Resveratrol Inhibits Electrical Activity and Insulin Release from Insulinoma Cells by Block of Voltage-Gated Ca^{2+} Channels and Swelling-Dependent Cl^- Currents

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Key Words

Insulin • Resveratrol • Voltage-dependent calcium channel • Swelling-dependent chloride current • INS-1E

Abstract

The phytoestrogen resveratrol (RV) improves the metabolic state in animal models by increasing the insulin responsiveness of tissues and there is evidence that RV affects insulin secretion from native β -cells and insulinoma cells. In whole cell patch clamp experiments on clonal rat INS-1E cells we used high extracellular glucose (20 mM), extracellular hypotonicity (30%) or tolbutamide (100 μM) to elicit membrane depolarizations and electrical activity. Application of RV (50 μM) repolarized the cells, terminated electrical activity and prevented the hypotonicity-induced depolarization. These effects were fully reversible and intermittent application of RV restored tolbutamide-induced electrical activity after desensitization. Glucose-induced depolarization was counteracted by RV in presence of iberiotoxin (50 nM), showing that the RV effect does not depend

on BK_{Ca} channel activation. RV dose-dependently inhibited K_{ATP} currents, L- and T-type Ca^{2+} currents and swelling-dependent Cl^- currents evoked by either hypotonicity or high extracellular glucose - ion conductances crucially involved in regulating the electrical activity of insulin secreting cells. We further show that RV blunts glucose-induced, but not basal insulin release. Our results indicate that RV counteracts/prevents stimulus-induced cell membrane depolarization and electrical activity by blocking voltage-gated Ca^{2+} - and swelling-dependent Cl^- currents despite the inhibition of K_{ATP} currents.

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Introduction

Insulin secretion is tuned in response to the plasma glucose concentration and other physiological secretagogues like amino acids, triglycerols and free fatty acids. Glucose triggers insulin release in a well characterized sequence of processes consisting of sugar uptake and metabolism resulting in an increased ATP/ADP ratio, closing of ATP-sensitive K^+ (K_{ATP} ; K_{IR} 6.2/

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SUR1) channels, membrane depolarization and stimulation of insulin exocytosis by Ca^{2+} influx through voltage-dependent Ca_v channels (Ca_v) [1-3]. In β -cells at least six different Ca_v α subunits are expressed that interact with different subunits to give rise to L-, P/Q-, N-, R- and T-type currents and which are involved in regulating the cells' electrical activity, insulin release and proliferation [4].

Distinctive Cl^- currents underlie the known anion dependence of the stimulus-secretion-coupling in β -cells. These currents are activated by cell swelling in response to glucose uptake or exposure to a hypotonic medium (referred to as $\text{ICl}_{\text{swell}}$, $\text{ICl}_{\text{glucose}}$ or $\text{ICl}_{\text{islet}}$) and by intracellular ATP and other adenosine nucleotides [5-10]. In a previous work we provided evidence that $\text{ICl}_{\text{glucose}}$ and $\text{ICl}_{\text{swell}}$ are identical currents and found that glucose uptake induces a volume increase sufficient to activate the conductance in INS-1E rat insulinoma cells [11]. Importantly, Cl^- efflux depolarizes the cell membrane which contributes to K_{ATP} channel-independent triggering of insulin release upon cell swelling. Hypotonic cell swelling *per se* induces depolarization, electrical activity and insulin release in clonal β -cells and isolated islets even under normo- or hypoglycemic conditions [6, 7, 9, 12-14]. The generation of an outwardly directed anion flux requires the intracellular Cl^- concentration in β -cells to be maintained above equilibrium which is accomplished by the $\text{Na}^+/\text{K}^+/\text{2Cl}^-$ cotransporter (NKCC) and the NKCC inhibitor bumetanide abolishes glucose-induced electrical activity [15]. Moreover glucose-induced β -cell depolarization and hormone secretion is abolished by the Cl^- channel blockers DIDS, NPPB, DCPIB or 4-hydroxytamoxifen which evidences the permissive role of $\text{ICl}_{\text{glucose}}/\text{ICl}_{\text{swell}}$ for insulin release [16-19]. Stimulus-secretion-coupling *via* $\text{ICl}_{\text{glucose}}/\text{ICl}_{\text{swell}}$ could be of particular importance during hyperglycemia in pathophysiological situations like in type 2 diabetes, but also under normoglycemic conditions when K_{ATP} channels are preferentially in the closed state and therefore cannot fully account for secretagogue-induced insulin secretion [20].

