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Oscillations of membrane potential and cytosolic Ca^{2+} concentration in SUR1$^{-/-}$ beta cells

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Abstract

Aims/hypothesis. SUR1(ABCC8)$^{-/-}$ mice lacking functional K_{ATP} channels are an appropriate model to test the significance of K_{ATP} channels in beta-cell function. We examined how this gene deletion interferes with stimulus-secretion coupling. We tested the influence of metabolic inhibition and galanin, whose mode of action is controversial.
Methods. Plasma membrane potential (Vm) and currents were measured with microelectrodes or the patch-clamp technique; cytosolic Ca^{2+} concentrations ($[Ca^{2+}]_c$) and mitochondrial membrane potential ($\Delta\Psi$) were measured using fluorescent dyes.
Results. In contrast to the controls, SUR1$^{-/-}$ beta cells showed electrical activity even at a low glucose concentration. Continuous spike activity was measured with the patch-clamp technique, but with microelectrodes slow oscillations in Vm consisting of bursts of Ca^{2+}-dependent action potentials were detected. $[Ca^{2+}]_c$ showed various patterns of oscillations or a sustained increase. Sodium azide did not hyperpolarize SUR1$^{-/-}$ beta cells. The depolarization of $\Delta\Psi$ evoked by sodium azide was significantly lower in SUR1$^{-/-}$ than SUR1$^{+/+}$ cells. Galanin transiently decreased action potential frequency and $[Ca^{2+}]_c$ in cells from both SUR1$^{-/-}$ and SUR1$^{+/+}$ mice.
Conclusion/interpretation. The strong dependence of Vm and $[Ca^{2+}]_c$ on glucose concentration observed in SUR1$^{+/+}$ beta cells is disrupted in the knock-out cells. This demonstrates that both parameters oscillate in the absence of functional K_{ATP} channels. The lack of effect of metabolic inhibition by sodium azide shows that in SUR1$^{-/-}$ beta cells changes in ATP/ADP no longer link glucose metabolism and Vm. The results with galanin suggest that this peptide affects beta cells independently of K_{ATP} currents and thus could contribute to the regulation of beta-cell function in SUR1$^{-/-}$ animals. [Diabetologia (2004) 47:488–498]

Keywords SUR1 (ABCC8) null mice · Plasma membrane potential · Cytosolic Ca^{2+} concentration · Mitochondrial membrane potential · Sodium azide · Galanin

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Abbreviations: Kir6.2, inward rectifying K^+ channel type 6.2 · SUR1, sulfonylurea receptor type1 · $\Delta\Psi$, mitochondrial membrane potential · Vm, plasmamembrane potential · $[Ca^{2+}]_c$, cytosolic free calcium concentration

K_{ATP} channels are essential for normal stimulus-secretion coupling in pancreatic beta cells because they link changes in electrical activity that trigger insulin secretion to beta-cell metabolism. Glucose influx into beta cells leads to enhanced glycolytic flux, an increased ATP/ADP ratio, closure of K_{ATP} channels, plasma membrane depolarization and opening of voltage-dependent Ca^{2+} channels. This results in a rise in the cytosolic free Ca^{2+} concentration, $[Ca^{2+}]_c$, which activates exocytosis of insulin-containing granules [1]. In addition to this well-characterized glucose-dependent triggering pathway, an amplifying pathway has been described [2] which is independent of K_{ATP} channels

and changes in membrane potential [3, 4]. Animals deficient in beta cell-type K_{ATP} channels provide a useful model in which to study the role of these channels in the metabolic and hormonal control of insulin secretion and in the feedback mechanism(s) driving oscillations in beta cells. Two mouse models have been developed which lack functional K_{ATP} channels as a consequence of genetic disruption of either the Kir6.2 (KCNJ11) gene, which encodes the K^+ ion-selective pore of the channel [5], or the Sur1 (ABCC8) gene which constitutes the neuroendocrine specific sulfonylurea receptor [6, 7]. Surprisingly, random-fed animals exhibit almost normal blood glucose concentrations while glucose-induced insulin secretion is decreased [5, 6]. This contrasts with loss-of-function mutations in human SUR1 and Kir6.2 which cause a recessive form of persistent hyperinsulinaemic hypoglycaemia of infancy (PHHI) characterised by oversecretion of insulin despite severe hypoglycaemia [8, for review see 9]. Thus in the animal models, K_{ATP} channel-independent mechanisms must prevent excessive insulin secretion. $SUR1^{-/-}$ mice show a reduced response to incretins, i.e., GLP-1 and GIP [7, 10] as a consequence of their impaired cAMP-induced potentiation of insulin secretion [10] which could contribute to their unexpected normoglycaemia. Moreover, metabolic and/or hormonal signals could contribute additional regulatory pathways. Glucose metabolism, via ATP production, affects not only K_{ATP} channel activity, but could alter insulin secretion by influencing the activity of ion pumps, protein phosphorylation, or exocytosis. We tried to evaluate whether in $SUR1^{-/-}$ beta cells a glucose dependence of membrane potential and $[Ca^{2+}]_c$ still exists.

The neuropeptide galanin is known to inhibit insulin secretion [11] and thus is a potential counterregulator of excessive insulin secretion. However, the mechanism of this inhibitory effect is controversial because it has been attributed to both K_{ATP} channel-dependent, via activation and hyperpolarization, and K_{ATP} channel-independent pathways [11, 12, 13, 14, 15, 16]. We have compared the effects of galanin and a metabolic inhibitor, NaN_3, on stimulus-secretion coupling in beta cells of SUR1 knock-out versus wildtype mice in a search for additional regulatory pathways.

Material and methods

Cell and islet preparation. Experiments were done on islets or single pancreatic beta cells isolated from fed $SUR1^{-/-}$, NMRI, or C57Bl/6 mice killed by cervical dislocation or CO_2. The principles of laboratory animal care were followed (NIH publication No. 85-23, revised 1985) and experiments were carried out according German laws. 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 done on islet cells isolated by collagenase digestion. Cells were dispersed in Ca^{2+}-free medium and cultured 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 [17].

Solutions and chemicals. Cell membrane potential and K_{ATP} current patch-clamp recordings were done at 32°C with amphotericin B (250 µg/ml) in the pipette solution which contained (in mmol/l): 10 KCl, 10 NaCl, 70 K_2SO_4, 4 $MgCl_2$, 2 $CaCl_2$, 10 EGTA, 20 HEPES, pH 7.15 adjusted with KOH. The bath solution contained (in mmol/l): 140 NaCl, 5 KCl, 1.2 $MgCl_2$, 2.5 $CaCl_2$, 0.5 glucose, 10 HEPES, pH 7.4 adjusted with NaOH. This bath solution was also used for determination of $[Ca^{2+}]_c$ and $\Delta\Psi$ at 37°C at the indicated glucose concentrations.

