The K_{ATP} Channel is Critical for Calcium Sequestration into Non-ER Compartments in Mouse Pancreatic Beta Cells

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Key Words

Cyclopiazonic acid • Cytosolic calcium concentration • FCCP • K_{ATP} channels • Mitochondrial Ca^{2+} storage • Ratiometric pericam • Sulfonylureas • Sur1KO mice

Abstract

K_{ATP} channel activity influences beta cell Ca^{2+} homeostasis by regulating Ca^{2+} influx through L-type Ca^{2+} channels. The present paper demonstrates that loss of K_{ATP} channel activity due to pharmacologic or genetic ablation affects Ca^{2+} storage in intracellular organelles. ATP depletion, by the mitochondrial inhibitor FCCP, led to Ca^{2+} release from the endoplasmic reticulum (ER) of wildtype beta cells. Blockade of ER Ca^{2+} ATPases by cyclopiazonic acid abolished the FCCP-induced Ca^{2+} transient. In beta cells treated with K_{ATP} channel inhibitors FCCP elicited a significantly larger Ca^{2+} transient. Cyclopiazonic acid did not abolish this Ca^{2+} transient suggesting that non-ER compartments are recruited as additional Ca^{2+} stores in beta cells lacking K_{ATP} channel activity. Genetic ablation of K_{ATP} channels in SUR1KO mice produced identical results. In INS-1 cells transfected with a mitochondrial-targeted Ca^{2+} -sensitive

fluorescence dye (ratiometric pericam) the increase in mitochondrial Ca^{2+} evoked by tolbutamide was 5-fold larger compared to 15 mM glucose. These data show that genetic or pharmacologic ablation of K_{ATP} channel activity conveys Ca^{2+} release from a non-ER store. Based on the sensitivity to FCCP and the property of tolbutamide to increase mitochondrial Ca^{2+} it is suggested that mitochondria are the recruited store. The change in Ca^{2+} sequestration in beta cells treated with insulinotropic antidiabetics may have implications for beta cell survival and the therapeutic use of these drugs.

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Introduction

An increase in the cytosolic Ca^{2+} concentration ($[Ca^{2+}]_i$) is essential for stimulus-secretion coupling in pancreatic beta cells because Ca^{2+} constitutes the trigger signal for the exocytosis of insulin. Glucose, the most important natural beta cell fuel, induces closure of K_{ATP} channels via changes in the ATP/ADP ratio which lead

to cell depolarization and opening of voltage-dependent L-type Ca^{2+} channels [1, 2]. The resulting increase in $[\text{Ca}^{2+}]_c$ elicits insulin release.

The homeostasis of beta cell Ca^{2+} depends on intracellular stores that modulate the ionic signal produced by the activation of voltage-dependent Ca^{2+} channels. The endoplasmic reticulum (ER) plays a role in the modulation of glucose-induced Ca^{2+} oscillations [3, 4] and receptor-mediated mobilization of intracellular Ca^{2+} [5, 6], while mitochondria provide a link between $[\text{Ca}^{2+}]_c$ and ATP production [7, 8].

An increase in glucose metabolism transiently lowers $[\text{Ca}^{2+}]_c$ [9] before opening of voltage-dependent Ca^{2+} channels. The initial drop in $[\text{Ca}^{2+}]_c$ is due to the activation of Ca^{2+} ATPases (SERCAs) which pump Ca^{2+} into the ER in response to enhanced ATP production. This glucose-dependent ER filling may provide Ca^{2+} for mobilization by hormones and neurotransmitters [5, 6] or for Ca^{2+} -induced Ca^{2+} release [10]. Ca^{2+} release from the ER may amplify depolarization-induced Ca^{2+} signalling in beta cells. Pharmacologic emptying of intracellular Ca^{2+} stores influences the shape and amplitude of glucose-induced Ca^{2+} oscillations probably because Ca^{2+} in the ER also oscillates due to regular uptake and efflux of the ion in the presence of glucose [3]. Moreover, the membrane potential of beta cells is influenced by the filling state of the ER since the inhibition of SERCAs results in membrane depolarization [3].