The diphenolic compound resveratrol (3,5,4'-trihydroxystilbene; RV) found in grape skins, olive oil, grapevine plants and peanuts has chemoprotective, cardio- and vasoprotective, anticarcinogenic, antiinflammatory and vasorelaxant properties. Effects of RV include inhibition of cyclooxygenases, ornithin decarboxylase and protein kinase C, inhibition of platelet aggregation and tumor angiogenesis, induction of cell cycle arrest and apoptosis as well as lifespan extension in diverse

organisms [21]. RV was found to decrease the plasma triglyceride content and cholesterol accumulation, to stimulate glucose uptake by hepatocytes, adipocytes and skeletal muscle and to retard the development as well as to meliorate complications associated with diabetes [22-24]. Further there is a growing amount of data demonstrating direct effects of RV on insulin secreting cells. Decreased as well as stimulated insulin secretion has been shown upon RV treatment in native β -cells, insulinoma cells and *in vivo* in normal rats [25-28]. Recently RV was found to inhibit the amplifying pathway of insulin secretion and to disturb the aerobic metabolism of β -cells [26]. Despite the broad variety of RV effects, comprehensive work addressing the effects of the stilbene on the electrical behavior and the triggering pathway of insulin release is scarce. An activation of large conductance Ca^{2+} -activated K^+ channels (BK_{Ca}) and a block of voltage-dependent K^+ (K_v)- and K_{ATP} currents has been shown in MIN6 insulinoma cells [28]. K_{ATP} channel inhibition, which was also described in isolated β -cells [29], along with the block of K_v has been suggested to contribute to the enhanced insulin secretion found in RV-treated MIN6, Hit-T15 and RIN-m5F cells [28].

RV is structurally related to inhibitors of $\text{ICl}_{\text{glucose}}/\text{ICl}_{\text{swell}}$ (like DIDS) and Ca_v (e.g. dihydropyridines). It was found to inhibit $\text{ICl}_{\text{swell}}$ in peritoneal mast cells [30] and L-type Ca_v in cultured bovine adrenal medullary cells [31], cardiac myocytes [32-34] and human platelets [35]. In cardiac myocytes the block of Ca_v by RV was found to influence the action potential duration and to reduce the incidence and duration of tachyarrhythmias - effects that might underlie the cardioprotective effect of the substance. In our present study on INS-1E rat insulinoma cells we therefore investigated the effects of RV on insulin release, stimulus-induced cell membrane depolarization as a fundamental step in stimulus-secretion-coupling as well as ion conductances involved in regulating insulin secretion; i.e., K_{ATP} -, Ca_v - and glucose/swelling-dependent Cl^- currents.

Materials and Methods

Cell culture

INS-1E cells [36] were grown in RPMI 1640 medium containing 1mM sodium pyruvate, 50 μM β -mercaptoethanol, 2mM glutamine, 10mM HEPES, 10% fetal calf serum (FCS), 100U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin at 37°C, 5% CO_2 and 95% air. Subcultures were established every second to third day by trypsin/EDTA treatment. Cells between passages 90 and 110 were used for this study.

Electrophysiology

INS-1E cells were seeded onto poly-D-lysine-coated glass coverslips and used for experiments after 24–48 h. Data were acquired and analyzed using an EPC-10 amplifier and Pulse/FitMaster software (HEKA, Germany). All experiments were performed at room temperature in the ‘perforated’ or conventional ‘ruptured’ patch clamp mode. For perforated patch experiments amphotericin B (120 µg/ml) was added to the pipette solution and recordings were started when the access resistance was <20 MΩ. Solution exchange was performed with a gravity flow perfusion system. The compositions of intracellular (pipette, P) and bath (extracellular, E) solutions are listed in Tab. 1. Osmolarities were measured by freezing point depression. Cell membrane potential recordings were performed in the perforated or conventional patch clamp configuration as indicated using the zero current clamp mode and solutions P1/E1–4. For recordings in the ruptured mode 2 mM Mg-ATP were added to the pipette solution. For the assessment of the whole cell iberiotoxin-sensitive calcium-activated K⁺ current the ruptured patch clamp configuration was applied using solution P1 in which EGTA was lowered to 0.1 mM and solution E1 to which 100 µM tolbutamide were added. Currents were elicited by 500-ms voltage steps from -60 to +60 mV (20-mV step increments) from a holding potential of -70 mV. Recordings of whole cell K_{ATP} currents were made in the conventional patch clamp configuration using solutions P1 (in absence or presence of 5 mM Mg-ATP as indicated) and E1. Currents were measured at a holding potential of -70 mV and during 500-ms pulses to -80 and -60 mV at 10-s intervals as previously described [29]. Whole cell Ba²⁺ currents were measured in the conventional patch clamp configuration using solutions P2/E5 in response to voltage ramps from -100 to +100 mV (500 ms duration, 10-s intervals) or 500-ms voltage pulses to -20 and 0 mV from a holding potential of -70 mV. Glucose- and swelling-activated Cl⁻ currents (I_{Cl_{glucose}}/I_{Cl_{swell}}) were recorded using the perforated patch clamp technique and solutions P3/E6–9. Currents were elicited by applying voltage ramps from -100 to +100 mV (500 ms duration, 10-s intervals) from a holding potential of 0 mV. Inward and outward currents were measured at -100 and +100 mV, respectively. Current-voltage relations were assessed by applying 500-ms voltage steps from -100 to +100 mV (20-mV step increments). Drugs were added to the extracellular solutions as indicated. Currents were normalized to the cell capacitances (8–15 pF) and are given as current densities (pA/pF).

