Original Paper

The H⁺/K⁺ ATPase Inhibitor SCH-28080 Inhibits Insulin Secretion and Induces Cell Death in INS-1E Rat Insulinoma Cells

Martin Jakab^a Nina Ketterl^{a,b} Johannes Fürst^c Marlena Beyreis^a Michael Kittl^a
Tobias Kiesslich^{a,d} Cornelia Hauser-Kronberger^e Martin Gaisberger^{a,f,g}
Markus Ritter^{a,f,g}

^aInstitute of Physiology and Pathophysiology, Laboratory of Functional and Molecular Membrane Physiology, Paracelsus Medical University, Salzburg, Austria, ^bExperimental and Clinical Cell Therapy Institute, Spinal Cord Injury and Tissue Regeneration Center, Paracelsus Medical University, Salzburg, Austria, ^cDepartment of Physiology and Medical Physics, Division of Physiology, Medical University of Innsbruck, Austria, ^dDepartment of Internal Medicine I, Salzburger Landeskliniken, Paracelsus Medical University, Austria, ^eDepartment of Pathology, University Hospital Salzburg, Paracelsus Medical University, Salzburg, Austria, ^fGastein Research Institute, Paracelsus Medical University, Salzburg, Austria, ^gDepartment for Radon Therapy Research, Ludwig Boltzmann Cluster for Arthritis and Rehabilitation, Salzburg, Austria

Key Words

H⁺/K⁺ ATPase • ATP12A • ATP1AL1 • INS-1E cells • Apoptosis • Cell volume • Insulin • Diabetes mellitus

Abstract

Background/Aims: Glucose-stimulated insulin secretion (GSIS) of pancreatic β -cells involves glucose uptake and metabolism, closure of K_{ATP} channels and depolarization of the cell membrane potential (V_{mem}), activation of voltage-activated Ca²⁺ currents (I_{Ca_v}) and influx of Ca²⁺, which eventually triggers hormone exocytosis. Beside this classical pathway, K_{ATP}-independent mechanisms such as changes in intracellular pH (pH_i) or cell volume, which also affect β -cell viability, can elicit or modify insulin release. In β -cells the regulation of pH_i is mainly accomplished by Na⁺/H⁺ exchangers (NHEs). To investigate if other proton extrusion mechanisms than NHEs are involved in pH regulation, we tested for the presence of the non-gastric H⁺/K⁺ ATPase in rat insulinoma cells and assessed effects of the H⁺/K⁺ ATPase inhibitor SCH-28080 on insulin secretion, cell viability and apoptosis. **Methods:** In INS-1E cell cultures, H⁺/K⁺ ATPase gene and protein expression was analyzed by reverse transcription PCR and Western blotting. Intracellular pH (pH_i) recovery after acute acidic load was measured by NH₄Cl prepulsing using BCECF. Insulin secretion was determined by ELISA from the cell culture supernatant. V_{mem} , K⁺ and Ca²⁺ currents were recorded using patch clamp. Overall cell responses were determined using resazurin (viability) and cytotoxicity assays. The mean cell volume

M. Jakab and N. Ketterl contributed equally to this work

Martin Jakab

Paracelsus Medical University, Institute of Physiology and Pathophysiology
Strubergasse 22, 5020 Salzburg (Austria)
Tel. +43-662-242080504, E-Mail martin.jakab@pmu.ac.at

(MCV), cell granularity (side-scatter; SSC), phosphatidylserine (PS) exposure, cell membrane integrity, caspase activity and the mitochondrial membrane potential ($\Delta\Psi_m$) were measured by flow cytometry. **Results:** We found that the α -subunit of the non-gastric H^+/K^+ ATPase (HK α 2) is expressed on mRNA and protein level. However, compared to rat colon tissue, in INS-1E cells mRNA abundance was very low. In NH_4Cl prepulsing experiments no K^+ -dependent pH_i recovery was observed under Na^+ -free extracellular conditions. Nonetheless within 1 h, 20 μM SCH-28080 inhibited GSIS by ~50%, while basal release was unaffected. The L-type ICa_v blocker nifedipine caused a full inhibition of GSIS at 10 and 20 μM . At 20 μM , SCH-28080 inhibited ICa_v comparable to 20 μM nifedipine and in addition augmented IK_{ATP} recorded at -60 mV and hyperpolarized V_{mem} by ~15 mV. Cell viability 2 and 24 h post treatment with SCH-28080 was dose-dependently inhibited with IC_{50} values of 22.9 μM and 15.3 μM , respectively. At 20 μM the percentages of Annexin-V+, caspase+ and propidium iodide+ cells were significantly increased after 24 and 48 h. Concurrently, the MCV was significantly decreased (apoptotic volume decrease, AVD) and the SSC signal was increased. At concentrations >40–50 μM , SCH-28080 became progressively cytotoxic causing a steep increase in necrotic cells already 2 h post treatment and a breakdown of $\Delta\Psi_m$ within 4 h under 50 and 100 μM while 10 and 20 μM had no effect on $\Delta\Psi_m$ within 24 h. **Conclusion:** We demonstrate expression of HK α 2 in rat INS-1E cells. However, the pump is apparently non-functional under the given conditions. Nonetheless the H^+/K^+ ATPase blocker SCH-28080 inhibits insulin secretion and induces cell death. Importantly, we show that SCH-28080 inhibits ICa_v - and activates K_{ATP} channels identifying them as novel “off-targets” of the inhibitor, causing hyperpolarization of V_{mem} and inhibition of insulin secretion.

© 2017 The Author(s)
Published by S. Karger AG, Basel

Introduction

Stimulus-secretion-coupling of pancreatic β -cells is the process by which insulin secretion is adjusted to the ambient concentration of nutrients such as glucose, amino acids and fatty acids. Glucose-stimulated insulin secretion (GSIS) is induced by sugar uptake *via* glucose transporter 2 (GLUT2), its metabolism leading to an increase in the cellular ATP/ADP ratio and closure of ATP-sensitive K^+ (K_{ATP}) channels. The depolarization of the cell membrane potential (V_{mem}) causes opening of voltage-gated Ca^{2+} channels, which triggers the exocytosis of the hormone [1, 2]. Besides the K_{ATP} -dependent mechanism, insulin release can be triggered or modified by β -cell swelling *per se* following glucose uptake or through activation of volume-regulated anion currents (VRAC) [3-7]. In addition, insulin secretion is sensitive to the intracellular pH (pH_i), which is modulated – amongst others – by both glucose metabolism and cell volume changes [3, 8-15]. Therefore, accurate regulation of pH_i and cell volume are mandatory for proper β -cell function and Na^+/H^+ exchangers (NHEs) play a major role here [3, 15]. Given the sensitivity of insulin secretion to intracellular ion homeostasis, pH_i and cell volume, it is important to identify additional ion transport mechanisms involved in these processes such as H^+/K^+ ATPases, which might also affect hormone release.

Previously, we have identified a role for H^+/K^+ ATPases in cell migration and apoptosis – processes which are also dependent on pH_i and cell volume regulation [16, 17]. H^+/K^+ ATPases comprise the gastric and non-gastric isoforms. The latter is also denoted as the renal and colonic H^+/K^+ ATPase and is encoded by gene ATP12A (ATP1AL1; human isoform)/Atp12a (Atp1al1; animal isoform) which is widely expressed in various mouse, rat, rabbit and human tissues [18-25]. We have described ATP12A expression in human prostate tissue and human myelomonocytic HL-60 cells. In prostate tissue we demonstrated deranged protein sorting in benign hyperplasia and protein upregulation in prostate cancer [26]. In HL-60 cells ATP12A seems to exert an anti-apoptotic function by counteracting apoptotic cation gradient reversal during apoptotic volume decrease [17].

