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Crosstalk between membrane potential and cytosolic Ca^{2+} concentration in beta cells from *Sur1*$^{-/-}$ mice

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Abstract *Aims/hypothesis:* Islets or beta cells from *Sur1*$^{-/-}$ mice were used to determine whether changes in plasma membrane potential (V_m) remain coupled to changes in cytosolic Ca^{2+} ($[Ca^{2+}]_i$) in the absence of K_{ATP} channels and thus provide a triggering signal for insulin secretion. The study also sought to elucidate whether $[Ca^{2+}]_i$ influences oscillations in V_m in sur1$^{-/-}$ beta cells. *Methods:* Plasma membrane potential and ion currents were measured with microelectrodes and the patch–clamp technique. $[Ca^{2+}]_i$ was monitored with the fluorescent dye fura-2. Insulin secretion from isolated islets was determined by static incubations. *Results:* Membrane depolarisation of Sur1$^{-/-}$ islets by arginine or increased extracellular K^+, elevated $[Ca^{2+}]_i$ and augmented insulin secretion. Oligomycin completely abolished glucose-stimulated insulin release from Sur1$^{-/-}$ islets. Oscillations in V_m were influenced by $[Ca^{2+}]_i$ as follows: (1) elevation of extracellular Ca^{2+} lengthened phases of membrane hyperpolarisation; (2) simulating a burst of action potentials induced a Ca^{2+}-dependent outward current that was augmented by increased Ca^{2+} influx through L-type Ca^{2+} channels; (3) Ca^{2+} depletion of intracellular stores by cyclopiazonic acid increased the burst frequency in Sur1$^{-/-}$ islets, elevating $[Ca^{2+}]_i$ and insulin secretion; (4) store depletion activated a Ca^{2+} influx that was not inhibitable by the L-type Ca^{2+} channel blocker D600. *Conclusions/interpretation:* Although V_m is largely uncoupled from glucose metabolism in the absence of K_{ATP} channels, increased electrical activity leads to elevations of $[Ca^{2+}]_i$ that are sufficient to stimulate insulin secretion. In Sur1$^{-/-}$ beta cells, $[Ca^{2+}]_i$ exerts feedback mechanisms on V_m by activating a hyperpolarising outward current and by depolarising V_m via store-operated ion channels.

Keywords Arginine · Cyclopiazonic acid · Cytosolic Ca^{2+} concentration · Insulin secretion · Plasma membrane potential · Sur1$^{-/-}$ mice

Abbreviations CPA: cyclopiazonic acid · $[Ca^{2+}]_i$: cytosolic free calcium concentration · FOPP: fraction of plateau phase · K_{ATP}: channel ATP-dependent K^+ channel · Kir6.2: inward rectifying K^+ channel type 6.2 · SUR1: sulphonylurea receptor type 1 · V_m: plasma membrane potential

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Introduction

In wild-type mice, glucose-stimulated insulin secretion involves two different mechanisms referred to as triggering and amplifying pathways (reviewed in [1]). The triggering pathway, which is well characterised and comprises closure of ATP-dependent K^+ channels (K_{ATP} channels), membrane depolarisation and Ca^{2+} influx through L-type Ca^{2+} channels, initiates coordinated oscillations of membrane potential (V_m), cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) and insulin secretion [2–6]. Loss of beta cell K_{ATP} channels results in persistent Ca^{2+}-dependent electrical activity and elevated $[Ca^{2+}]_i$ [7, 8], including oscillation of V_m [9]. In humans, impairment of the triggering pathway by loss-of-function mutations in either the *Kir6.2* or the *SUR1* genes encoding the K_{ATP} channel subunits [10] results in a severe disregulation of glucose homeostasis known as congenital hyperinsulinism [11–13], while gain-of-function mutations

in *Kir6.2* can produce neonatal diabetes [14]. The knockout mouse models are only mildly glucose-intolerant [7, 8, 15] pointing to activation of compensatory mechanisms. Recent studies suggest that excessive secretion of insulin may be prevented by a reduced sensitivity of $Sur1^{-/-}$ beta cells to incretins [8, 16, 17]. In addition, inhibitory signals via the sympathetic nervous system or neuropeptides are still operative. It has been shown that epinephrine and galanin could hyperpolarise V_m in $sur1^{-/-}$ beta cells [9, 18]. Thus these mechanisms may counterbalance the loss of K_{ATP} channel-mediated regulation. Several groups have reported that glucose stimulation of insulin release is reduced in $Sur1^{-/-}$ mice [8, 15] and in their isolated islets [8, 15, 17]. Some have argued for a compensatory increase in sensitivity to cholinergic stimulation [8, 19] and/or amino acid stimulation [20, 21] contributing to the euglycaemia observed in fed animals. A recent report [21] described persistent, elevated insulin secretion in low glucose (1 mmol/l) that was stimulated by high glucose secondary to a transient increase in $[Ca^{2+}]_i$, consistent with an intact amplifying pathway.

Beta cell K_{ATP} channels are considered essential for the generation of effective triggering signals, i.e. membrane depolarisation and Ca^{2+} influx, and have been suggested to underlie V_m oscillations [22, 23]. Early studies on isolated $Sur1^{-/-}$ [8, 15, 24] and $Kir6.2^{-/-}$ [7] beta cells described Ca^{2+}-dependent action potentials and elevated, oscillating $[Ca^{2+}]_i$, in the absence of V_m oscillations. Subsequent recording from $sur1^{-/-}$ beta cells in intact islets demonstrated V_m oscillations [9], but the extent to which changes in membrane potential or $[Ca^{2+}]_i$ participate in regulating insulin secretion in the absence of these channels is unclear. Our recent study showing that the neuropeptide galanin terminates not only oscillations of V_m, but also decreases $[Ca^{2+}]_i$ in $sur1^{-/-}$ beta cells [9] indicates that $[Ca^{2+}]_i$ is determined by V_m. The aim of the present study was to evaluate the coupling between V_m and $[Ca^{2+}]_i$ in K_{ATP} channel-deficient mice and its influence on insulin secretion.