For cell membrane potential measurements with intracellular microelectrodes the extracellular fluid contained (in mmol/l): 120 NaCl, 5 KCl, 2.5 $CaCl_2$, 1.2 $MgCl_2$, 24 $NaHCO_3$, 15 glucose and was gassed with 95% O_2 and 5% CO_2 to maintain a pH of 7.4 at 37°C.

Fura-2AM and rhodamine 123 were obtained from Molecular Probes (Eugene, Ore., USA). RPMI1640 medium, penicillin/streptomycin and glutamine were from GIBCO/BRL (Karlsruhe, Germany). Galanin was from Bachem (Heidelberg, 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 with an EPC-9 patch-clamp amplifier and software "Pulse" (HEKA, Lambrecht, Germany) in the voltage-clamp or current-clamp mode, respectively. Whole-cell K_{ATP} currents were measured at a holding potential of −70 mV and during 300 ms voltage pulses to −80 mV and −60 mV at 15-s intervals. Under these experimental conditions the currents measured with control NMRI beta cells are due almost entirely to K_{ATP} channels and are blocked by tolbutamide [18].

Membrane potential measurements. Vm was determined using high resistance microelectrodes [19]. Beta cells from $SUR1^{+/+}$ mice were identified by their characteristic membrane potential oscillations. Beta cells from $SUR1^{-/-}$ islets cells were discriminated from α-cells by their lack of Na^+ action potentials and their contrary glucose dependence of electrical activity [20].

Measurement of [Ca2+]c. The cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_c$) was measured in single cells or small clusters by the fura-2 method according to [21] using equipment and software from TILL photonics (Gräfelfing, Germany). Cells were considered to be beta cells when $[Ca^{2+}]_c$ was not decreased by 15 mmol/l glucose as described for α-cells [22]. 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 then directed through the objective (PlanNeofluar40x objective, Zeiss, Stuttgart, Germany) by means of a glass fiber 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 $[Ca^{2+}]_c$ using an in vitro calibration with fura-2 K^+-salt.

Measurement of ΔΨ. The mitochondrial membrane potential ($\Delta\Psi$) was measured using the same equipment as for $[Ca^{2+}]_c$. Rhodamine 123 (Rh123) fluorescence was excited at 480 nm

and the intensity of the emitted light (LP515 nm) was measured and given in arbitrary units (a.u.) (12-bit grey values of the CCD camera). Cells were loaded with Rh123 (10 µg/ml) for 10 min at 37°C. An increase of Rh123 fluorescence corresponds to a decrease in mitochondrial membrane potential [23, 24].

Presentation of results. Electrophysiological experiments, $[Ca^{2+}]_c$ and $\Delta\Psi$ are illustrated by recordings representative of the indicated number of experiments carried out with different cells. At least three different cell preparations have been used for each series of experiments. If possible the means ± SEM are given in the text for the indicated number of experiments. For evaluation of electrical activity in microelectrode experiments the last 4 to 6 min prior to changes in glucose concentration were analysed. The frequency of Ca^{2+} action potentials was calculated by the number of action potentials during the first 5 s of each burst phase. 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

Comparison of membrane potential and [Ca2+]c in cells from SUR1+/+ and SUR1−/− mice. Figure 1 shows the effect of glucose stimulation on the plasma membrane potential and whole-cell K_{ATP} currents of beta cells from $SUR1^{+/+}$ mice (Fig. 1a) versus $SUR1^{-/-}$ (Fig. 1b) mice measured with the perforated-patch technique. The traces illustrate the membrane potential recorded in the current-clamp mode. At the intervals indicated by VC the amplifier was switched to the voltage-clamp mode to register currents, illustrated at the right side, during voltage pulses from −70 mV to −60 mV. In $SUR1^{+/+}$ mice, with functional K_{ATP} channels, the plasma membrane potential changed in response to an increase in glucose concentration (Fig. 1a). On average, the plasma membrane potential was −72±2 mV at a glucose concentration of 0.5 mmol/l (*n*=9). Increasing the glucose concentration to 15 mmo/l led to depolarization of the plasma membrane potential and the occurrence of Ca^{2+}-dependent action potentials. The plateau potential (potential from which the spikes start) was −46±2 mV (*n*=9, $p \leq 0.001$). The K_{ATP} current amplitude was 10.6±1.7 pA in the presence of 0.5 mmol/l glucose and 2.0±0.4 pA after switching to 15 mmol/l glucose (*n*=9, $p \leq 0.001$). In all isolated $SUR1^{-/-}$ beta cells the membrane potential was persistently depolarized in 0.5 mmol/l glucose and continuous Ca^{2+} action potentials were observed. This pattern did not change with increasing glucose concentration (Fig. 1b). The plateau potential was −38±2 mV (*n*=12) at the low glucose concentration and −40±1 at 15 mmol/l glucose (*n*=14). No current similar to the K_{ATP} current was detectable in voltage-clamp mode, i.e., 1.8±0.3 pA leak current at 0.5 mmol/l glucose (*n*=10) and 1.1±0.2 pA

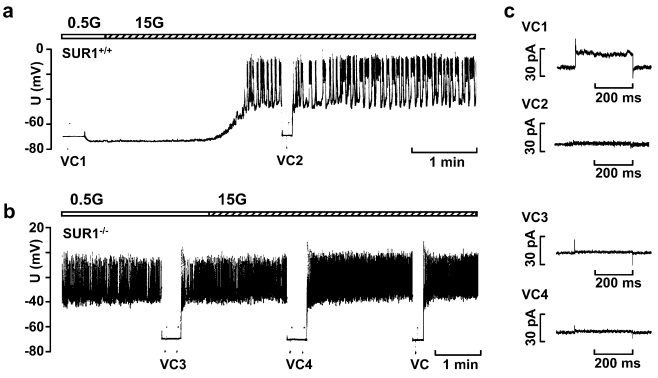

Fig. 1a, b. Comparison of the glucose dependence of membrane potential and current in $SUR1^{+/+}$ (**a**) and $SUR1^{-/-}$ beta cells (**b**) measured in the current-clamp mode with the perforated-patch technique to maintain normal cell metabolism. The change in glucose concentration from 0.5 mmol/l (0.5G) to 15 mmol/l (15G) is indicated by the horizontal bars. At the intervals indicated by VC the amplifier was switched to the voltage-clamp mode to record the current at −70 mV (continuous trace) and during 300 ms voltage steps to −60 mV and −80 mV applied every 15 s (upper and lower deflections). The currents measured during the pulses to −60 mV, marked VC1 to VC4, are illustrated at the right side of the figure (**c**). VC1 and VC3: 0.5 mmol/l glucose, VC2 and VC4: 15 mmol/l glucose, VC1 and VC2: $SUR1^{+/+}$ beta cell, VC3 and VC4: $SUR1^{-/-}$ beta cells. In contrast to $SUR1^{+/+}$ beta cells the $SUR1^{-/-}$ beta cells showed electrical activity at the low glucose concentration and the corresponding leak current was small. The recordings are representative of nine ($SUR1^{+/+}$) and fourteen ($SUR1^{-/-}$) experiments

at 15 mmol/l glucose (*n*=10) (compare VC3 and VC4 with VC2).

