_{basal} is outwardly rectifying as shown by the current-voltage (I/V) relation and the current tracing in fig. 3b and c. The normalized current amplitudes are 22.87 ± 1.32 pA/pF at -100 mV ($n=29$) and -2.46 ± 0.46 pA/pF at +100 mV ($n=29$), respectively. The current remains stable

Fig. 1. High glucose concentrations or hypotonicity induce swelling of INS-1E insulinoma cells. The cell volume of individual cells (estimated from the cell area as described in materials and methods) was determined under control conditions (0 min) as well as 1, 2, 3, 5, 15 and 20 min after solution exchange. The arrow indicates solution exchange. Data are presented as cell volumes measured at a given point of time (V) with respect to the volume measured

under control conditions (V_0 at 0 min). Asterisks denote a significant volume increase over control values ($p < 0.01$), # denotes a significant volume reduction (RVD) compared to peak values measured after 5 min under 30% hypotonicity ($p < 0.05$).

Fig. 2. Effect of hypotonicity on the Cl^- conductance of INS-1E cells. Conventional (ruptured) whole-cell patch clamp recordings. a Time-course of RVDC activation upon exposure to a 30% hypotonic extracellular solution ($n=12-20$). Currents were recorded in 10 second intervals in response to voltage ramps (insert) from -100 mV to +100 mV (ramp duration 500 ms, holding potential 0 mV). Data are given as normalized currents (pA/pF) determined at ± 100 mV. b Peak current-voltage relations recorded in response to voltage steps between -100 mV and +100 mV (step duration 500 ms, step increments 20 mV, holding potential of 0 mV; insert) under isotonic conditions and after 10 min exposure to 30% hypotonicity, as indicated by 1 and 2 in a ($n=13-24$). c NPPB significantly inhibits both the hypotonicity-induced outward (black bars) and inward (grey bars) Cl^- current measured at ± 100 mV ($n=9$; *, $p=0.02$).

Fig. 3. Perforated whole-cell patch clamp recordings of hypotonicity-activated Cl^- currents. **a** Stable basal currents are recorded under isotonic conditions over 10 min (open circles; $n=3-7$). Exposure to 30% hypotonicity leads to the activation of an outwardly rectifying Cl^- current (closed circles; $n=29$). Currents were recorded in 10 second-intervals in response to voltage ramps (insert) from -100 mV to +100 mV (ramp duration 500 ms, holding potential 0 mV). Normalized currents (pA/pF) determined at ± 100 mV. **b** Peak current-voltage relations recorded under isotonic conditions and after 10 min exposure to 30% hypotonicity, as indicated by 1 and 2 in **a** ($n=29$). Normalized currents (pA/pF). **c** Current tracings under isotonic and hypotonic conditions and after application of 4-hydroxytamoxifen (tamox; 10 μM) and NPPB (100 μM). **d** Relative inhibition of RVDC by extracellularly applied ATP (1 mM) and 4-hydroxytamoxifen (10 μM) at +100 mV (black bars) and -100 mV (grey bars). Asterisks denote significant inhibition.

for at least 10 min when cells are kept under isotonicity (fig. 3a). Hypotonic cell swelling by withdrawal of 80 mM mannitol from the bath solution leads to the generation of $\text{ICl}_{\text{swell}}$ with a gradual timecourse of activation (fig. 3a). Within 5 min the peak currents at +100 mV and -100 mV reach 73.78 ± 6.68 pA/pF ($n=29$) and -15.27 ± 2.02 pA/pF ($n=29$), respectively (fig. 3b). $I_{\text{ss}}/I_{\text{peak}}$ at +100 mV is 0.87 ± 0.04 ($n=26$), indicating time-dependent current inactivation. Fig. 3c shows typical current tracings obtained under isotonic and hypotonic conditions in response to 500 ms voltage pulses to clamp potentials