Wild-type beta cells exhibit a strong glucose dependence on V_m that is close to the K^+ equilibrium potential at low glucose concentrations, oscillates between 7 and 18 mmol/l and shows continuous electrical activity at higher concentrations [25]. During glucose stimulation transient openings of K_{ATP} channels [23, 26, 27] and activation of a small Ca^{2+}-dependent K^+ current [28–30] produce hyperpolarised intervals of V_m that transiently reduce $[Ca^{2+}]_i$. Interestingly, $Sur1^{-/-}$ beta cells also show oscillations in V_m and $[Ca^{2+}]_i$ [8, 9]. These oscillations are not driven by glucose metabolism [9]. We therefore investigated whether there is a direct feedback of $[Ca^{2+}]_i$ on V_m in $Sur1^{-/-}$ beta cells by testing the effect of increased Ca^{2+} influx through voltage-dependent L-type Ca^{2+} channels and the influence of store-operated ion channels on V_m, $[Ca^{2+}]_i$ and insulin secretion.

Material and methods

Cell and islet preparation Experiments were performed using islets, clusters or single pancreatic beta cells isolated from fed C57BL/6 (Charles River, Sulzfeld, Germany) or $Sur1^{-/-}$ (breeding facility of the Dept of Pharmacology, University of Tübingen, Germany) mice that had been killed by cervical dislocation or CO_2. For measurements of cell membrane potential with microelectrodes, a piece of pancreas was fixed in a perifusion chamber and islets were microdissected by hand. Other experiments were performed with islets isolated by collagenase digestion. For generation of clusters or single cells islets were dispersed in Ca^{2+}-free medium and cultured for up to 4 days in RPMI 1640 medium (11.1 mmol/l glucose) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin [31]. The principles of laboratory animal care (NIH publication nos. 85–23, revised 1985) and German laws were followed.

Solutions and chemicals Cell membrane potential and current patch–clamp recordings were done with amphotericin B (250 μg/ml) in the pipette solution which contained: 10 mmol/l KCl, 10 mmol/l NaCl, 70 mmol/l K_2SO_4, 4 mmol/l $MgCl_2$, 2 mmol/l $CaCl_2$, 10 mmol/l EGTA, 5 mmol/l HEPES, pH 7.15 adjusted with KOH. The bath solution contained: 140 mmol/l NaCl, 3.6 mmol/l KCl, 2 mmol/l $NaHCO_3$, 0.5 mmol/l NaH_2PO_4, 0.5 mmol/l $MgSO_4$, 1 mmol/l $CaCl_2$, 15 mmol/l glucose, 5 mmol/l HEPES, pH 7.4 adjusted with NaOH. $[Ca^{2+}]_i$ was determined in a bath solution containing: 140 mmol/l NaCl, 5 mmol/l KCl, 1.2 mmol/l $MgCl_2$, 2.5 mmol/l $CaCl_2$, 15 mmol/l glucose, 10 mmol/l HEPES, pH 7.4 adjusted with NaOH. Cell membrane potential measurements with intracellular microelectrodes were done in a bath solution containing: 120 mmol/l NaCl, 5 mmol/l KCl, 2.5 mmol/l $CaCl_2$, 1.2 mmol/l $MgCl_2$, 24 mmol/l $NaHCO_3$, 15 mmol/l glucose, gassed with 95% O_2 and 5% CO_2 to maintain a pH of 7.4. The incubation medium for determining insulin secretion contained (in mmol/l): 122 NaCl, 4.8 KCl, 2.5 $CaCl_2$, 1.1 $MgCl_2$, 10 HEPES, 0.5% bovine serum albumin, pH 7.4.

Fura-2AM was obtained from Molecular Probes (Eugene, OR, USA). RPMI 1640 medium was from PromoCell (Heidelberg, Germany), penicillin/streptomycin was from GIBCO/BRL (Karlsruhe, Germany). All other chemicals were purchased from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany) in the purest form available.

Patch–clamp recordings Patch pipettes were pulled from borosilicate glass capillaries (Clark, Pangbourne, UK) and had resistances between 3 and 5 MΩ when filled with pipette solution. Membrane currents and potentials were recorded at 32°C with an EPC-9 patch–clamp amplifier using 'Pulse' software (HEKA, Lambrecht, Germany) in the voltage-clamp or current-clamp mode respectively. Measurement of $I_{K,slow}$ currents was done using a modified protocol described by Göpel et al. [28]: After a depolarising step from −70 to −40 mV, a train of 26 voltage ramps (−40 to 0 to −40 mV within 200 ms) was applied. This train of simulated action potentials was followed by a period of at least 10 s at −40 mV prior to a voltage step back to −70 mV. Data were analysed with 'Chart' software (ADInstruments, Spechbach, Germany).

Membrane potential measurements with microelectrodes V_m was determined at 37°C with high-resistance microelectrodes [25], except for the data in Fig. 4 where the perforated-patch configuration was used. Beta cells from Sur1$^{-/-}$ islets were discriminated from non-beta cells by their lack of Na^+ action potentials and the opposite glucose dependence of their electrical activity [32].

Measurement of [Ca^{2+}]$_i$ The cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_i$) was measured at 37°C in single cells or small clusters by the fura-2 method according to Grynkiewicz et al. [33] using equipment and software from TILL photonics (Gräfelfing, Germany). Cells were identified as beta cells when $[Ca^{2+}]_i$ was not decreased by 15 mmol/l glucose, as described for alpha cells [34]. The cells were loaded with fura-2AM (5 μmol/l) for 30 min at 37°C. Intracellular fura-2 was excited alternately at 340 nm or 380 nm by means of an oscillating diffraction grating. The excitation light was directed through the objective (PlanNeofluar ×40 objective, Zeiss, Stuttgart, Germany) by means of a glass fibre light guide and a dichroic mirror. The emitted light was filtered (LP515 nm) and measured by a digital camera. The ratio of the emitted light intensity at 340 nm/380 nm excitation was used to calculate $[Ca^{2+}]_i$ following an in vitro calibration with fura-2 K^+-salt.

Measurement of insulin secretion Batches of five islets were incubated for 60 min at 37°C with the indicated substances. Insulin was determined by radioimmunoassay using rat insulin (Crystal Chem, Downers Grove, IL, USA) as the standard.

