

RESEARCH PAPER

Pharmacological stimulation and inhibition of insulin secretion in mouse islets lacking ATP-sensitive K⁺ channels

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Background and purpose: ATP-sensitive potassium channels (K_{ATP} channels) in beta cells are a major target for insulinotropic drugs. Here, we studied the effects of selected stimulatory and inhibitory pharmacological agents in islets lacking K_{ATP} channels.

Experimental approach: We compared insulin secretion (IS) and cytosolic calcium ([Ca²⁺]_c) changes in islets isolated from control mice and mice lacking sulphonylurea receptor1 (SUR1), and thus K_{ATP} channels in their beta cells (*Sur1*KO).

Key results: While similarly increasing [Ca²⁺]_c and IS in controls, agents binding to site A (tolbutamide) or site B (meglitinide) of SUR1 were ineffective in *Sur1*KO islets. Of two non-selective blockers of potassium channels, quinine was inactive, whereas tetraethylammonium was more active in *Sur1*KO compared with control islets. Phentolamine, efaroxan and alinidine, three imidazolines binding to K_{IR}6.2 (pore of K_{ATP} channels), stimulated control islets, but only phentolamine retained weaker stimulatory effects on [Ca²⁺]_c and IS in *Sur1*KO islets. Neither K_{ATP} channel opener (diazoxide, pinacidil) inhibited *Sur1*KO islets. Calcium channel blockers (nimodipine, verapamil) or diphenylhydantoin decreased [Ca²⁺]_c and IS in both types of islets, verapamil and diphenylhydantoin being more efficient in *Sur1*KO islets. Activation of α₂-adrenoceptors or dopamine receptors strongly inhibited IS while partially (clonidine > dopamine) lowering [Ca²⁺]_c (control > *Sur1*KO islets).

Conclusions and implications: Those drugs retaining effects on IS in islets lacking K_{ATP} channels, also affected [Ca²⁺]_c, indicating actions on other ionic channels. The greater effects of some inhibitors in *Sur1*KO than in control islets might be relevant to medical treatment of congenital hyperinsulinism caused by inactivating mutations of K_{ATP} channels.

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Abbreviations: [Ca²⁺]_c, cytosolic free Ca²⁺ concentration; K_{ATP} channel, ATP-sensitive potassium channel; SUR1, sulphonylurea receptor 1

Introduction

ATP-sensitive potassium channels (K_{ATP} channels) are critical for the control of insulin secretion by beta cells. Their role in stimulus-secretion coupling, which was initially established by various experimental approaches, has been strikingly highlighted by recent evidence that mutations in the genes of the channel subunits may result in congenital hyperinsulinism with life-threatening hypoglycaemia or insulin-deficient neonatal diabetes (Dunne *et al.*, 2004; Bryan *et al.*, 2007; Girard *et al.*, 2009). K_{ATP} channels are complex octameric assemblies of two proteins. In beta cells, the pore consists of K_{IR}6.2, and

the regulatory subunit consists of the sulphonylurea receptor 1 (SUR1/ABCC8), while other isoforms encoded by *ABCC9*, SUR2A or SUR2B, are present in most other tissues (Miki *et al.*, 1999; Gribble and Reimann, 2003; Bryan *et al.*, 2007). In low glucose concentrations, enough K_{ATP} channels are open to hold the beta cell membrane hyperpolarized and prevent Ca²⁺ influx into the cell. When the glucose concentration increases, the channels close, permitting depolarization to the potential of activation of voltage-gated calcium channels. The influx of Ca²⁺ then leads to an increase in the cytosolic concentration of ionized Ca²⁺ ([Ca²⁺]_c) that serves as an essential triggering signal for exocytosis of insulin granules. Full expression of the secretory response also requires generation by glucose of amplifying signals that augment the efficacy of [Ca²⁺]_c on exocytosis (Henquin, 2009).

Many pharmacological agents lower or increase blood glucose levels by stimulating or inhibiting insulin secretion through a direct action on beta cells. It is hardly surprising

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that most clinically useful drugs aiming at increasing or decreasing insulin secretion exert their effects through an action on K_{ATP} channels. However, actions at other sites have not always been ruled out, and alternative targets might prove useful for therapeutic control of beta cell secretory function (Morgan and Chan, 2001; Doyle and Egan, 2003; Gribble and Reimann, 2003; MacDonald and Wheeler, 2003; Henquin, 2004; Farret *et al.*, 2005; Jacobson and Philipson, 2007).