ranging from -100 mV to +100 mV. The activated current is significantly inhibited below basal values by the $\text{ICl}_{\text{swell}}$ blocker 4-hydroxytamoxifen (10 μM). Application of 100 μM NPPB on top of 4-hydroxytamoxifen causes an almost complete inhibition of the current (fig. 3c). $\text{ICl}_{\text{swell}}$ is inhibited by 4-hydroxytamoxifen to 0.32 ± 0.06 at +100 mV and 0.34 ± 0.06 at -100 mV ($n=6$; $p < 0.01$; fig. 3d). Extracellularly applied ATP (1 mM), which is known to inhibit $\text{ICl}_{\text{swell}}$ in different cell types [15], causes a significant inhibition of the hypotonicity-activated outward current to 0.65 ± 0.02 ($n=5$; $p=0.03$; fig. 3d). Tolbutamide

Fig. 4. High glucose concentrations and 3-O-methylglucose induce an outwardly rectifying Cl^- conductance in INS-1E cells under isotonic conditions. **a** Timecourses of current activation by 20 mM glucose (full circles; $n=3-6$) and 3-O-methylglucose (empty triangles; $n=4-8$). Currents are stable for at least 10 min under substimulatory glucose concentrations (5 mM; open circles; $n=3-5$). Activated currents are not significantly reduced 10 min after switching back to the isotonic control solution containing 5 mM glucose (ns, not significant). NPPB inhibits both the glucose- and 3-O-methylglucose-activated Cl^- current. Currents were recorded in 10 second intervals in response to voltage ramps (insert) and are given as normalized currents (pA/pF) at ± 100 mV. **b** Peak current-voltage relations ($n=5-6$) recorded at the points of time indicated in **a** (1-4). Normalized currents (pA/pF). **c** Current tracings of a single experiment (numbers correspond to the points of time indicated in **a**).

(100 μM) does not significantly affect $\text{ICl}_{\text{swell}}$ ($n=4$).

Since an increase of the glucose concentration from 5 mM to 20 mM in the isotonic extracellular solution causes swelling of INS-1E cells (fig. 1), whole-cell current recordings were performed under the same experimental conditions. $\text{ICl}_{\text{basal}}$ under isotonic conditions and substimulatory concentrations of glucose (5 mM) is outwardly rectifying with normalized current amplitudes of 19.28 ± 1.00 pA/pF and -1.47 ± 0.33 pA/pF at +100 mV and -100 mV, respectively ($n=6$; fig. 4b). Current amplitudes remain stable for at least 10 min under 5 mM glucose (fig. 4a) and are not different from basal currents recorded in the absence of glucose (fig. 3b; $p=0.25$).

Elevation of the extracellular glucose concentration to 20 mM leads to the activation of an outwardly rectifying Cl^- current ($\text{ICl}_{\text{glucose}}$) with peak current amplitudes of 50.74 ± 6.55 pA/pF and -7.14 ± 1.26 pA/pF at +100 mV and -100 mV, respectively ($n=6$; fig. 4b). Current activation reaches its maximum after 5-6 min (fig. 4a). At positive membrane potentials time-dependent current inactivation is evident ($I_{\text{ss}}/I_{\text{peak}} = 0.82 \pm 0.03$ at +100 mV; $n=21$). Fig. 4 (a-c) shows, that the current activated by 20 mM glucose remains activated for at least 10 min after stepping back to the control solution containing 5 mM glucose, with no significant current reduction being evident ($p=0.69$). Subsequent application of NPPB (100 μM) blocks the

Fig. 5. Effects of anion current blockers and tolbutamide on $ICl_{glucose}^-$. a Current tracings of an individual experiment: basal current (isotonic 5 mM glucose), current after 10 min in isotonic 20 mM glucose, after 5 min under 20 mM glucose in the presence of tolbutamide (tol; 100 μ M) and after application of DIDS (200 μ M). b Relative currents at +100 mV and -100 mV (black and grey bars, respectively) under tolbutamide (tol; 100 μ M), DIDS (200 μ M) in presence of tolbutamide (100 μ M) and 4-hydroxytamoxifen (tamox; 10 μ M) in presence of tolbutamide (100 μ M). Asterisks denote significant inhibition.

