Effect of the AMP-Kinase Modulators AICAR, Metformin and Compound C on Insulin Secretion of INS-1E Rat Insulinoma Cells under Standard Cell Culture Conditions

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Key Words

AMP kinase • AICAR • Metformin • Compound C • β -cell • K_{ATP} channels • $ICl_{glucose}$ • Insulin release

Abstract

Background/Aims: The function of β -cells is regulated by nutrient uptake and metabolism. The cells' metabolic state can be expressed as concentration ratios of AMP, ADP and ATP. Relative changes in these ratios regulate insulin release. An increase in the intracellular ATP concentration causes closure of K_{ATP} channels and cell membrane depolarization, which triggers stimulus-secretion coupling (SSC). In addition to K_{ATP} channels, the AMP-dependent protein kinase (AMPK), a major cellular fuel sensor in a variety of cells and tissues, also affects insulin secretion and β -cell survival. In a previous study we found that the widely used AMPK inhibitor compound C retards proliferation and induces apoptosis in the rat β -cell line INS-1E. We therefore tested the effects of AMPK activators (AICAR and metformin), and compound C on AMPK phosphorylation, insulin secretion, K_{ATP} channel currents, cell membrane potential, intracellular calcium concentration, apoptosis and cell

cycle distribution of INS-1E cells under standard cell culture conditions (11 mM glucose). **Methods:** Western blotting, ELISA, patch-clamp, calcium imaging and flow cytometry. **Results:** We found that basal AMPK phosphorylation is enhanced by AICAR (1 mM) and metformin (1 mM) but remained unaffected by compound C (10 μ M). Both AICAR and compound C stimulated basal insulin secretion whereas metformin had no effect. Pre-incubation with AICAR (1 mM) caused an inhibition of K_{ATP} currents but did not significantly alter the average cell membrane potential (V_m) or the threshold potential of electrical activity. Acute administration of AICAR (300 μ M) led to a depolarization of V_m , which was not due to an inhibition of the basal- or glucose-induced chloride conductance, and was not accompanied by elevations of intracellular calcium (Ca_i). AICAR had no additive blocking effect on K_{ATP} currents when applied together with tolbutamide. Compound C applied over 24 hours induced an increase in the percentage of cells positive for caspase activity, whereas AICAR (1 mM) applied for 48 hours was without effect. Medium glucose concentration <3 mM caused cell cycle arrest, caspase activation and an increase of cell granularity.

Conclusion: We conclude that under standard cell culture conditions the AMPK modulators AICAR and compound C, but not metformin, stimulate insulin secretion by AMPK-independent mechanisms.

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Introduction

Insulin secretion from pancreatic β -cells requires precise cellular mechanisms sensing the ambient concentrations of the cellular nutrients glucose, amino acids and free fatty acids by adjusting hormone exocytosis in response to the prevailing metabolic demands. On the cellular level the metabolic status is reflected by the generation of ATP, ADP and AMP, which play a crucial role in stimulus-secretion coupling (SSC). SSC in pancreatic β -cells involves both ATP-sensitive potassium (K_{ATP}) channel-dependent- and independent pathways [1]. The K_{ATP} channel-dependent pathway involves GLUT-2 mediated uptake and rapid metabolism of glucose leading to an increase in the cellular ATP/ADP ratio, which results in the closure of K_{ATP} channels, membrane depolarization, calcium influx *via* voltage-dependent Ca^{2+} channels and exocytosis of insulin [2, 3].

The K_{ATP} channels in pancreatic β -cells consist of two subunits, the sulfonylurea receptor SUR1 and the inwardly rectifying Kir6.2 potassium channel, which are assembled to a hetero-octameric complex forming a functional K_{ATP} channel. ATP directly inhibits the Kir6.2 channel subunit. SUR1 comprises two nucleotide-binding domains and functions as a modulatory element of the K_{ATP} channel. It is suggested that SUR1 monitors cellular energy levels and modulates the K_{ATP} channels by affecting the affinity of ATP binding to Kir6.2, thus adapting insulin release to the prevailing metabolic demands (reviewed in [4]).

Beside K_{ATP} channels the adenosine 5' monophosphate-activated protein kinase (AMPK), a main homeostasis regulator of systemic and cellular energy, acts as a cellular energy status-monitoring element in pancreatic β -cells [5]. The AMPK complex is a heterotrimeric serine/threonine kinase, which is activated by an increase in the cellular AMP/ATP ratio by stress factors like glucose deprivation, hypoxia, ischemia or inflammation. By this process AMPK protects the cell from ATP-depletion by down-regulating energy-consuming processes such as operation of ion transporters and secretion. Simultaneously ATP generation is favored, thereby restoring cellular energy balance. AMPK becomes activated by phosphorylation of the alpha-subunit

on amino acid Thr¹⁷² by upstream kinases like LKB1 or the calcium/calmodulin-dependent protein kinase kinase 2 (CaMKK2). AMPK can also be activated independently from the AMP/ATP ratio *via* CaMKK2 by an increase in the intracellular Ca^{2+} concentration (reviewed in [5, 6]). Active AMPK has short- as well as long term effects on cellular energy homeostasis, insulin sensitivity and insulin secretion [7]. In general, activation and inhibition of AMPK in the β -cell are expected to cause inhibition and stimulation of insulin secretion, respectively [5]. However, the reports about AMPK activation on insulin release are controversial describing either inhibition [8-12], or stimulation [13-15].