Changes in membrane potential normally control the free cytosolic Ca^{2+} concentration ($[Ca^{2+}]_c$) which triggers insulin secretion in beta cells. Figure 2 illustrates the effects of different glucose concentrations on $[Ca^{2+}]_c$ in isolated beta cells or small cell clusters from $SUR1^{+/+}$ (Fig. 2a) and $SUR1^{-/-}$ (Fig. 2b–e) mice. As described previously [24, 25], control cells respond to an increase of the glucose concentration from 0.5 mmol/l to 15 mmol/l with an initial decrease (due to an activation of Ca^{2+} pumps) followed by a longer period with increased $[Ca^{2+}]_c$ and finally slow oscillations of $[Ca^{2+}]_c$ (Fig. 2a). On average $[Ca^{2+}]_c$ was 81±3 nmol/l (*n*=13) at a glucose concentration of 0.5 mmol/l in control cells, decreasing to 57±4 nmol/l after switching to 15 mmol/l glucose ($p \leq 0.001$), then abruptly rising to a peak value of 406±23 nmol/l (*n*=13, $p \leq 0.001$). Cells from $SUR1^{-/-}$ mice exhibited variable patterns of $[Ca^{2+}]_c$, but importantly the direct correlation between glucose concentration and $[Ca^{2+}]_c$ was lost (Fig. 2). The only consistent response of $[Ca^{2+}]_c$ to changes in glucose was a drop upon shifting from 0.5 mmol/l to 15 mmol/l (see arrowheads in Fig. 2b,c,e). A spontaneous rise in $[Ca^{2+}]_c$ in $SUR1^{-/-}$, but not wildtype beta cells, often occurred in the presence of 0.5 mmol/l glucose (*n*=11, see Fig. 2b). In

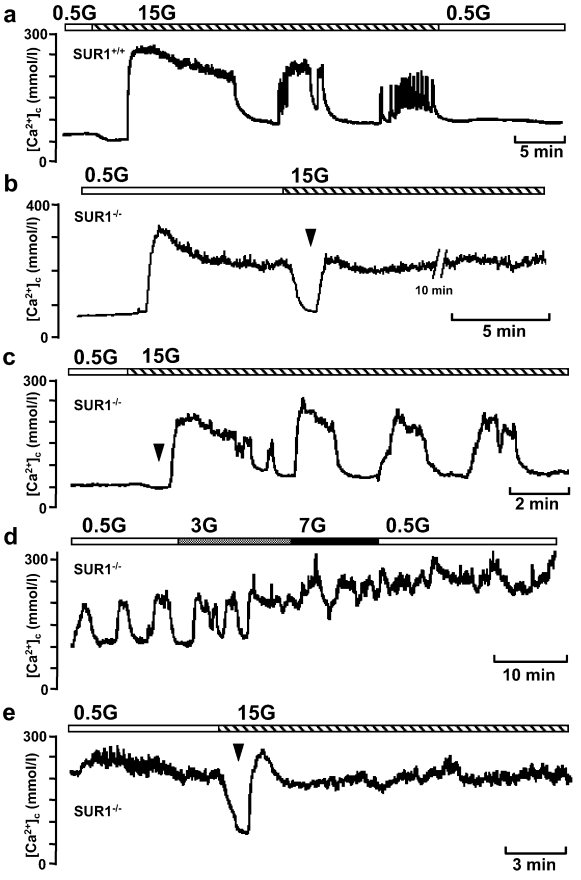

Fig. 2a–e. Comparison of the glucose dependence of $[Ca^{2+}]_c$ in $SUR1^{+/+}$ (**a**) and $SUR1^{-/-}$ beta cells (**b–e**). (**a**) In beta cells from $SUR1^{+/+}$ mice increasing the glucose concentration from 0.5 mmol/l to 15 mmol/l produced an initial decrease in $[Ca^{2+}]_c$ followed by a rapid increase at the beginning of the first phase and finally a second phase with oscillations. This recording is representative of thirteen experiments with similar results. (**b–e**) In $SUR1^{-/-}$ beta cells the pattern of the $[Ca^{2+}]_c$ response to glucose was quite variable. (**b**) In eleven cells $[Ca^{2+}]_c$ rose spontaneously in low glucose (0.5 mmol/l) and oscillated in four cells. (**c**) In three cells $[Ca^{2+}]_c$ was low at 0.5 mmol/l glucose and increasing glucose produced a result similar to that seen in control cells. (**d**) In three other cells $[Ca^{2+}]_c$ was oscillating in the presence of 0.5 mmol/l glucose and increased to a sustained level when glucose was increased and remained increased when glucose was lowered to 0.5 mmol/l. (**e**) In six cells, a sustained increase was observed at 0.5 mmol/l glucose and increasing the concentration of glucose produced a transient reduction in $[Ca^{2+}]_c$

four of eleven cells increased glucose produced spontaneous oscillations in $[Ca^{2+}]_c$, in the remaining cells the increase was sustained without oscillations. In three cells or cell clusters the behavior was similar to that observed in wildtype cells (Fig. 2c). In three other cells $[Ca^{2+}]_c$ was oscillating in 0.5 mmol/l glucose and increased to a sustained level after changing the glucose concentration to 3 mmol/l and 7 mmol/l. These cells sustained the higher level of $[Ca^{2+}]_c$ upon returning the glucose concentration to 0.5 mmol/l (Fig. 2d). In another series $[Ca^{2+}]_c$ was increased, but not oscillating, in 0.5 mmol/l glucose (Fig. 2e, n=6). In these cells $[Ca^{2+}]_c$ was 213±9 nmol/l in the presence of 0.5 mmol/l glucose. Raising the glucose concentration to 15 mmol/l evoked a transient drop in $[Ca^{2+}]_c$ to 89±9 nmol/l ($p \leq 0.001$) corresponding to the initial decrease observed in Fig. 2b, c.