Previous studies, using islets isolated from mice with a genetic deletion of *Sur1* (*Sur1KO*) (Seghers *et al.*, 2000), have shown that beta cells lacking K_{ATP} channels are depolarized and display oscillations of $[Ca^{2+}]_c$ that explain their high rate of insulin secretion in the presence of low glucose concentrations, which are non-stimulatory for normal beta cells (Düfer *et al.*, 2004; Nenquin *et al.*, 2004; Szollosi *et al.*, 2007). When challenged with high glucose, *Sur1KO* beta cells do not further depolarize, but the frequency of their membrane potential and $[Ca^{2+}]_c$ oscillations increases. At the same time, a major increase in insulin secretion occurs, which is due to both this small elevation of average $[Ca^{2+}]_c$ and the amplifying action of glucose (Szollosi *et al.*, 2007). Beta cells lacking K_{ATP} channels are thus responsive to glucose and also known to be unresponsive to sulphonylureas and diazoxide (Seghers *et al.*, 2000; Düfer *et al.*, 2004; Nenquin *et al.*, 2004), prototypical inhibitors and activators of K_{ATP} channels in normal islets. *Sur1KO* islets have however not previously been used for a systematic characterization of the effects of insulinotropic drugs. In the present study, therefore, we used *Sur1KO* islets to gain a better insight into the mode of action of various pharmacological stimulators and inhibitors of insulin secretion. The effect of the different drugs on islet $[Ca^{2+}]_c$ was also measured to determine whether the observed changes in secretion result from changes in the triggering Ca^{2+} signal produced by K_{ATP} channel-independent mechanisms or occur independently of $[Ca^{2+}]_c$ changes.

Methods

The study was approved by, and the experiments were conducted in accordance with the guidelines of the Animal Research Committee of our institution.

Animals

Sur1KO mice generated (Seghers *et al.*, 2000) and provided by J. Bryan were maintained in our animal facilities. Controls were C57Bl/6 mice originally obtained from Charles River Laboratories, Brussels, Belgium. All experiments were performed with adult female mice of 10–14 months. *Sur1KO* mice were slightly heavier than controls (30.2 ± 0.5 g, $n = 89$ versus 26.9 ± 0.7 g, $n = 59$, $P < 0.001$), but their morning blood glucose was similar (7.5 ± 0.2 mmol·L⁻¹ in both groups).

Solutions and reagents

The control medium used for islet isolation was a bicarbonate-buffered solution containing (mmol·L⁻¹): NaCl 120, KCl 4.8, CaCl₂ 2.5, MgCl₂ 1.2 and NaHCO₃ 24. It was gassed with O₂: CO₂ (94%: 6%) to maintain pH 7.4 and was supplemented

with 1 mg·mL⁻¹ bovine serum albumin and 10 mmol·L⁻¹ glucose. A similar medium was used for all experiments after adjustment of the glucose concentration and addition of test substances.

Preparation of islets

Islets from *Sur1KO* and control mice were aseptically isolated by collagenase digestion of the pancreas followed by hand selection. The islets were then cultured for ~18 h in RPMI1640 medium (Invitrogen, Belgium) kept at 37°C in a 95% air: 5% CO₂ atmosphere. The culture medium contained 10 mmol·L⁻¹ glucose, 10% heat-inactivated fetal calf serum, 100 IU·mL⁻¹ penicillin and 100 µg·mL⁻¹ streptomycin (Nenquin *et al.*, 2004).

Measurements of insulin secretion

After overnight culture, the islets were distributed in batches of 15 before being transferred into perfusion chambers (Henquin *et al.*, 2006). The islets were then perfused (flow rate of 1 mL·min⁻¹) at 37°C with a control medium containing 3 or 15 mmol·L⁻¹ glucose, with or without test substance. Effluent fractions were collected at 2 min intervals and saved for insulin assay using rat insulin as a standard (Henquin *et al.*, 2006).

Measurements of islet $[Ca^{2+}]_c$

After overnight culture, islets were loaded with the Ca^{2+} indicator fura-PE3-AM (2 µmol·L⁻¹, Teflabs, Austin, TX, USA) for 2 h in control medium containing 10 mmol·L⁻¹ glucose. Loaded islets were then placed into the perfusion chamber of a spectrofluorimetric system, equipped with a camera, and with which $[Ca^{2+}]_c$ was measured at 37°C (Nenquin *et al.*, 2004).

Data presentation and analysis

All experiments have been performed with islets from three to five different preparations. Typical changes in $[Ca^{2+}]_c$ produced by selected drugs are illustrated by representative experiments. Otherwise, results are presented as means \pm SE. The statistical significance of differences between means of non-stimulated islets and islets challenged by a drug was assessed by ANOVA followed by Dunnett's test. $P < 0.05$ was considered to indicate a statistically significant difference between means.

Materials

Tolbutamide, quinine hydrochloride, tetraethylammonium chloride (TEA), efaroxan hydrochloride, compound UCL-1684, verapamil hydrochloride, dopamine hydrochloride and hydrochlorothiazide were obtained from Sigma (St. Louis, MO, USA). Alinidine hydrobromide and clonidine hydrochloride were from Boehringer-Ingelheim (Ingelheim, Germany). Phentolamine mesylate was from Novartis-Pharma (Basel, Switzerland). Meglitinide was from Hoechst AG (Frankfurt, Germany), pinacidil from Leo Pharmaceuticals (Ballerup,

Denmark), nimodipine from Bayer (Wuppertal, Germany), diphenylhydantoin from Fluka (Buchs, Switzerland), and diazoxide was a gift from Schering-Plough (Rathdrum, Ireland). Tolbutamide, meglitinide, diazoxide, diphenylhydantoin and hydrochlorothiazide were added from 500 to 1500 \times concentrated stock solutions freshly prepared in 0.1N NaOH, and pH of the perfusion media was adjusted back to 7.4 when required. Stock solutions of UCL-1684 and nimodipine were prepared in dimethyl sulphoxide, the maximum amount of which (0.5 $\mu\text{L}\cdot\text{mL}^{-1}$) had no effect alone. The other substances were dissolved in H_2O either directly in perfusion media or as a concentrated stock solution. Drug and molecular target nomenclature follows Alexander *et al.* (2008).