Fig. 6. Effect of hypertonicity on $ICl_{glucose}^-$. a Whole-cell Cl^- current recordings under basal conditions, after 10 min of current activation by 20 mM glucose and after 10 min superfusion with 20% hypertonic bath solution containing 20 mM glucose. Currents were recorded in response to voltage steps from -100 mV to +100 mV in 20 mV increments (step duration 500 ms, holding potential 0 mV) as shown by the insert. b $ICl_{glucose}^-$ is significantly reduced to basal current levels by 20% hypertonicity in presence of 20 mM glucose (gluc) within 10 min (*, $p=0.03$). In contrast, Cl^- currents activated by 20 mM glucose or 3-O-methylglucose (m-gluc) are not significantly reduced after 10 min exposure to isotonic 5 mM glucose (ns). Data are given as normalized currents (I/I_{max} , where I_{max} is the maximum current during an individual experiment; black bars). Values for the experimental series 'isotonic 5 gluc - 20 gluc - 5 gluc' and 'isotonic 5 gluc - 20 m-gluc - 5 gluc' were calculated from the timecourses shown in fig. 4a. Numbers in the bars are the numbers of individual observations.

peak current at +100 mV to 10.08 ± 2.79 pA/pF, which is significantly lower compared to basal current amplitudes ($n=5$; $p<0.01$).

As shown in fig. 4a, the non-metabolizable glucose analogue 3-O-methylglucose (20 mM in isotonic extracellular solution) induces a Cl^- current with similar

Fig. 7. CFP-ICln-YFP-Mem FRET images acquired in living INS-1E cells in the absence (upper panels) and 2, 5, 10 and 20 min after addition of 20 mM D-glucose (lower panels). Cells were cotransfected with CFP-ICln (ECFP fused to the N-terminus of ICln) and YFP-Mem (EYFP attached to the membrane). Left panels show a merge of the transmission image and the YFP fluorescence image (ex. 515 nm, em. 560 nm). Numbers are the pixel values determined at the indicated positions (bars). Right panels show the NFRET images. Note the glucose-induced cell swelling and the regions with increased (hot spots, red arrow) and decreased (cold spots, blue arrow) NFRET activity.

Fig. 8. Change of CFP-ICln-YFP-Mem NFRET in INS-1E cells. The FRET signals were collected from the images of whole cells before and 2, 5, 10 and 20 min after addition of 20 mM glucose (*, $p < 0.05$).

kinetics as $ICl_{glucose}^-$: the current is outwardly rectifying (45.71 ± 6.40 pA/pF at +100 mV and -7.34 ± 1.30 pA/pF at -100 mV; $n=8$), inactivates at positive holding potentials ($I_{ss}/I_{peak} = 0.90 \pm 0.04$; $n=8$; not significantly different from $ICl_{glucose}^-$; $p=0.20$), remains activated for at least 10 min after withdrawal of 3-O-methylglucose and is inhibited below basal levels by 100 μ M NPPB at +100 mV (7.50 ± 1.81 pA/pF; $n=6$; $p<0.01$).

ICl_{basal} recorded under 5 mM glucose is not affected by tolbutamide. In the presence of 100 μ M tolbutamide the current is 16.68 ± 1.88 pA/pF at +100 mV and -1.33 ± 0.28 pA/pF at -100 mV ($n=10$), which is not significantly different from the basal currents shown in fig. 4 recorded in the absence of tolbutamide ($p=0.33$ and 0.77, respectively). When applied after full activation of $ICl_{glucose}^-$, tolbutamide is ineffective as well (fig. 5). Current amplitudes are not significantly altered by the application of 100 μ M tolbutamide for 5 min in the continued presence of 20 mM glucose. Relative current amplitudes at +100 mV and -100 mV are 1.38 ± 0.15 and 1.22 ± 0.10 ($n=6$), respectively (fig. 5b). The anion current blocker DIDS, applied on top of tolbutamide (100 μ M), significantly blocks $ICl_{glucose}^-$ to 0.45 ± 0.06 ($n=6$; $p=0.01$) at +100 mV and 0.76 ± 0.07 ($n=6$; $p=0.03$) at -100 mV. 4-hydroxytamoxifen only inhibits the outward current (0.30 ± 0.05 at +100 mV; $n=6$; $p<0.01$), whereas the inward current at -100 mV remains unaffected (1.27 ± 0.18 ; $n=6$; $p=0.22$).