In a previous study we have shown that inhibition of AMPK by compound C leads to caspase activation in rat INS-1E insulinoma cells, indicating the induction of apoptosis [16]. This effect eventually involves interference of AMPK with the auto/paracrine actions of insulin on the survival of the cells [16, 17]. In the present study we therefore investigated the effect of AMPK activity on insulin secretion, K_{ATP} channel currents, cell membrane potential, intracellular calcium concentration and apoptosis of INS-1E cells. Experiments were performed under standard cell culture conditions (11 mM medium glucose concentration) [18] in absence or presence of pharmacological AMPK modulators; i.e., the AMPK activators AICA riboside (AICAR), or metformin, and the AMPK inhibitor compound C.

Materials and Methods

Chemicals and Reagents

All salts and chemicals used for the preparation of experimental solutions and cell culture media were p.a. grade. 6-[4-(2-Piperidin-1-yl-ethoxy)-phenyl]-3-pyridin-4-yl-pyrazolo[1,5-a]pyrimidine (compound C), AICA riboside (AICAR), 1,1-dimethylbiguanide hydrochloride (metformin) were obtained from Calbiochem. Stock solutions of AICAR (30 mM) and metformin (30 mM) were prepared in deionized water, and stored in aliquots at -20°C, stock solutions of compound C (12.5 mM) and diazoxide (40 mM) were prepared in di-methyl-sulphide-oxide (DMSO) and stored at 4°C in the dark. Tolbutamide was purchased from Sigma-Aldrich. The primary rabbit anti-AMPK α , anti-phospho-AMPK α (Thr172) and anti- β -actin, and secondary goat anti-rabbit IgG HRP-linked antibodies were from Cell Signaling Technology. Fura-2/AM was from Molecular Probes (Invitrogen, Oregon, USA).

Cell Culture

INS-1E cells were grown in RPMI 1640 medium containing 11 mM D-glucose, 1 mM sodium pyruvate, 50 μ M β -mercaptoethanol, 2 mM glutamine, 10 mM HEPES, 10% fetal

calf serum (FCS), 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml amphotericin B at 37°C, 5% CO₂ and 95% air. Subcultures were established once a week by trypsin/EDTA treatment. Cells between passages 90 and 110 were used.

Immunoblotting

For Western blot experiments cells were grown in 9-cm polystyrene petri dishes for 3-5 days with feeding every second day with 10% FCS-supplemented RPMI-medium containing 11 mM glucose. To investigate the effect of reduced medium glucose concentration on AMPK phosphorylation the standard culture medium was replaced for 24 or 1 hour with serum-free RPMI 1640 medium containing 2.8 mM glucose. To study the effects of pharmacological AMPK activation or inhibition under basal conditions, the medium was replaced for 1 hour with serum-free RPMI 1640 medium (11 mM glucose) containing 1 mM AICAR, 1 mM metformin, 10 µM compound C, or solvents alone (deionized water or 0.8% DMSO). After the incubation period culture media were aspirated and cells were washed with ice cold PBS. Cells were scraped off with lysis buffer composed of 150 mM NaCl, 50 mM TRIS-HCl (pH 7.5), 1 mM EGTA, 1% (v/v) Triton X-100, 0.1% (w/v) SDS and proteinase inhibitor cocktail containing AEBSF, pepstatinA, E-64, bestatin, leupeptin, aprotinin (Sigma-Aldrich). Cell lysates were stored on ice and sonicated (five 1-second pulses) to achieve complete cell lysis. Lysates were centrifuged at 14,000×g for 10 minutes. The protein concentration in the supernatants was measured with a BCA (bicinchoninic acid) protein assay kit (Pierce-Thermo Scientific). Samples were mixed with 2× sample buffer containing 125 mM TRIS-HCl, 4% (w/v) SDS, 0.7% (v/v) β-mercaptoethanol, 20% (v/v) glycerol and 0.004% (w/v) bromophenol blue, and 16 µg protein/sample were separated by SDS-PAGE on 4-15% TRIS-HCl gels (Bio-Rad). β-actin served as loading control. The Spectra™ Multicolor Broad Range Protein Ladder (Fermentas) was used as protein size marker. Proteins were transferred onto 0.45-µm nitrocellulose membranes (Whatman). For immunodetection polyclonal rabbit anti-AMPKα and phospho-AMPKα (Thr172) (~62 kD) antibodies, and a rabbit anti-β-actin (~45 kD) antibody were used at a dilution of 1:500. Antibody binding was detected with a horseradish peroxidase (HRP)-conjugated secondary goat anti-rabbit antibody (1:1000) and SuperSignal® West Pico Chemiluminescent Substrate (Pierce). 16-bit TIFF images were acquired using a BioSpectrum AC Imaging System and VisionWorks LS Software (UVP). Densitometric analysis was carried out using ImageJ 1.416a (NIH, Bethesda, Maryland, USA, <http://imagej.nih.gov/ij/>). For each experimental condition the phosphorylated-to-total protein ratio (pAMPKα^{Thr172}/AMPKα) was calculated and normalized to the ratio obtained under control conditions (11 mM medium glucose in the absence of drugs) (Fig. 1B).

Insulin ELISA

For ELISA experiments cells were seeded in poly-D-lysine covered 96-well plates (Sarstedt) at a density of 40,000 cells/ml and grown under standard culture conditions for 48 hours before starting the experiments. To avoid FCS-induced cross-reaction of serum insulin with the ELISA, experiments were

performed in serum-free RPMI 1640 medium. In this series of experiments cells were incubated for 1 hour in serum-free medium containing 11 mM glucose in absence (control), or presence of 1 mM AICAR, 1 mM metformin, or 10 µM compound C, respectively. The insulin released into the medium during the 1-hour incubation period was measured using a rat insulin ELISA kit (Mercodia) according to the manufacturer's instructions. Samples were measured in quadruplicates and data (ng insulin/ml medium) were normalized to the release under control conditions in the absence of drugs (Fig. 2).