Presentation of results Electrophysiological and $[Ca^{2+}]_i$ experiments are illustrated by recordings representative of the indicated number of experiments carried out with different cells. At least three different cell preparations were

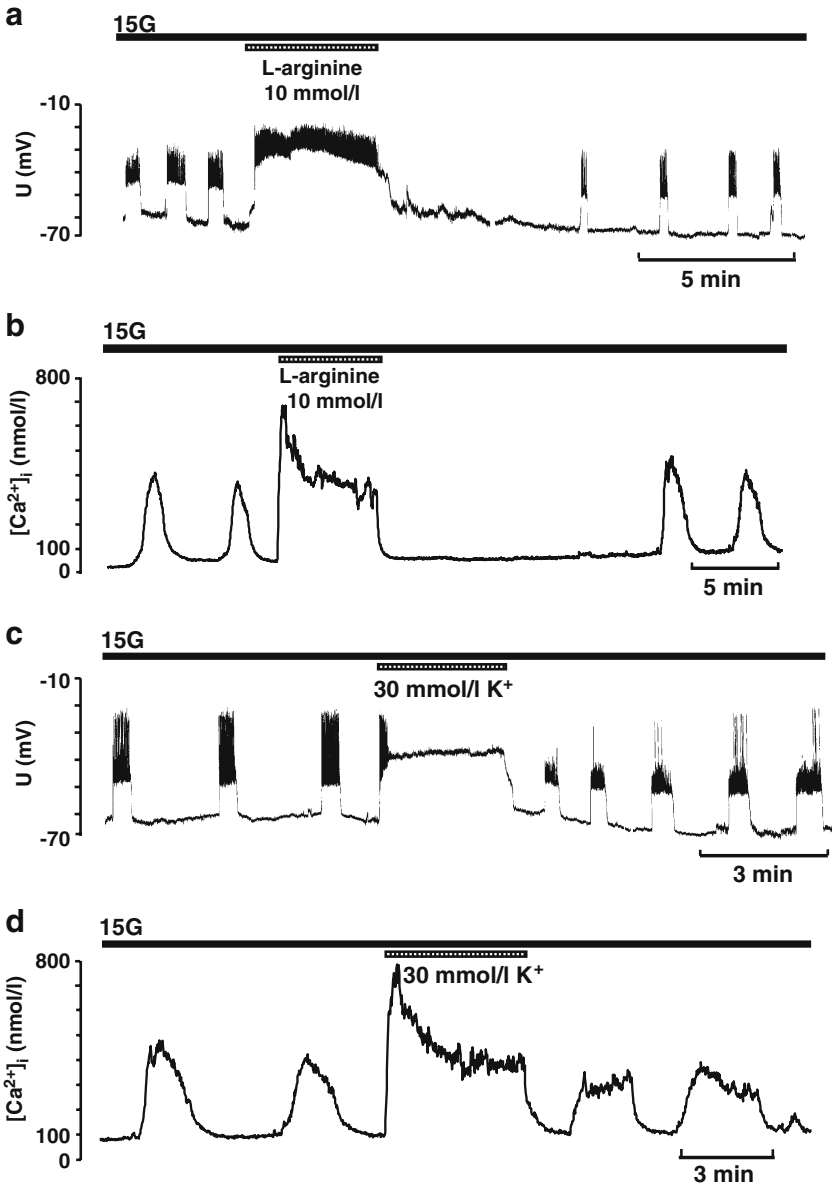

Fig. 1 Influence of 10 mmol/l arginine (**a**, **b**) and 30 mmol/l K^+ (**c**, **d**) on V_m (**a**, **c**) and $[Ca^{2+}]_i$ (**b**, **d**) in Sur1$^{-/-}$ beta cells. Experiments were done in 15 mmol/l glucose (15G). V_m was determined with intracellular microelectrodes. In the presence of 10 mmol/l arginine (**a**) or 30 mmol/l K^+ (**c**) oscillations of V_m were terminated and the plasma membrane was persistently depolarised, displaying continuous Ca^{2+} action potentials in the presence of arginine. Oscillations of $[Ca^{2+}]_i$ (**b**, **d**) were also abrogated by addition of the depolarising agents that induced a biphasic rise of $[Ca^{2+}]_i$ with an initial peak followed by a plateau phase with steadily elevated $[Ca^{2+}]_i$. The recordings are representative of four (**a**), eleven (**b**), five (**c**) and ten (**d**) experiments

used for each series of experiments. Where possible the means±SEM are given in the text for the indicated number of experiments. In microelectrode experiments recordings during the 4 to 6 min prior to changes in glucose concentration were analysed. The statistical significance of differences between means was assessed by a one sample *t*-test or Student's *t*-test for paired values when two samples were compared; multiple comparisons were made by ANOVA followed by Student–Newman–Keuls test. A p value of less than 0.05 was considered significant.

Results

Influence of membrane depolarisation on $[Ca^{2+}]_i$ and insulin secretion in Sur1$^{-/-}$ beta cells To investigate whether $[Ca^{2+}]_i$ is controlled by membrane potential, pancreatic beta cells devoid of K_{ATP} channels were depolarised by 10 mmol/l arginine or 30 mmol/l K^+ respectively. V_m recordings from intracellular microelectrodes in islets perfused with 15 mmol/l glucose displayed regular oscillations (slow waves) as described recently [9]. The addition of arginine (Fig. 1a) or an increase in extracellular K^+ (Fig. 1c) resulted in depolarisation of V_m; continuous Ca^{2+} action potentials were present when arginine was added. The depolarising agents terminated oscillations in $[Ca^{2+}]_i$ by inducing a biphasic rise in $[Ca^{2+}]_i$ with an initial peak followed by an increased steady-state level (Fig. 1b and d).

On average, the fraction of plateau phase (FOPP; percentage of time when the cell membrane is depolarised) increased from 44±6% to 100% after addition of arginine ($p\leq0.01$, n=4) and from 37±4% to 100% after elevation of extracellular K^+ ($p\leq0.001$, n=5) respectively. Under control conditions (15 mmol/l glucose) $[Ca^{2+}]_i$ oscillated between a peak value of 378±31 nmol/l and a nadir of 66±8 nmol/l (n=12). Application of 10 mmol/l arginine induced a peak increase in $[Ca^{2+}]_i$ of 614±41 nmol/l followed by a plateau of 293±32 nmol/l (n=11). With 30 mmol/l K^+ $[Ca^{2+}]_i$ reached a peak value of 705± 46 nmol/l and a sustained plateau of 339±20 nmol/l (n=10).