Mitochondria also serve as a Ca^{2+} store in pancreatic beta cells and glucose metabolism triggers Ca^{2+} uptake into both the ER and mitochondria in insulin-secreting INS-1 cells [11–13]. Ca^{2+} uptake into mitochondria is strongly coupled to ATP production. The high electrochemical gradient across the inner mitochondrial membrane favours ATP production and entry of Ca^{2+} into the mitochondrial matrix. Ca^{2+} influx into the mitochondria affects ATP production [8] and thus influences beta cell electrical activity via plasma membrane K_{ATP} channels. We have proposed previously that cyclic alternations in mitochondrial Ca^{2+} uptake are critically involved in beta cell oscillatory activity by inducing fluctuations in mitochondrial ATP production which in turn provoke periodic changes in the opening and closure of the K_{ATP} channels [8]. Thus, K_{ATP} channel activity, Ca^{2+} homeostasis and cell metabolism are tightly coupled in beta cells. We used pharmacologic inhibitors of K_{ATP} channels that bind to the SUR1 subunit and SUR1KO mice lacking functional K_{ATP} channels [14] to evaluate changes in beta cell Ca^{2+} handling induced by ablation of K_{ATP} channel activity.

The present paper demonstrates that the ER is the

major Ca^{2+} store in wildtype (WT) mouse beta cells. Disruption of K_{ATP} channel function recruits additional non-ER Ca^{2+} stores, most likely mitochondria. The results show that pharmacologic reduction of K_{ATP} channel activity or genetic ablation of the SUR1 subunit changes Ca^{2+} sequestration in pancreatic beta cells and thus may severely alter beta cell metabolism.

Materials and Methods

Cell and islet preparation

Experiments were performed using clusters or single pancreatic beta cells isolated from fed C57Bl/6 (Charles River, Sulzfeld, Germany) or SUR1KO mice killed by CO_2 . Islets were isolated by collagenase digestion of the pancreata. For generation of clusters or single cells, islets were dispersed in Ca^{2+} -free medium and cultured up to 4 days in RPMI 1640 medium (11.1 mM glucose) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 $\mu\text{g}/\text{ml}$ streptomycin [15]. INS-1 cells were cultured in RPMI 1640 supplemented with 10% fetal calf serum, 1 mM HEPES, 1 mM glutamine, 1 mM pyruvate, 10 μM beta mercaptoethanol and antibiotics as described by Ullrich et al. [16]. The principles of laboratory animal care (NIH publication no. 85-23, revised 1985) and German laws were followed. Animals were maintained in the breeding facility of the Dept. of Pharmacology, University of Tübingen, Germany.

Measurement of $[\text{Ca}^{2+}]_c$

The cytosolic free Ca^{2+} concentration ($[\text{Ca}^{2+}]_c$) was measured at 37 °C in single cells or small clusters by the fura-2 method according to Grynkiewicz *et al.* [17] using equipment and software from TILL photonics (Gräfelfing, Germany). Cells were identified as beta cells when $[\text{Ca}^{2+}]_c$ was not decreased by 15 mM glucose as described for alpha cells [18]. The cells were loaded with fura-2AM (5 μM) 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 40x 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 340nm/380nm excitation was used to calculate $[\text{Ca}^{2+}]_c$ following an *in vitro* calibration with fura-2 K^+ -salt.

Measurement of $[\text{Ca}^{2+}]_m$

Mitochondrial Ca^{2+} was measured in INS-1 cells 36 to 60 h after transfection with ratiometric pericam targeted to mitochondria by encoding the N-terminal 12-aa presequence of subunit IV of cytochrom c oxidase [19]. Transfection was performed with Effectene reagent (Qiagen) as described in the Effectene Transfection Reagent Handbook. Ratiometric pericam was excited alternately at 410 nm and 480 or 490 nm and the emitted light was filtered through a bandpass filter of 530/50 nm.