Electrophysiology

INS-1E cells were seeded on poly-D-lysine-coated glass coverslips and used for experiments after 24-48 hours. The coverslips were transferred into the recording chamber mounted on a Zeiss IM 35 inverted microscope. All experiments were performed at room temperature in the perforated patch clamp configuration with amphotericin B (120 µg/ml) added to the pipette solutions. Recordings were started when the access resistance was <20 MΩ. Patch pipette resistances were 4-6 MΩ. Data were acquired/analyzed using an EPC-10 amplifier and Pulse/Fitmaster software (HEKA). Currents were normalized to the cell capacitances and are given as current densities (pA/pF). Osmolalities of the solutions were measured with a vapor pressure osmometer (Wescor). Solution exchange was performed with a gravity-flow perfusion system. The pipette solution for recordings of whole-cell K_{ATP} currents and cell membrane potentials (V_m) consisted of (mM) 70 K₂SO₄, 25 NaCl, 10 KCl, 2 MgCl₂, 10 HEPES-FA, 5 EGTA and 40 raffinose (296 mOsm/kg). The pH was titrated to 7.2 with KOH. The extracellular solution contained (mM): 137 NaCl, 5.6 KCl, 2.5 CaCl₂, 1.5 MgCl₂, 10 HEPES-FA, 11 glucose (296 mOsm/kg), pH 7.4 (adjusted with NaOH). To match the experimental conditions used for immunoblotting and insulin release studies, cells were incubated for 1 hour at 37°C in presence of 1 mM AICAR, 1 mM metformin, or 10 µM compound C before starting the measurements, during which drugs were present in the extracellular solution (data shown in Fig. 3). Recordings lasted at least 10 minutes. The effect of acutely applied AICAR or high extracellular glucose was investigated under continuous perfusion (Fig. 4). After establishing stable recordings, cells were superfused with an extracellular solution containing 300 µM AICAR. For the experiments under 5 and 25 mM glucose the NaCl concentration was increased or decreased, respectively, thereby maintaining the osmolality of the extracellular solution. K_{ATP} currents were measured at a holding potential of -70 mV and during 500-ms pulses to -60 mV and -80 mV at 10-second intervals; in this range of potentials the membrane conductance is predominately determined by K_{ATP} currents [19]. Cell membrane potentials were recorded in the zero-current clamp mode. To determine action potential firing thresholds cells were clamped at -70 mV before switching into the current clamp mode. After 10 seconds of membrane potential recording a 20-second current ramp was performed ranging from the respective clamp current to +10 pA (ΔI=+10 pA). The point of initiation of the first action potential evoked by the current ramp was determined. Cl⁻ currents were measured as previously described [20] using a pipette solution

containing (mM) 100 CsCl, 5 MgCl₂, 10 HEPES-FA, 11 EGTA, 65 raffinose, pH 7.2 (adjusted with CsOH), 306 mOsm/kg, and an extracellular solution composed of (mM) 100 NaCl, 0.5 CaCl₂, 2.5 MgCl₂, 10 HEPES-FA, 5 glucose, 82.5 mannitol, pH 7.2 (adjusted with NaOH), 295 mOsm/kg. Glucose-activated Cl⁻ currents (ICl_{glucose}) were elicited by superfusion with a solution containing 25 mM glucose at the expense of mannitol. 10 μM nifedipine was included to block residual voltage-activated (L-type) Ca²⁺ currents. AICAR (300 μM) was added as indicated. Current-voltage relations were assessed by applying 500-ms voltage steps from -100 to +100 mV (20-mV step increments) from a holding potential of 0 mV (Fig. 5).

Measurement of intracellular Ca²⁺

INS-1E cells were seeded on poly-D-lysine-coated glass coverslips and used for experiments after 24-48 hours. Cells were loaded with Fura-2/AM (4 μM) for 30 minutes at room temperature and then transferred to an open incubation chamber. All experiments were performed at room temperature on an Olympus IX70 inverted microscope equipped with a monochromator (Polychrome 4, TILL Photonics) and a cooled CCD camera (TILL Imago SVGA) controlled by TILLvisION software. The extracellular solution was composed of (mM) 140 NaCl, 5.6 KCl, 2.5 CaCl₂, 1.5 MgCl₂, 10 HEPES-FA, 11 glucose. Time-lapse series were performed for 30 minutes in absence or presence of 1 mM AICAR. Fura-2 was alternately excited at 340 and 380 nm at 15-second intervals. Data are presented as 340/380 nm ratio of emitted light detected at 510 nm.