Insulin secretion

Experiments were performed on INS-1E cells in 12-well plates in 1 ml of medium (3 × 10⁵ cells/well). Before the experiments cells were maintained for 2 h in glucose-free Krebs-Ringer bicarbonate HEPES buffer (KRBH) containing (in mM): 135 NaCl, 3.6 KCl, 5 NaHCO₃, 0.5 NaH₂PO₄, 0.5 MgCl₂, 1.5 CaCl₂, 10 HEPES, pH 7.4. BSA (0.1%) was added as an insulin carrier. Then cells were washed twice in PBS and preincubated for 30 min in glucose-free medium, washed once with glucose-free KRBH and then incubated for 30 min successively in basal and stimulating medium as indicated. Incubation media: Basal medium (Krebs-Ringer HEPES buffer, KRH; in mM): 133.75 NaCl,

Intracellular (Pipette) solutions (mM)											pH	mOsm
	CsCl	K ₂ SO ₄	NaCl	KCl	MgCl ₂	Mg-ATP	HEPES	EGTA	raffinose			
P1	-	70	25	10	-	-	10	5	40	7.2 (KOH)	295	
P2	100	-	-	-	5	2	10	11	65	7.2 (CsOH)	313	
P3	100	-	-	-	5	-	10	11	65	7.2 (CsOH)	305	

Extracellular solutions (mM)											pH	mOsm
	NaCl	KCl	CaCl ₂	MgCl ₂	TEA-Cl	BaCl ₂	HEPES	glucose	mannitol			
E1	140	5.6	2.5	1.5	-	-	10	5	-	7.4 (NaOH)	297	
E2	90	5.6	2.5	1.5	-	-	10	5	100	7.4 (NaOH)	313	
E3	90	5.6	2.5	1.5	-	-	10	5	-	7.4 (NaOH)	211	
E4	90	5.6	2.5	1.5	-	-	10	20	85	7.4 (NaOH)	311	
E5	-	-	-	-	80	10	10	5	120	7.2 (TEA-OH)	302	
E6	100	-	2.5	2.5	-	-	10	-	80	7.2 (NaOH)	304	
E7	100	-	2.5	2.5	-	-	10	-	-	7.2 (NaOH)	221	
E8	100	-	2.5	2.5	-	-	10	5	75	7.2 (NaOH)	300	
E9	100	-	2.5	2.5	-	-	10	20	60	7.2 (NaOH)	298	

Table 1. Experimental solutions used for patch clamp experiments

3.6 KCl, 5 NaHCO₃, 0.5 NaH₂PO₄, 0.5 MgCl₂, 1.5 CaCl₂, 10 HEPES, 2.5 glucose, BSA (0.1%), pH 7.4; 292 mosm/l. A high glucose stimulation medium was prepared by increasing glucose to 15 mM. To maintain physiological osmolarity, NaCl was reduced to 127.5 mM. Other components were the same as in the basal medium. Resveratrol (50 µM) was added to the basal and stimulation medium as indicated. Insulin release into the cell culture medium was determined by specific radioimmunoassay [14]. All samples from each experiment were analyzed in the same assay to avoid interassay variation. Rat insulin standards were prepared in each type of medium we used and thus the correction for potential effect of different media on antibody binding was included. ¹²⁵I-monoiodoinsulin was prepared using lactoperoxidase. Rabbit anti-insulin antibody was provided by Dr. Štolba (Institute of Endocrinology, Prague). Synthetic insulin was obtained from Novo Nordisk (USA). All assays were performed in a total volume of 400 µl 0.01 M PBS (pH 7.4). After overnight incubation of samples with the antibody at 4 °C, bound and free peptides were separated by cold 200 µl dextran coated charcoal (500 mg Norit+50 mg dextran in 100 ml H₂O). After 15 min centrifugation at 3000 g the radioactivity of the pellet was measured by a RIA multidetector counter (JNG-402, URVJT, Prague). Insulin release is given as ng insulin/10⁶ cells/100 µl of incubation medium over an incubation period of 30 min.

Salts, Chemicals and Drugs

All salts and chemicals were p.a. grade. *Trans*-resveratrol, DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid), NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid), nifedipine, tolbutamide and iberiotoxin were from Sigma-Aldrich (Germany).

Data analysis

Data are given as mean ± SEM. Statistical comparisons were performed using paired or unpaired t-tests, where applicable. Means were considered significantly different at *p*-values <0.05. (*, #), (**, ##) and (***, ###) denotes *p*<0.05, *p*<0.01 and *p*<0.001, respectively. ns = not significant. N refers to the number of independent experiments. Data were analysed and plotted using GraphPad Prism version 5.00 (GraphPad Software, San Diego California USA).

Fig. 1. Inhibition of glucose- and hypotonicity-induced electrical activity and insulin secretion in INS-1E cells by resveratrol (RV). A, C: Changes of the MP induced by raising the glucose concentration from 5 to 20 mM or by a 30 % reduction of the extracellular osmolarity in absence and presence of 50 μM RV. Each data point represents the average MP of a 5 second-interval. Perforated patch clamp recordings. B,D: Tracings taken from the continuous recordings at the intervals indicated by grey bars and numbers 1-5 in A and C. Dashed lines: MPs under initial experimental condition (5 mM glucose or isotonicity, respectively). E: Inhibition of glucose-induced insulin release. Basal insulin release measured after a 30-min incubation in Krebs-Ringer HEPES buffer (KRH) containing 2.5 mM glucose (open bars, basal) and stimulated release determined after a 30 min in KRH with 15 mM glucose (grey bars, gluc) in absence or presence of 50 μM RV. Data are given as release/10⁶ cells/100 μl of incubation medium (n=6).