Table 1. Effect of glucose concentration on the frequency of Ca^{2+} action potentials, fraction of plateau phase (FOPP) and duration of burst or interburst phases in $SUR1^{-/-}$ islets. Values are expressed as percentage increase or decrease ± SEM versus 15 mmol/l glucose

Glucose concentration:	0.5 mmol/l	25 mmol/l	40 mmol/l
$SUR1^{-/-}$			
Action potential frequency	67±3[b]	134±11[a]	150±27[a]
FOPP	170±12[b]	97±7	178±41
Burst duration	195±27[a]	164±27[a]	102±15
Interburst duration	80±9	201±34	59±22
	[% vs 15 mmol/l glucose]		
	n=9	n=5–8	n=4

[a] $p \leq 0.05$; [b] $p \leq 0.001$

It is commonly accepted that the oscillations in $[Ca^{2+}]_c$ in wildtype beta cells are linked to glucose metabolism via oscillations in electrical activity [26]. The persistent Ca^{2+}-dependent action potentials with variable $[Ca^{2+}]_c$ observed here raise the question of whether $[Ca^{2+}]_c$ and membrane potential remain coupled in $SUR1^{-/-}$ beta cells. The patch-clamp experiments done with single isolated beta cells suggest the strong coupling between changes in membrane potential and $[Ca^{2+}]_c$ observed in cells with functional K_{ATP} channels could be abrogated in $SUR1^{-/-}$ beta cells. To test this point we measured the cell membrane potential in intact islets using intracellular microelectrodes since oscillatory activity is difficult to detect in single beta cells [27, 28, 29]. Surprisingly, the membrane potential of $SUR1^{-/-}$ beta cells oscillated under these conditions in the presence of 15 mmol/l glucose (n=8, Fig. 3b, c) resembling recordings from wildtype cells in intact islets (Fig. 3a). In contrast to wildtype cells the oscillations persisted in 0.5 mmol/l (n=9, Fig. 3b), 25 mmol/l (n=8, Fig. 3c, d), and 40 mmol/l glucose (n=4, Fig. 3d).

Measurements with intracellular microelectrodes on intact islets clearly show oscillations in the absence of K_{ATP} channels. To exclude the possibility that the different ionic compositions of the bath solutions contribute to the conflicting result, additional patch-clamp experiments were done with the bicarbonate buffer used for intracellular recording. In this series of experiments the membrane potential did not oscillate and was persistently depolarized at 0.5 mmol/l (−38±3 mV, n=4) and 15 mmol/l glucose (−43±2 mV; n=7; not shown).

The microelectrode experiments showed that $SUR1^{-/-}$ beta-cell activity could be modulated by glucose (Fig. 3 and Table 1): The reduction of glucose

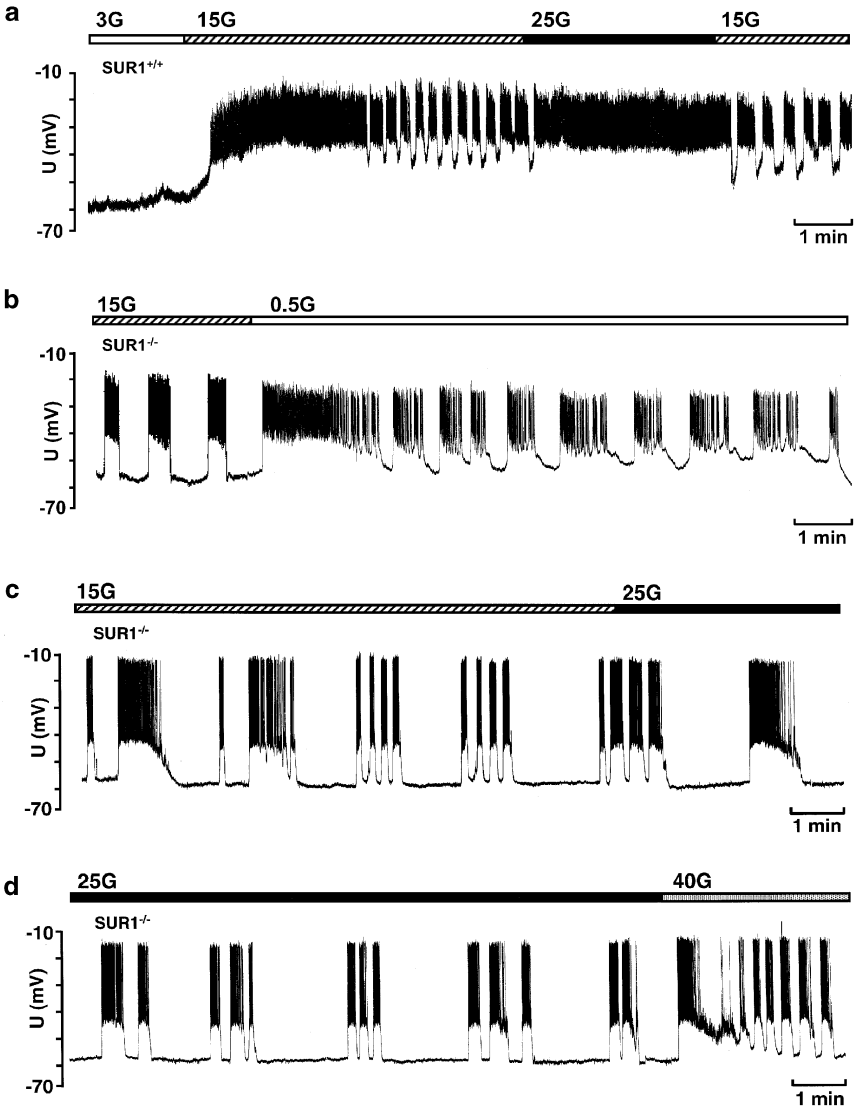

Fig. 3a–d. Recording of plasma membrane potential using intracellular microelectrodes on intact islets. (**a**) SUR1$^{+/+}$ islets from C57Bl/6 mice in the presence of 0.5 mmol/l, 15 mmol/l and 25 mmol/l glucose. (**b–d**) Continuous oscillations in SUR1$^{-/-}$ islets perifused with 0.5 mmol/l, 15 mmol/l, 25 mmol/l and 40 mmol/l glucose. Record (**d**) is the direct continuation of (**c**). The recordings are representative of five (**a**), nine (**b**), eight (**c**) and four (**d**) experiments

concentration from 15 to 0.5 mmol/l led to a decrease in the frequency of Ca^{2+}-dependent action potentials whereas the fraction of plateau phase (FOPP; percentage of time with spike activity) and burst duration were increased (Fig. 3b, n=9). Increasing the glucose concentration from 15 to 25 mmol/l or 40 mmol/l did not change the FOPP, but did increase the frequency of Ca^{2+}-dependent action potentials (n=5 with 25 mmol/l glucose and n=4 with 40 mmol/l glucose). The influence of glucose concentration on the electrical activity in SUR1$^{-/-}$ islets is summarized in Table 1.