Insulin release from Sur1$^{-/-}$ islets was augmented when the glucose concentration was increased from 0.5 up to 40 mmol/l (Fig. 2a). Insulin secretion in 0.5 and 3 mmol/l glucose was significantly lower compared to 15 mmol/l glucose (G0.5: 0.23±0.05 ng·h^{-1}·islet^{-1}, n=6; G3: 0.24±0.06 ng·h^{-1}·islet^{-1}, n=5; G15: 0.48±0.06 ng·h^{-1}·islet^{-1}, n=6; $p\leq0.05$). Stimulation with 25 mmol/l or 40 mmol/l glucose increased insulin release to 0.67±0.08 ng·h^{-1}·islet^{-1} (n=5) or 0.74±0.11 ng·h^{-1}·islet^{-1} (n=5; $p\leq0.05$) respectively. Addition of arginine (10 mmol/l) or K^+ (30 mmol/l) in the presence of 15 mmol/l glucose resulted in a rise in secretion consistent with increased Ca^{2+} influx acting as a stimulus for exocytosis. On average, insulin release was elevated from 0.48±0.06 (15 mmol/l glucose, n=6) to 1.10±0.14 ng·h^{-1}·islet^{-1} by 10 mmol/l arginine (n=5; $p\leq0.01$ compared to 15 mmol/l glucose) and to 1.02±0.10 ng·h^{-1}·islet^{-1} by 30 mmol/l K^+ ($p\leq0.001$ compared to 15 mmol/l glucose n=5–6). Glucose-induced insulin release was also determined in wild-type islets. Basal secretion of wild-type islets (0.5 mmol/l glucose) was significantly lower, but stimulation with 15 or 40 mmol/l glucose potentiated the secretory response much more efficiently compared to Sur1$^{-/-}$ islets. The increase from 0.5 to 15 mmol/l glucose induced a 2-fold vs 7-fold elevation of insulin secretion in Sur1$^{-/-}$ and wild-type mice respectively. On average, insulin release in wild-type islets was elevated from 0.06±0.02 (0.5 mmol/l glucose, n=8; $p\leq0.01$ compared to Sur1$^{-/-}$ islets) to 0.43±0.06 ng·h^{-1}·islet^{-1} by 15 mmol/l glucose and to 0.64±0.08 ng·h^{-1}·islet^{-1} by 40 mmol/l glucose (n=8; $p\leq0.001$ and n=3; $p\leq0.05$ compared to 15 mmol/l glucose in wild-type islets respectively). Insulin content amounted to 27.4±1.9 ng/islet in Sur1$^{-/-}$ and 26.8±1.5 ng/islet in wild-type islets (n=4 and n=8 independent preparations respectively).

The contribution of ATP production to glucose-stimulated insulin secretion of Sur1$^{-/-}$ islets was assessed by inhibiting mitochondrial ATP production in the presence of 30 mmol/l K^+ to depolarise V_m and maintain elevated $[Ca^{2+}]_i$. Oligomycin (2 μg/ml) had only marginal effects on basal

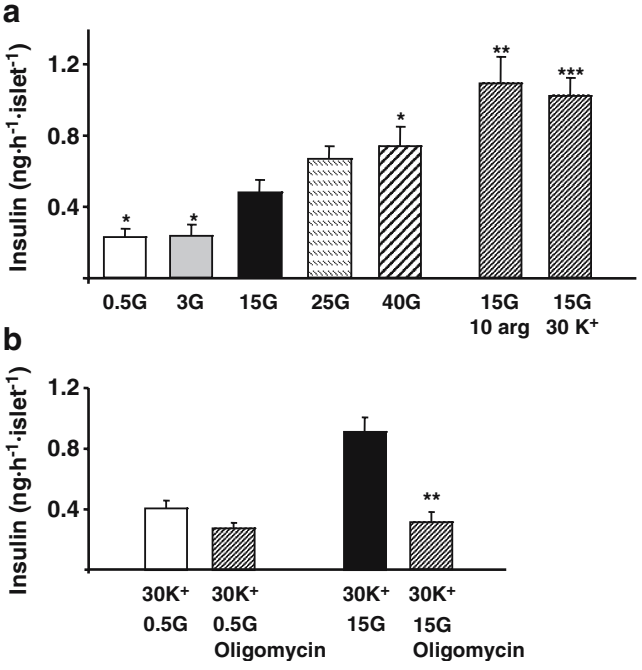

Fig. 2 Insulin secretion of isolated Sur1$^{-/-}$ islets with different glucose concentrations, depolarising agents and inhibition of glucose metabolism. Batches of five islets were incubated with the indicated substances for 60 min. **a** Insulin release was increased by elevating the glucose concentration. Compared with 15 mmol/l glucose (*15G*), insulin secretion in 0.5 (*0.5G*) or 3 mmol/l (*3G*) glucose was significantly lower, whereas increasing the glucose concentration to 40 mmol/l (*40G*) significantly increased secretion. The addition of 10 mmol/l arginine or 30 mmol/l K^+ to Sur1$^{-/-}$ islets already stimulated with 15 mmol/l glucose led to a further rise in insulin release. *$p\leq0.05$; **$p\leq0.01$; ***$p\leq0.001$ vs 15 mmol/l glucose. **b** The inhibition of mitochondrial ATP production by 2 μg/ml oligomycin had no effect on Sur1$^{-/-}$ islets incubated in 0.5 mmol/l glucose (*0.5G*), but decreased insulin release in islets stimulated with 15 mmol/l glucose (*15G*). Throughout this series of experiments 30 mmol/l K^+ (*30 K$^+$*) was present. **$p\leq0.01$ vs 15 mmol/l glucose + 30 mmol/l K^+. Insulin secretion was calculated as ng·h^{-1} ·islet^{-1}±SEM of five to six independent series of experiments with different preparations