Results

Resveratrol reversibly suppresses electrical activity of INS-1E cells and inhibits insulin release. Representative membrane potential (MP) recordings of cells stimulated with high glucose, tolbutamide or hypotonicity in absence and presence of 50 μM resveratrol (RV) are shown in Fig. 1 and 2. The results are summarized in Fig. 3. Unless stated otherwise, the recordings were performed in the perforated patch clamp configuration using pipette solution P1 (Tab. 1).

An increase of the glucose concentration in the isotonic extracellular solution from 5 to 20 mM (solutions E2 and E4, respectively; Tab. 1) led to a depolarization and the appearance of action potentials (Fig. 1A and B).

RV (50 μM) in the continued presence of high glucose caused a repolarization and suppressed spike activity. The effect was reversible and could be repeated for at least a second time. In a control series the MP remained unchanged during 15 min of superfusion with isotonic extracellular solution containing 5 mM glucose (-60.1 ± 3.6 mV vs. -67.4 ± 3.4 mV after 15 min; n=5; ns).

To test if the effect of RV is due to the stimulation of Ca²⁺-activated K⁺ channels (K_{Ca}), we applied RV in the presence of the maxi-K_{Ca} (BK_{Ca}) channel inhibitor iberiotoxin (IbTX; 50 nM). IbTX did not diminish the potency of RV to repolarize the MP (Fig. 3A). IbTX alone did not significantly affect the MP (-53.0 ± 7.5 mV; n=6). The presence of IbTX-sensitive BK_{Ca} currents in INS-1E cells was confirmed by voltage clamp experiments

Fig. 2. Effect of resveratrol (RV) on the electrical activity of an INS-1E cell induced by tolbutamide added to the extracellular solution containing 5 mM glucose. Perforated patch clamp recording. A: Time course of the MP in absence and presence of 100 μ M tolbutamide and 50 μ M RV. Each data point represents the average of a 5 second-interval. B: Tracings taken from the continuous recording at the intervals indicated by grey bars and numbers 1-5 in A. Dashed lines: MP under initial 5 mM glucose in the absence of tolbutamide.

shown in Fig. 4A and B. The mean whole cell current density at +40 and +60 mV under 100 μ M extracellular tolbutamide was significantly reduced by 50 nM IbTX. The differential current reflecting the IbTX-sensitive BK_{Ca} channel activity was 13.8 ± 3.7 pA/pF at +60 mV, similar as previously reported for MIN6 insulinoma cells [28].

In absence of RV stimulation of INS-1E cells with 15 mM glucose for 30 min resulted in a 2.9-fold increase of insulin release (Fig. 1E). The release induced by high glucose was completely inhibited by RV (50 μ M), whereas the basal release under 2.5 mM glucose was unaffected (Fig. 1E).

Swelling-induced depolarization and electrical activity of INS-1E cells was evoked by exchanging the isotonic extracellular solution (E2; Tab. 1) for a 30% hypotonic solution (E3) under normoglycemic conditions. Hypotonicity induced a transient hyperpolarization to -76.2 ± 2.9 mV ($n=6$; $p<0.01$) reflecting the activation of Ca^{2+} -activated K^{+} channels during the regulatory volume decrease, followed by a depolarization and the appearance of action potentials (Fig. 1C, D and 3B). This biphasic response is in line with previous work on isolated β -cells [7] and RINm5F insulinoma cells [37]. Under RV electrical activity ceased and the MP was reversibly repolarized. Further the hypotonicity-induced depolarization was completely suppressed by RV but could be restored by washout of RV with hypotonic solution (Fig. 3B).

Addition of tolbutamide (100 μ M) to the isotonic bath

solution (E1; Tab. 1) induced a rapid depolarization leading to the appearance of action potentials, which was counteracted by 50 μ M RV (Fig. 2 and 3C). Likewise, in a separate series of experiments performed in the ruptured whole cell patch clamp configuration, RV counteracted the tolbutamide-induced depolarization and electrical activity. This shows that the effect of RV persists under a condition where K_{ATP} channels are completely blocked (2 mM intracellular ATP + 100 μ M extracellular tolbutamide) and implies that the effect does not depend on the integrity of the intracellular compartment. The MP recorded in the absence and presence of 100 μ M tolbutamide was -54.8 ± 4.5 and -36.38 ± 6.0 mV, respectively ($n=5$; $p<0.01$). In the presence of both tolbutamide and 50 μ M RV the MP was -52.9 ± 4.2 mV ($p<0.01$). Tolbutamide-induced depolarization and spike activity was further abrogated by the anion channel blocker NPPB (100 μ M), which completely repolarized the MP in the continued presence of tolbutamide (Fig. 3C).

To test if the inhibition of L-type Ca^{2+} channels alone is sufficient to counteract tolbutamide-induced depolarizations, we applied nifedipine in the presence of the sulfonylurea. Tolbutamide depolarized the cells by $+21.9 \pm 3.0$ mV, while nifedipine (10 μ M) caused only a slight repolarization of -6.5 ± 3.4 mV ($n=7$; ns).