Measurement of caspase activation, membrane integrity and cell granularity

Caspase activation was measured using CaspACE™ FITC-VAD-FMK *in situ* marker, a fluoroisothiocyanate (FITC)-conjugate of the membrane permeable pan-caspase inhibitor VAD-FMK (Promega). The excitation wavelength was 288 nm, emission was measured at 525 nm. Cells treated for 24 hours with 10 μM compound C or 48 hours 1 mM AICAR were washed once with PBS, detached from the culture dish with trypsin/EDTA and centrifuged for 5 minutes at 300×g, washed twice with PBS, incubated with caspase inhibitor reagent for 30 minutes at 37°C, washed twice and resuspended to 1-2×10⁶ cells/ml for measuring caspase activity by flow cytometry. Flow cytometry was performed on a Cell Lab Quanta™ SC flow cytometer (Beckman Coulter) equipped with a 488 nm laser. Particles with diameters <7 μm were regarded as debris and excluded from analysis. Data are shown as the percentage of cells positive for FITC fluorescence. 20,000-30,000 cells per sample were analyzed. Experiments were repeated 3-5 times. Cell membrane integrity was analyzed by staining with the DNA intercalating exclusion dye propidium iodide (PI). The excitation wavelength was 288 nm, PI emission was measured at 670 nm. Side (or orthogonal) scatter (SSC) was determined as a measure of cell granularity.

Cell cycle measurements

For assessing the cell cycle distribution cells were seeded in 9.2-cm petri dishes at a density of 100,000/ml and grown for 24 hours at 37°C, 5% CO₂ and 95% air in RPMI 1640 medium

containing 11 or 2.8 mM glucose. Cells were detached by accutase treatment, centrifuged and washed twice in PBS. 10⁶-2×10⁶ cells/sample were incubated in nuclear isolation and staining medium containing DAPI (NPE systems) for 10 minutes at room temperature. Isolated nuclei were filtered through a 40-μm nylon mesh and analyzed by flow cytometry. The excitation light from the mercury arc lamp was passed through a 355/37 nm band-pass filter. The emission light was directed towards the photomultiplier tube by a dichroic mirror (cut-off 550 nm) and passed through a 465/30 nm band-pass filter. 20,000-40,000 single nuclei were analyzed per sample.

Statistics and data presentation

Data are expressed as mean ± SEM. Statistical comparisons were made using 1-way ANOVA and Bonferroni's multiple comparison post test, or unpaired or paired Student's t-test, as applicable. P values <0.05 between groups were considered as statistically significant. Since in none of the experimental series a significant difference between untreated and vehicle treated samples was found, the data obtained from untreated cells were omitted from all graphs for simplicity. Data were analyzed and plotted using GraphPad Prism version 5.0d for Mac (GraphPad Software).

Results

Effect of AICAR, metformin, compound C and reduced medium glucose concentration on AMPKα phosphorylation

To assess activation or inhibition of AMPK, western blot analysis using antibodies against AMPKα, its phosphorylated form pAMPKα^{Thr172} and the housekeeping protein β-actin was performed after 1 hour of pre-incubation in serum-free RPMI medium containing 11 mM glucose in absence and presence of 1 mM AICAR, 1 mM metformin, or 10 μM compound C. The amount of Thr¹⁷²-phosphorylated AMPKα (pAMPKα^{Thr172}) was significantly increased in cell preparations treated with AICAR and metformin compared to untreated cells, or cells treated with compound C (Fig. 1A). The amounts of total AMPKα and β-actin were unaffected by either treatment. Normalized AMPKα phosphorylation (Fig. 1B) was significantly increased ~2.9-fold under AICAR (2.87 ± 0.80, n=3) and ~1.4-fold under metformin (1.42 ± 0.09, n=3) compared to control levels (n=3). Compound C did not significantly reduce AMPK phosphorylation under the prevailing conditions (0.95 ± 0.21, n=3). A reduction of the glucose concentration in the culture medium from 11 mM to 2.8 mM resulted in a significant ~1.3-fold (1.28 ± 0.06; n=3) increase of AMPKα phosphorylation within 1 hour. After 24 hours of culturing in 2.8 mM glucose AMPKα

phosphorylation was ~1.3-fold increased (1.27 ± 0.10 , n=3, p=0.056).

Insulin release

Insulin released into the culture medium with 11 mM glucose over an incubation period of 1 hour was significantly increased in presence of 1 mM AICAR to $130.40 \pm 11.12\%$ (n=13) compared to basal in absence of drugs (Fig. 2). In contrast metformin failed to stimulate insulin release ($106.10 \pm 13.50\%$; n=7). Compound C significantly increased insulin secretion to $132.50 \pm 7.82\%$ (n=8) compared to basal release in absence of drugs (n=13). The data are shown in Fig. 2.

Effect of pre-incubation with AICAR, metformin and compound C on K_{ATP} currents and cell membrane potential and excitability

In this series of experiments cells were pre-treated with drugs for 1 hour to match the experimental conditions to those used for Western blot and insulin release studies, and to avoid possible data variations as a consequence of varying exposure times to the drugs. Recordings were done in the perforated patch clamp configuration in the absence and presence of 1 mM AICAR, 1 mM metformin, or 10 μ M compound C. K_{ATP} current densities at -60 and -80 mV were 0.49 ± 0.05 pA/pF and -0.78 ± 0.08 pA/pF (n=39), respectively, under control conditions (Fig. 3A). After AICAR treatment, current densities were significantly reduced to 0.33 ± 0.03 pA/pF and -0.51 ± 0.06 pA/pF (n=28), respectively. Currents measured after pre-incubation with metformin or compound C did not significantly differ from currents measured under control conditions, but were significantly higher compared to currents measured in AICAR treated cells. The respective values were 0.63 ± 0.06 pA/pF and -0.94 ± 0.08 pA/pF for metformin (n=25), and 0.64 ± 0.10 pA/pF and -0.87 ± 0.15 pA/pF for compound C (n=13) at -60 and -80 mV, respectively. Under control conditions V_m was -48.34 ± 2.80 mV (n=39). In presence of AICAR, metformin and compound C V_m was -44.31 ± 3.64 mV (n=28), -42.50 ± 3.08 mV (n=25) and -46.62 ± 4.70 mV (n=13), respectively. Individual V_m