In a further series of experiments we investigated the effect of hypertonicity on $ICl_{glucose}^-$. After full activation of the Cl^- current by 20 mM glucose in isotonic extracellular solution, cells were superfused for 10 min with a 20% hypertonic solution containing 20 mM glucose. As shown in fig. 6a, this maneuver causes a current reduction back to basal values measured under 5 mM glucose. This is clearly different from the results obtained when stepping back from isotonic 20 mM glucose or 20 mM 3-O-methylglucose to isotonic 5 mM glucose, as shown in fig. 4. In both cases the current remains significantly increased above basal levels. The data obtained from the three experimental series are summarized in fig. 6b. Currents are given as I/I_{max} , where I_{max} is the respective maximum current recorded in individual experiments under 20 mM glucose or 20 mM 3-O-methylglucose. In all experimental series an outwardly rectifying current is activated within 10 min with amplitudes significantly higher compared to basal currents ($p<0.05$). Switching back to an isotonic extracellular solution containing 5 mM glucose does not lead to a significant current reduction within 10 min (currents remain

significantly increased over basal levels; $p<0.05$), whereas stepping to a 20% hypertonic solution containing 20 mM glucose causes a significant current reduction to basal levels ($p<0.05$).

FRET measurements

Interaction of $ICln$ with the cell membrane during glucose stimulation was determined using the FRET technique as described earlier [30]. The association of $ICln$ with the cell membrane is evident in the absence of glucose (fig. 7, right panel, 0 minutes). Addition of 20 mM glucose leads to a clear change in the distribution pattern of membrane-associated $ICln$, showing regions of both enhanced (hot spots, red arrows) and reduced (cold spots, blue arrows) NFRET activity. NFRET signals calculated from the total cell area of single cells change transiently during the 20 min lasting glucose stimulation (fig. 8). As shown in the left panel of fig. 7 the changes of the NFRET signals are paralleled by a shape change of the cells due to glucose-induced cell swelling. Both the NFRET changes and the shape changes of the cells are detectable after 2 min and appear to be most prominent after 5 and 10 min, whereas 20 min after addition of glucose the cells attain a shape and $ICln$ -membrane NFRET pattern comparable to the situation before glucose stimulation.

Discussion

The metabolic coupling of insulin secretion by pancreatic beta cells is accomplished by evocation of electrical activity due to glucose-driven increased ATP production, closure of K_{ATP} channels (Kir 6.2/SUR1) and membrane depolarization. Studies on isolated pancreatic beta cells have shown, however, that K_{ATP} channel activity is maximally inhibited in the presence of substimulatory concentrations of glucose (5 mM) with no further effect of higher concentrations [25]. In addition, glucose is able to stimulate beta cell electrical activity and insulin release even under conditions when K_{ATP} channel activity is completely blocked by tolbutamide [32, 33], or when K_{ATP} channels are opened by diazoxide [33, 34]. These findings suggest the existence of a K_{ATP} channel-independent stimulus secretion coupling of pancreatic beta cells. In addition it has been shown that cell swelling *per se* is a potent stimulus for insulin secretion that can be triggered independently from K^+ channel activity and even in the absence of glucose [18, 35].

Cell swelling is usually accompanied by the activation

of a distinct class of Cl⁻ channels (ICl_{swell}) to drive the process of regulatory volume decrease (RVD). Since in many cells these channels are not exclusively permeable to Cl⁻ ions, but also to other anions and even cations and organic osmolytes like taurine, these channels and their currents have recently been named regulatory volume decrease channels/currents (RVDC) [15]. The molecular identity of RVDC is still elusive.