Fig. 1. FCCP-inducible Ca^{2+} release in WT beta cells is abolished by CPA. *A*, $[\text{Ca}^{2+}]_c$ was oscillating in the presence of 15 mM glucose (15 G). The time in which a particular substance is added to the bath solution is indicated in all figures by horizontal bars. Addition of the mitochondrial uncoupler FCCP (0.5 μM) resulted in a transient increase in $[\text{Ca}^{2+}]_c$ which is due to Ca^{2+} release from the endoplasmic reticulum (ER) as a result of ATP depletion. The trace is representative for six experiments. The grey field highlights the amount of released Ca^{2+} (AUC_{Ca}). This also applies for Figs. 3, 4 and 5. *B*, Inhibition of SERCA induces Ca^{2+} release from ER stores (arrow in Fig. 1B) and elevates $[\text{Ca}^{2+}]_c$. *C*, $[\text{Ca}^{2+}]_c$ raised after addition of CPA (10 μM). Additional application of FCCP did not induce a transient Ca^{2+} increase but reduced $[\text{Ca}^{2+}]_c$ to basal levels. The experiments are representative for seven with similar results.

Solutions and chemicals

$[\text{Ca}^{2+}]_c$ and $[\text{Ca}^{2+}]_m$ were determined in a bath solution containing (in mM): 140 NaCl, 5 KCl, 1.2 MgCl_2 , 2.5 CaCl_2 , 10 HEPES, glucose as indicated, pH 7.4 adjusted with NaOH. Fura-2AM was obtained from Molecular Probes (Eugene, Oregon, USA). RPMI 1640 medium was from PromoCell (Heidelberg, Germany); foetal bovine serum and penicillin/streptomycin were from GIBCO/BRL (Karlsruhe, Germany). All other chemicals were purchased from Sigma (Deisenhofen, Germany) and Merck (Darmstadt, Germany) in the purest form available.

Presentation of results

Experiments are illustrated by recordings representative of the indicated number of experiments carried out with different cells. At least three different cell preparations were used for each series of experiments. To quantify the relative amount of Ca^{2+} released by FCCP the area under the curve (AUC_{Ca}) was calculated (see grey shaded area in Figs. 1, 3, 4 and 5). Changes of $[\text{Ca}^{2+}]_m$ are expressed as changes in fluorescence ratio (F_{490}/F_{410}). Where possible the means $\pm$ SEM are given in the text for the indicated number of experiments. The statistical significance of differences between means was assessed by a one sample t-test or Student's t-test 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 K_{ATP} channel activity on intracellular Ca^{2+} sequestration in mouse pancreatic beta cells

The ER is a predominant site of Ca^{2+} storage in pancreatic beta cells. Raising the extracellular glucose concentration leads to ATP-dependent Ca^{2+} storage via increased SERCA activity in the ER [9]. Inhibition of SERCA either by thapsigargin or depletion of ATP by the protonophore FCCP or the cytochrome a_3 inhibitor NaN_3 , respectively, induces Ca^{2+} release from the ER [20]. Despite the different mode of action on mitochondrial function FCCP and NaN_3 elicit almost identical effects on the mitochondrial membrane potential and ER Ca^{2+} release indicating that impairment of mitochondrial function is the common property which induces Ca^{2+} release from the ER [8, 20]. Figure 1A confirms that uncoupling mitochondria with FCCP elicits a large transient increase in $[\text{Ca}^{2+}]_c$ (grey shaded area). FCCP (0.5 μM) transiently elevated $[\text{Ca}^{2+}]_c$ by 251 ± 36 nM (from 355 ± 39 nM to a peak value of 606 ± 74 nM; $n=6$, $p \leq 0.05$), although

Fig. 2. Summary of effects of tolbutamide and CPA on FCCP-induced Ca²⁺ transients in WT beta cells. Column I and III represent Ca²⁺ release induced by ATP depletion in WT beta cells with intact ER stores in the absence (I) or presence (III) of tolbutamide. After SERCA inhibition Ca²⁺ release is completely prevented when K_{ATP} channels are operative, i.e. the release is zero (II) but ER-independent Ca²⁺ release is evoked in the presence of K_{ATP} channel blocker (IV).

inhibition of ATP synthesis leads to activation of K_{ATP} current and interruption of Ca²⁺ influx. The area under the curve (AUC_{Ca}) was used as an indicator for the amount of released Ca²⁺ (grey shaded area in Fig 1A). The mean value amounted to 9594±572 nMs (Fig. 2 column I, n=6).