Results and discussion

Experimental design and control values

The concentrations of the tested drugs were selected on the basis of their *in vitro* effects in normal islets reported in previous publications by ourselves and other groups. Figure 1 shows how the effects of the drugs on islet $[\text{Ca}^{2+}]_c$ (panel A) or insulin secretion (panel B) were measured. The concentration of glucose in the perfusion medium was kept constant (here at 15 $\text{mmol}\cdot\text{L}^{-1}$) throughout, and tested drugs (here 100 $\mu\text{mol}\cdot\text{L}^{-1}$ tolbutamide or diazoxide) were applied from 15 to 40 min (A) or 15 to 45 min (B). The steady state effect of each drug was computed during the last 10 (A) or 15 min (B) of application, and expressed as a percentage of the pre-

stimulatory reference value (between 5 and 15 min) in each individual experiment. These normalized values were then averaged, and means are presented in Tables. In control islets perfused with 15 $\text{mmol}\cdot\text{L}^{-1}$ glucose, tolbutamide increased $[\text{Ca}^{2+}]_c$ by 67% and insulin secretion by 161% (Figure 1 and Table 1, line 2). These values must be compared to the spontaneous small increase in $[\text{Ca}^{2+}]_c$ (+13%) and small decrease in insulin secretion (–18%) that occurred when no drug was applied (Figure 1 and Table 1, line 1). Diazoxide lowered $[\text{Ca}^{2+}]_c$ by 75% and inhibited insulin secretion by 96% in these control islets (Figure 1 and Table 2, line 2).

In control islets perfused with 15 $\text{mmol}\cdot\text{L}^{-1}$ glucose alone, reference values (100%) were $227 \pm 10 \text{ nmol}\cdot\text{L}^{-1}$ $[\text{Ca}^{2+}]_c$ ($n = 30$) and $67 \pm 7 \text{ pg insulin per islet}\cdot\text{min}^{-1}$ ($n = 12$). In untreated *Sur1KO* islets, $[\text{Ca}^{2+}]_c$ averaged $194 \pm 6 \text{ nmol}\cdot\text{L}^{-1}$ ($n = 29$) and $157 \pm 7 \text{ nmol}\cdot\text{L}^{-1}$ ($n = 23$) in 15 and 3 $\text{mmol}\cdot\text{L}^{-1}$ glucose, respectively, while insulin secretion rates averaged $149 \pm 11 \text{ pg per islet}\cdot\text{min}^{-1}$ ($n = 10$) and $52 \pm 9 \text{ pg per islet}\cdot\text{min}^{-1}$ ($n = 9$). High glucose thus slightly increased $[\text{Ca}^{2+}]_c$ ($P < 0.01$) and markedly augmented insulin secretion ($P < 0.001$) in these islets lacking K_{ATP} channels (Nenquin *et al.*, 2004; Szollosi *et al.*, 2007).

Effects of putative stimulators of insulin secretion

Blockers of K_{ATP} channels. Tolbutamide closes K_{ATP} channels by interacting with binding site A that is present in SUR1 only (Gribble and Reimann, 2003; Bryan *et al.*, 2005). As previously reported (Seghers *et al.*, 2000; Düfer *et al.*, 2004; Nenquin