Beta cell depolarization has been observed in response to glucose- or hypotonicity-induced cell swelling [6, 11, 18, 36] and was found to involve the activation of an anion conductance with features resembling RVDC [5, 6, 9, 13]. The importance of RVDC for the excitability and secretory response of beta cells is highlighted by the fact that common blockers of RVDC including DIDS, NPPB, 4-hydroxytamoxifen and DCPIB are potent inhibitors of insulin secretion [6, 8, 12, 13, 18, 22-24, 26] and further emphasized by work showing RVDC inhibition and reduction of insulin secretion by certain HIV protease inhibitors [16, 37], which could explain the adverse side effects of these antiviral substances on glucose homeostasis.

The results of the present study show that application of isotonic 20 mM glucose induces a 10% volume increase of INS-1E insulinoma cells which is paralleled by the generation of an outwardly rectifying Cl⁻ current (ICl_{glucose}). Likewise, 30% hypotonicity induces cell swelling (20% volume increase) and activation of a swelling-dependent Cl⁻ current (ICl_{swell}, RVDC). ICl_{glucose} and ICl_{swell} show identical biophysical and pharmacological properties, i.e. outward rectification and time-dependent inactivation at positive membrane potentials. ICl_{glucose} can only be activated if cells are adapted to 'normoglycaemic' glucose concentrations (5 mM) prior to the experiment. Both currents are blocked below basal levels by the anion channel blockers 4-hydroxytamoxifen, NPPB and DIDS, indicating that basal currents measured under isotonicity in the absence or presence of substimulatory glucose concentrations are mainly carried by Cl⁻ ions (ICl_{basal}). The small shift of E_{rev} towards negative potentials found by substitution of extracellular NaCl for choline-Cl⁻ unmasks the involvement of a small Cs⁺ background conductance. This is in agreement with a previous study showing that RVDC in NIH 3T3 fibroblasts are also permeable to Cs⁺ with a pCs⁺/pCl⁻ of ~ 0.4 [15]. From the present experiments it is, however, not possible to distinguish whether in INS-1E cells this Cs⁺ current is mediated by the anion conductive pathway or *via* separate cation channels. Since intracellular Cs⁺ is known to block most of the K⁺ channels described so far [38] and the

fact that ICl_{swell} and ICl_{glucose} are not inhibited by tolbutamide, it is highly unlikely that these currents arise from incomplete activation of K_{ATP} channels in the perforated patch clamp mode.

Glucose-induced cell swelling shows a peak within 5 minutes and the cell volume remains increased for at least 15 minutes (fig. 1). The timecourse of ICl_{glucose} activation reaches a maximum within 6 minutes and the current remains activated, even when the extracellular glucose concentration is reduced from 20 mM to 5 mM (fig. 4). This is reminiscent of ICl_{swell} in NIH 3T3 fibroblasts, which also remains activated for several minutes after reestablishing isotonic conditions [39]. In contrast to hypotonic cell swelling no significant RVD is evident during glucose-induced cell swelling (fig. 1). In a previous study the glucose induced cell swelling was discussed as the result of a persistent challenge for the cell volume due to sugar metabolism and accumulation of acidic metabolites [9, 13]. This assumption has been strengthened by experiments using 3-O-methylglucose, a glucose analogue that is taken up but not metabolized by beta cells and which induced a much smaller volume increase than equivalent amounts of glucose and did not exert an effect on the electrical behaviour of beta cells [9]. In our experiments we found, however, the activation of a Cl⁻ current by 3-O-methylglucose with the same features as ICl_{glucose}. The current has an identical timecourse and extent of activation, shows outward rectification and time-dependent inactivation at positive holding potentials and can be blocked by NPPB. Therefore it is reasonable to assume that under the given experimental conditions in INS-1E cells, swelling and current activation can be attributed to the concentrative uptake of glucose or 3-O-methylglucose and osmotically obliged water. This does by no means exclude that metabolic effects are involved in glucose-induced cell swelling and Cl⁻ current activation in INS-1E cells.