Emptying the Ca²⁺ stores by inhibition of SERCAs with a reversible blocker, cyclopiazonic acid (CPA, 10 μM) [21], induced an initial rise due to Ca²⁺ release from the ER (arrow in Fig. 1B) and changed the oscillatory pattern of [Ca²⁺]_c. Under these conditions FCCP failed to elicit Ca²⁺ release (Figure 1C, n=7). Instead, [Ca²⁺]_c immediately dropped after application of FCCP from 330±50 nM to 129±21 nM (n=7, p<0.01) due to opening of K_{ATP} channels and subsequent closure of voltage-dependent Ca²⁺ channels [20]. Ca²⁺ extrusion may occur via the Na⁺/Ca²⁺ exchanger [22]. Before addition of the uncoupler [Ca²⁺]_c amounted to the same level as in the experiments without CPA (330±50 vs 355±39 nM) demonstrating that the lack of the Ca²⁺ peak was not a result of variations in [Ca²⁺]_c prior to FCCP application. In conclusion, with operative K_{ATP} channels the FCCP-

Fig. 3. Inhibition of K_{ATP} channels evoked an additional Ca²⁺ store in WT beta cells. *A*, In beta cells treated with tolbutamide (tolb, 100 μM) which abolishes K_{ATP} current the FCCP-induced Ca²⁺ release was markedly enhanced. The trace is representative of three recordings. *B*, FCCP elicited a smaller but marked Ca²⁺ release in the presence of CPA, when the cells were treated with 100 μM tolbutamide. One experiment out of three with similar results.

induced Ca²⁺ transient is solely due to Ca²⁺ release from ER Ca²⁺ stores.

Tolbutamide (1 mM) administered approximately 5 min before addition of FCCP markedly increased FCCP-induced Ca²⁺ release giving a mean AUC_{Ca} of 20522±4030 nMs, (Fig. 2 column III, n=5, p<0.001), more than twice the value of control beta cells (compare Fig. 2 column I and III). Most strikingly, CPA (10 μM) did not prevent FCCP-induced Ca²⁺ release in tolbutamide-

Fig. 4. FCCP-induced Ca^{2+} release in CPA-treated SUR1KO beta cells. *A*, In SUR1KO beta cells FCCP ($0.5 \mu\text{M}$) elicited a Ca^{2+} release which is approximately twice as large as in WT cells not treated with CPA and tolbutamide. One experiment out of five with similar results. *B*, In SUR1KO cells FCCP also induces Ca^{2+} release when the ER Ca^{2+} stores were emptied with CPA. The amount of released Ca^{2+} is, however, considerably lower. This indicates that beta cells lacking K_{ATP} channels have an additional, non-ER Ca^{2+} store. The result is representative for thirteen experiments. *C*, Comparable to pharmacological inhibition of K_{ATP} channel activity with tolbutamide (Fig. 2 column III and IV) Ca^{2+} release is increased (I) and partly ER-independent (II) in

treated beta cells (Fig. 2 column IV), but the AUC_{Ca} of 8157 ± 2173 nMs was considerably lower than with tolbutamide alone (compare Fig. 2 column IV and III, $n=4$, $p \leq 0.01$). In addition to AUC , we quantified the relative amount of Ca^{2+} released by FCCP by determining the full width (time) at half maximum of the release peak ($\text{width}_{0.5\text{P}}$, dashed line in Fig. 1A). This method provided comparable changes. $\text{Width}_{0.5\text{P}}$ was 32 ± 4 s under control conditions (Fig. 1A), increased with 1 mM tolbutamide to 89 ± 10 s ($P \leq 0.001$) and amounted to 35 ± 7 s with CPA and tolbutamide. Thus blockade of beta cell K_{ATP} channels enhanced the amount of Ca^{2+} released, presumably from non-ER pools. Concerning tolbutamide, similar results were achieved with a lower concentration of tolbutamide ($100 \mu\text{M}$) or with nateglinide, another K_{ATP} channel blocker structurally unrelated to sulfonylureas. With $100 \mu\text{M}$ tolbutamide the AUC_{Ca} amounted to 18455 ± 4457 nMs (Fig. 3A). After addition of CPA the FCCP-induced Ca^{2+} release was reduced to 6614 ± 2522 nMs (Fig. 3B, $n=3$, not significant vs 1 mM tolbutamide). In the presence of $50 \mu\text{M}$ nateglinide and $10 \mu\text{M}$ CPA FCCP produced a Ca^{2+} release with a mean AUC_{Ca} of 4353 ± 629 nMs ($n=4$, not significant vs data with tolbutamide, data not shown). This confirms that the results are not restricted to high concentrations of tolbutamide.