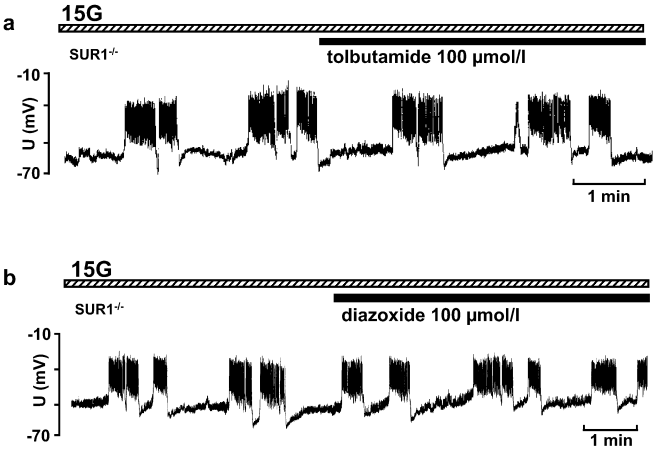

Fig. 4a, b. Recordings of plasma membrane potential using intracellular microelectrodes on intact SUR1$^{-/-}$ islets in the presence of (**a**) 100 µmol/l tolbutamide or (**b**) 100 µmol/l diazoxide. The recordings are representative of nine (**a**) or seven (**b**) experiments

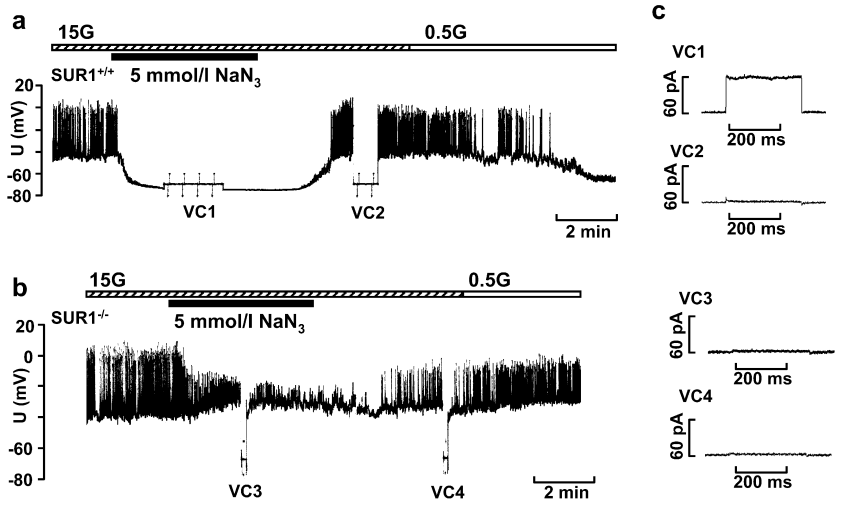

Fig. 5a–c. Effects of NaN_3 on beta-cell membrane potential and currents. (**a**) $SUR1^{+/+}$ and (**b**) $SUR1^{-/-}$ beta cells. Recordings were made using the perforated patch mode on beta cells in 15 mmol/l glucose. In control beta cells 5 mmol/l NaN_3 hyperpolarised the membrane potential and increased the K_{ATP} current amplitude (**a**). This recording is representative of four experiments. In $SUR1^{-/-}$ beta cells 5 mmol/l NaN_3 did not alter the membrane potential (n=5) or current (n=3), but suppressed spike activity (**b**). The currents measured during voltage pulses to −60 mV (VC1 to VC4) are illustrated in (**c**). VC1 and VC3: 15 mmol/l glucose and 5 mmol/l NaN_3, VC2 and VC4: 15 mmol/l glucose, VC1 and VC2: $SUR1^{+/+}$ beta cell, VC3 and VC4: $SUR1^{-/-}$ beta cell

The membrane potential oscillations of $SUR1^{-/-}$ beta cells were not influenced by 100 µmol/l tolbutamide (n=9) or 100 µmol/l diazoxide (n=7) (Fig. 4).

Effects of metabolic inhibition on SUR1+/+ and SUR1−/− beta cells. Figure 5 compares the changes in beta-cell membrane potential and current induced by the metabolic inhibitor, NaN_3. When applied in the presence of 15 mmol/l glucose NaN_3 markedly increased K_{ATP} currents in $SUR1^{+/+}$ beta cells, hyperpolarizing their plasma membranes, and suppressing spike activity (Fig. 5a). The plateau potential was −52±4 mV in the presence of 15 mmol/l glucose and decreased to −76±1 mV after addition of NaN_3 (n=4, $p \leq 0.002$). The K_{ATP} current was essentially zero in 15 mmol/l glucose (VC2), but increased to 12.0±4.4 pA during treatment with NaN_3 (VC1; n=4, $p \leq 0.01$). NaN_3 did not influence the remaining leak currents or plateau potential in $SUR1^{-/-}$ beta cells, but did partially inhibit spike activity (Fig. 5b). The plateau potential was −42±2 mV in 15 mmol/l glucose and −34±3 mV after addition of NaN_3 (n=5). The corresponding leak current amplitudes were 0.9±0.2 pA (VC3) and 1.0±0.3 pA (VC4), respectively (n=3). The inhibitory effect of NaN_3 in $SUR1^{-/-}$ beta cells is unlikely to be mediated by glucose metabolism as it also appeared in the presence of 0.5 mmol/l glucose (n=4).