Resveratrol inhibits ATP-sensitive K^{+} currents. Representative tracings of whole cell K_{ATP} currents in the absence and presence of blockers are shown in Fig.

4A. Currents were recorded in the ruptured patch clamp configuration in absence of intracellular ATP (solutions P1/E1; Tab. 1). After breaking the cell membrane, the current gradually increased due to dialysis of the cell interior with ATP free pipette solution. Blockers were applied after the current had reached a stable plateau. When applied on top of 100 μ M tolbutamide, RV (50 μ M) caused a significant further current inhibition. Upon removal of tolbutamide but in the continued presence of RV a ~35% block persisted (Fig. 4B). After washout of both substances the current amplitude nearly returned to control levels. Likewise, without prior application of tolbutamide, RV caused a reversible inhibition to ~63% of control currents (Fig. 4C). Since the effects of tolbutamide and RV were additive under the given conditions we further tested if RV acts on the same or distinct conductances. Under complete inhibition of K_{ATP} channels (5 mM intracellular ATP + 100 μ M extracellular tolbutamide) the current density of the background current was 0.5 ± 0.1 pA/pF ($n=5$) at -60 mV. Application of RV (50 μ M) did not significantly inhibit this conductance (0.4 ± 0.1 pA/pF at -60 mV; $n=5$; $p=0.48$) which indicates that both RV and tolbutamide act on K_{ATP} channels.

Resveratrol blocks voltage-gated Ca^{2+} currents (Ca_v). Whole cell currents were recorded in the conventional patch clamp configuration with 10 mM Ba^{2+} in the extracellular solution (solutions P2/E5). The control tracing in Fig. 5A and B show two subpopulations of inward currents reflecting transient (T)- and slowly inactivating (L)-type Ca_v . Both current subtypes were inhibited by 50 μ M RV (Fig. 5B). The dose dependence of the block is shown in Fig. 5C. T- and L-type currents had their maxima at -33.4 ± 1.5 ($n=22$) and -4.2 ± 1.3 mV ($n=31$). 10 and 50 μ M RV reduced peak T-type currents to 58% and 38% of control currents, respectively. L-type currents were only slightly reduced by 10 μ M RV (15% inhibition), but a 53% block was evident in the presence of 50 μ M. Compared to RV the L-type channel blocker nifedipine (10 μ M) caused a 30% inhibition of L-type currents without affecting T-type currents (Fig. 5D). At -100 and +100 mV no inhibition by RV was evident. In absence of RV the reversal potential (E_{rev}) of the Ba^{2+} current was $+40.1 \pm 2.0$ mV ($n=37$). Since Ba^{2+} currents were recorded under symmetrical intra- and extracellular Cl^- , a basal background Cl^- conductance with an E_{rev} of 0 mV was present. If this Cl^- current was unaffected or affected to a lesser extent by RV, the inhibition of Ba^{2+} currents is expected to shift E_{rev} towards zero. Indeed 10 and 50 μ M RV caused a shift to $+36.8 \pm 3.1$ mV ($n=13$) and $+28.8 \pm 3.2$ mV ($n=24$; $p<0.01$), respectively, indicating

Fig. 3. Effect of resveratrol (RV; 50 μ M) alone, iberiotoxin (IbTX; 50 nM) in presence of RV and NPPB (100 μ M) on membrane potential alterations induced by (A) 20 mM glucose (glc), (B) 30% hypotonicity or (C) 100 μ M tolbutamide (tol) in perforated patch clamp recordings. For the different experimental series performed the order of bars from left to right corresponds to the chronological sequence of experimental conditions. # denotes significant stimulus-induced depolarization compared to the respective basal conditions (open bars). *: significant difference to the data depicted by the second bar or significant effect of washout. Numbers in bars indicate the number of experiments.

Fig. 4. Iberitoxin (IbTX)-sensitive Ca^{2+} -activated K^+ currents in INS-1E cells and inhibition of K_{ATP} currents in INS-1E cells by resveratrol (RV). **A:** Current-voltage relations of whole cell currents recorded in the ruptured patch clamp configuration in presence of 100 μM tolbutamide alone (tol; open circles) and tolbutamide together with IbTX (50 nM; closed circles). **B:** Representative current tracings from a single experiment under the indicated conditions. The differential current (a-b; lower tracings) corresponds to the IbTX-sensitive BK_{Ca} current. **C:** Conventional whole cell patch clamp recordings of K_{ATP} currents measured in response to 500-ms voltage pulses from -70 to -60 and -80 mV under control conditions, in presence of 100 μM tolbutamide (tol), both tol and 50 μM RV, 50 μM RV alone and after washout of inhibitors. **D and E:** Mean $\pm$ SEM of current densities measured in response to voltage pulses from -70 to -60 mV. Numbers in bars indicate the number of individual experiments. *: significant differences between experimental conditions as indicated by horizontal lines.

that RV has a stronger inhibitory effect on Ca_v than on the basal Cl^- current.