FCCP-induced Ca^{2+} release in K_{ATP} channel-deficient beta cells.

To further analyze the role of K_{ATP} channels in Ca^{2+} handling, we tested the effect of FCCP in K_{ATP} channel-deficient SUR1KO beta cells. FCCP ($0.5 \mu\text{M}$) elevated $[\text{Ca}^{2+}]_{\text{c}}$ by 328 ± 27 nM (Fig. 4A) ($n=5$) in SUR1KO beta cells, equally to wildtype (WT) cells. However, the Ca^{2+} transient evoked by FCCP (grey shaded area Fig. 4A) was larger than in WT beta cells (compare Fig. 4C column I to Fig. 2 column I) but similar to WT cells treated with tolbutamide (compare Fig. 4C column I to Fig. 2 column III). On average, the AUC_{Ca} amounted to 23402 ± 4462 nMs ($n=5$) in SUR1KO beta cells ($n=6$, $p \leq 0.01$ vs WT). Thus the FCCP-induced Ca^{2+} transient is increased by K_{ATP} channel knock-out. In contrast to WT cells emptying ER Ca^{2+} stores with CPA did not suppress the FCCP-induced Ca^{2+} transient in SUR1KO beta cells but reduced it (Fig. 4B). The AUC_{Ca} of the Ca^{2+} transient was 13519 ± 1824 nMs, significantly lower compared to the value without CPA (Fig. 4C, $n=13$, $p \leq 0.05$). The maximal increase was not altered (285 ± 19 nM, $n=13$). These data demonstrate large ER-independent Ca^{2+} transients in SUR1KO beta cells and WT cells treated with K_{ATP} channel blockers suggesting increased Ca^{2+} sequestration into non-ER compartments.

Fig. 5. High $[Ca^{2+}]_c$ is not sufficient to sequester Ca^{2+} into non-ER Ca^{2+} stores. *A*, In WT beta cells which were treated with 10 mM arginine (arg) in the presence of 15 G and CPA (10 μ M) $[Ca^{2+}]_c$ amounted to the same value as in the cells treated with tolbutamide (1 mM) and 15 G. The addition of FCCP reduced $[Ca^{2+}]_c$. One out of four similar experiments. *B*, When WT beta cells were bathed in 15 G, CPA, arginine and tolbutamide, Ca^{2+} release was evoked by FCCP, pointing to the blockade of K_{ATP} channels rather than a high $[Ca^{2+}]_c$ as the reason for non-ER Ca^{2+} storage. One representative trace out of four.

High $[Ca^{2+}]_c$ is not decisive for recruitment of non-ER Ca^{2+} stores

Treatment of beta cells with tolbutamide [23] induces permanent membrane depolarisation and enhances $[Ca^{2+}]_c$ due to the increased Ca^{2+} influx. To test whether a permanently elevated $[Ca^{2+}]_c$ may be the reason for Ca^{2+} storage in non-ER organelles, $[Ca^{2+}]_c$ in WT beta cells was increased by three different manoeuvres: L-arginine (10 mM) in the presence of 15 mM glucose (Fig. 5 and table), elevated glucose (table) and a high extracellular K^+ concentration (table). Under each condition ER stores were emptied by CPA and the rapid FCCP-induced change in $[Ca^{2+}]_c$ was monitored with and without sulfonylurea (tolbutamide or nateglinide). Data are presented as FCCP-induced decrease and increase, respectively, in the table. L-arginine, being positively charged at physiological pH, depolarizes beta cells because it enters cells through an electrogenic pathway [23]. With L-arginine in the presence of CPA $[Ca^{2+}]_c$ was permanently increased similar to the level in CPA-treated SUR1KO beta cells (275 ± 32 nM; $n=4$ vs 220 ± 13 nM, $n=13$; compare Fig. 5A to Fig. 4B). FCCP failed to elicit Ca^{2+} release under these conditions, producing an immediate drop in $[Ca^{2+}]_c$ to 55 ± 13 nM (Fig. 5A, $n=4$, $p \leq 0.001$). A Ca^{2+} release was induced by FCCP when