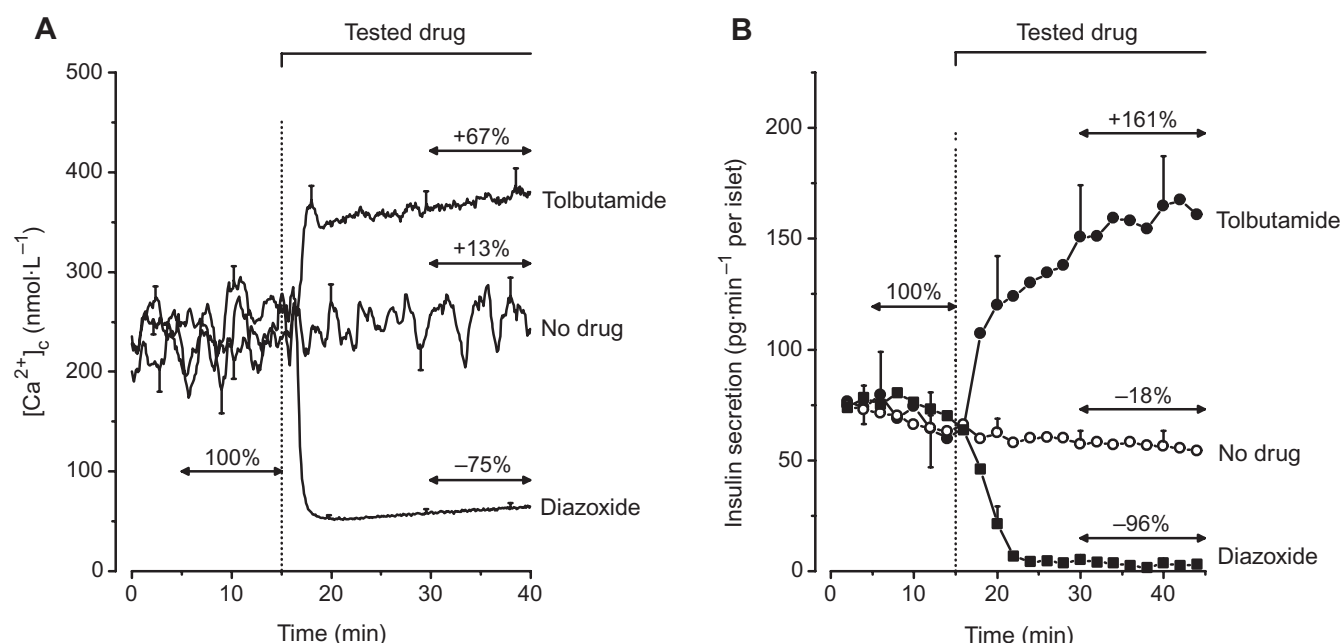


Figure 1 Effects of tolbutamide (100 $\mu\text{mol}\cdot\text{L}^{-1}$) and diazoxide (100 $\mu\text{mol}\cdot\text{L}^{-1}$) on $[\text{Ca}^{2+}]_c$ (panel A) and insulin secretion (panel B) in control islets perfused with a medium containing 15 $\text{mmol}\cdot\text{L}^{-1}$ glucose throughout. Values are means \pm SE for 16–30 islets from 3–5 preparations in $[\text{Ca}^{2+}]_c$ experiments, and 5–12 perfusions in insulin secretion experiments. The figure illustrates how the effects of the drugs were computed for presentation in tables. In each individual experiment, $[\text{Ca}^{2+}]_c$ and the insulin secretion rate were averaged between 5 and 15 min to obtain the reference value (100%). Steady-state effects of drugs were averaged between 30 and 40 min for $[\text{Ca}^{2+}]_c$, and 30–45 min for insulin secretion, and expressed relative to the reference value in the same experiment. The mean effect of the drug was then calculated. Panel A thus shows that $[\text{Ca}^{2+}]_c$ increased by 13% in the absence of drug and by 67% after addition of tolbutamide, and that it decreased by 75% after addition of diazoxide.

Table 1 Effects of stimulatory agents on $[Ca^{2+}]_c$ and insulin secretion in control and *Sur1KO* islets, in 15 or 3 mmol·L⁻¹ glucose

Line	Test agent ($\mu\text{mol}\cdot\text{L}^{-1}$)	Control islets in 15 mmol·L ⁻¹ glucose		<i>Sur1KO</i> islets in 15 mmol·L ⁻¹ glucose		<i>Sur1KO</i> islets in 3 mmol·L ⁻¹ glucose	
		$\Delta [Ca^{2+}]_c$ (%)	$\Delta \text{Insulin}$ (%)	$\Delta [Ca^{2+}]_c$ (%)	$\Delta \text{Insulin}$ (%)	$\Delta [Ca^{2+}]_c$ (%)	$\Delta \text{Insulin}$ (%)
1	None	+13 ± 2	-18 ± 3	+10 ± 1	-2 ± 3	+10 ± 3	-20 ± 3
2	Tolbutamide (100)	+67 ± 7**	+161 ± 41**	+14 ± 2	-1 ± 5	+3 ± 3	-19 ± 7
3	Meglitinide (10)	+61 ± 5**	+148 ± 35**	+14 ± 2	-3 ± 5	+16 ± 2	-27 ± 3
4	Quinine (50)	-	+175 ± 31**	-	+13 ± 4	-	-
5	Tetraethylammonium (10 ⁴)	-9 ± 3**	+24 ± 5	-2 ± 2**	+82 ± 12**	-1 ± 2	+20 ± 1**
6	UCL-1684 (0.5)	+20 ± 4	-6 ± 3	+10 ± 2	+2 ± 5	-	-
7	Phentolamine (100)	+70 ± 5**	+181 ± 42**	+29 ± 4**	+39 ± 6**	+34 ± 6**	+46 ± 9**
8	Efaroxan (100)	+60 ± 3**	+187 ± 30**	+18 ± 3	+17 ± 13	-	-
9	Alinidine (100)	+33 ± 5**	+57 ± 7*	+12 ± 2	+10 ± 14	+17 ± 2	-9 ± 19

Values are means ± SEM for 16–30 islets from 3–5 preparations in $[Ca^{2+}]_c$ experiments and for 4–12 separate perfusions in insulin secretion experiments. Absolute reference values (100%) for $[Ca^{2+}]_c$ and insulin secretion were similar between all test groups of control and *Sur1KO* islets.

* $P < 0.05$, ** $P < 0.01$ or less versus 15 or 3 mmol·L⁻¹ glucose alone (first line in the same column) by ANOVA.