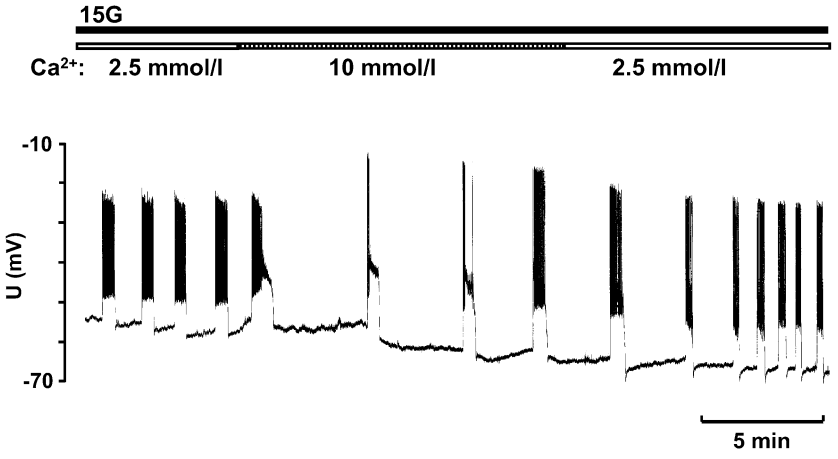

Fig. 3 Hyperpolarising effect of a rise in the extracellular Ca^{2+} concentration in $Sur1^{-/-}$ islets. Experiments were performed with intracellular microelectrodes. Increasing the extracellular Ca^{2+} concentration from 2.5 to 10 mmol/l decreased the fraction of the plateau phase and increased the length of interburst phases, indicating activation of a hyperpolarising ion current. The recording is representative of 11 experiments

insulin secretion in 0.5 mmol/l glucose, but significantly lowered exocytosis from islets stimulated with 15 mmol/l glucose (Fig. 2b). On average, in 15 mmol/l glucose and 30 mmol/l K^+ insulin release was 0.92±0.09 ng·h^{-1}·$islet^{-1}$ in the absence, versus 0.31±0.06 ng·h^{-1}·$islet^{-1}$ in the presence of oligomycin ($p \leq 0.01$; $n=6$).

Effect of Ca^{2+} influx on membrane potential and ion currents in $Sur1^{-/-}$ beta cells In wild-type beta cells, changes in external Ca^{2+} concentration, which are assumed to determine $[Ca^{2+}]_i$, play a crucial role in the regulation of membrane potential oscillations. The influence of increased Ca^{2+} influx on V_m oscillations in $Sur1^{-/-}$ beta cells was

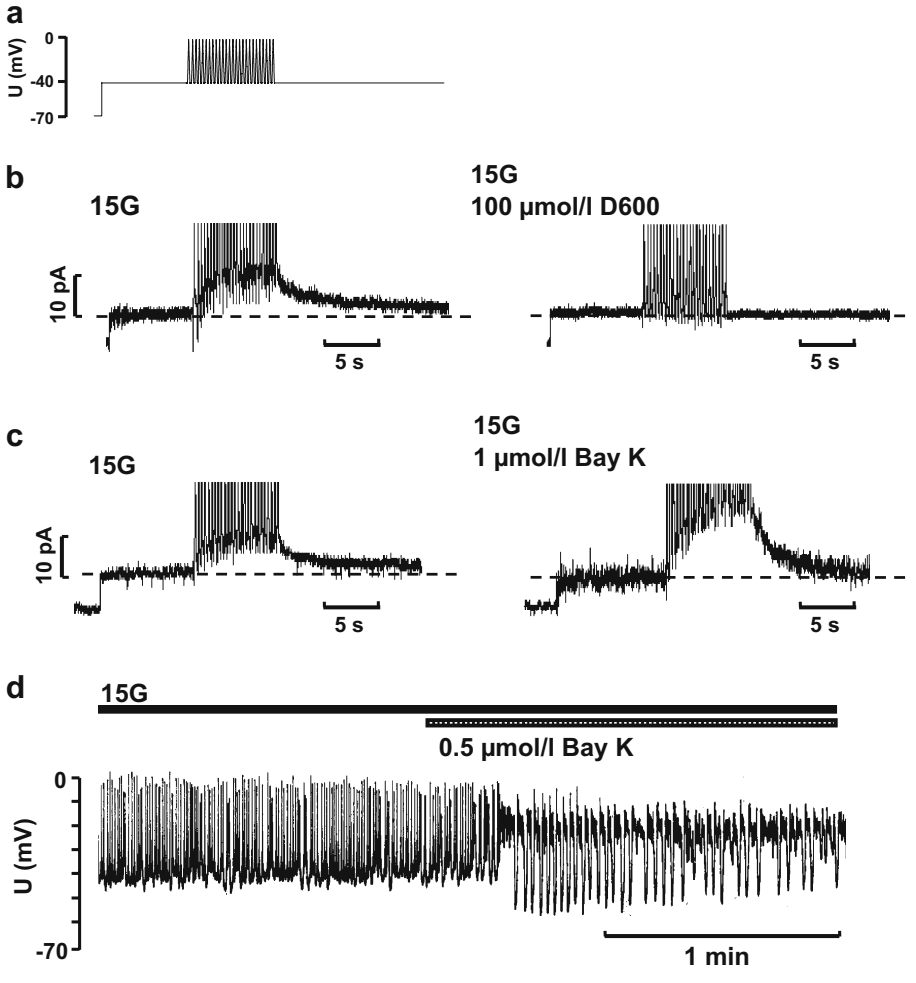

Fig. 4 Activation of Ca^{2+}-dependent ion currents in isolated $Sur1^{-/-}$ beta cells. Experiments were performed in the perforated-patch configuration. The bath solution contained 15 mmol/l glucose (*15G*) in all experiments. **a** Pulse protocol for activation of ion currents recorded in (**b**) and (**c**). The pulse protocol consisted of a depolarising step from −70 to −40 mV, followed by 26 voltage ramps from −40 to 0 mV and vice versa. **b**, **c** The simulated burst of action potentials induced an outward current that was completely prevented by 100 µmol/l D600. The application of 1 µmol/l of the L-type Ca^{2+}-channel opener BayK 8644 (**c**) significantly elevated the outward current. **d** The increased outward current in the presence of BayK 8644 coincided with hyperpolarisation of V_m. Current measurements were representative of 20 with 15G, four with D600 (**b**) and 13 with BayK 8644 (**c**). The recording of V_m (**d**) was representative of 13 experiments with similar results

investigated with intracellular microelectrodes. Figure 3 shows that elevation of the extracellular Ca^{2+} concentration from 2.5 to 10 mmol/l markedly altered the pattern of oscillations. FOPP was reduced from 32±3% to 23±4% ($p \leq 0.05$; $n=11$). The average burst duration was not significantly changed, while the length of the interburst phase was increased from 62±7 s to 143±29 s ($p \leq 0.05$; $n=11$), consistent with the induction of a Ca^{2+}-dependent hyperpolarising current.