Deranged insulin secretion and apoptosis in β -cells are crucial events in the development of both type-1 and type-2 diabetes mellitus [27-29]. Therefore we tested for expression of the non-gastric H^+/K^+ ATPase in insulin-secreting INS-1E rat insulinoma cells, a well-established

β -cell model [30] and for the effect of SCH-28080, a widely-used inhibitor of both gastric and non-gastric H^+/K^+ ATPases [31–36]. We investigated H^+/K^+ ATPase activity by measuring K^+ -dependent H^+ extrusion after cellular acidification as well as the effects of SCH-28080 on insulin secretion, cell viability and induction of apoptosis. The effects on V_{mem} , K^+ currents and ICa_v were tested by patch clamping. We found that INS-1E cells express Atp12a at low abundance and that SCH-28080 reduces GSIS. Further we show that over time SCH-28080 induces apoptosis and is cytotoxic at higher doses. Despite Atp12a expression, however, no K^+ -dependent H^+ extrusion was apparent under the prevailing experimental conditions. Instead we found that SCH-28080 inhibits ICa_v , activates K^+ currents and hyperpolarizes V_{mem} and thereby inhibits insulin secretion.

Materials and Methods

Salts, chemicals and drugs

All salts and chemicals were p.a. grade. SCH-28080 (2-methyl-8-(phenylmethoxy)imidazopyridine-3-acetonitrile), CCCP (carbonyl cyanide 3-chlorophenylhydrazone), nifedipine and nigericin were purchased from Sigma-Aldrich. Stock solutions of SCH-28080 and CCCP (40 and 50 mM, respectively) and nifedipine (100 mM) were prepared in dimethyl sulfoxide (DMSO). Resazurin (7-hydroxy-3H-phenoxazin-3-on-10-oxid; Alfa Aesar) was dissolved in phosphate buffered saline (PBS) to give a 2.5 mM stock solution. Nigericin was dissolved in ethanol to a 5 mg/ml stock solution. Stocks were stored in aliquots at -20°C until use.

Cell culture

Rat INS-1E cells [30] were cultured in RPMI 1640 medium containing 24 mM sodium bicarbonate, 2 mM L-glutamine and 10 mM HEPES (Biochrom), supplemented with 1 mM sodium pyruvate, 50 μM 2-mercaptoethanol, 10% fetal bovine serum (FBS), 100 U/mL penicillin, 100 $\mu\text{g}/\text{mL}$ streptomycin and 250 ng/mL amphotericin B at humidified 37°C and 5% CO_2 (standard conditions). Subcultures were established by trypsin/EDTA treatment once a week. Cells within passages 62 and 148 were used for the experiments. Cells for insulin secretion measurements were grown in RPMI 1640 medium containing 80 μM 2-mercaptoethanol and 5% FBS.

Reverse transcription PCR

Total RNA from INS-1E cells was isolated using the spin-column based SurePrep™ TrueTotal™ RNA purification kit (Fisher BioReagents) according to the manufacturer's recommendations. RNA from rat colon and stomach was isolated using RNeasy reagent (ams biotechnology). RNA content and purity of the samples was determined photometrically. For cDNA synthesis, 1 μg of RNA was reverse-transcribed with RevertAid Premium Reverse Transcriptase (RT), RiboLock RNase inhibitor and oligo(dT)18 primers (Thermo Scientific). Expression of gastric H^+/K^+ ATPase α -subunit mRNA (HK α 1; Atp4a; accession number NM_012509.1) was tested by end-point PCR (forward primer: 5'-GACCAGTGCCACCAAGGCCTG-3', binding position 198–219; reverse primer: 5'-GCTGCCGGGCGAAGTTCACG-3', binding position 297–316; amplicon size 119 base pairs) on a Labcycler (SensoQuest). Real-time quantitative PCR (qPCR) was performed on a Rotorgene Q 6000 thermal cycler (QIAGEN) using predesigned 5' nuclease (TaqMan®) gene expression assays (Applied Biosystems). Two different exon boundary-spanning assays were used for rat non-gastric H^+/K^+ ATPase α -subunit Atp12a (HK α 2; accession number NM_133517.1), assay A targeting the N-terminus (Rn00591698_m1; probe sequence 5'-CATCATCCAGGGTCTCTCCAGCGTC-3'; position 487–511; amplicon length 130 bp) and assay B targeting the C-terminus (Rn01481934_m1; probe sequence 5'-ACAGACATTATCCCCCTCCATTGCCT-3'; position 2750–2774; amplicon length 142 bp). Rat glyceraldehyde 3-phosphate dehydrogenase (Gapdh; accession number NM_017008.3) was chosen as reference gene and probed with an endogenous control primer-probe set (Applied Biosystems). Non-template controls (NTCs) were included in every run. To test for genomic DNA contamination, no-reverse-transcriptase (–RT) samples were prepared. PCR reactions were separated on 2% agarose gels and visualized by Midori Green (NIPPON Genetics) staining on a UV transilluminator (Alpha Innotech Corporation). Data are presented as differences of threshold cycles (ΔCt) between Atp12a and Gapdh ($\text{Ct}_{\text{Atp12a}} - \text{Ct}_{\text{Gapdh}}$). To test for the specificity of the Atp12a qPCR product, the amplicon of assay B was gel extracted using the GeneJET™

extraction kit (Thermo Scientific) and used as a reverse “megaprimer” in conjunction with a forward primer (5′-GGCTGTCAGAGGCAGGACGC-3′; binding position 2750–2774). The reaction yielded a ~400 base pair fragment, which was gel extracted and RsiI-digested. The cleavage products had the expected sizes of ~220 and ~180 base pairs.

Western blot

For immunoblotting, INS-1E cells were grown in 6-well plates under standard culture conditions to ~80% confluency. For cell lysis, cells were incubated for 5 min on ice in a buffer containing 150 mM NaCl, 50 mM TRIS-HCl, 1 mM EGTA, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin and aprotinin, then scraped off and sonicated briefly (five 1-s pulses) using a Sonopuls HD 70 (Bandelin). Rat colon tissue was homogenized with a Kontes Pellet Pestle motor tissue grinder for 5 min and then mixed with lysis buffer. Both crude INS-1E cell lysates and colon samples were centrifuged for 10 min (14,000×g) at 4°C. The protein concentration in the supernatants was determined using a bicinchoninic acid (BCA) assay (Pierce, Thermo Scientific). Samples were mixed 1:1 with a Laemmli sample buffer containing 125 mM TRIS-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 0.004% (w/v) bromophenol blue and 0.7% (v/v) β-mercaptoethanol and proteins (10 μg/sample) were separated by SDS-PAGE on 7% gels using a Mini-PROTEAN® 3 electrophoresis cell (Bio-Rad). Proteins were blotted onto 0.2-μm nitrocellulose membranes in a Trans-Blot® electrophoretic transfer cell (Bio-Rad). For immunodetection, a primary polyclonal rabbit-anti-ATP1A1 (N-12) IgG antibody raised against human ATP1A1 (Santa Cruz Biotechnology) and a polyclonal rabbit-anti-β-actin antibody (Cell Signaling Technology) was used at a dilution of 1:200 and 1:1,000, respectively. The secondary antibody was an IRDye® 800CW infrared dye-conjugated polyclonal goat-anti rabbit IgG (LI-COR) at a dilution of 1:10,000. Secondary antibody controls were performed to show that the label is specific to the primary antibody. Fluorescence was detected on a LI-COR Odyssey® infrared imaging system.