Table 2 Effects of inhibitory agents on $[Ca^{2+}]_c$ and insulin secretion in control and *Sur1KO* islets, in 15 or 3 mmol·L⁻¹ glucose

Line	Test agent ($\mu\text{mol}\cdot\text{L}^{-1}$)	Control islets in 15 mmol·L ⁻¹ glucose		<i>Sur1KO</i> islets in 15 mmol·L ⁻¹ glucose		<i>Sur1KO</i> islets in 3 mmol·L ⁻¹ glucose	
		$\Delta [Ca^{2+}]_c$ (%)	$\Delta \text{Insulin}$ (%)	$\Delta [Ca^{2+}]_c$ (%)	$\Delta \text{Insulin}$ (%)	$\Delta [Ca^{2+}]_c$ (%)	$\Delta \text{Insulin}$ (%)
1	None	+13 ± 2	-18 ± 3	+10 ± 1	-2 ± 3	+10 ± 3	-20 ± 3
2	Diazoxide (100)	-75 ± 2**	-96 ± 1**	+4 ± 2	+2 ± 5	+14 ± 5	-20 ± 5
3	Pinacidil (250)	-37 ± 3**	-69 ± 3**	+2 ± 2	-3 ± 7	+2 ± 5	-11 ± 6
4	Nimodipine (0.1)	-67 ± 4**	-76 ± 3**	-63 ± 2**	-85 ± 6**	-64 ± 4**	-91 ± 3**
5	Verapamil (20)	-38 ± 2**	-21 ± 8	-52 ± 2**	-79 ± 4**	-58 ± 2**	-78 ± 16**
6	Verapamil (100)	-64 ± 1**	-65 ± 5**	-61 ± 3**	-82 ± 4**	-	-
7	Diphenylhydantoin (20)	-28 ± 2**	-64 ± 3**	-51 ± 2**	-82 ± 1**	-55 ± 3**	-67 ± 5**
8	Hydrochlorothiazide (1)	+20 ± 3	-14 ± 3	+13 ± 2	+1 ± 6	+14 ± 4	-26 ± 5
9	Clonidine (1)	-59 ± 4**	-96 ± 2**	-35 ± 2**	-87 ± 3**	-43 ± 2**	-92 ± 3**
10	Dopamine (10)	-51 ± 3**	-88 ± 4**	-27 ± 3**	-68 ± 3**	-23 ± 2**	-80 ± 7**

Values are means ± SEM for 16–30 islets from 3–5 preparations in $[Ca^{2+}]_c$ experiments and for 4–12 separate perfusions in insulin secretion experiments. Absolute reference values (100%) for $[Ca^{2+}]_c$ and insulin secretion were similar between all test groups of control and *Sur1KO* islets.

* $P < 0.05$, ** $P < 0.01$ or less versus 15 or 3 mmol·L⁻¹ glucose alone (first line in the same column) by ANOVA.

et al., 2004), *Sur1KO* islets did not display any change in $[Ca^{2+}]_c$ or insulin secretion when challenged by tolbutamide (Table 1, line 2). Meglitinide, the non-sulphonylurea moiety of glibenclamide (also known as HB699), is the mother compound of the glinide family. It mimics the effects of tolbutamide on K_{ATP} channels (Garrino *et al.*, 1985; Panten *et al.*, 1989), but does so by interacting with binding site B, that is present in both SUR1 and SUR2 (Bryan *et al.*, 2005). In control islets, meglitinide suppressed $[Ca^{2+}]_c$ oscillations induced by glucose and caused a sustained elevation (Figure 2A), which resulted in marked increases in average $[Ca^{2+}]_c$ and insulin secretion rate (Table 1, line 3). In *Sur1KO* islets, $[Ca^{2+}]_c$ was also oscillatory in the presence of 15 mmol·L⁻¹ glucose alone (Nenquin *et al.*, 2004; Szollosi *et al.*, 2007), but it was unaffected by meglitinide (Figure 2A), so that average $[Ca^{2+}]_c$ and insulin secretion were not different in the presence or absence of the drug (Table 1, line 3). The lack of effects of tolbutamide and meglitinide in *Sur1KO* islets was confirmed in the presence of low glucose (Table 1, lines 2 and 3, right-hand columns). Glibenclamide that interacts with both binding sites A and B of SUR1 (Bryan *et al.*, 2005) is also without effect in *Sur1KO* islets (Henquin, 2004). Altogether, these results show that neither sulphonylureas nor glinides have effects on insulin secretion in the absence of K_{ATP} channels.

Quinine has long been known to augment insulin secretion by decreasing the potassium permeability of the beta cell membrane (Henquin, 1982). This effect results from closure of K_{ATP} channels (Bokvist *et al.*, 1990) via a direct interaction with $K_{IR6.2}$ (Gribble *et al.*, 2000) and inhibition of several other types of potassium channels (Bokvist *et al.*, 1990). The fluorescence of quinine precluded reliable measurements of $[Ca^{2+}]_c$, but the drug potently increased insulin secretion in control islets and was without effect in *Sur1KO* islets (Table 1, line 4). This indicates that blockage of K_{ATP} channels accounts for the effects of the drug on insulin secretion in control islets, and that beta cells lacking K_{ATP} channels are unlikely to express other channels incorporating $K_{IR6.2}$. In this context, it is relevant that antibacterial fluoroquinolones, which also increase insulin secretion by blocking K_{ATP} channels at the $K_{IR6.2}$ level (Zünkler and Wos, 2003; Saraya *et al.*, 2004), have recently been reported to be inefficient in *Sur1KO* islets (Ghaly *et al.*, 2009).