	FCCP-induced decrease [nM]	FCCP-induced transient increase [nM]	n
WT + CPA			
15 mM G	-201 $\pm$ 42		7
15 mM G + nate		141 $\pm$ 39	5
15 mM G + tol		175 $\pm$ 20	5
40 mM G	-257 $\pm$ 22		5
40 mM G + tol		152 $\pm$ 22	5
10 mM arg	-220 $\pm$ 26		4
10 mM arg + tol		138 $\pm$ 14	4
30 mM K^+	-127 $\pm$ 11		4
30 mM K^+ + tol		173 $\pm$ 11	4
SUR1KO + CPA			
15 mM G		285 $\pm$ 19	13

Table. Change in $[Ca^{2+}]_c$ after addition of FCCP (0.5 μ M) under different conditions. In all experiments 10 μ M CPA was present. Tolbutamide (tolb) was used in a concentration of 1 mM, nateglinide (nate) in a concentration of 50 μ M. The data give the change in $[Ca^{2+}]_c$ immediately after addition of FCCP. G = glucose; arg = arginine.

additionally K_{ATP} channels were inhibited by 1 mM tolbutamide (Fig. 5B). The AUC_{Ca} was 5079 ± 1005 nMs ($n=4$) in this series of experiments. This effect was not owing to elevated $[Ca^{2+}]_c$ which amounted to 296 ± 37 nM after application of the sulfonylurea ($n=4$, ns vs arginine/CPA). Similarly, in experiments with a high glucose concentration (40 mM) and a high K^+ concentration (30 mM) the FCCP-induced Ca^{2+} release was only evoked in sulfonylurea-treated beta cells (table). In the presence of 40 mM glucose and tolbutamide the AUC_{Ca} was 7871 ± 1491 nMs ($n=5$, not shown) and with 30 mM K^+ and tolbutamide the Ca^{2+} release amounted to 6265 ± 987 nMs in the first 60 s after addition of FCCP ($n=4$, not shown). These experiments suggest that suppression of K_{ATP} channel activity, not elevation of $[Ca^{2+}]_c$, triggers altered Ca^{2+} release.

The table summarizes the changes in $[Ca^{2+}]_c$ observed directly after addition of FCCP. The data illustrate that the immediate response to FCCP was a decrease in $[Ca^{2+}]_c$ in cells with functional K_{ATP} channels, but an increase after pharmacologic or genetic deletion of the channels. This confirms the release of Ca^{2+} from ATP-dependent non-ER organelles in the absence of functioning K_{ATP} channels.

Fig. 6. Elevation of mitochondrial Ca^{2+} influx in response to glucose and tolbutamide in INS-1 cells. *A*, Increasing glucose from 0.5 to 15 mM induced oscillations of $[\text{Ca}^{2+}]_{\text{m}}$. *B*, Compared to 15 mM glucose, 1 mM tolbutamide elevated $[\text{Ca}^{2+}]_{\text{m}}$ to a much larger extent. Mitochondrial Ca^{2+} stores were emptied by 0.5 μM FCCP. The recordings are representative for seven (15 G), six (tolbutamide) and three (FCCP) experiments, respectively.

Increase of mitochondrial Ca^{2+} storage by K_{ATP} channel inhibition in INS-1 cells

To elucidate to what extent mitochondria serve as intracellular Ca^{2+} stores, INS-1 cells were transiently transfected with a mitochondrial-targeted Ca^{2+} -sensitive fluorophore, ratiometric pericam. Elevating glucose from 0.5 to 15 mM induced small oscillations of mitochondrial Ca^{2+} ($[\text{Ca}^{2+}]_{\text{m}}$) (Fig. 6A). By contrast, 1 mM tolbutamide evoked an increase of $[\text{Ca}^{2+}]_{\text{m}}$ (Fig. 6B) that was about 5-fold larger compared to the amplitude of glucose-induced oscillations. On average, $[\text{Ca}^{2+}]_{\text{m}}$ was elevated by 56 ± 3 a.u. in the presence of 15 mM glucose ($n=7$) and by 246 ± 41 a.u. after addition of tolbutamide ($n=9$, $p < 0.001$). These data show that mitochondrial Ca^{2+} accumulation is drastically increased in response to sulfonylurea application. The mitochondrial Ca^{2+} accumulated in the presence of tolbutamide could be completely released by FCCP (0.5 μM , Fig. 6B) and $[\text{Ca}^{2+}]_{\text{m}}$ dropped by 254 ± 58 a.u. immediately after addition of FCCP ($n=3$).