Measurement of intracellular pH (pH_i)

Measurements of pH_i were performed using the cell permeable pH-sensitive dye BCECF-AM (2′,7′-bis(2-carboxyethyl)-5(6)-carboxyfluorescein-acetoxymethylester; Calbiochem). Cells grown on poly-D-lysine coated 3-cm glass bottom imaging dishes (MatTek Corporation) were loaded with 10 μM BCECF-AM at 37°C for 30 min in the dark in a solution containing (in mM) 135 NaCl, 5.6 KCl, 2.5 CaCl₂, 1.5 MgCl₂, 10 HEPES FA, 5 D-glucose (pH 7.4 adjusted with NaOH), denoted as 135 Na⁺/5.6 K⁺ (control) solution. After two washing steps the dishes were mounted on an inverted microscope (iMIC) controlled by the Life Acquisition (LA) software (FEI/TILL Photonics). The fluorescence filter set of the iMIC consisted of a 460/80 nm bandpass excitation filter, a 506-nm dichroic mirror and a 535/22 nm emission filter (AHF Analysentechnik). A Polychrome V monochromator served as light source and an IMAGO QE cooled 12-bit CCD camera was used as image capturing device (FEI/TILL Photonics). BCECF was alternately excited every 10 s at 440 nm and 490 nm through a 40× objective. The fluorescence emission at 535 nm was recorded from regions of interest and the raw intensity data were background corrected. Experiments were performed at room temperature. A gravity-driven perfusion system (ALA Scientific Instruments) with a flow-rate of 3–5 ml/min was used for extracellular solution exchange. NH₄Cl prepulsing experiments were performed to test for the cells’ ability to recover from an acute acid load as described earlier [17]. Briefly, after achieving stable baseline signals under 135 Na⁺/5.6 K⁺ control conditions, cells were exposed for ~5 min to a solution containing 20 mM NH₄Cl. Thereafter, a rapid intracellular acidification was achieved by removal of NH₄Cl in the absence of Na⁺ and K⁺ (0 Na⁺/0 K⁺ solution; NaCl and KCl replaced by 140 mM choline-Cl). The pH_i time-course was subsequently monitored for ~15 min under Na⁺-free conditions in the presence of K⁺ (0 Na⁺/5.6 K⁺ solution; NaCl replaced by 135 mM choline-Cl) and for additional ~15 min upon re-addition of Na⁺ (135 Na⁺/5.6 K⁺ control solution). Every experiment was followed by an *in situ* pH calibration with high (140 mM) K⁺/nigericin (10 μg/ml) solutions adjusted to pH 7.4 or 6.0 and 490/440 nm excitation ratios detected at 535 nm were transformed into absolute pH values.

Glucose-stimulated insulin secretion assay and ELISA

On day 1, INS-1E cells were detached by Trypsin/EDTA, counted using a CASY TT cell counter (OMNI Life Science) and seeded into 35-mm cell culture dishes at a density of ~400,000 cells per dish. On day 4, 60–80% confluent cells were gently washed with PBS and then preconditioned for 30 min in 2 ml per cell

culture dish of glucose-free buffer (0 Gluc) containing (in mM): 145 NaCl, 3.6 KCl, 1.5 CaCl_2 , 0.5 MgCl_2 , 10 HEPES FA, 0.5 NaH_2PO_4 , 5 NaHCO_3 and 0.1% BSA (fraction V, fatty acid free), pH 7.4 (adjusted with NaOH). For a first series of experiments (data in Fig. 3A) the preconditioning buffer contained 0.04% DMSO (solvent control) or 20 μM SCH-28080 and preconditioning was followed by treatment of cells for 1 h with 1 ml per cell culture dish of 0 gluc buffer or a 20-mM glucose buffer (20 Gluc), each containing 0.04% DMSO or 20 μM SCH-28080. For a second series of experiments (data in Fig. 3B) preconditioning was followed by a 1 h treatment of cells with 1 ml per cell culture dish of 0 Gluc buffer, or 20 Gluc buffer containing 0.05% DMSO (solvent control) or either 10 or 20 μM nifedipine. In the 20 Gluc buffer NaCl was reduced to 132 mM. Preconditioning and treatment of cells was carried out at 37°C in humidified air. At the end of the treatment period, 200 μl of each cell culture supernatant was collected, centrifuged (800×g, 5 min, 4°C) and stored at -20°C until further analysis. Cells were then detached from each cell culture dish by Trypsin/EDTA and the number of cells per dish was counted as described above. Insulin concentration in the cell culture supernatants was determined by a rat Insulin ELISA (Mercodia) according to the supplier's protocol and normalized to the number of cells determined at the end of the treatment period in the corresponding cell culture dishes. Data are given as $\mu\text{g/l}$ per 10^5 cells.

Electrophysiology

INS-1E cells were seeded on poly-D-lysine-coated glass coverslips, cultured under standard conditions and used for patch clamp experiments after 24–48 h at 60–80% confluency. The coverslips were transferred to a recording chamber and mounted on a TE2000-U inverted microscope (Nikon). Data were acquired and analyzed using an EPC-10 amplifier and Pulse/FitMaster software (HEKA). Cell membrane potentials and whole-cell currents were recorded in the “perforated” patch clamp mode using a pipette solution containing (in mM) 120 potassium D-gluconate, 5 NaCl, 10 KCl, 2 CaCl_2 , 4 MgCl_2 , 5 HEPES FA, 10 EGTA, 5 raffinose, 120 $\mu\text{g/ml}$ amphotericin B (pH 7.2 adjusted with KOH, 300 mOsm/kg) and a bath solution containing (in mM) 140 NaCl, 5.6 KCl, 2.5 CaCl_2 , 1.5 MgCl_2 , 10 HEPES FA, 4.5 D-glucose, 5 mannitol (pH 7.4 adjusted with NaOH, 300 mOsm/kg). For current recordings 500-ms pulses to -60 mV from a holding potential (V_{hold}) of -80 mV were delivered at 10-s intervals. Ca^{2+} currents were recorded in the “ruptured” patch clamp mode using Ba^{2+} as charge carrier. The pipette solution contained (in mM): 100 CsCl, 5 MgCl_2 , 2 ATP (Mg^{2+} salt), 10 HEPES FA, 11 EGTA, 65 raffinose (pH 7.2 adjusted with CsOH, 310 mOsm/kg). The extracellular solution consisted of (in mM): 80 tetraethylammonium (TEA)-Cl, 10 BaCl_2 , 10 HEPES FA, 5 D-glucose, 120 mannitol (pH 7.2 adjusted with TEA-OH, 303 mOsm/kg). Ba^{2+} currents were elicited by voltage ramps from -100 to +100 mV (500 ms duration) from a V_{hold} of -80 mV. Osmolalities were measured with a vapor pressure osmometer (Wescor). Experiments were performed at room temperature. Patch electrode resistances were 3–5 M Ω . A gravity-driven perfusion system (ALA Scientific Instruments) was used for bath solution exchange (flow-rate of 3–5 ml/min). SCH-28080 and nifedipine were added to the extracellular solutions as indicated.

Cell viability and cytotoxicity

For cell viability measurements, INS-1E cells were seeded in black, clear bottom, tissue culture treated 96-well microplates (Greiner Bio-One) at a density of 12,500 cells/well. After over-night incubation under standard conditions cells were either left untreated, or incubated in culture medium containing (in μM) 1.5, 3.1, 6.25, 12.5, 25, 50 and 100 SCH-28080, or 0.1% (v/v) DMSO (solvent control). For quantification of cell viability, a resazurin (alamar blue) assay was used [37], which determines the number of viable, metabolically active cells, given that only viable cells are able to metabolize resazurin. The reduction of resazurin to resorufin is irreversible and fluorescence intensity is proportional to the number of viable cells. Resazurin signals were recorded after 1 h incubation with 0.5 mM resazurin at 2 and 24 h post treatment with SCH-28080 on a Zenyth 3100 multimode reader (Anthos Labtec Instruments GmbH). Excitation and emission wavelength were 535 and 595 nm, respectively. For background subtraction, background fluorescence was determined for each experimental treatment from blank, cell-free wells containing incubation medium with drugs only. Apoptosis was discriminated from necrotic cell death as previously described [38] by calculating the difference curve (Δ) of the dose-response relations obtained 2 h and 24 h post treatment. Positive Δ (2–24 h) values indicate apoptosis at doses where cells are still metabolically active at the early time point, which is prerequisite for apoptosis as an active, energy-dependent process, to occur. Δ (2–24 h) values of ~ 0 at high doses point towards necrotic cell death characterized by an early loss of metabolic activity and cell membrane integrity. Values ≤ 0 at the lower doses indicate cell proliferation/survival. Cytotoxicity was

measured using the CytoTox-Fluor™ assay (Promega) following the manufacturer's protocol. Data are given as relative fluorescence units (RFU).