In isolated pancreatic beta cells the sulfonylurea tolbutamide has been reported both to be without an effect [10, 11] as well as to augment the glucose-induced anion current in a glucose concentration-dependent manner [40]. Therefore we tested for a possible inhibitory effect of tolbutamide on the Cl⁻ currents in INS-1E cells. Neither ICl_{basal} nor ICl_{glucose} or ICl_{swell} were inhibited by tolbutamide. However, when the ICl_{swell} inhibitor tamoxifen was added on top of tolbutamide, an inhibition of ICl_{glucose} by tamoxifen was only observed for outward currents. In addition, in the study showing volume-regulated anion current potentiation, measurements were performed in the current clamp mode, whereas in our experiments the

voltage clamp mode was used, where cells were clamped at a holding potential of 0 mV. Thus from the present study it cannot be excluded that tolbutamide interferes with the molecular entity mediating $ICl_{glucose}$ and exerts an effect on the basal electrical activity of beta cells.

From the results obtained in this study as well as from previous studies discussed above it can be assumed that $ICl_{glucose}$ and ICl_{swell} represent currents conducted by identical or at least closely related channels, namely RVDC. This hypothesis is further substantiated by the finding that counteracting the glucose-induced cell swelling by establishing slightly hypertonic conditions in the continued presence of a high glucose concentration effectively diminishes $ICl_{glucose}$ (fig. 6). It is noteworthy to mention that extracellular hyperosmolarity has been found to inhibit insulin secretion from pancreatic islets [22, 41] and to reduce prolactin release from GH4/C1 cells [42, 43]. These findings further underline the importance of osmotic events in the regulation of exocytosis.

In a previous work we found that osmotic swelling of NIH 3T3 fibroblast causes a cytosol-to-membrane transposition and cellular redistribution of ICln and that this transposition coincides with altered activation of RVDC in these cells [30]. ICln is a multifunctional 'connector hub' protein, which plays a crucial role in the evocation of RVDC upon cell swelling [15]. ICln highly specifically interacts with integral membrane proteins like integrin $\alpha_{IIb}\beta_3$ and plays an important role in integrin-mediated platelet activation, a process branded by massive changes in cell shape and cell volume [29]. Recent NMR analysis revealed that soluble ICln folds into a PH-domain structure [27]. PH domains are highly conserved regulatory proteins involved in intracellular signaling by linking host proteins to the cell membrane. Moreover ICln has been shown to spontaneously insert into membranes to form functional ion channels in artificial lipid bilayers [28]. Similarly to the cell swelling-induced redistribution in NIH 3T3 fibroblasts we found a cellular redistribution of ICln in glucose-stimulated INS-1E cells by NFRET analysis. As shown in fig. 7, NFRET signals based on ICln-membrane association can be detected in unstimulated cells. Upon stimulation with glucose, regions

of increased (hot spots) and decreased (cold spots) NFRET signals can be visualized and the overall membrane NFRET activity increases, which indicates a reorganization of the ICln-membrane interaction and transposition of ICln from the cytosol to the cell membrane. Despite the persistent activation of Cl^- currents and the shallow RVD in cells swollen by addition of glucose (fig. 7, left panel) the detected increase of the NFRET signals is transient under the given experimental conditions. Possible explanations are that ICln might act as an initiator signal for the generation of $ICl_{glucose}$ and/or ICl_{swell} /RVDC (i.e. it acts as a regulator thereof) or that a conformational change of the membrane-associated ICln molecules leads to a separation of the donor (CFP) and acceptor (YFP) dyes, thereby reducing the efficiency of the energy transfer.

In conclusion, INS-1E rat insulinoma cells respond to high levels of glucose and 3-O-methylglucose with cell swelling which can both at least in part be attributed to a cellular uptake of the molecules and the activation of an anion conductive pathway that resembles RVDC. In addition we demonstrate a cellular redistribution of the membrane-associated portion of the ICln protein as measured by FRET. Given the crucial function of ICln in cell volume regulation and the pivotal role of cell volume in hormone secretion it can be assumed that this interaction is an important step in mediating swelling-induced insulin secretion of beta cells. The functional significance of the ICln-membrane interaction in beta cells and the contribution of RVDC activation needs to be investigated in further studies.

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