Discussion

The present paper demonstrates that pharmacologic inhibition or knock-out of the SUR1 subunit of K_{ATP} channels dramatically affects beta cell Ca^{2+} handling.

We have used FCCP to elucidate Ca^{2+} transients from ATP-dependent intracellular Ca^{2+} stores. FCCP rapidly depolarizes the mitochondrial membrane potential thereby reducing the intracellular ATP concentration in WT and SUR1KO beta cells [8, 24]. In WT cells with intact K_{ATP} channels the Ca^{2+} transient elicited by ATP depletion critically depends on SERCA activity. When ER stores are emptied the FCCP-induced elevation of $[\text{Ca}^{2+}]_{\text{c}}$ is completely absent consistent with transient Ca^{2+}

release from the ER. This observation implies the ER to be the predominant Ca^{2+} store in WT beta cells. However, in cells that lack functioning K_{ATP} channels the Ca^{2+} transient evoked by ATP depletion is partly ER-independent showing intracellular Ca^{2+} release from non-ER compartments. Evidently, the altered release in response to reduced ATP synthesis is the result of a rise in the total amount of stored Ca^{2+} when beta cell K_{ATP} channels are inactivated. This suggestion is supported by the following observations: 1) In WT cells treated with tolbutamide the Ca^{2+} transient is higher than without tolbutamide. 2) Emptying the ER store completely prevented the FCCP-induced Ca^{2+} transient in WT cells, but only partially reduced the FCCP-induced Ca^{2+} transient in WT cells treated with tolbutamide or with nateglinide. 3) The FCCP-induced Ca^{2+} transient is larger in SUR1KO cells than in WT cells and treatment of WT cells with tolbutamide produced an increase in the FCCP-induced Ca^{2+} transient in the same order of magnitude as in SUR1KO cells. 4) FCCP induced a Ca^{2+} transient from SUR1KO cells when ER Ca^{2+} stores were emptied.

In other tissues like vascular smooth muscle cells the contribution of the sarcoplasmic reticulum and mitochondria to Ca^{2+} storage depends on the cytosolic Ca^{2+} concentration [25, 26]. After pharmacologic inhibition of beta cell K_{ATP} channels the plasma membrane is more depolarized, does not display oscillations and $[\text{Ca}^{2+}]_{\text{c}}$ is higher than in untreated WT cells. Consequently, we investigated whether the increase in $[\text{Ca}^{2+}]_{\text{c}}$ is sufficient to alter Ca^{2+} storage. To address this question we elevated $[\text{Ca}^{2+}]_{\text{c}}$ either by treating WT cells with 10 mM arginine or with 40 mM glucose in the presence of CPA to empty ER stores. Importantly, under these conditions FCCP was unable to induce a Ca^{2+} transient and $[\text{Ca}^{2+}]_{\text{c}}$ rapidly decreased after addition of FCCP, expected to activate K_{ATP} channels and strongly hyperpolarize beta cells. A

decrease was also observed when FCCP was added in the presence of 30 mM K^+ , i.e. under conditions where changes of membrane potential in response to increased K_{ATP} channel activity are minimized. However, Ca^{2+} transients were elicited when the same manoeuvres were repeated with blocked K_{ATP} channels. These results are consistent with the idea that Ca^{2+} storage in non-ER compartments occurs upon suppression of K_{ATP} channels and not in response to an increased Ca^{2+} concentration. This hypothesis is further supported by our experiments with SUR1KO beta cells: Although $[Ca^{2+}]_c$ of these cells oscillates [24, 27, 28] the FCCP-induced Ca^{2+} release amounted to the same value as in tolbutamide-treated WT cells with steadily elevated $[Ca^{2+}]_c$.