Flow cytometry

Mitochondrial membrane potential ($\Delta\Psi_m$), phosphatidylserine (PS) exposure, caspase activity, cell membrane integrity, cell granularity (side-scatter, SSC) and mean cell volume (MCV) were measured by flow cytometry. Cells were seeded in 35-mm petri dishes or 6-well plates (400,000 or 600,000 cells/dish or well) and cultured 24–72 h to 70–80% confluency under standard conditions. Thereafter cells were incubated in culture medium containing (in μM) 10, 20, 50, or 100 SCH-28080, or 0.1% (v/v) DMSO (solvent control) for further 4, 24 or 48 h, or left untreated (untreated control). For the assessment of $\Delta\Psi_m$, cells were loaded with 5 μM JC-1 (5, 5',6, 6'-tetrachloro-1, 1',3, 3'-tetraethylbenzimidazoly carbocyanine iodide; Merck-Calbiochem). Stock solutions (5 mM in DMSO) were freshly prepared prior to use, vigorously vortexed for 5 min and centrifuged for 5 min at 13,000 rpm. After staining for 30 min at 37°C, cell culture supernatants were aspirated and cells were harvested on ice by trypsin/EDTA. Cell suspensions were centrifuged (5 min at 300×g at 4°C), washed twice with ice-cold PBS, diluted to a density of $\sim 1 \times 10^6$ cells/ml and measured immediately. PS exposure was determined by staining with FITC (fluorescein isothiocyanate)-conjugated Annexin-V (BioLegend). Caspase activity was quantified using the membrane permeable CaspACE™ FITC-VAD-FMK *in situ* marker (Promega). Cell membrane integrity was assessed by staining with 7-actinomycin D (7-AAD) or propidium iodide (PI) (BioLegend and Sigma-Aldrich, respectively). Co-stainings of FITC-Annexin-V with 7-AAD and CaspACE™ FITC-VAD-FMK marker with PI were analyzed. Prior to staining, cell culture supernatants were collected and cells were harvested by Accutase® (Sigma-Aldrich) treatment. Cells were centrifuged for 5 min at 300×g, washed twice in PBS and diluted to a density of $\sim 1 \times 10^6$ cells/ml in PBS or Annexin binding buffer. Stainings were performed according to the manufacturers' protocols. Measurements were performed on a Cell Lab Quanta™ SC flow cytometer (Beckman Coulter). Debris (particles with a diameter <7 μm) and cell aggregates (diameter >20 μm) were excluded from analysis and 20,000–50,000 single cells were analyzed per sample. Dyes were excited with a 488-nm argon laser and emissions were measured at 525 nm (FL1), 575 nm (FL2), or 670 nm (FL3). Emission of JC-1 in its monomeric- and J-aggregate form was detected separately at 525 nm (FL1) and 575 nm (FL2), respectively, as described by Salvioli et al [39]. The mitochondrial uncoupler CCCP (applied at 50 μM over the 30-min staining period) was used as positive control for $\Delta\Psi_m$ depolarization and for adjusting FL1 and FL2 PMT-voltages. Data are given as FL2/FL1 ratios. Fluorescence emissions of FITC-Annexin-V/ CaspACE™ FITC-VAD-FMK marker and 7-AAD/PI were detected at 525 nm (FL1) and 670 nm (FL3), respectively. MCV, given in femtoliters (fl), was directly measured employing the Coulter principle for volume assessment. The electronic volume (EV) channel was calibrated using 10- μm Flow-Check fluorospheres (Beckman-Coulter).

Statistics and data presentation

Data are expressed as mean±SEM of at least three independent biological replicates ($n \geq 3$). In all experimental series, solvent control samples were included. Statistical analysis was carried out using Student's t-test, Wilcoxon test or one-way ANOVA with Dunnett's, Tukey's, or Bonferroni's post-hoc tests, as applicable. Means were considered significantly different at p -values <0.05. */#, **/##/••, ***/###/•••, ****/**** denotes $p < 0.05$, $p < 0.01$, $p < 0.001$ and $p < 0.0001$ respectively. Data were analyzed and plotted using GraphPad Prism 6 (GraphPad Software) or Igor Pro 6 (WaveMetrics).

Results

Expression of the non-gastric H^+/K^+ ATPase α -subunit mRNA in rat INS-1E cells

While INS-1E cells do not express the gastric H^+/K^+ ATPase α -subunit isoform (HK α 1 encoded by gene Atp4a; rat stomach tissue was used as positive control; Fig. 1A), we could detect the non-gastric isoform Atp12a (HK α 2; Fig. 1B), although at low abundance compared to rat distal colon where Atp12a is functionally expressed [19] (ΔCt : 5.9 ± 0.3 vs. 21.5 ± 0.2 , respectively; Fig. 1C). The two gene expression assays used (assay A and B targeting the N- and C-terminus of Atp12a, respectively) gave identical results. Despite low mRNA expression levels, Atp12a protein was detectable (Fig. 1D). As in rat colon tissue, a specific band is

Fig. 1. H^+/K^+ ATPase expression analysis in INS-1E cells. (A) PCR results for the gastric H^+/K^+ ATPase α -subunit (HK α 1). Rat stomach tissue was used as positive control yielding a specific 119 base pair (bp) amplicon. M, 100-bp size marker (GeneRuler; Thermo Scientific); -RT, no-reverse-transcriptase control. (B) Results from qPCR in INS-1E cells using predesigned TaqMan® gene expression assays (Applied Biosystems) for rat non-gastric H^+/K^+ ATPase α -subunit Atp12a (HK α 2). Agarose gel electrophoresis showing specific bands for N-terminal assay A (~130 bp) and C-terminal assay B (~142 bp). (C) ΔC_t -values of rat distal colon and INS-1E cells using assay A. (D) Western blot of INS-1E cells and rat distal colon tissue using a primary antibody raised against the human isoform of Atp12a (ATP1A1). Incubation with secondary antibody alone yielded no bands (not shown). β -actin served as loading control. kDa, kilodalton.

Fig. 2. Measurements of pH_i using BCECF. (A) Time-course of pH_i of a single NH_4^+ prepulsing experiment under the indicated extracellular conditions (mean response of 44 cells). Absolute pH data were obtained by high K^+ /nigericin calibration. (B) Results of 6 independent experiments. Data are given as pH_i differences (ΔpH_i) to control conditions (135 Na^+ /5.6 K^+) before NH_4^+ prepulsing. Concentrations in mM.

visible in INS-1E cells along with a second discrete band of lower molecular weight. It is unclear if this second band results from expression of a truncated splice variant in INS-1E cells as previously described in rat kidney and rat exocrine pancreas [24, 40], from antibody cross-reaction with a second target, or from Atp12a protein cleavage.

Intracellular pH measurements

Intracellular pH (pH_i) measurements (BCECF fluorescence) were performed to test for H^+/K^+ ATPase activity; i.e., K^+ -dependent H^+ extrusion after cellular acidification (NH_4^+ prepulsing). Fig. 2A shows an original recording, the results are summarized in Fig. 2B (n=6). Application of 20 mM NH_4^+ induced an intracellular alkalization by ~0.5 pH units. Subsequent withdrawal of NH_4^+ , Na^+ and K^+ caused a rapid acidification by 1.0 pH unit from initial control conditions (135 Na^+ /5.6 K^+). While no pH_i recovery occurred over 15 min after re-addition of 5.6 mM K^+ in the continued absence of Na^+ (cells even further acidified by ~0.1 pH units), re-application of Na^+ was followed by a significant increase in pH_i .