Blockers of other potassium channels. In addition to K_{ATP} channels, tetraethylammonium inhibits voltage-gated and Ca^{2+} -activated potassium channels (Bokvist *et al.*, 1990) presumably through a direct action on the pore of the channels. In control islets, tetraethylammonium produces

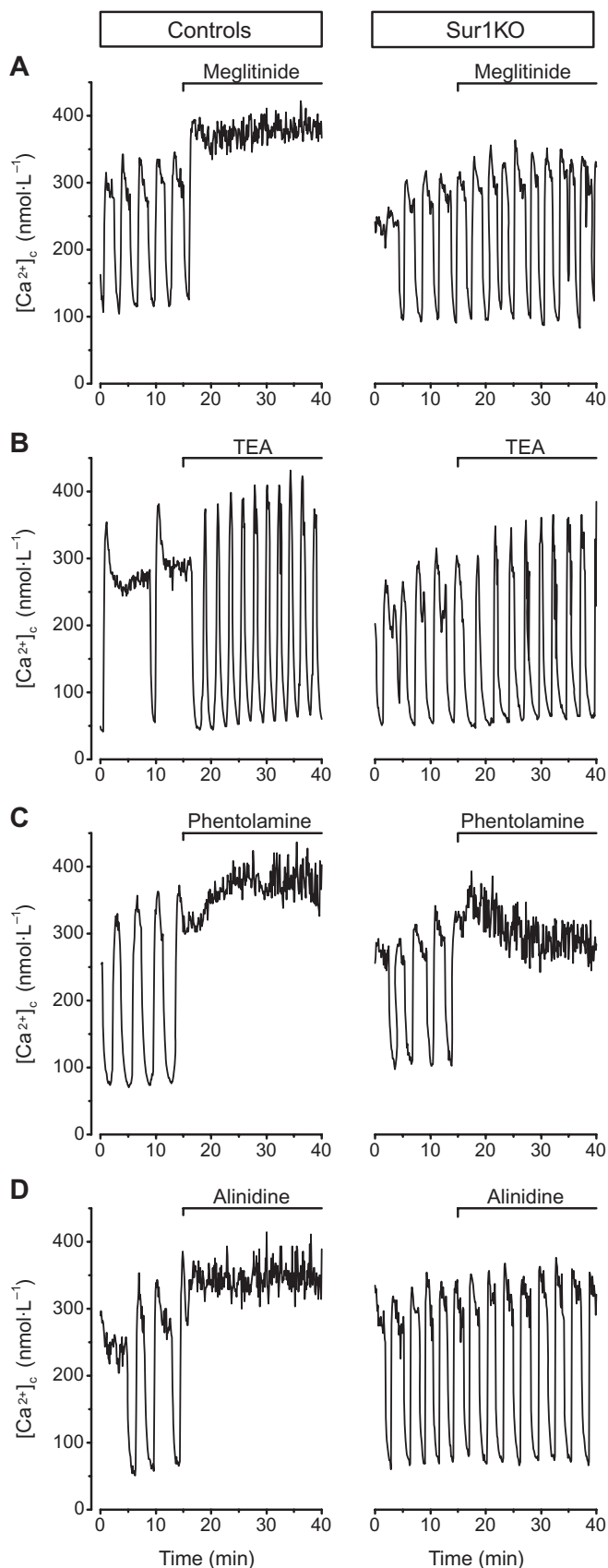


Figure 2 Effects of selected stimulators of insulin secretion on $[Ca^{2+}]_i$ in control and *Sur1KO* islets perfused with 15 mmol·L⁻¹ glucose. Meglitinide (100 μ mol·L⁻¹), tetraethylammonium (TEA, 10 mmol·L⁻¹), phentolamine (100 μ mol·L⁻¹) or alinidine (100 μ mol·L⁻¹) was added as indicated at the top of each panel. Traces illustrate the changes occurring in representative islets. Quantification of the changes is presented in Table 1.

typical shortening, acceleration and increase in amplitude of the oscillations of membrane potential induced by glucose (Henquin, 1990). The oscillations of $[Ca^{2+}]_i$ changed in a similar way (Roe *et al.*, 1996) (Figure 2B), which resulted in a minor decrease in average $[Ca^{2+}]_i$ (Table 1, line 5) with, however, a tendency to increase insulin secretion (the difference with untreated islets did not reach statistical significance by ANOVA, but it was $P < 0.0001$ by Student's *t*-test). Qualitatively similar changes occurred in *Sur1KO* islets (Figure 2B), but the increase in insulin secretion was greater (Table 1, line 5). This trend and this increase in insulin secretion in face of slightly lower average $[Ca^{2+}]_i$ are paradoxical and suggest that the profile and amplitude of $[Ca^{2+}]_i$ oscillations may be relevant for stimulus-secretion coupling. The effectiveness of tetraethylammonium in *Sur1KO* islets suggests that potassium channels other than K_{ATP} channels are potential targets of pharmacological secretagogues, as previously proposed by other approaches (MacDonald and Wheeler, 2003; Jacobson and Philipson, 2007), if sufficient tissue selectivity can be achieved (Henquin, 2004).