Fig. 3. Perforated patch clamp recordings of whole-cell K_{ATP} current densities (A) and membrane potentials (B) of INS-1E cells pre-incubated for 1 hour with 1 mM AICAR, 1 mM metformin (MET), or 10 μ M compound C (CompC). Drugs were present during the recordings. (C and D) Action potential thresholds were measured under control conditions and AICAR treatment as the point of initiation of the first action potential evoked by a 20-second current ramp to +10 pA (dotted lines). (E) The depolarization required to elicit an action potential is given as the difference between the threshold potential and the cell's membrane potential (V_m) measured in the zero-current clamp mode. Mean $\pm$ SEM. 1-way ANOVA and Bonferroni's multiple comparison post test (A), or unpaired t-tests (B, C). Asterisks and + signs indicate statistically significant differences from CONTROL or AICAR, respectively (*, $p < 0.05$; ++, $p < 0.01$, ns, not significant).

values show similar distributions in the four groups and the respective means are not significantly different from each other (Fig. 3B). The membrane potentials measured under these conditions show a strong variability spanning a range of ~ -10 to -75 mV with spontaneous action potential firing evident in strongly depolarized cells (identifying them as viable cells). This reflects a higher average electrical activity of cells kept under 11 mM glucose as compared to cells superfused for ~ 10 –15 minutes with an extracellular solution containing sub-stimulatory 5 mM glucose before stimulation with high glucose (data shown in Fig. 4A). In spontaneously depolarized cells the pattern of electrical activity frequently consisted in wave-like behavior with broadened action potentials of reduced amplitude (comparable to trace 3 in Fig. 4D). This pattern of activity precluded an accurate calculation of plateau phase fractions under the different treatments. To test if the inhibitory effect of AICAR on K_{ATP} currents might be reflected in an altered excitability of the cells, we measured action potential firing thresholds. For this purpose current ramps were applied after clamping the cells to a sub-threshold potential of -70 mV. Tracings of such recordings are shown in Figs. 3C and D. The depolarization required to elicit action

potentials was not significantly different between control cells and AICAR treated cells (9.05 ± 1.14 mV, $n=9$, and 7.92 ± 1.49 mV, $n=7$, respectively; $p=0.55$) (Fig. 3E).

Effect of acute administration of glucose, AICAR and/or tolbutamide on the electrophysiological behavior of INS-1E cells

INS-1E cells respond to glucose stimulation with cell membrane depolarization and electrical activity [18, 21]. As shown in Fig. 4A the increase in the glucose concentration in the extracellular solution from 5 to stimulatory 25 mM led to a significant depolarization of V_m from -57.31 ± 2.11 mV to -46.19 ± 3.37 mV ($n=4$) within 2–5 minutes. Switching back to 5 mM extracellular glucose significantly repolarized the cells to -52.54 ± 2.48 mV ($n=4$). Superfusion of INS-1E cells with a solution containing 300 μ M AICAR induced a depolarization in 4 out of 6 cells within 3–12 minutes, resulting in an average depolarization from -58.6 ± 5.42 mV to -48.93 ± 5.78 mV ($n=6$; Fig. 4B). Wash out of AICAR was not followed by a significant repolarization within the observed period (-51.55 ± 9.13 mV; $n=4$). Glucose- and AICAR-induced depolarizations were accompanied by action potential firing (Figs. 4C and D). To further investigate the effect

Fig. 4. Perforated patch clamp recordings on INS-1E cells exposed to 25 mM glucose (A), or 300 μ M AICAR (B-D). (A) Increasing the extracellular glucose concentration from 5 to 25 mM glucose (GLC) depolarized the cells by $\sim$ 11 mV. Stepping back to 5 mM glucose (washout, WO) caused repolarization. Asterisks and + signs indicate statistically significant differences from control (5 mM GLC) and 25 mM glucose, respectively; **, $p < 0.01$; +, $p < 0.05$; WO (5 mM GLC) was not

significantly different from 5 mM GLC; 1-way ANOVA and Bonferroni's multiple comparison post test. (B) Acute exposure to AICAR (300 μ M) induced a response in 4 out of 6 cells, resulting in an average $\sim$ 10 mV depolarization from control conditions (11 mM extracellular glucose); *, $p < 0.05$; ns, not significant; paired t-tests. (C and D) Vm over time and action potentials recorded at the indicated time-points (1-6) of a single INS-1E cell in which AICAR induced a reversible depolarization. The responsiveness of the cell to high glucose (25 mM) was retained after wash out of AICAR. Each data point in C represents the average Vm over 5 seconds.

of AICAR on the electrical behavior of INS-1E cells we examined its effect in the presence of the K_{ATP} channel inhibitor tolbutamide. As shown in Fig. 5A, superfusion of cells with extracellular solution containing 11 mM glucose and 200 μ M tolbutamide led to a significant inhibition of the K_{ATP} current density measured at -60 mV ($\sim$ 36% inhibition; $n=5$). At -80 mV the reduction of the K_{ATP} current was not significant at the concentration used ($\sim$ 13% inhibition; $n=5$). However, at -80 mV the application of AICAR (1 mM) on top of tolbutamide resulted in a significant inhibition of the conductance by $\sim$ 21% ($n=3$). At -60 mV the conductance was reduced by $\sim$ 41% in the presence of both substances ($n=3$). Neither tolbutamide alone, nor AICAR applied together with tolbutamide had a significant effect on Vm (-68.94 ± 1.62 mV under control conditions, $n=5$; -69.46 ± 1.53 mV under tolbutamide, $n=5$; -69.33 ± 1.27 mV in presence of both tolbutamide and AICAR, $n=3$; Fig. 5B), or depolarizations required to elicit electrical activity (-9.66 ± 1.35 mV under control conditions, $n=5$; -9.17 ± 1.64 mV under tolbutamide, $n=5$; -9.94 ± 1.01 mV in presence of both tolbutamide and AICAR, $n=3$; Fig. 5C). In one out of five experiments tolbutamide induced action potential firing, which continued in the presence of AICAR.