Fig. 3. Inhibition of glucose-stimulated insulin secretion (GSIS) by SCH-28080 and nifedipine. (A) Effect of 20 μ M SCH-28080 (SCH) on basal (0 Gluc) and glucose-stimulated insulin secretion (20 gluc) (n=9). (B) Inhibition of GSIS by 10 and 20 μ M nifedipine (NIF) (n=5). Cumulative insulin release was measured over 1 h. Asterisks and # indicate statistically significant differences to basal release (0 Gluc) and GSIS under 20 Gluc in the absence of drugs, respectively.

Fig. 4. Effect of SCH-28080 on V_{mem} and IK_{ATP} . (A) V_{mem} recording of an electrically active cell stimulated with 20 mM glucose (the inset shows a series of action potentials in higher temporal resolution). (B) Results from V_{mem} recordings under control conditions (DMSO solvent control; n=11), 20 μ M SCH-28080 (SCH; n=11) and after wash-out of SCH-28080 (wash; n=10). (C) IK_{ATP} recordings under control conditions (DMSO solvent control), 20 μ M SCH-28080 (SCH) and after wash-out of SCH-28080 (wash). 500-ms voltage steps to -60 mV, V_{hold} -80 mV, 10 s step-intervals. (D) Summary of current recordings (n=7).

Inhibition of insulin secretion by SCH-28080 and nifedipine

To study if the H^+/K^+ ATPase inhibitor SCH-28080 affects INS-1E cell function, we measured basal and glucose-stimulated insulin secretion (GSIS) by ELISA. As shown in Fig. 3A, cells responded to 20 mM glucose with a ~ 3.3 -fold increase in insulin secretion to 3.5 ± 0.5 μ g/l per 10^5 cells. 20 μ M SCH-28080 diminished GSIS by $\sim 50\%$ to 1.9 ± 0.3 μ g/l per 10^5 cells (n=9), whereas basal release was not significantly affected. In comparison, the L-type Ca^{2+} channel blocker nifedipine at 10 and 20 μ M completely inhibited GSIS (Fig. 3B).

Effects of SCH-28080 on electrophysiological properties of INS-1E cells

In view of inhibited GSIS by SCH-28080 we tested for short-term effects of the drug on the cell membrane potential (V_{mem}). As shown in Fig. 4A, 20 μ M SCH-28080 reversibly hyperpolarized V_{mem} and abrogated action potential firing in electrically active cells. V_{mem} under SCH-28080 (-75.9 ± 1.3 mV vs. -61.4 ± 2.4 mV under control conditions, n=11; Fig. 4B) was close to the calculated reversal potential for K^+ (~ -79 mV), which suggested a K^+ -dependent mode of action. Indeed, we observed a massive and reversible current activation from 9.6 ± 1.5 pA

Fig. 5. Inhibition of I_{Ca_v} by SCH-28080 and nifedipine. (A) Original current tracings of an individual experiment in absence (DMSO solvent control) and presence of 20 μ M SCH-28080 (SCH), and after wash-out of SCH-28080 (wash). The voltage-ramp protocol is shown as inset, V_{hold} between ramps was -80 mV, ramp duration was 500 ms. (B) Effect of SCH-28080 (SCH) on I_{Ca_v} (n=5). (C+D) Inhibition of I_{Ca_v} by 10 and 20 μ M nifedipine (NIF; n=4 and n=5, respectively).

to 343.6 ± 33.9 pA by SCH-28080 (n=7; Fig. 4C+D) in this V_{mem} range where the membrane conductance is predominately determined by K_{ATP} currents [41]. Insulin release is ultimately triggered by opening of voltage-gated Ca^{2+} channels and Ca^{2+} influx [1, 2]. Therefore, we recorded voltage-activated Ca^{2+} current (I_{Ca_v}). Using Ba^{2+} as charge carrier, voltage ramps elicited low- and high-voltage (T- and L-type)-activated Ca^{2+} currents, as previously described in INS-1E and INS-1 cells [42, 43]. While T-type currents ran down upon repeated activation, L-type currents persisted with peak amplitudes at -13.3 mV (n=14), as shown in Fig. 5A. SCH-28080 (20 μ M) reversibly inhibited I_{Ca_v} by ~22% from -812.4 ± 124.4 pA to -630.6 ± 103.5 pA (n=5; Fig. 5B). By comparison, the L-type channel blocker nifedipine reduced I_{Ca_v} by ~12% at 10 μ M from -929.3 ± 165.1 pA to -822.3 ± 151.7 pA, (n=4; Fig. 5C) and ~18% at 20 μ M from -817.9 ± 116.5 pA to -673.6 ± 111.6 pA, (n=5; Fig. 5D).

Fig. 6. Effect of SCH-28080 on INS-1E cell viability. (A) Dose-response relations obtained by resazurin-based assays 2 and 24 h post treatment (n=4; four parameters variable slope curve fits) and Δ (2–24 h) differential curve (manual fit) calculated for cell death discrimination [38]. CTL, DMSO (solvent) control. RFU, relative fluorescence units. Asterisks and • indicate significant differences to CTL after 2 and 24 h, respectively. # indicates a significant difference between 2 and 24 h values at the same concentration of SCH-28080. (B) Results from the CytoTox-Fluor™ cytotoxicity assay (Promega). Second order polynomial fit (quadratic).

Fig. 7. Effects of SCH-28080 on apoptotic cell death related parameters. (A) JC-1 red/green (FL2/FL1) fluorescence ratio after 4 h (n=3) and 24 h (n=4) under control conditions (DMSO, open bars), in the presence of 10, 20, 50 or 100 μ M SCH-28080 (SCH; gray bars) and in the presence of 50 μ M of the mitochondrial uncoupler CCCP applied for 30 min (black bars). (B) Percentage of the Annexin-V+/7-AAD- (early apoptotic) sub-population of cells under control conditions (DMSO) and 20 μ M SCH-28080 (SCH) after 24 h (open bars) and 48 h (gray bars) (n=6–8). (C) Effects of SCH-28080 on activation of key caspases (left panel), mean cell volume (MCV; middle panel) and cell granularity (side-scatter, SSC; right panel) in caspase+/PI- and caspase+/PI+ cells under control conditions (DMSO) and after 24 h (open bars) and 48 h (gray bars) of treatment with 10 or 20 μ M SCH-28080 (SCH; n=5–7). Asterisks and # indicate statistically significant differences to control conditions (DMSO) and to 10 μ M SCH-28080 at the same time point, respectively.

Effect of SCH-28080 on cell viability, mitochondrial membrane potential, phosphatidylserine exposure, cell granularity and mean cell volume

Since SCH-28080 has distinct effects on different levels of stimulus-secretion coupling and inhibits GSIS, we investigated its effects on INS-1E viability, cytotoxicity and apoptosis. Fig. 6A depicts the results from resazurin-based viability analysis obtained at 2 and 24 h post treatment. SCH-28080 caused a dose-dependent reduction in cell viability with IC_{50} values of 22.9 μ M and 15.3 μ M after 2 and 24 h, respectively (n=7) and cell viability was below 10% at 100 μ M already after 2 h. Differential curve analysis [38] yielded Δ (2–24 h) values ≤ 0 up to 12.5 μ M consistent with cell proliferation/survival in this concentration range. At 20–30 μ M, the curve shows a positive peak indicating the range of concentrations where SCH-28080 exerted a pro-apoptotic effect. At higher doses, Δ (2–24 h) values decreased back to $\sim 0\%$ at concentrations where cells lost their metabolic activity/viability early after application of the drug and necrosis became the prevailing form of cell death. In line with this, cytotoxicity (necrosis *via* loss of membrane integrity) measured 2 h post treatment steeply increased at concentrations >40 –50 μ M (n=4; Fig. 6B).