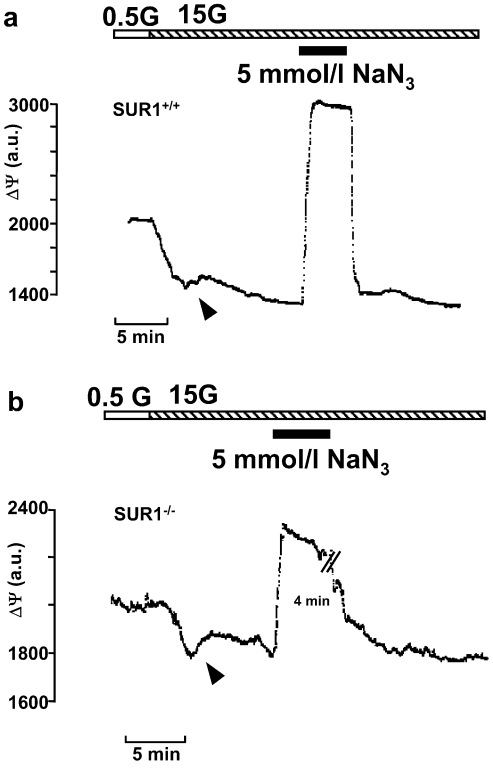

Fig. 6a, b. Effects of glucose concentration and NaN_3 on mitochondrial membrane potential ($\Delta\Psi$). (**a**) $SUR1^{+/+}$ (**b**) and $SUR1^{-/-}$ beta cells. Increasing glucose from 0.5 to 15 mmol/l hyperpolarised $\Delta\Psi$ in both cell types. This is indicated by the decrease in fluorescence (a.u.=arbitrary units) followed by a small increase (*arrowheads*) due to Ca^{2+} influx into cytosol and mitochondria. In 15 mmol/l glucose, NaN_3 depolarized $\Delta\Psi$. The effects of glucose and NaN_3 were smaller in $SUR1^{-/-}$ versus $SUR1^{+/+}$ beta cells. These recordings are representative of twenty-one (glucose) and six (NaN_3) experiments with $SUR1^{+/+}$ beta cells and thirteen (glucose) and twelve (NaN_3) experiments with $SUR1^{-/-}$ beta cells

Ca^{2+} homeostasis and ATP production are tightly coupled in normal beta cells raising the question of how the loss of glucose-dependent regulation of $[Ca^{2+}]_c$ in isolated $SUR1^{-/-}$ beta cells influences their

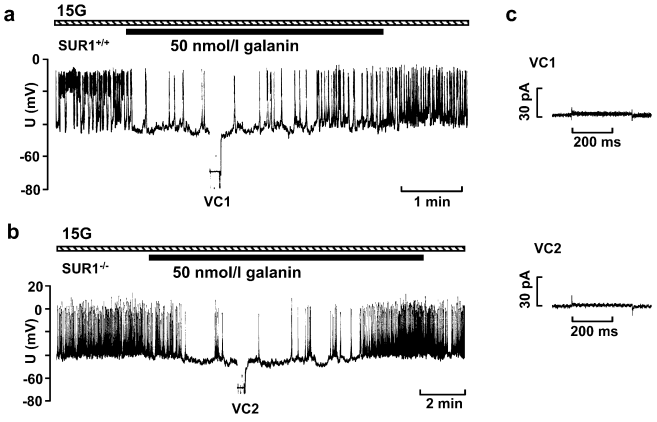

Fig. 7. Effects of galanin on beta-cell membrane potential and currents. (**a**) SUR1$^{+/+}$ (**b**) and SUR1$^{-/-}$ beta cells. Galanin (50 nmol/l) slightly hyperpolarized the membrane potential and transiently suppressed electrical activity in both cell types. The currents measured during voltage pulses to –60 mV (VC1 and VC2) in the presence of galanin are illustrated in (**c**). These recordings are representative of nine experiments with SUR1$^{+/+}$ beta cells and five experiments with SUR1$^{-/-}$ beta cells

metabolism. The mitochondrial membrane potential, $\Delta\Psi$, reflects ATP production and thus changes in beta-cell metabolism in response to glucose. Increasing the glucose concentration from 0.5 mmol/l to 15 mmol/l induced a clear hyperpolarization of $\Delta\Psi$ in both SUR1$^{+/+}$ (Fig. 6a) and SUR1$^{-/-}$ beta-cells (Fig. 6b) followed by a smaller depolarization. The hyperpolarization is caused by an increase in ATP production while the depolarization (arrow heads in Fig. 6) is a consequence of Ca^{2+} influx [24]. The addition of NaN_3 in 15 mmol/l glucose depolarized $\Delta\Psi$ in both cell types. The glucose-evoked hyperpolarization was larger in wildtype (647±49 a.u., n=21, or 48±3% compared to maximal depolarization achieved with 5 mmol/l NaN_3, respectively, n=6) than knock-out beta cells (365±74 a.u., n=13; $p \leq 0.002$, or 26±2% compared to maximal depolarization by 5 mmol/l NaN_3, respectively, n=12; $p \leq 0.0001$). The result suggests that the mitochondria of SUR1$^{-/-}$ beta cells do not hyperpolarize to the same extend as in wildtype beta cells and implies a lower rate of ATP production. This might, at least in part, be due to the higher $[Ca^{2+}]_c$ in the knock-out beta cells which is expected to induce a depolarizing current in the inner mitochondrial membrane [24]. However, hyperpolarization of SUR1$^{-/-}$ beta cell mitochondria was not increased by removing extracellular Ca^{2+} (n=14), thus additional factors contribute to this difference.

Effects of galanin on SUR1+/+ and SUR1–/– beta cells. In addition to metabolic signals, beta-cell activity is regulated by hormones and neuropeptides. The inhibitory effect of galanin on insulin secretion has been suggested to require activation of K_{ATP} channels in various insulin-secreting cell lines [12, 30]. We tested the ability of this neuropeptide to hyperpolarize SUR1$^{+/+}$ vs SUR1$^{-/-}$ beta cells and affect $[Ca^{2+}]_c$. Galanin slightly hyperpolarized the membrane potential transiently suppressing spike activity in both SUR1$^{+/+}$ (Fig. 7a) and SUR1$^{-/-}$ beta cells (Fig. 7b) (–10±3 mV, n=9 vs –7±1 mV, n=5, respectively). The galanin-induced current is too small to be detected under these conditions even in the absence of K_{ATP} currents. The average membrane conductance in SUR1$^{+/+}$ beta cells amounted to 0.20±0.04 nS in the presence of 15 mmol/l glucose and 0.31±0.07 nS after addition of 50 nmol/l galanin (n=9). In SUR1$^{-/-}$ beta cells the