Resveratrol inhibits swelling- and glucose-activated Cl^- currents. The effect of RV on $\text{ICl}_{\text{swell}}$ and $\text{ICl}_{\text{glucose}}$ was studied in whole cell perforated patch clamp experiments using symmetrical Cl^- concentrations (solutions P3/E6, E7 for $\text{ICl}_{\text{swell}}$ and P3/E8, E9 for $\text{ICl}_{\text{glucose}}$; Tab. 1). Under isotonic and normoglycemic conditions and 2.5 mM extracellular Ca^{2+} , superimposing Ca^{2+} inward currents (Ca_v) were evident which gradually inactivated within ~ 3 minutes at a holding potential of 0 mV (data not shown). The remaining basal Cl^- current was outwardly rectifying and increased upon hypotonic exposure or a raise of the extracellular glucose concentration as previously described [11].

Exposure to 30% hypotonicity led to the progressive activation of $\text{ICl}_{\text{swell}}$ with pronounced outward rectification which reached a stable plateau after ~ 9 min (Fig. 6A, B). Addition of RV (50 and 100 μM) concentration-dependently and reversibly inhibited both the inward and

outward component, whereas 10 μM had no effect. At 50 μM the current measured at -100 and +100 mV was reduced to 85 and 82%, respectively. 100 μM RV inhibited both the inward and outward current by $\sim 40\%$. The block was reversible upon washout with hypotonic solution. Subsequent application of DIDS (100 μM) reduced the current voltage-dependently to 75 and 29% at -100 and +100 mV, respectively. Although a substantial contribution of Ca^{2+} currents to the apparent $\text{ICl}_{\text{swell}}$ is unlikely (both T- and L-type Ca_v are closed at ± 100 mV [4]), we performed a series of experiments to exclude an eventual overestimation of the RV effect on $\text{ICl}_{\text{swell}}$ due to its action on Ca_v . Exposure of cells to 30% hypotonicity increased the Cl^- current from -8.3 ± 1.2 pA/pF to -15.9 ± 2.1 pA/pF at -100 mV and from 12.6 ± 1.6 pA/pF to 43.0 ± 7.7 pA/pF at +100 mV ($n=6$). While the current amplitude was not significantly reduced by 10 μM nifedipine alone, 100 μM RV in presence of nifedipine blocked both the inward and outward current to 68% (-10.4 ± 1.3 pA/pF and 27.6 ± 4.6 pA/pF, respectively; $n=6$; $p < 0.05$).

Fig. 5. Inhibition of voltage-gated Ca^{2+} currents in INS-1E cells by resveratrol (RV) and nifedipine. Conventional whole cell patch clamp recordings. A: Current-voltage relations of Ca_v recorded under 10 mM extracellular Ba^{2+} in absence (control) and presence of 50 μM RV. Voltage ramps from -100 to +100 mV (holding potential -70 mV; duration 500 ms). B: T- and L-type currents elicited by 500-ms voltage pulses to -20 mV (upper tracings) or 0 mV (lower tracings) from a holding potential of -70 mV, respectively in absence (control) and presence of RV. Same experiment as shown in A. C and D: Effects of RV (10 and 50 μM) and nifedipine (10 μM) on T- and L-type Ca^{2+} currents. Normalized currents. *: significant difference from control. Numbers in bars indicate the number of individual experiments.

The inhibition was fully reversible and equally efficient as compared to the block by 100 μM RV in absence of nifedipine ($p=0.88$ and 0.62 at -100 mV and $+100$ mV, respectively).

$\text{ICl}_{\text{glucose}}$ was induced by increasing the extracellular glucose concentration from 5 to 20 mM. Stable currents developed within ~ 11 min. Addition of 50 μM RV reversibly inhibited the current measured at -100 and $+100$ mV by 9 and 17%, respectively (Fig. 6C). 100 μM RV caused a 25 and 36% inhibition at -100 and $+100$ mV, respectively. DIDS (100 μM) blocked the inward and outward component of $\text{ICl}_{\text{glucose}}$ by 28 and 75%. Current tracings of a representative experiment are shown in Fig. 6D.

Discussion

Secretagogue-induced insulin release is strictly regulated by contemporary electrical and non-electrical

events referred to as triggering and amplifying pathway, respectively [3]. Our present data show that resveratrol (RV; 10–100 μM) counteracts the triggering pathway in clonal INS-1E cells by blocking ion conductances involved in cell membrane depolarization during stimulus-secretion-coupling; i.e., voltage-gated Ca^{2+} (Ca_v) and swelling/glucose-dependent Cl^- currents. This is consistent with the inhibition of glucose- and tolbutamide-induced electrical activity and insulin release in isolated β -cells by the anti-estrogen tamoxifen through simultaneous inhibition of these currents [18].