Next, we analyzed apoptotic cell death-related parameters including the mitochondrial membrane potential ($\Delta\Psi_m$), phosphatidylserine (PS) exposure at the outer leaflet of the cell membrane (Annexin-V binding), key caspase activity, cell membrane integrity (7-AAD and PI exclusion), cell granularity (side-scatter, SSC) and mean cell volume (MCV). The results are shown in Fig. 7. After only 4 h of treatment with 50 or 100 μ M SCH-28080 we observed a significant reduction of the JC-1 red/green fluorescence ratio, indicating a dissipation of $\Delta\Psi_m$, whereas 10 and 20 μ M did not significantly affect the ratio even after 24 h (Fig. 7A).

After 24 and 48 h, 20 μ M SCH-28080 significantly increased the fraction of Annexin-V+/7-AAD- (early apoptotic) cells (Fig. 7B) and of both caspase+/PI- and caspase+/PI+ cells (Fig. 7C, left panel). MCV was significantly decreased by 10 and 20 μ M SCH-28080 in caspase+/PI- cells after 48 h and by 20 μ M in caspase+/PI+ cells after 24 and 48 h (Fig. 7C, middle panel). Similarly, cell granularity (SCC) was increased by 20 μ M SCH-28080 after 48 h in caspase+/PI- cells and after 24 and 48 h in caspase+/PI+ cells (Fig. 7C, left panel).

Discussion

In this study we show by RT-PCR and Western blotting that Atp12a, the rat isoform of the non-gastric H⁺/K⁺ ATPase α -subunit (HK α 2) is expressed in rat INS-1E insulinoma cells. The basal transcription of Atp12a, however, is low, as indicated by the shallow abundance of Atp12a mRNA/cDNA compared to rat distal colon tissue.

In spite of protein expression, we could not measure K⁺-dependent pH_i recovery after acute acidification in the absence of extracellular Na⁺ within 15 min indicating that the H⁺/K⁺ pump does not contribute to proton extrusion in INS-1E cells under the given experimental conditions. However, it has been shown that the rat isoform of the pump present in distal colonic apical membrane vesicles – or if expressed in insect Sf9 cells – can function also as an NH₄⁺/H⁺ ATPase [34, 35, 44]. Moreover, the rat, rabbit and human colonic H⁺/K⁺ ATPases have been reported not only to catalyze the exchange of H⁺ for K⁺ ions but also for Na⁺, thus acting as a Na⁺/H⁺ ATPase [45–47]. Therefore, functions other than ATP-driven H⁺/K⁺ exchange cannot be ruled out. While no apparent proton extrusion occurs in the absence of extracellular Na⁺, re-addition of Na⁺ is followed by rapid pH_i recovery, indicating that Na⁺/H⁺ exchangers (NHEs) are mainly involved in cytosolic pH regulation. This is in line with previous findings in HIT-T15 hamster insulinoma cells and mouse islets [9, 15]. Accordingly, in INS-1E cells we found transcripts of the Na⁺/H⁺ exchanger isoforms NHE1, NHE2 and NHE7 (not shown).

To test for a possible involvement of the H⁺/K⁺ ATPase on INS-1E cell function and viability despite its apparently lacking contribution to cellular acid extrusion, we tested for the effects of the inhibitor SCH-28080. For insulin release measurements, we chose a concentration of 20 μ M. In this context it is important to mention that SCH-28080 does not as potently inhibit the rat isoform of the non-gastric H⁺/K⁺ ATPase as the human enzyme; at 20 μ M it only inhibits approximately 20% of the ATPase activity when expressed in Sf9 cell membranes [34, 35]. Nonetheless, at this concentration we observed morphological alterations and reduced cell proliferation and found a strong (~50%) inhibition of GSIS within 1 h. The fact that SCH-28080 inhibits GSIS and cell viability by approximately 50% at a concentration which exerts only a shallow inhibition of H⁺/K⁺ ATPase activity suggests that the drug might affect other cellular targets relevant for GSIS and cell survival. Such “off target” effects of SCH-28080 have been described also in other systems: in mouse inner medullary collecting duct cells (mIMCD-3), SCH-28080 has been shown to deplete intracellular ATP at 200 μ M within 30 min causing a deactivation of the Na⁺/K⁺ ATPase as shown by an almost complete inhibition of ⁸⁶Rb⁺ uptake [48]. In renal cortical, medullary and liver endosomes, the drug inhibits vacuolar (V-type) H⁺ ATPases at 100 μ M but not at 10 μ M [32] and in hog gastric vesicles it inhibits phospholipid flippase activity with an IC₅₀ value of 0.13 μ M [49, 50]. In the sub- μ M range, SCH-28080 also inhibits neuronal and cardiac G protein-activated inwardly rectifying K⁺ (GIRK) channels [51]. In mouse pancreatic β -cells, GIRK channels have been shown to mediate adrenalin-induced hyperpolarization and these channels are expressed in INS-1E cells [52]. Importantly, we show that SCH-28080 may act as an activator of ATP-dependent K⁺ channels thereby causing a hyperpolarization of V_{mem}. At 20 μ M, SCH-28080 massively augmented the conductance recorded at -60 mV, most likely IK_{ATP}, which predominantly determines the whole-cell conductance in this range of potentials [41] near to the resting membrane potential of these cells. In how far eventual GIRK channel inhibition influences the overall effect in this setting remains to be clarified. It also needs

to be evaluated if SCH-28080 could be useful as a channel activator in ion channel research. Besides K_{ATP} channels we could identify voltage-gated Ca^{2+} channels as novel targets of SCH-28080. At 20 μM ICa_v was inhibited by ~20% comparable to 20 μM of the L-type channel inhibitor nifedipine. This might in part explain the inhibitory effect of the drug on GSIS, although the dominant effect of SCH-28080 is probably the hyperpolarization of V_{mem} via K^+ current activation. Interestingly, although nifedipine and SCH-28080 inhibited ICa_v to approximately the same extent, this resulted in a complete inhibition of GSIS by nifedipine, but only ~50% inhibition by SCH-28080 despite the massive additional activation of IK_{ATP} by the latter. Therefore, it can be assumed that either nifedipine acts on (an) additional ICa_v -independent mechanism(s) inhibiting insulin secretion, or insulin secretion is triggered by glucose via (a) mechanism(s) bypassing classical stimulus-secretion-coupling; e.g., by cell swelling [4-7]. The effects of SCH-28080 on IK_{ATP} , V_{mem} and ICa_v were reversible, which argues against cytotoxicity and metabolic derangement to underlie the short-term effects of the drug on the electrophysiological behavior (e.g., ICa_v rundown). Current recordings were usually completed within 20 min and even in case of V_{mem} recordings which exceeded 30 min, the hyperpolarizing effect of the drug was fully reversible and cells were still capable of firing regular action potentials.

We further show here that SCH-28080 causes a dose-dependent reduction in cell viability with pro-apoptotic doses in the range of the IC_{50} of the drug (20–30 μM) – inferred from the difference curve calculated from early (2 h) and late (24 h) viability readouts [38]. This could be confirmed by increased phosphatidylserine (PS) exposure, caspase activation, loss of cell membrane integrity, an increase in cell granularity and apoptotic cell volume decrease in cells treated with 20 μM SCH-28080 after 24 and 48 h. Compared to the increase in caspase+ cells, the increase in PS (Annexin-V)+ cells appears small but might perhaps have been dampened by inhibition of flippases [49, 50]. Regarding the mode of action, disruption of autocrine/paracrine action of insulin [53, 54] as prime course for the pro-apoptotic effect seems unlikely since the basal insulin release is not significantly affected at that drug concentration. At doses >40–50 μM , necrosis predominated, with cells losing their metabolic activity/viability already 2 h post treatment and concentration-dependent loss of membrane integrity. This was associated with a dissipation of the mitochondrial membrane potential. In this concentration range we have described similar cytotoxic effects on INS-1E and INS-1 cells for the phytoestrogen resveratrol and the flavonoids rutin and quercetin [43, 55]. We also tested SCH-28080 on RIN-5F rat pancreatic islet cells and NIH-3T3 fibroblasts: in RIN-5F cells, the dose-response curve was shifted towards higher concentrations with significantly reduced viability $\geq 50 \mu M$, whereas in NIH-3T3 fibroblasts the drug had no effect on cell viability within 24 h (data not shown). The viability of myelomonocytic HL-60 cells was also not affected by the drug up to a concentration of 100 μM [17]. Whether SCH-28080 acts specifically on β -cells, however, needs continuative studies on further insulinoma cell lines and primary β -cells.