To characterise this hyperpolarising current induced in $Sur1^{-/-}$ beta cells by increased Ca^{2+} influx, ion currents, were recorded in the perforated-patch configuration. As described previously [9, 15], single, cultured $Sur1^{-/-}$ beta cells are persistently depolarised and show slow waves of $[Ca^{2+}]_i$ in the absence of V_m oscillations. Stimulation of $Sur1^{-/-}$ beta cells with simulated bursts of action potentials, following the protocol described by Göpel et al. [Fig. 4a; 28], elicited a small outward current that could be inhibited by D600, an L-type Ca^{2+} channel blocker (Fig. 4b). This outward current amounted to 5.00±0.64 pA ($n=20$) and was reduced to 0.03±0.41 pA in the presence of 50–100 µmol/l D600 ($p \leq 0.01$; $n=4$). Figure 4c shows that BayK 8644, an L-type Ca^{2+} channel opener in beta cells [35], significantly increased the Ca^{2+}-dependent outward current (3.67±0.46 pA without BayK 8644 vs 10.66±2.07 pA after the addition of 1 µmol/l BayK 8644; $p \leq 0.01$; $n=13$). In Fig. 4d the effect of BayK 8644 on V_m was measured in isolated patch-clamped $Sur1^{-/-}$ beta cells. Prior to the addition of BayK 8644 persistent Ca^{2+} action potentials occurred. Application of BayK 8644 hyperpolarised the beta cell membrane potential by ~8 mV (−49±1 mV vs an interburst potential of −57±2 pA in 0.5–1 µmol/l BayK 8644; $p \leq 0.001$; $n=18$) converting the continuous Ca^{2+} action potentials, characteristic of single $Sur1^{-/-}$ beta cells [9, 15], to an oscillatory pattern. This pattern was remotely reminiscent of the slow waves seen in whole islets, but with a much higher frequency. However, as already mentioned above, electrical activity of isolated beta cells markedly differs from whole islets, so that the frequency of oscillations cannot be directly compared.

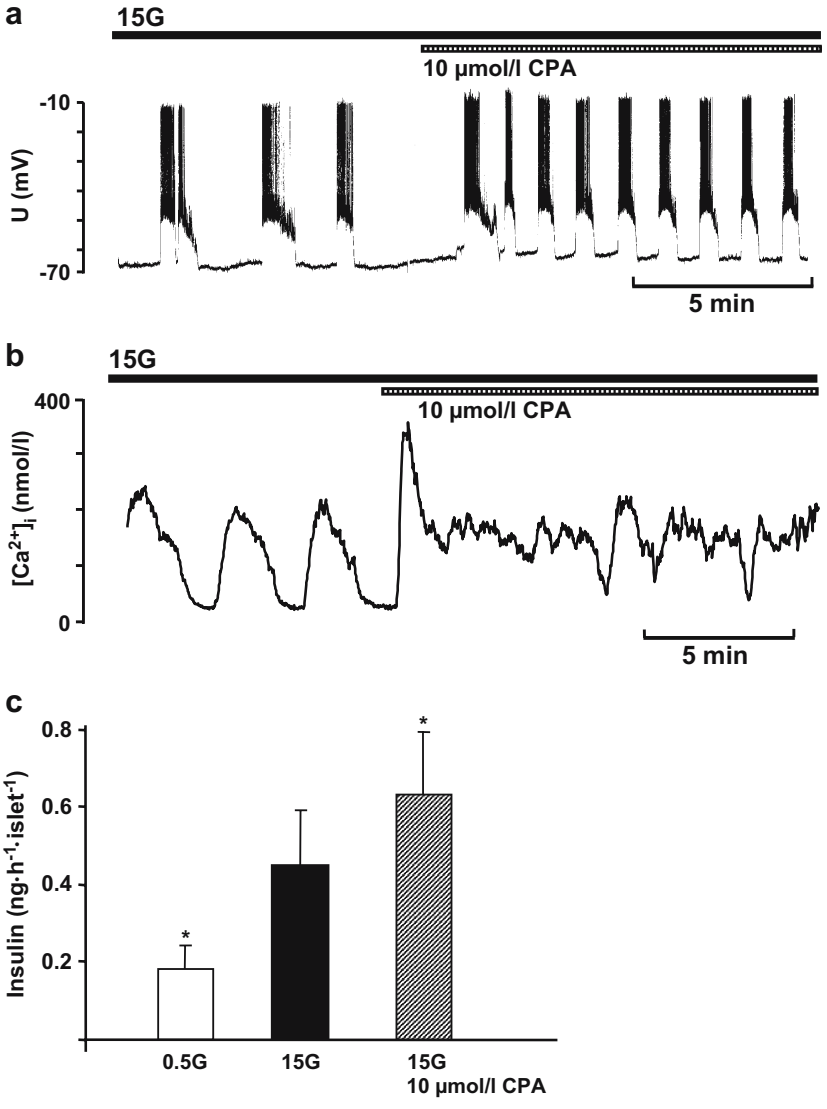

Fig. 5 Influence of store depletion on V_m, $[Ca^{2+}]_i$ and insulin release in $Sur1^{-/-}$ islets. Intracellular Ca^{2+} stores were emptied by 10 µmol/l cyclopiazonic acid (CPA). **a** Determination of V_m with intracellular microelectrodes showed that addition of CPA accelerated burst frequency and decreased interburst intervals. **b** $[Ca^{2+}]_i$ of isolated clusters displayed regular oscillations in the presence of 15 mmol/l glucose. After application of CPA, $[Ca^{2+}]_i$ was elevated and oscillation frequency increased. The traces are representative of five (**a**), and 12 (**b**) experiments. **c** Isolated $Sur1^{-/-}$ islets were incubated with 0.5 mmol/l glucose (*0.5G*), 15 mmol/l glucose (*15G*) and 15 mmol/l glucose + 10 µmol/l CPA for 60 min. Compared to 0.5 mmol/l glucose, insulin secretion was stimulated by 15 mmol/l glucose and further increased in the presence of CPA. The diagram shows the average insulin release±SEM of five independent experiments with different preparations. *$p \leq 0.05$ vs 15 mmol/l glucose