Effect of acute administration of AICAR on basal and glucose-induced chloride currents ($ICl_{glucose}$)

The Cl^- conductance activated by hypotonicity- or glucose-induced cell swelling (VRAC, ICl_{swell} , ICl_{islet} , $ICl_{glucose}$) in pancreatic β -cells is ATP-sensitive and might act as a sensor of the cells' metabolic status [22-24]. Therefore we tested if the AMPK activator AICAR affects this current, which we have previously characterized in INS-1E cells [20, 21]. As shown in Fig. 6A, basal Cl^- peak-current densities measured in the presence of 5 mM glucose were -2.52 ± 0.98 pA/pF and 7.12 ± 0.64 pA/pF ($n=5$) at -100 and +100 mV, respectively. Addition of 300 μ M AICAR to the bath solution did not significantly affect basal chloride currents, which were -2.19 ± 0.91 pA/pF and 5.80 ± 0.80 pA/pF at -100 and +100 mV, respectively ($n=4$). Superfusion of INS-1E cells with a bath solution containing 25 mM glucose led to the activation of an outwardly rectifying chloride current ($ICl_{glucose}$), which reached its maximum of -4.46 ± 0.16 pA/pF at -100 mV and 19.39 ± 3.44 pA/pF ($n=4$) at +100 mV after 5-10 minutes. $ICl_{glucose}$ current densities measured in presence of 300 μ M AICAR were not significantly different, amounting to -4.53 ± 0.86 pA/pF and 27.50 ± 5.50 pA/pF at -100 and +100 mV, respectively ($n=3$).

Fig. 5. Effect of AICAR in the presence of tolbutamide (TOLBUT; 200 μ M). Perforated patch clamp recordings. (A) K_{ATP} current densities measured at -80 and -60 mV (HP, holding potential). (B) Membrane potentials and (C) depolarizations required to evoke electrical activity determined in the same cells. *, $p < 0.05$; ns, not significant; 1-way ANOVA and Bonferroni's multiple comparison post test.

Effect of short-term AICAR application on the intracellular calcium concentration (Ca_i)

Ca_i was measured over a period of 30 minutes in absence (control) and presence of 1 mM AICAR. Under both conditions INS-1E cells exhibited spontaneous oscillations of Ca_i without apparent difference with respect to frequency and amplitude. The mean ratio of Fura-2 fluorescence did not change significantly within 30 minutes of observation and was not significantly different between control and AICAR treated cells (Fig. 6B).

Effects of long-term application of compound C, AICAR and reduced medium glucose concentration on apoptosis and cell cycle distribution of INS-1E cells

By inducing insulin release glucose acts as a β -cell growth factor. Insulin secreted in response to elevated glucose exerts auto/paracrine effects to protect against apoptosis and stimulate proliferation [25, 26]. Given that AICAR and compound C stimulate insulin release by approximately the same extent (Fig. 2), we investigated long-term effects of these substances on INS-1E cells. As shown in Fig. 7A, culturing of cells in presence of 10 μ M compound C added to the standard culture medium containing 11 mM glucose led to a slight but significant increase of caspase positive cells within 24 hours ($7.34 \pm 1.37\%$ in absence versus $10.73 \pm 1.70\%$ in presence of compound C; $n=5$). The percentage of caspase positive cells, however, was unaffected even after 48 hours of treatment with AICAR ($7.32 \pm 1.36\%$ in absence versus $7.15 \pm 1.76\%$ in presence of the drug; $n=3$). As evident from our present data, a reduction of

Fig. 6. Measurements of short-term AICAR effects on (A) basal and glucose-induced chloride currents ($I_{Cl}^{glucose}$), and (B) the intracellular calcium concentration (Ca_i) in INS-1E cells. (A) Basal and glucose-activated chloride currents were recorded under 5 and 25 mM glucose (GLC), respectively, in absence (open circles/rectangles) and presence (closed circles/rectangles) of 300 μ M AICAR. (B) Time-lapse imaging of Fura-2-loaded cells over 30 minutes in absence (CONTROL, open circles; 56 cells of 4 experiments), and presence of 1 mM AICAR (closed circles; 55 cells of 4 experiments).

the glucose concentration in the culture medium from 11 to 2.8 mM causes a stimulation of AMPK α phosphorylation (Fig. 1). In a previous work we could

Fig. 7. Measurements of caspase activation, cell membrane integrity, cell granularity and cell cycle distribution in INS-1E cells. (A) Caspase activation after treatment for 24 hours with compound C (CompC, 10 μ M), or 48 hours with AICAR (1 mM) was measured by flow cytometry using CaspACE™ FITC-VAD-FMK marker (Promega). (B) Caspase activation and membrane integrity tested by double staining with CaspACE™ FITC-VAD-FMK marker and propidium iodide (PI) after culturing cells for 24 hours in standard medium (11 mM glucose) or 2.5 mM glucose. (C) Cell granularity assessed by side scatter (SSC) measurements after 24 hours in 2.5 mM glucose. (D) Measurement of the cell cycle distribution after 24 hours under 11 or 2.8 mM glucose. *, $p < 0.05$; **, $p < 0.01$; ns, not significant; paired t-tests.