SCH-28080 is frequently used to functionally characterize H^+/K^+ ATPases in cell types and tissues. In the light of our current data as well as previous findings by others, such studies need to be cautiously interpreted considering off-target drug effects on e.g., ion channels or transporters other than H^+/K^+ ATPases and on cell viability. Therefore, these findings stress the importance of using low concentrations of SCH-28080 in studies on H^+/K^+ ATPases in non-gastric tissues to avoid misinterpretation of the data due to unspecific effects.

In conclusion, we demonstrate that in rat INS-1E cells the H^+/K^+ ATPase inhibitor SCH-28080 inhibits insulin secretion by activation of IK_{ATP} and inhibition of L-type voltage-gated Ca^{2+} channels, reduces cell viability and dose-dependently induces apoptosis/necrosis. Even though these cells express the non-gastric H^+/K^+ ATPase Atp12a at low abundance, a functional role of Atp12a in regulation of intracellular pH in INS-1E cells could not be disclosed under the given experimental conditions and the effects of SCH-28080 rather arise from off-target actions of the drug than specific inhibition of H^+/K^+ ATPase activity.

Acknowledgements

The skilled technical and administrative assistances of Katharina Schuhbeck, Leman Emin, Anna Baumgartner and Brigitte Maier are gratefully acknowledged. This project was supported by grants R-09/04/009-JAK, R-11/02/024-JAK and K-11/01-JAK to MJ.

Disclosure Statement

The authors declare that there are no conflicts of interest

References

- 1 Henquin JC: Regulation of insulin release by ionic and electrical events in B cells. *Horm Res* 1987;27:168-178.
- 2 Ashcroft FM, Rorsman P: Electrophysiology of the pancreatic beta-cell. *Prog Biophys Mol Biol* 1989;54:87-143.
- 3 Lang F, Busch GL, Ritter M, Volkl H, Waldegger S, Gulbins E, Haussinger D: Functional significance of cell volume regulatory mechanisms. *Physiol Rev* 1998;78:247-306.
- 4 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 ICl_{in} in INS-1E rat insulinoma cells. *Cell Physiol Biochem* 2006;18:21-34.
- 5 Orecna M, Hafko R, Bacova Z, Podskocova J, Chorvat D, Jr., Strbak V: Different secretory response of pancreatic islets and insulin secreting cell lines INS-1 and INS-1E to osmotic stimuli. *Physiol Res* 2008;57:935-945.
- 6 Orecna M, Hafko R, Toporcerova V, Strbak V, Bacova Z: Cell swelling-induced insulin secretion from INS-1E cells is inhibited by extracellular Ca²⁺ and is tetanus toxin resistant. *Cell Physiol Biochem* 2010;26:197-208.
- 7 Best L, Brown PD, Sener A, Malaisse WJ: Electrical activity in pancreatic islet cells: The VRAC hypothesis. *Islets* 2010;2:59-64.
- 8 Best L, Yates AP, Gordon C, Tomlinson S: Modulation by cytosolic pH of calcium and rubidium fluxes in rat pancreatic islets. *Biochem Pharmacol* 1988;37:4611-4615.
- 9 Trebilcock R, Lynch A, Tomlinson S, Best L: Na⁺/H⁺ exchange is responsible for intracellular pH regulation in insulin-secreting HIT-T15 cells. *Mol Cell Endocrinol* 1990;71:21-25.
- 10 Juntti-Berggren L, Arkhammar P, Nilsson T, Rorsman P, Berggren PO: Glucose-induced increase in cytoplasmic pH in pancreatic beta-cells is mediated by Na⁺/H⁺ exchange, an effect not dependent on protein kinase C. *J Biol Chem* 1991;266:23537-23541.
- 11 Best L, Ammala C, Rorsman P, Tomlinson S: Intracellular pH, cytosolic calcium concentration and electrical activity in RINm5F insulinoma cells. *Biochim Biophys Acta* 1994;1192:107-111.
- 12 Ritter M, Fuerst J, Woll E, Chwatal S, Gschwentner M, Lang F, Deetjen P, Paulmichl M: Na⁺/H⁺ exchangers: Linking osmotic dysequilibrium to modified cell function. *Cell Physiol Biochem* 2001;11:1-18.
- 13 Gunawardana SC, Sharp GW: Intracellular pH plays a critical role in glucose-induced time-dependent potentiation of insulin release in rat islets. *Diabetes* 2002;51:105-113.
- 14 Stiernet P, Guiot Y, Gilon P, Henquin JC: Glucose acutely decreases pH of secretory granules in mouse pancreatic islets. Mechanisms and influence on insulin secretion. *J Biol Chem* 2006;281:22142-22151.
- 15 Stiernet P, Nenquin M, Moulin P, Jonas JC, Henquin JC: Glucose-induced cytosolic pH changes in beta-cells and insulin secretion are not causally related: studies in islets lacking the Na⁺/H⁺ exchanger NHE1. *J Biol Chem* 2007;282:24538-24546.
- 16 Ritter M, Schratzberger P, Rossmann H, Woll E, Seiler K, Seidler U, Reinisch N, Kahler CM, Zwierzina H, Lang HJ, Lang F, Paulmichl M, Wiedermann CJ: Effect of inhibitors of Na⁺/H⁺-exchange and gastric H⁺/K⁺ ATPase on cell volume, intracellular pH and migration of human polymorphonuclear leucocytes. *Br J Pharmacol* 1998;124:627-638.