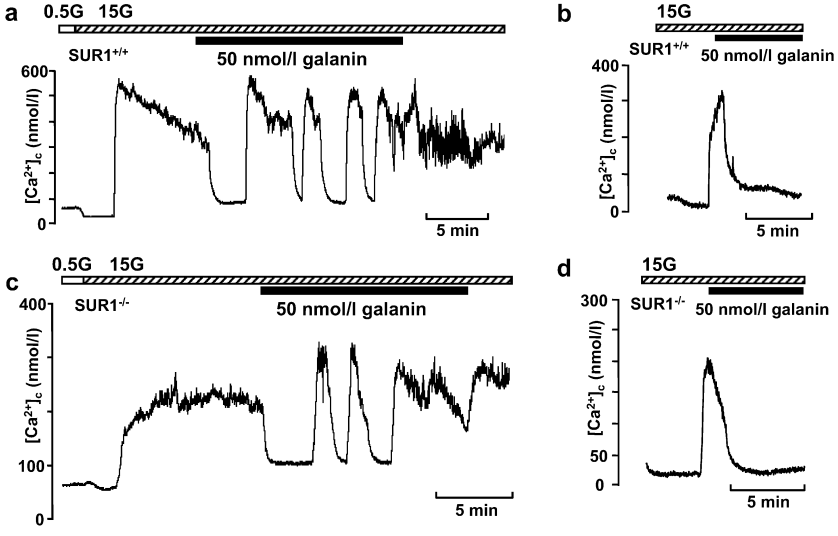

Fig. 8a–d. Effects of galanin on beta-cell $[Ca^{2+}]_c$. (**a, b**) SUR1$^{+/+}$ and (**c, d**) SUR1$^{-/-}$ beta cells. 50 nmol/l galanin transiently decreased $[Ca^{2+}]_c$ and subsequently induced oscillations in SUR1$^{+/+}$ as well as SUR1$^{-/-}$ beta cells in 15 mmol/l glucose. In **b** and **d** galanin was added immediately after the first rise of $[Ca^{2+}]_c$ in 15 mmol/l glucose to show that the effect of galanin is independent of the point of application. The recordings are representative of thirteen (**a**) and three (**b**) experiments for SUR1$^{+/+}$ and six (**c**) and three (**d**) experiments for SUR1$^{-/-}$ beta cells

average conductance was 0.09±0.03 nS before and 0.11±0.02 nS after treatment with galanin (n=3). As the beta-cell membrane resistance is high, even small changes in current are sufficient to evoke membrane hyperpolarization. Galanin lowered $[Ca^{2+}]_c$ in both cell types (Fig. 8a–d), but consistent with the incomplete suppression of spike activity the effect was transient. In $SUR1^{+/+}$ beta cells the first peak after increasing the glucose concentration from 0.5 mmol/l to 15 mmol/l reached 407±21 nmol/l (n=16). Application of galanin led to a rapid, transient decrease to 96±8 nmol/l (n=16; $p \leq 0.0001$) followed by $[Ca^{2+}]_c$ oscillations and/or a steady sustained level. The galanin induced changes in $[Ca^{2+}]_c$ in $SUR1^{-/-}$ beta cells were similar; before addition, $[Ca^{2+}]_c$ was 276±21 nmol/l (n=9) in the presence of 15 mmol/l glucose, then dropped to 105±19 nmol/l (n=9; $p \leq 0.0001$) after application before oscillations resumed. To ensure the lowering of $[Ca^{2+}]_c$ was not coincidental with the end of a phase with increased $[Ca^{2+}]_c$, galanin was applied immediately after the beginning of the first rise in $[Ca^{2+}]_c$ observed with 15 mmol/l glucose in 3 out of the 16 experiments with $SUR1^{+/+}$ cells (Fig. 8b) and three out of the nine experiments with $SUR1^{-/-}$ beta cells (Fig. 8d).

Discussion

In SUR1 knock-out mice the beta-cell membrane potential, measured using the whole-cell configuration of the patch-clamp technique, was depolarized with continuous spike activity occurring in all cells independent of the glucose concentration. This is in agreement with the occurrence of spontaneous, Ca^{2+}-dependent action potentials observed in all beta cells lacking functional K_{ATP} channels, i.e., beta cells from Kir6.2 [5] and SUR1 knock-out mice [6, 7] or beta cells from patients with PHHI (persistent hyperinsulinaemic hypoglycaemia of infancy) [8], a disorder which can be caused by the loss of functional K_{ATP} channels [8]. The depolarized membrane potential, Ca^{2+}-dependent action potentials, and increased $[Ca^{2+}]_c$ suggest that insulin secretion should be increased and result in hypoglycaemia. This is the case in patients with PHHI, but not in $SUR1^{-/-}$ or $Kir6.2^{-/-}$ mice which are mildly glucose intolerant, but not hypoglycaemic [5, 6, 7]. Thus rodents are able to compensate for the lack of functional K_{ATP} channels.

The $[Ca^{2+}]_c$ oscillations that occur in some $SUR1^{-/-}$ beta cells were unexpected for two reasons: First, the generally accepted assumption is that changes in beta-cell membrane potential govern changes in $[Ca^{2+}]_c$ [26] and thus insulin secretion [31]. Second, K_{ATP} channels have been considered to play a key role in the oscillatory activity of beta cells [24, 32, 33, 34]. For example, it has been reported recently that a rise in $[Ca^{2+}]_c$ lowers ATP production evoking an increase in the K_{ATP} currents which act as a negative feedback to reduce Ca^{2+} influx by hyperpolarizing the beta cell [35]. In normal beta cells oscillations in $[Ca^{2+}]_c$ are thought to be necessary for pulsatile insulin secretion [36]. Whether this applies to $SUR1^{-/-}$ beta cells remains to be verified. Our patch-clamp experiments could be interpreted to suggest that $[Ca^{2+}]_c$ oscillations in isolated $SUR1^{-/-}$ beta cells are regulated independently of membrane potential. However, it is often difficult to detect membrane potential oscillations when patch-clamping single or small clusters of beta cells [27, 28, 29], although they invariably occur in experiments with intact islets [37], when electrically coupled cells, surrounded by nerve endings and blood vessels, are impaled by intracellular microelectrodes. In short, microelectrode impalements are a better reflection of the situation in vivo than patch-clamp experiments. Of interest, it has been reported [38] that the pattern of $[Ca^{2+}]_c$ oscillations observed in single beta cells can differ markedly from those recorded with intact islets. Our data show that the membrane potential of $SUR1^{-/-}$ beta cells can oscillate in the absence of functional K_{ATP} channels. This points to the existence of a second underlying oscillator able to substitute in $SUR1^{-/-}$ mice for the one in WT beta cells involving K_{ATP} channels. Several lines of evidence suggest that this secondary oscillator may not be operative in normal mouse beta cells. First, it is almost impossible to obtain continuous oscillations of membrane potential or $[Ca^{2+}]_c$ in wildtype islets comparable to the behaviour of $SUR1^{-/-}$ islets when K_{ATP} channels are inhibited by tolbutamide and the perifusion time with glucose-free medium is sufficient to exclude the influence of glucose metabolism [39]. Second, oscillations are not observed when K_{ATP} channels are opened with diazoxide and cells are depolarized by increasing the external K^+ concentration [40, 41]. Recently, it has been shown that oscillatory activity in wildtype beta cells involves activation of a low-conductance Ca^{2+}-dependent K^+ current ($I_{K,slow}$) [42, 43].