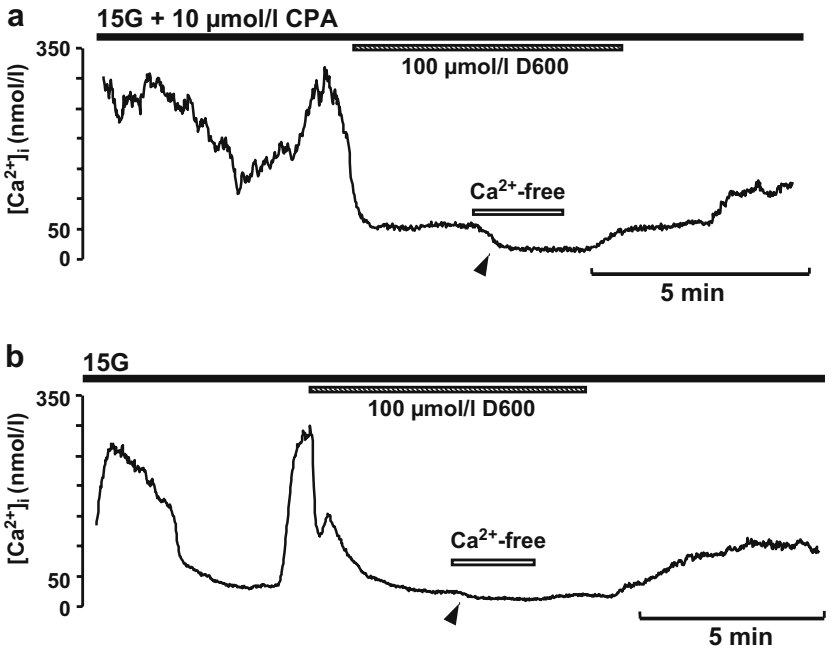

Fig. 6 Activation of Ca^{2+} influx by store depletion of $Sur1^{-/-}$ beta cells. Blockage of L-type Ca^{2+} channels by 100 µmol/l D600 terminated oscillations and decreased $[Ca^{2+}]_i$ (**a**, **b**). In cells treated with 10 µmol/l CPA (**a**), the omission of extracellular Ca^{2+} further reduced $[Ca^{2+}]_i$. This reduction was significantly greater than in the absence of CPA (**b**). The recordings represent eight experiments with and eight experiments without CPA

Regulation of membrane potential oscillations, $[Ca^{2+}]_i$ and insulin secretion by intracellular Ca^{2+} stores In wild-type beta cells Ca^{2+} uptake and release from intracellular stores is known to influence V_m via changes in $[Ca^{2+}]_i$ [36, 37]. Figure 5a shows that the addition of 10 µmol/l cyclopiazonic acid (CPA), a reversible inhibitor of ATP-dependent Ca^{2+} uptake into the endoplasmic reticulum [38], significantly increased the frequency of V_m oscillations and shortened the duration of the interburst phase (frequency=0.43±0.03 vs 1.06±0.06 bursts/min in 10 µmol/l CPA, $p \leq 0.001$, n=6; interburst intervals=97±25 s vs 27±4 s, $p \leq 0.05$, n=5; FOPP=44±7% vs 58±5%, $p \leq 0.05$, n=5). CPA increased $[Ca^{2+}]_i$ consistent with coupling of V_m and $[Ca^{2+}]_i$ in $Sur1^{-/-}$ beta cells (Fig. 5b) and increased insulin secretion from $Sur1^{-/-}$ islets stimulated with 15 mmol/l glucose (Fig. 5c). To quantify the effect of CPA on $[Ca^{2+}]_i$ the area under the curve, $AUC_{[Ca2+]i}$, was calculated for 5 min prior to changing the bath solution. In 10 µmol/l CPA the $AUC_{[Ca2+]i}$ increased by 3895±897 $nmol \cdot l^{-1} \cdot min^{-1}$ ($p \leq 0.01$; n=12) corresponding to an elevation of ~52% compared to the value in 15 mmol/l glucose. Insulin secretion increased from 0.45±0.14 $ng \cdot h^{-1} \cdot islet^{-1}$ in 15 mmol/l glucose to 0.63± 0.17 $ng \cdot h^{-1} \cdot islet^{-1}$ in 15 mmol/l glucose plus 10 µmol/l CPA ($p \leq 0.05$; n=5).

The results with CPA suggest that Ca^{2+} store depletion can activate a depolarising current that counteracts the hyperpolarising current maintaining the interburst phases. To determine the contribution of Ca^{2+} influx via store-operated channels, L-type Ca^{2+} channels were inhibited by D600 in the presence of CPA. Figure 6 shows that addition of 100 µmol/l D600 immediately abrogated oscillations and drastically decreased $[Ca^{2+}]_i$. $[Ca^{2+}]_i$ could be reduced further by omission of extracellular Ca^{2+} (Fig. 6a, arrowhead), but the effect was marginal in the absence of CPA (Fig. 6b, arrowhead). Omission of extracellular Ca^{2+} in the presence of CPA and D600, decreased $[Ca^{2+}]_i$ by 34±4 nmol/l vs 8±1 nmol/l ($p \leq 0.001$; n=8) in D600 alone. This series of experiments was also performed with thapsigargin, another potent inhibitor of sarcoplasmic Ca^{2+} ATPases, leading to similar results: with 1 µmol/l thapsigargin plus 100 µmol/l D600, $[Ca^{2+}]_i$ was reduced by 33±3 nmol/l after omission of extracellular Ca^{2+} (n=3; not shown).