We find that Ca_v are dose-dependently inhibited by RV with T-type currents showing a higher sensitivity than L-type currents (inhibition by 10 and 50 μM , respectively; Fig. 5). In the same concentration range RV has previously been found to inhibit Ca^{2+} influx and catecholamine release in bovine adrenal medullary cells [31]. In insulin secreting cells Ca_v trigger the secretory response and are involved in repetitive action potential firing [2, 4, 38, 39]. T-type currents display a low threshold potential ($> \sim -65$ mV)

Fig. 6. Inhibition of ICl_{swell} (A, B) and $ICl_{glucose}$ (C, D) by resveratrol (RV). Perforated patch clamp recordings. A: Effect of 10–100 μ M RV and DIDS (100 μ M) on ICl_{swell} . B: Current tracings of a single experiment under isotonicity (iso), 30% hypotonicity (hypo), in presence of 10–100 μ M RV and after washout with hypotonic solution. Inset: Current-voltage relation of a single cell under isotonic and hypotonic conditions. 500-ms voltage pulses from -100 to -100 mV (20 mV increments; holding potential 0 mV). C: Inhibition of $ICl_{glucose}$ by 50 and 100 μ M RV and 100 μ M DIDS. D: Current tracings of a representative experiment under 20 mM glucose, in presence of 50 and 100 μ M RV and after washout. Currents were induced by 30% hypotonicity or by increasing the extracellular glucose concentration from 5 to 20 mM, respectively, and measured in response to voltage ramps from -100 to +100 mV (500 ms duration, holding potential 0 mV). Normalized currents at -100 and +100 mV are shown. #: significant current activation by hypotonicity or high glucose. *: significant inhibition as compared to fully activated currents under hypotonic conditions or in presence of 20 mM glucose, respectively, in absence of blockers. ns: not significantly different from fully activated currents. Numbers in bars indicate the number of individual experiments.

and contribute to the regulation of basal β -cell excitability by mediating a steady state Ca^{2+} influx near the resting MP [39]. In our measurements T-type current activation appears at potentials $> \sim 50$ mV with peak currents at ~ 33 mV. It is therefore unlikely that the sole inhibition of Ca_v by RV accounts for a complete repolarization back to the resting membrane potential (~ 60 mV in our experiments), even though their inhibition suppresses action potential firing. Support for this assumption comes from our data showing that L-type current inhibition by nifedipine does not induce a significant repolarization.

The K_{ATP} current of INS-1E cells is additively inhibited by 50 μ M RV when applied on top of tolbutamide (Fig. 4). In line with our results a block of K_{ATP} channels by RV was found in MIN6 insulinoma cells and isolated β -cells [28, 29]. Therefore our data rule out the possibility that the repolarizing effect of RV is due to a relief of the

tolbutamide block, but discloses the K_{ATP} channel as a target for inhibition by the phytostilbene, which would rather support depolarization. Importantly, even when K_{ATP} channels are completely inhibited (dialysis of the cell interior with a pipette solution containing 2 mM Mg-ATP using the ruptured patch clamp configuration and 100 μ M extracellular tolbutamide) the MP is fully repolarized by RV. Also an activation of large conductance Ca^{2+} -activated K^+ currents (BK_{Ca}) by RV as shown in MIN6 insulinoma cells [28] and vascular smooth muscle cells [40, 41] does obviously not underlie the repolarization, since a block of BK_{Ca} by iberiotoxin does not abrogate the RV effect (Fig. 3A).

In INS-1E cells hypotonic stress induces membrane depolarization and electrical activity (Fig. 1) like in native β -cells. The depolarization is triggered by Cl^- efflux through swelling-activated Cl^- channels [6, 7, 10, 15] which

serve as an extrusion pathway for Cl^- , other anions and osmolytes. The parallel movement of osmotically obliged water counteract excessive swelling and cell damage in a process known as regulatory volume decrease (RVD) [42, 43]. Previously we have shown that the swelling-dependent Cl^- current ($\text{ICl}_{\text{swell}}$) in INS-1E cells is identical to the Cl^- current evoked by cell swelling upon concentrative glucose uptake ($\text{ICl}_{\text{glucose}}$). Apart from common biophysical and pharmacological properties this is supported by our finding that $\text{ICl}_{\text{glucose}}$ is counteracted by extracellular hypertonicity [11] - an effect that might account to the suppression of glucose-induced insulin release in pancreatic islets upon exposure to a hypertonic extracellular medium [44]. $\text{ICl}_{\text{glucose}}/\text{ICl}_{\text{swell}}$ is reversibly inhibited by 50-100 μM RV (Fig. 6). Since the inhibition is voltage-independent, the drug can be expected to counteract stimulus-induced depolarization at potentials near to the resting MP. This is confirmed by our data showing a complete suppression of hypotonicity-induced depolarization by RV (Fig. 3B). Furthermore tolbutamide-induced depolarization and electrical activity of INS-1E cells is abolished by the anion channel blocker NPPB (Fig. 3C). This is in line with studies showing an inhibition of stimulus-induced membrane depolarization, electrical activity and insulin release by blockers of $\text{ICl}_{\text{swell}}/\text{ICl}_{\text{glucose}}$ [16-19]. In murine β -cells the block of $\text{ICl}_{\text{swell}}$ by the HIV protease inhibitors ritonavir and nelfinavir was found to suppress electrical activity and intracellular Ca^{2+} oscillations. The authors suggest that this effect contributes to glucose intolerance as a common adverse side effect of HIV therapy [45]. A basal $\text{ICl}_{\text{glucose}}$ is active under isotonic, normoglycemic conditions as evident from its sensitivity to the anion channel blockers DIDS, NPPB and tamoxifen (Fig. 6 and [11]). Moreover, Cl^- channel inhibition under isotonic was found to cause swelling of clonal HIT cells [9]. These findings point at the function of the current to control cell volume and β -cell excitability as similarly discussed for $\text{ICl}_{\text{swell}}$ in cardiac myocytes [46].