A hyperpolarizing current (IK_{slow}) produced by small conductance, Ca^{2+} -activated and sulphonylurea-insensitive potassium channels could be implicated in the termination of membrane potential and $[Ca^{2+}]_i$ oscillations in glucose-stimulated normal beta cells (Göpel *et al.*, 1999; Zhang *et al.*, 2005) and in *Sur1KO* beta cells (Haspel *et al.*, 2005). However, compound UCL-1684, a selective blocker of these channels (Zhang *et al.*, 2005), had no effect on $[Ca^{2+}]_i$ or insulin secretion in either control or *Sur1KO* islets (Table 1, line 6), which questions the role of these SK_{Ca} channels in beta cell function.

Imidazolines. Stimulation of insulin secretion by various compounds belonging to the family of imidazolines has been attributed to blockage of K_{ATP} channels through a direct interaction with $K_{IR6.2}$ (Jonas *et al.*, 1992; Proks and Ashcroft, 1997), but mechanisms independent of the generation of the triggering Ca^{2+} signal have also been proposed (Morgan and Chan, 2001; Efendic *et al.*, 2002). In control islets, phentolamine (Figure 2C), efaroxan (not illustrated) and alinidine (Figure 2D) caused a sustained increase in $[Ca^{2+}]_i$ and insulin secretion (Table 1, lines 7, 8, 9). In *Sur1KO* islets perfused with 15 mmol·L⁻¹ glucose, efaroxan and alinidine were ineffective, but phentolamine still increased both $[Ca^{2+}]_i$ (Figure 2C) and insulin secretion (Table 2, line 7), albeit to a much smaller extent than in controls. Similar results were obtained in *Sur1KO* islets perfused with 3 mmol·L⁻¹ glucose. The effects of phentolamine on $[Ca^{2+}]_i$ in beta cells lacking K_{ATP} channels can be attributed to blockage of other types of potassium channels (Jonas *et al.*, 1992). It thus appears that classical imidazolines augment insulin secretion via an increase in $[Ca^{2+}]_i$, which is mainly mediated by an action on

K_{ATP} channels. Newer imidazolines, such as compound RX871024, may have other, intracellular sites of action, but their effects are notably smaller in *Sur1KO* than control islets (Efanov *et al.*, 2001).

Effects of putative inhibitors of insulin secretion

Activators of K_{ATP} channels. Diazoxide opens K_{ATP} channels containing either SUR1 or SUR2 (Gribble and Reimann, 2003; Bryan *et al.*, 2005; Moreau *et al.*, 2005). As shown in Figure 1, diazoxide markedly decreased $[Ca^{2+}]_c$ and abolished insulin secretion in control islets, but it was ineffective in *Sur1KO* islets (Table 2, line 2), as previously reported (Nenquin *et al.*, 2004). Pinacidil also inhibits insulin secretion by opening beta cell K_{ATP} channels (Garrino *et al.*, 1989; Lebrun *et al.*, 1989), but its affinity is much greater for SUR2 than SUR1 (Gribble and Reimann, 2003; Bryan *et al.*, 2005; Moreau *et al.*, 2005). Although used at a higher concentration than diazoxide, pinacidil was less potent on $[Ca^{2+}]_c$ and insulin secretion in control islets, and, like diazoxide, totally ineffective in *Sur1KO* islets (Figure 3A) (Table 2, line 3). While there is no dispute that SUR1 is the major sulphonylurea receptor in beta cells, SUR2A is moderately expressed in mouse islets (Miki *et al.*, 1999), and the ubiquitous SUR2B is present in the pancreas (Isomoto *et al.*, 1996). It is, however, uncertain whether these two isoforms are present in beta cells or in endocrine non-beta cells or non-endocrine (vascular) cells of the islets. Taken together, our experiments using meglitinide, diazoxide and pinacidil show that no alternative form of K_{ATP} channels is expressed in these *Sur1KO* islets, and thus presumably in normal beta cells. The results also reinforce the common, though not unanimous (Grimmsmann and Rustenbeck, 1998; Geng *et al.*, 2007), view that these drugs and sulphonylureas do not influence insulin secretion by interacting with intracellular targets.

Blockers of voltage-gated calcium channels. Nimodipine potently decreased $[Ca^{2+}]_c$ and inhibited insulin secretion in control and *Sur1KO* islets perfused with 15 mmol·L⁻¹ glucose (Table 2, line 4). Qualitatively similar effects were produced by verapamil (Figure 3B) (Table 2, lines 5, 6). Both agents were also efficient in *Sur1KO* islets perfused with only 3 mmol·L⁻¹ glucose. This shows that continuous influx of Ca^{2+} through voltage-gated calcium channels of the L-type is required for the high rate of insulin secretion in *Sur1KO* islets at both low and high glucose. Unexpectedly, the inhibition of insulin secretion by verapamil was greater in *Sur1KO* than control islets, particularly at 20 μ mol·L⁻¹. We tentatively attribute this difference to the fact that, besides their inhibitory action via blockage of calcium channels, phenylalkylamines such as verapamil also have a positive effect via blockage of K_{ATP}