While both membrane potential and $[Ca^{2+}]_c$ clearly oscillate in $SUR1^{-/-}$ beta cells in islets, determining whether membrane potential and $[Ca^{2+}]_c$ are as tightly coupled as in control beta cells [26] will require simultaneous measurement of Vm and $[Ca^{2+}]_c$. The experiments with galanin support the view that changes in $[Ca^{2+}]_c$ in $SUR1^{-/-}$ beta cells, like $SUR1^{+/+}$ beta cells, are governed by changes in membrane potential since galanin-induced hyperpolarization caused a decrease in $[Ca^{2+}]_c$ in both. Patch-clamp recordings from isolated beta cells from PHHI patients exhibit Ca^{2+}-dependent spikes or action potentials equivalent to those observed in $SUR1^{-/-}$ beta cells. We are unaware of any intracellular microelectrode studies on PHHI islets. One plausible explanation for the severity of the disturbance in insulin secretion in PHHI patients versus the knock-out mouse models is that PHHI beta

cells are persistently depolarized in islets. Corroboration for this speculation will require measurement of the beta-cell membrane potential in PHHI islets using intracellular microelectrodes.The discrepancy in glucose homeostasis between animal models lacking functional beta-cell K_{ATP} channels and PHHI patients raises interesting questions about the mechanisms involved in normalising insulin secretion in the knock-out animals. We used the metabolic inhibitor NaN_3 to test the possibility that metabolic factors influence beta-cell activity and thus insulin secretion at a site distinct from K_{ATP} channels. NaN_3 increased the K_{ATP} current amplitude and thus hyperpolarized the plasma membrane potential of beta cells from wildtype mice (Fig. 5, [44]), an effect attributed to reduced ATP production and diminished ATP/ADP. Remarkably, NaN_3 influenced the electrical activity of $SUR1^{-/-}$ beta cells by decreasing spike activity without concomitant membrane hyperpolarization. We recently showed that NaN_3 inhibits L-type Ca^{2+} currents in $SUR1^{+/+}$ beta cells using the standard whole-cell configuration [44]. This implies the reduction of Ca^{2+} action potentials in $SUR1^{-/-}$ beta cells could result from a direct effect of NaN_3 on the L-type Ca^{2+} channel. We cannot exclude that inhibition of mitochondrial metabolism has additional effects on L-type Ca^{2+} channel activity as suggested by [45] in experiments with oligomycin . The data imply that the membrane hyperpolarization induced by decreasing the metabolic rate in $SUR1^{+/+}$ beta cells is mediated by openings of K_{ATP} channels, and that decreased ATP/ADP alone does not influence the membrane potential in $SUR1^{-/-}$ beta cells. Activation of other K^+ channels by adrenaline or neuropeptides, e.g. somatostatin or galanin, known to reduce glucose-induced insulin secretion, are candidates for suppression of the hyperinsulinaemia observed in PHHI and could contribute to glucose homeostasis in K_{ATP}-channel deficient mice. Somatostatin is used to treat PHHI patients [46] where it is suggested to activate a K^+ channel distinct from the K_{ATP} channel. Galanin has been reported to lower insulin secretion via a direct effect on exocytosis [13, 16] and has been suggested to increase the K^+ permeability of beta cells [11, 15]. One report [15] shows that clonidine induces a K^+ current insensitive to sulphonylureas in mouse beta cells. For galanin a direct proof of the nature of the K^+ channel underlying membrane hyperpolarisation is still lacking. Direct activation of K_{ATP} channels has been described for various tumour cell lines [12, 30], but these results may not directly transfer to the normal situation. Our experiments indicate that there is no significant difference in the action of galanin on $SUR1^{+/+}$ compared with $SUR1^{-/-}$ beta cells and exclude the possibility that galanin evokes membrane hyperpolarisation and lowering of $[Ca^{2+}]_c$ via K_{ATP} channels.

The mitochondrial membrane potential, $\Delta\Psi$, governs ATP production and thus reflects cellular metabolic status. The glucose-induced hyperpolarization and NaN_3-induced depolarization of $\Delta\Psi$ are markedly lower in beta cells from $SUR1^{-/-}$ mice implying reduced ATP synthesis in the knock-out beta cells. This suggests that $[Ca^{2+}]_c$ may not be strongly coupled to glucose metabolism in $SUR1^{-/-}$ beta cells. Changes in $[Ca^{2+}]_c$ are known to influence $[Ca^{2+}]_m$, the mitochondrial Ca^{2+} concentration [47, 48] and persistent increased $[Ca^{2+}]_c$ in the presence of a low glucose could provoke Ca^{2+} influx into mitochondria and result in their depolarization. This would increase the open probability of the mitochondrial transition pore (mPTP) [49] and would explain the decreased hyperpolarization of $\Delta\Psi$ in $SUR1^{-/-}$ beta cells. This hypothesis is not fully supported by our experiments with Ca^{2+}-free medium, therefore additional factors appear to contribute to impaired mitochondrial function.

In conclusion, the new insights gained from these experiments with K_{ATP}-channel deficient mice are: (i) Oscillations in plasma membrane potential and $[Ca^{2+}]_c$ can occur despite the loss of channels presumed to play a key role in normal beta-cell oscillations; (ii) the oscillations of Vm and $[Ca^{2+}]_c$ in $SUR1^{-/-}$ beta cells are not driven by glucose metabolism, but changes in $[Ca^{2+}]_c$ seem to be coupled to Vm; (iii) the loss of K_{ATP} channels affects mitochondrial metabolism; (iv) the action of galanin does not require K_{ATP} channels. Consequently, this neuropeptide is able to influence membrane potential and $[Ca^{2+}]_c$ in $SUR1^{-/-}$ beta cells and could constitute a K_{ATP} channel independent contribution to the maintenance of glucose homeostasis in these cells.

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