Our insulin release data confirm the maintenance of the secretory response of INS-1E cells to glucose and demonstrate a complete suppression of stimulated hormone release under 50 μM RV (Fig. 1E). This is consistent with a series of studies showing a reversible inhibition of glucose, amino-acid and glibenclamide-induced release from rat pancreatic islets by 1-100 μM RV [25-27]. In these studies, most notably, at 1 and 10 μM the inhibitory effect was counteracted by activation of protein kinase C, indicating an effect on the amplifying pathway of insulin release, whereas at 100 μM the inhibition persisted. This could be due to the

'electrical silencing' of cells at high RV concentrations as described here. Moreover, the time course (significant reduction of glucose-induced release by 100 μM RV after 8 min) and the reversibility of the RV effect shown in this work [25] correlate well with our patch clamp data (reversible inhibition of electrical activity and repolarization within 1.5-5 minutes; Fig. 1 and 2). The effect of RV on insulin release under substimulatory glucose concentrations is less consistent and ranges from no effect in INS-1E cells as shown here, in INS-1 cells [47] and in pancreatic islets [27] to stimulated secretion in MIN6, Hit-T15, RIN-m5F cells [28]. In the latter work RV (3-100 μM) was shown to depolarize unstimulated MIN6 cells which discloses marked differences in the behavior of tumor cell lines. In this context it is notable that extracellular hypotonicity, which is a potent stimulus for insulin release in native- and clonal β -cells [6, 7, 9, 12-14], induces secretion in INS-1 cells but not in the derivative INS-1E cell line ([44] and own observations). However, regardless of the dissipation of hypotonicity-induced electrical activity and insulin secretion in INS-1E cells our patch clamp data demonstrate the general ability of RV to suppress the depolarization-mediated triggering pathway of insulin release independently of nutrient uptake and metabolic processes.

Besides representing a useful pharmacological tool to dissect the complex process of stimulus-secretion-coupling in β -cells, RV or RV-derivatives might exert beneficial effects in diabetic subjects upon nutritional uptake. After ingestion the substance is metabolically converted in enterocytes. In the plasma RV is mainly circulating as glucuronide and sulfate conjugates and only to a low extent in its authentic form [48]. Peak serum concentrations of ~2.4 nM unconjugated and ~180 nM total RV are reached by a daily intake of 2 glasses of red wine (~375 ml), the richest source of RV. At a high but pharmacologically relevant dose of 100 mg/kg body weight, serum concentrations of ~9 μM authentic and ~680 μM total RV are obtained (based on rodent data) [21]. However, considerable amounts of unconjugated RV can be accumulated and delivered to body tissues by binding to plasma albumin and lipoproteins. A ~30-fold accumulation over plasma concentrations has been found in some organs as the liver and kidneys [21, 49]. Apart from that, biotransformation of RV does not necessarily mean a loss of pharmacological efficiency. After tolbutamide application we frequently observed persistent depolarizations following a period of high frequency action potential firing. In the continued presence of tolbutamide, however, the intermittent

RV-induced repolarization reestablished the sensitivity of cells to the antidiabetic drug (Fig. 2, trace 5). Loss of β -cell glucose sensitivity and exhaustion of insulin secretion is a common drawback of antidiabetic drugs that stimulate insulin secretion by persistent depolarization through K_{ATP} channel inhibition like sulfonylureas, imidazolines and quinine [50, 51]. Therefore RV might eventually counteract the drug-induced β -cell desensitization. Such an effect would compare to the K_{ATP} channel opener diazoxide, which causes a recovery of glucose responsiveness after chronic hyperglycemia in diabetic rats [52]. Future work should be aimed at identifying effects of RV conjugates and derivatives that are resistant to biotransformation. Moreover, although INS-1E cells have been described as a stable β -cell surrogate for *in vitro* studies on secretagogue-induced electrical activity and insulin release [36], further studies are needed to test if our findings are applicable also for native β -cells.

We conclude that the suppression of electrical activity by RV in clonal INS-1E cells is due to an inhibition of voltage-gated Ca^{2+} channels and swelling/glucose-dependent Cl^- currents despite the block of K_{ATP} channels. This effect might significantly contribute to the

suppression of insulin release by RV as shown here and as observed in native β -cells and animal models.

Abbreviations

BK_{Ca} channels (Ca^{2+} -activated K^+ channels); Ca_v currents (voltage-gated Ca^{2+} currents); DCPIB (4-(2-butyl-6,7-dichloro-2-cyclopentylindan-1-on-5-yl)oxybutyric acid); DIDS (4,4'-diisothiocyanatostilbene-2,2'-disulphonic acid); $ICl_{swell}/ICl_{glucose}$ (swelling/glucose-activated Cl^- currents); K_{ATP} channels (ATP-sensitive K^+ channels (K_{IR} 6.2/SUR1)); MP (cell membrane potential); NKCC ($Na^+/K^+/2Cl^-$ cotransporter); NPPB (5-nitro-2-(3-phenylpropylamino)benzoic acid)); RV (*trans*-resveratrol).

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