In other cell systems mitochondria are an important store with a high Ca^{2+} buffering capacity [29-33]. The capability of mitochondrial Ca^{2+} release to influence global $[Ca^{2+}]_c$ has been shown, e.g. for T-lymphocytes and neurons [34, 35]. In WT beta cells mitochondria store Ca^{2+} , although to a lesser extent than the ER [20]. This study and previous data of Kennedy *et al.* [13] demonstrate that glucose stimulation of INS-1 cells triggers Ca^{2+} -influx into mitochondria. Consistent with a feedback mechanism in which fluctuations of $[Ca^{2+}]_m$ are a key factor producing oscillatory K_{ATP} channel activity [8], $[Ca^{2+}]_m$ was not stably elevated during glucose stimulation, but oscillated in the majority of cells investigated. Sulfonylurea treatment significantly increased mitochondrial Ca^{2+} storage in INS-1 cells. This suggests that augmented Ca^{2+} release from mitochondria is the main reason for the elevation of FCCP-induced Ca^{2+} transients in beta cells with inactive K_{ATP} channels. The uncoupler FCCP directly interferes with mitochondrial function. The decrease in mitochondrial membrane potential $\Delta\Psi$ evoked by FCCP [8] induces the opening of the permeability transition pore (PTP) and in turn Ca^{2+} release [36-38]. Consistently, we observed a drastic decrease of $[Ca^{2+}]_m$ in INS-1 cells after addition of the uncoupler. At present we cannot exclude that inhibition of mitochondrial ATP production by FCCP may influence Ca^{2+} storage in other compartments where ATP-driven pumps create a driving force for Ca^{2+} uptake, for example, granules, lysosomes or ATP-driven Ca^{2+} transport across the plasma membrane.

The alteration in Ca^{2+} handling induced by K_{ATP}

channel inhibition seems to influence beta cell metabolism. This is supported by the observation that the mitochondrial membrane potential, $\Delta\Psi$, which reflects ATP production, is more depolarized in SUR1KO beta cells compared to WT cells [24]. Interference with intracellular Ca^{2+} handling of drugs acting on the SUR1 subunit of the K_{ATP} channels could possibly contribute to the secondary failure of sulfonylureas. Moreover, Ca^{2+} overload especially in mitochondria could induce apoptosis [39, 40] which would reduce beta cell mass and decrease insulin secretion. It has been reported that sulfonylureas provoke apoptosis in insulin-secreting cells and native beta cells [41-43]. In the light of our results one could speculate that this effect is not primarily caused by increased Ca^{2+} concentration due to increased Ca^{2+} influx as a consequence of the depolarization but rather by altered Ca^{2+} storage into the intracellular compartments.

To elucidate how suppression of functioning K_{ATP} channels could influence Ca^{2+} storage in intracellular organelles further studies are required. One possibility is that intracellular organelles possess K_{ATP} channels which are hit by K_{ATP} channel blockers or knock-out of SUR1. This would decrease a depolarizing current and result in enhanced hyperpolarization which would favour Ca^{2+} uptake. However, present data argue against (SUR1/Kir6.2)- K_{ATP} channels in mitochondria or insulin containing granules [44, 45]. A second possibility is that the SUR1 subunit of plasma membrane K_{ATP} channels is a member of a protein network which couples to intracellular organelles and influences their function. Such a network is suggested by Eliasson *et al.* [45] for the SUR1 subunit, the cAMP receptor protein cAMP-GEFII and exocytosis regulating proteins in insulin containing granules. cAMP-GEFII has been shown to be involved in Ca^{2+} release from intracellular Ca^{2+} stores in INS-1 cells [46] supporting the idea that SUR1 could influence beta cell Ca^{2+} handling via cAMP-GEFII and its connection to intracellular Ca^{2+} stores.

We demonstrate in the present paper that suppression of functional SUR1 subunits of K_{ATP} channels influence intracellular Ca^{2+} storage in pancreatic beta cells by recruiting non-ER Ca^{2+} stores. This effect was observed after pharmacologic inhibition of the channels as well as after deletion of the SUR1 gene and may severely influence beta cell metabolism.

Abbreviations

[Ca²⁺]_c (cytosolic free calcium concentration); [Ca²⁺]_m (mitochondrial calcium concentration); CPA (cyclopiazonic acid); ER (endoplasmic reticulum); FCCP (carbonyl cyanide 4-trifluoromethoxyphenylhydrazone); K_{ATP} channel (ATP-dependent K⁺ channel); SUR1 (sulfonylurea receptor type 1).

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