Discussion

In contrast to what has been observed in beta cells from patients with congenital hyperinsulinism, several studies from K_{ATP}-channel-deficient mice [7, 8, 15] have shown that the loss of these channels, which play a key role in the triggering pathway of insulin secretion, does not result in hyperinsulinaemic hypoglycaemia. This implies that ablation of K_{ATP} channels in mice leads to compensatory mechanisms that allow for the maintenance of euglycemia. The observation that isolated $sur1^{-/-}$ beta cells were persistently depolarised while $[Ca^{2+}]_i$ oscillated [8, 15], suggested that the coupling between V_m and $[Ca^{2+}]_i$ was disrupted in murine K_{ATP}-channel-deficient beta cells [8]. By contrast, we have demonstrated regular oscillations in beta cells when V_m measurements were done on intact $sur1^{-/-}$ islets [9], suggesting V_m is involved in the regulation of intracellular Ca^{2+} homeostasis. The present study extends this analysis showing that V_m and $[Ca^{2+}]_i$ are closely coupled in $Sur1^{-/-}$ beta cells. Arginine or high extracellular K^+ abrogate V_m oscillations, depolarise the beta cell membrane and provide a strong triggering signal for Ca^{2+} influx [Figs. 1 and 2], which further increases insulin release from $Sur1^{-/-}$ islets incubated with 15 mmol/l glucose (Fig. 2a).

Several studies demonstrate that insulin secretion from $Sur1^{-/-}$ islets remains sensitive to glucose stimulation, although the sensitivity appears reduced [8, 15, 17, 21]. We

show that secretion from Sur1$^{-/-}$ islets can be elevated over a broad range of glucose concentrations (0.5–40 mmol/l; Fig. 2a). One prerequisite for exocytosis is the activation of mitochondrial ATP synthesis, and oligomycin completely suppressed glucose-stimulated insulin secretion under conditions where $[Ca^{2+}]_i$ was elevated by application of 30 mmol/l K^+ (Fig. 2b). This agrees with the recent report that elevated insulin secretion from sur1$^{-/-}$ islets can be suppressed with NaN_3 [21]. Nenquin et al. [21] largely ascribed the regulatory role of glucose to an effective amplifying pathway, but found that elevating glucose induced an unexpected transient rise in $[Ca^{2+}]_i$. Together with our previous observation that oscillations of V_m and $[Ca^{2+}]_i$ were modified by glucose [9], this suggests a V_m/$[Ca^{2+}]_i$-dependent pathway that contributes to glucose-regulated exocytosis in Sur1$^{-/-}$ mice.

In wild-type beta cells, repetitive activation of K_{ATP} channels, due to Ca^{2+}-induced changes in the ATP:ADP ratio, contributes to the maintenance of V_m oscillations [23, 26]. In addition, it has been shown that increased Ca^{2+} influx can induce hyperpolarisation of V_m that is not mediated by K_{ATP} channels [39] and that a small Ca^{2+}-dependent K^+ current, referred to as $I_{K,slow}$, is activated when $[Ca^{2+}]_i$ is increased in response to simulated bursts of action potentials [28, 30]. As $I_{K,slow}$ in wild-type beta cells contains a tolbutamide-resistant component of ~52% of the K^+ current induced by a train of depolarisations [29], we examined this component in Sur1$^{-/-}$ beta cells. Application of a train of depolarising pulses revealed an outward current of ~5 pA that was inhibited by D600 and potentiated by BayK 8644 (Fig. 4b, c). V_m was significantly hyperpolarised by BayK 8644, demonstrating that the outward current was sufficient to interrupt the persistent depolarisation observed in isolated Sur1$^{-/-}$ beta cells (Fig. 4d). Together with the observation that an increase in extracellular Ca^{2+} markedly prolonged the interburst phase of V_m in microelectrode experiments (Fig. 3), our data strongly support the view that the interplay between Ca^{2+} influx and activation of Ca^{2+}-dependent K^+ channels constitutes a feedback loop that maintains V_m oscillations in K_{ATP}-channel-deficient islets. Whether the conductance we describe for Sur1$^{-/-}$ beta cells is identical with the tolbutamide-insensitive component of $I_{K,slow}$ as well as detailed characterisation of the underlying ion channel(s) remain subjects for further investigation.

A second mechanism that can influence intracellular Ca^{2+} dynamics is based on ATP-dependent Ca^{2+} sequestration by the endoplasmic reticulum (ER). Previous studies in wild-type beta cells demonstrated that emptying intracellular stores depolarised V_m and affected the amplitude and frequency of oscillations in $[Ca^{2+}]_i$ [36, 37, 40]. Our data show that V_m in Sur1$^{-/-}$ beta cells is responsive to changes in Ca^{2+} stores. Store depletion with the SERCA-blocker CPA led to an increase in burst frequency that was coupled to elevated Ca^{2+} influx (Fig. 5a, b). The CPA-induced Ca^{2+} influx was resistant to D600 but sensitive to extracellular Ca^{2+} (Fig. 6), which is consistent with the activation of store-operated channels as described previously for wild-type beta cells [41]. We suggest that this store-operated depolarising current is sufficient to promote membrane depolarisation and thus increase the frequency of V_m oscillations. Consistent with the idea that the V_m/$[Ca^{2+}]_i$-dependent pathway is K_{ATP}-channel-independent, we were able to demonstrate that the CPA-induced increase in $[Ca^{2+}]_i$ stimulated insulin secretion (Fig. 5c).

Our data show that the coupling of V_m and $[Ca^{2+}]_i$ can determine insulin secretion in Sur1$^{-/-}$ beta cells. This illustrates that signals of a triggering pathway exist in the absence of functional K_{ATP} channels. The central role in regulation of V_m is provided by $[Ca^{2+}]_i$. Dependent on the origin of the $[Ca^{2+}]_i$ increase, Ca^{2+} exerts a feedback on V_m by activating depolarising and hyperpolarising ion currents that maintain membrane potential oscillations in beta cells of K_{ATP}-channel-deficient mice.

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Duality of interest.

There was no duality of interest in this study.

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