show that a similar reduction in medium glucose causes a complete suppression of INS-1E cell proliferation [27]. Here we performed additional series of experiments in which we investigated the effects of lowering the extracellular glucose concentration on caspase activation, cell membrane integrity (PI staining), cell granularity (SSC) and cell cycle distribution. As shown in Fig. 7B, the percentage of viable cells, characterized by low caspase activity and lack of PI staining (caspase-/PI-), was reduced by ~4% after 24 hours under 2.5 mM glucose ($n=4$). Simultaneously the percentage of caspase+/PI+ cells is slightly, but significantly increased by ~1% ($n=4$) and the side scatter (SSC) signal as a measure of cell granularity was significantly increased in cells kept under low glucose (Fig. 7C). Within the same period we measured a significant increase of cells in the G1 phase (from $75.93 \pm 1.88\%$ to $79.90 \pm 1.17\%$; $n=3$) paralleled by a decrease in the S phase (from $15.08 \pm 0.60\%$ to $12.27 \pm 0.19\%$; $n=3$) upon reduction of the medium glucose concentration from 11 to 2.8 mM (Fig. 7D).

Discussion

In the present study we show that INS-1E cells respond to both AMPK activators AICAR and metformin with a ~3-fold and ~1.4-fold increase in AMPK α phosphorylation, respectively, whereas the ATP-

competitive AMPK inhibitor compound C has no apparent effect (Fig. 1). The inability of compound C to suppress AMPK phosphorylation is not surprising, since it can be assumed that baseline phosphorylation under these culture conditions is low due to sufficiently high cellular ATP levels, so that a further reduction cannot be detected at the drug concentration used. AMPK activation by AICAR, but not by metformin was paralleled by enhanced basal insulin secretion (Fig. 2). The unchanged insulin release under metformin contrasts data showing inhibited insulin secretion from β -cells and β -cell lines [10-12, 28], but is in line with a recent study in mouse pancreatic β -cells showing that although both metformin and AICAR caused similar effects on the electrical activity, the metformin effect did not result in a stimulation of insulin release [15]. From our observation that both AICAR and compound C enhance insulin release and that compound C, but not AICAR, leads to caspase activation in INS-1E cells when applied for 24 or 48 hours (Fig. 6 and [16]), it can be concluded, that the pro-apoptotic effect of compound C does not result from AMPK inhibition and/or reduced insulin release under the chosen experimental conditions. Under the same conditions metformin does not stimulate insulin release. Given their regulatory effects on both AMPK and K_{ATP} channels, it is obvious that the cellular ATP/ADP/AMP ratios play an important role in the regulation of insulin release. AICAR and metformin differ in their modes of action, leading to different outcomes in

the cellular ATP/ADP/AMP ratios. AICAR is converted to its metabolite ZMP (AICAR monophosphate) [29], which mimics the effects of AMP on AMPK kinase while the cellular levels of AMP, ADP and ATP remain unaffected [30, 31]. Metformin can activate AMPK *via* its upstream kinase LKB1, mitochondrial respiratory chain block and reduction of the intracellular ATP concentration [32, 33], or inhibition of AMP-deaminase [34], which leads to increased levels of AMP and reduced ATP. This lowering of intracellular ATP might impede insulin secretion by keeping K_{ATP} channels in the open state, thereby overcoming a possible inhibitory action of AMPK on these channels under metformin [14]. However, in mouse β -cells metformin did not alter mitochondrial membrane potential, which is an indirect measure of ATP production [15]. We found that insulin release was enhanced under compound C, although AMPK α phosphorylation was unaffected, indicating an AMPK-independent effect. This differs from a work on INS 832/13 cells showing increased glucose-induced insulin release linked to reduced AMPK phosphorylation [28].

To test for a possible involvement of the K_{ATP} channel-dependent pathway in AICAR and/or compound C-stimulated insulin secretion we performed patch clamp experiments measuring K_{ATP} currents in the absence and presence of the drugs. To correlate the patch clamp data with the data on AMPK α phosphorylation and insulin release, we chose identical experimental conditions; i.e., 1 hour of pre-incubation with drugs. These experiments showed that treatment of the cells with AICAR leads to a significant inhibition of K_{ATP} currents, whereas compound C and metformin are without effect (Fig. 3A). Therefore the stimulatory effect of AICAR, but not of compound C, could at least in part be due to activation of the K_{ATP} channel-dependent pathway of SSC. Recently it has been shown, that the AMPK is able to directly phosphorylate the Kir6.2 subunit of the K_{ATP} channel thereby inhibiting the channel [14]. Alternatively AICAR itself or its metabolites ZMP or ZTP could directly act on K_{ATP} channels. In inside-out patches of rat pancreatic islets it has been found that ZTP at concentrations of 0.4 mM exerts moderate inhibitory effects on K_{ATP} channels [35]. As evident from Fig. 3B, however, the distribution of the cells' resting membrane potentials (V_m) in absence and presence of the drugs is virtually identical among the four groups, and the mean V_m of AICAR treated cells is not significantly different compared to untreated cells, or cells treated with metformin or compound C. The action potential threshold was also unchanged under AICAR. The unaltered V_m and