- 17 Jakab M, Hofer S, Ravasio A, Huber F, Schmidt S, Hitzl W, Geibel JP, Furst J, Ritter M: The putative role of the non-gastric H⁺/K⁺-ATPase ATP12A (ATP1AL1) as anti-apoptotic ion transporter: effect of the H⁺/K⁺ ATPase inhibitor SCH28080 on butyrate-stimulated myelomonocytic HL-60 cells. *Cell Physiol Biochem* 2014;34:1507-1526.
- 18 Crowson MS, Shull GE: Isolation and characterization of a cDNA encoding the putative distal colon H⁺,K⁺-ATPase. Similarity of deduced amino acid sequence to gastric H⁺,K⁺-ATPase and Na⁺,K⁺-ATPase and mRNA expression in distal colon, kidney, and uterus. *J Biol Chem* 1992;267:13740-13748.
- 19 Takahashi Y, Sakai H, Kuragari M, Suzuki T, Tauchi K, Minamimura T, Tsukada K, Asano S, Takeguchi N: Expression of ATP1AL1, a non-gastric proton pump, in human colorectum. *Jpn J Physiol* 2002;52:317-321.
- 20 Pestov N, Korneenko T, Shakhparonov M, Shull G, Modyanov N: Loss of acidification of anterior prostate fluids in Atp12a-null mutant mice indicates that nongastric H-K-ATPase functions as proton pump *in vivo*. *Am J Physiol Cell Physiol* 2006;291:C366-374.
- 21 Pestov NB, Korneenko TV, Adams G, Tillekeratne M, Shakhparonov MI, Modyanov NN: Nongastric H-K-ATPase in rodent prostate: lobe-specific expression and apical localization. *Am J Physiol Cell Physiol* 2002;282:C907-916.
- 22 Pestov NB, Romanova LG, Korneenko TV, Egorov MV, Kostina MB, Sverdlov VE, Askari A, Shakhparonov MI, Modyanov NN: Ouabain-sensitive H,K-ATPase: tissue-specific expression of the mammalian genes encoding the catalytic alpha subunit. *FEBS Lett* 1998;440:320-324.
- 23 Johansson M, Jansson T, Pestov NB, Powell TL: Non-gastric H⁺/K⁺ ATPase is present in the microvillous membrane of the human placental syncytiotrophoblast. *Placenta* 2004;25:505-511.
- 24 Novak I, Wang J, Henriksen KL, Haanes KA, Krabbe S, Nitschke R, Hede SE: Pancreatic bicarbonate secretion involves two proton pumps. *J Biol Chem* 2011;286:280-289.
- 25 Pestov NB, Korneenko TV, Shakhparonov MI, Modyanov NN: Postnatal regulation of X,K-ATPases in rat skin and conserved lateroapical polarization of Na,K-ATPase in vertebrate epidermis. *Exp Dermatol* 2013;22:423-425.
- 26 Streif D, Iglseider E, Hauser-Kronberger C, Fink KG, Jakab M, Ritter M: Expression of the non-gastric H⁺/K⁺ ATPase ATP12A in normal and pathological human prostate tissue. *Cell Physiol Biochem* 2011;28:1287-1294.
- 27 Butler AE, Janson J, Bonner-Weir S, Ritzel R, Rizza RA, Butler PC: β -cell deficit and increased β -cell apoptosis in humans with type 2 diabetes. *Diabetes* 2003;52:102-110.
- 28 Chang-Chen KJ, Muller R, Bernal-Mizrachi E: β -cell failure as a complication of diabetes. *Rev Endocr Metab Disord* 2008;9:329-343.
- 29 Eizirik D, Colli M, Ortis F: The role of inflammation in insulinitis and β -cell loss in type 1 diabetes. *Nat Rev Endocrinol* 2009;5:219-226.
- 30 Merglen A, Theander S, Rubi B, Chaffard G, Wollheim CB, Maechler P: Glucose sensitivity and metabolism-secretion coupling studied during two-year continuous culture in INS-1E insulinoma cells. *Endocrinology* 2004;145:667-678.
- 31 Modyanov NN, Mathews PM, Grishin AV, Beguin P, Beggah AT, Rossier BC, Horisberger JD, Geering K: Human ATP1AL1 gene encodes a ouabain-sensitive H-K-ATPase. *Am J Physiol* 1995;269:C992-997.
- 32 Sabolic I, Brown D, Verbavatz JM, Kleinman J: H⁺-ATPases of renal cortical and medullary endosomes are differentially sensitive to Sch-28080 and omeprazole. *Am J Physiol* 1994;266:F868-877.
- 33 Silver RB, Frindt G: Functional identification of H-K-ATPase in intercalated cells of cortical collecting tubule. *Am J Physiol* 1993;264:F259-266.
- 34 Swarts HG, Koenderink JB, Willems PH, De Pont JJ: The non-gastric H,K-ATPase is oligomycin-sensitive and can function as an H⁺,NH₄⁺-ATPase. *J Biol Chem* 2005;280:33115-33122.
- 35 Swarts HG, Koenderink JB, Willems PH, De Pont JJ: The human non-gastric H,K-ATPase has a different cation specificity than the rat enzyme. *Biochim Biophys Acta* 2007;1768:580-589.
- 36 Farley RA, Schreiber S, Wang SG, Scheiner-Bobis G: A hybrid between Na⁺,K⁺-ATPase and H⁺,K⁺-ATPase is sensitive to palytoxin, ouabain, and SCH 28080. *J Biol Chem* 2001;276:2608-2615.
- 37 Nakayama GR, Caton MC, Nova MP, Parandoosh Z: Assessment of the Alamar Blue assay for cellular growth and viability *in vitro*. *J Immunol Methods* 1997;204:205-208.
- 38 Helm K, Beyreis M, Mayr C, Ritter M, Jakab M, Kiesslich T, Plaetzer K: *In vitro* Cell Death Discrimination and Screening Method by Simple and Cost-Effective Viability Analysis. *Cell Physiol Biochem* 2017;41:1011-1019.

- 39 Salvioli S, Dobrucki J, Moretti L, Troiano L, Fernandez MG, Pinti M, Pedrazzi J, Franceschi C, Cossarizza A: Mitochondrial heterogeneity during staurosporine-induced apoptosis in HL60 cells: analysis at the single cell and single organelle level. *Cytometry* 2000;40:189-197.
- 40 Kone BC, Higham SC: A Novel N-terminal Splice Variant of the Rat H⁺-K⁺-ATPase α 2 Subunit. Cloning, functional expression, and renal adaptive response to chronic hypokalemia. *J Biol Chem* 1998;273:2543-2552.
- 41 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.
- 42 Jakab M, Lach S, Bacova Z, Langeluddecke C, Strbak V, Schmidt S, Iglseider E, Paulmichl M, Geibel J, Ritter M: Resveratrol inhibits electrical activity and insulin release from insulinoma cells by block of voltage-gated Ca²⁺ channels and swelling-dependent Cl⁻ currents. *Cell Physiol Biochem* 2008;22:567-578.
- 43 Kittl M, Beyreis M, Tumurkhuu M, Furst J, Helm K, Pitschmann A, Gaisberger M, Glasl S, Ritter M, Jakab M: Quercetin Stimulates Insulin Secretion and Reduces the Viability of Rat INS-1 Beta-Cells. *Cell Physiol Biochem* 2016;39:278-293.
- 44 Codina J, Pressley TA, DuBose TD, Jr.: The Colonic H⁺,K⁺-ATPase Functions as a Na⁺-dependent K⁺(NH₄⁺)-ATPase in Apical Membranes from Rat Distal Colon. *J Biol Chem* 1999;274:19693-19698.
- 45 Cougnon M, Bouyer P, Planelles G, Jaisser F: Does the colonic H,K-ATPase also act as an Na,K-ATPase? *Proc Natl Acad Sci U S A* 1998;95:6516-6520.
- 46 Grishin AV, Caplan MJ: ATP1A1, a Member of the Non-gastric H,K-ATPase Family, Functions as a Sodium Pump. *J Biol Chem* 1998;273:27772-27778.
- 47 Peti-Peterdi J, Bebok Z, Lapointe JY, Bell PD: Novel regulation of cell [Na⁺] in macula densa cells: apical Na⁺ recycling by H-K-ATPase. *Am J Physiol Renal Physiol* 2002;282:F324-329.
- 48 Codina J, Cardwell J, Gitomer JJ, Cui Y, Kone BC, Dubose TD, Jr.: SCH-28080 depletes intracellular ATP selectively in mIMCD-3 cells. *Am J Physiol Cell Physiol* 2000;279:C1319-1326.
- 49 Suzuki H, Kamakura M, Morii M, Takeguchi N: The phospholipid flippase activity of gastric vesicles. *J Biol Chem* 1997;272:10429-10434.
- 50 Suzuki H, Morii M, Takeguchi N: Effects of ionophores on the phospholipid flippase activity of gastric vesicles. *Jpn J Physiol* 1999;49:431-436.
- 51 Walsh KB: Targeting GIRK Channels for the Development of New Therapeutic Agents. *Front Pharmacol* 2011;2:64.
- 52 Iwanir S, Reuveny E: Adrenaline-induced hyperpolarization of mouse pancreatic islet cells is mediated by G protein-gated inwardly rectifying potassium (GIRK) channels. *Pflugers Arch* 2008;456:1097-1108.
- 53 Leibiger IB, Leibiger B, Berggren PO: Insulin feedback action on pancreatic β -cell function. *FEBS Lett* 2002;532:1-6.
- 54 Muller D, Jones PM, Persaud SJ: Autocrine anti-apoptotic and proliferative effects of insulin in pancreatic β -cells. *FEBS Lett* 2006;580:6977-6980.
- 55 Bortolotti C, Kunit T, Moder A, Hufnagl C, Schmidt S, Hartl A, Langeluddecke C, Furst J, Geibel J, Ritter M, Jakab M: The Phytostilbene Resveratrol Induces Apoptosis in INS-1E Rat Insulinoma Cells. *Cell Physiol Biochem* 2009;23:245-254.