excitability of cells pre-treated with AICAR might be caused by an effect of AICAR on another ion conductance, which overcomes the depolarizing effect of K_{ATP} channel inhibition; e.g., activation of other K^+ channels. Alternatively AICAR could act *via* an inhibition of a chloride conductance, which would drive V_m close to the equilibrium potential for potassium, irrespective of the inhibition of K_{ATP} currents. Recently it has been shown that AMPK inhibits CFTR [36] and that hypoxia inhibits colonic chloride secretion *via* AMPK mediated inhibition of CFTR [37]. Previous studies from our group and others have shown that glucose induces cell swelling in INS-1E cells and β -cells, which is paralleled by the activation of a chloride conductance ($ICl_{glucose}$, ICl_{islet}) with features of swelling-activated chloride currents (ICl_{swell}) [20, 21, 23]. ICl_{swell}/ICl_{islet} requires intracellular ATP for activation [22-24]. Given the influence of $ICl_{glucose}$ on the electrical properties of β -cells and insulin release it is likely that this system acts as a K_{ATP} channel-independent nutrient sensing system for SCC [23], which might take over a key role in SSC under conditions when K_{ATP} channels are closed [38], e.g. under hyperglycemia. We could show that inhibition of $ICl_{glucose}$ hyperpolarizes V_m and so counteracts depolarization evoked by K_{ATP} channel inhibition [21]. Therefore we tested for a possible inhibitory effect of AICAR on $ICl_{glucose}$. As shown in Fig. 6A, however, AICAR did neither inhibit the basal chloride conductance nor $ICl_{glucose}$ so that it unlikely that inhibition of chloride currents account for the observed effect. Regardless of the prevailing ion conductance, the given V_m under these conditions is apparently not suitable to favor classical SSC of insulin release, which requires depolarization-driven opening of voltage-gated calcium channels. Despite the apparent lack of effect on V_m in cells pre-treated with AICAR for 1 hour, a possible explanation for the observed enhanced cumulative insulin release is that AICAR elicits a rapid transient depolarization which is followed by activation of hyperpolarizing or inhibition of a depolarizing conductance by AMPK and/or AICAR leading to a repolarization of V_m . This might overcome the still prevailing reduced K_{ATP} channel conductance in the majority of the cells. To test for this, we measured V_m of the cells during acute administration of AICAR and glucose. In fact 4 out of 6 cells responded to AICAR with a rapid depolarization paralleled by the generation of action potentials (Fig. 4), which may trigger insulin secretion. It remains to be determined whether this effect is due to K_{ATP} channel inhibition by activated AMPK, AICAR or its metabolites. Notably, another member of the Kir family

(Kir2.1) has recently been shown to be inhibited by AMPK [39]. Despite the generation of action potentials we did not observe an overall elevated intracellular calcium concentration in cells exposed to 1 mM AICAR over a period of 30 minutes (Fig. 6B). To further investigate the effect of AICAR on K_{ATP} channels, we tested in a separate series of experiments its effect in the presence of the K_{ATP} channel blocker tolbutamide. Under the given experimental conditions tolbutamide at a concentration of 200 μ M blocked the conductance at -60 mV, but not at -80 mV (Fig. 5). AICAR applied on top of tolbutamide did not further enhance the block at -60 mV, but notably led to a significant inhibition at -80 mV. At -60 mV there was no difference between the effects of tolbutamide alone and tolbutamide in the presence of AICAR, suggesting different modes of action of the two drugs on K_{ATP} channels. Neither applying tolbutamide alone, nor together with AICAR significantly depolarized the cells or altered their excitability, which is most likely due to the weak inhibitory effect of tolbutamide under the prevailing conditions.

Rodent β -cell lines including INS-1E cells are routinely cultured in standard RPMI 1640 medium containing 11 mM glucose (e.g., [15, 16, 28, 40]). In fasted rats and mice plasma glucose concentrations of 5-7.5 mM are regarded as normo-glycemic [41]. β -cells kept in culture under 11 mM glucose over many passages might thus be metabolically adapted to “hyper-glycemic” condition. This is evidenced by data showing that a reduction of the glucose concentration in the growth medium to “normo-glycemic” levels induces apoptosis in murine β -cells [26]. In line with these findings we could show in a previous study that a reduction of the medium glucose concentration to 5 mM causes a complete suppression of INS-1E cell proliferation [27]. Here we further show that a lowering of the medium glucose concentration to 2.5, or 2.8 mM causes increased caspase activation, increased cell granularity and a measurable G1-phase arrest already after 24 hours (Fig. 7B and C).

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This clearly shows, that medium glucose concentrations below ~5 mM are not tolerated by INS-1E cells. In most studies investigating the effects of drugs interfering with AMPK activity at “normo- or hypo-glycemic” conditions, cells are acutely exposed to reduced glucose concentrations, which is *per se* the master stimulus for AMPK activation [6, 40]. Here we show that within 1 hour of starvation AMPK α phosphorylation is significantly increased over control levels measured under standard culture conditions (Fig. 1). Therefore these cells might show considerably different responses to these drugs even at nominally “normo-glycemic” conditions compared to responses under conditions to which they are chronically adapted; i.e., standard cell culture conditions.

We conclude that under standard cell culture conditions (11 mM glucose), the AMPK modulators AICAR and compound C, but not metformin, stimulate insulin release of INS-1E cells by AMPK-independent mechanisms.

Abbreviations

AICAR (5-amino-4-imidazole carboxamide riboside); PI (propidium iodide); SSC (stimulus-secretion coupling); Vm (cell membrane potential); ZTP (5-amino-4-imidazolecarboxamide riboside 5'-triphosphate).

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