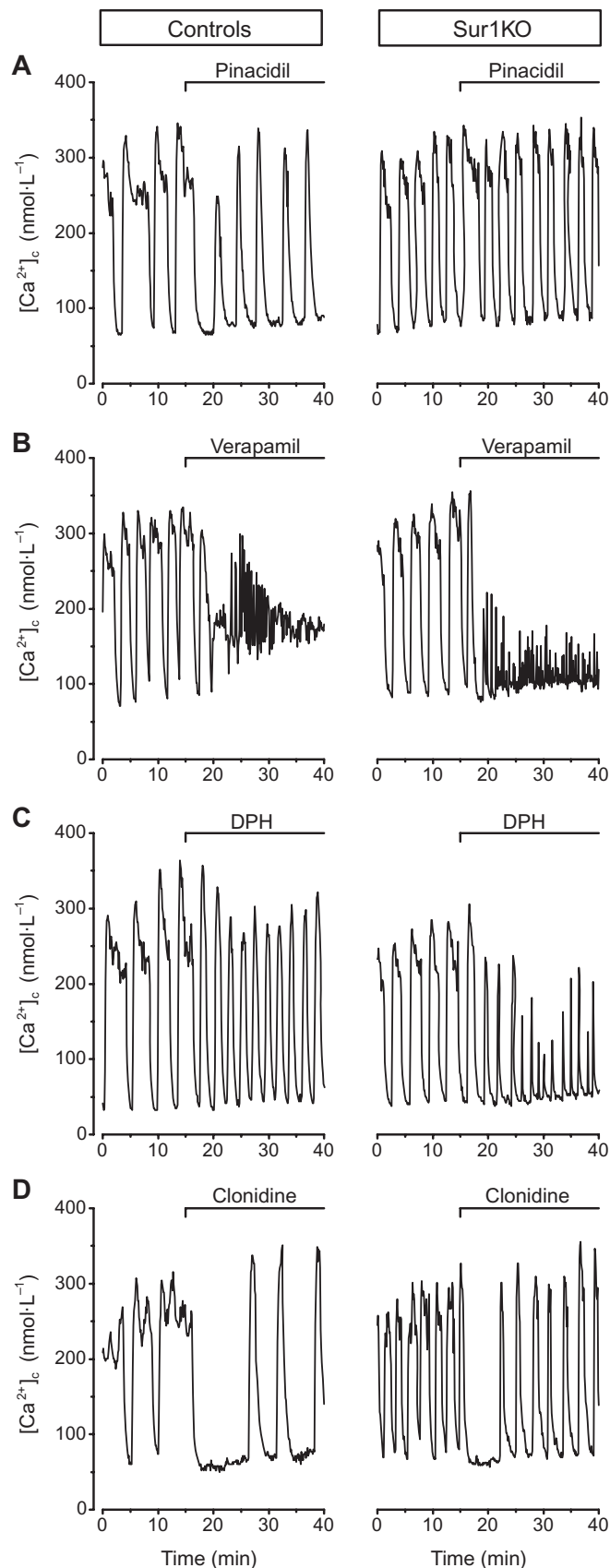


Figure 3 Effects of selected inhibitors of insulin secretion on $[Ca^{2+}]_c$ in control and *Sur1KO* islets perfused with 15 mmol·L⁻¹ glucose. Pinacidil (250 μ mol·L⁻¹), verapamil (20 μ mol·L⁻¹), diphenylhydantoin (DPH, 20 μ mol·L⁻¹) or clonidine (1 μ mol·L⁻¹) was added as indicated at the top of each panel. Traces illustrate the changes occurring in representative islets. Quantification of the changes is presented in Table 2.

channels in control islets (Lebrun *et al.*, 1997). In *Sur1KO* islets, only the inhibitory action can manifest itself. In contrast, dihydropyridines such as nimodipine, do not affect K_{ATP} channels (Lebrun *et al.*, 1997), which explains their similar action in both types of islets. Calcium channel blockers are sometimes used in the medical treatment of congenital hyperinsulinism (Aynsley-Green *et al.*, 2000). It might be worth testing whether phenylalkylamines have advantages over dihydropyridines in cases where a focal lesion lacking functional K_{ATP} channels coexists with normal islets (Sempoux *et al.*, 2003). Their effect might be greater in the lesion than in normal beta cells.

The antiepileptic agent diphenylhydantoin is known to inhibit glucose-induced insulin secretion (Levin *et al.*, 1972). This effect has been attributed to a decrease in Ca²⁺ influx into beta cells (Herchuelz *et al.*, 1981), but this view was challenged recently and the effect of diphenylhydantoin attributed to an alkalization of beta cell cytosol with secondary decrease in Ca²⁺ action on exocytosis (Nabe *et al.*, 2006). As shown in Figure 3C, diphenylhydantoin decreased amplitude and duration of [Ca²⁺]_c oscillations, which resulted in a decrease in average [Ca²⁺]_c and insulin secretion in control and *Sur1KO* islets perfused with 15 mmol·L⁻¹ glucose (Table 2, line 7). Interestingly, the effects of diphenylhydantoin were greater in *Sur1KO* than control islets. Because the inhibition of insulin secretion was also observed in low glucose, we suggest that diphenylhydantoin might be tested as an adjunct therapy in diazoxide-resistant congenital hyperinsulinism due to inactivating mutations of K_{ATP} channel subunits. Diphenylhydantoin has occasionally been found useful in the medical treatment of insulinomas (Imanaka *et al.*, 1986). In the same perspective, we tested the effects of hydrochlorothiazide, a diuretic drug that is often associated with diazoxide (Aynsley-Green *et al.*, 2000), and has been reported to inhibit insulin secretion by islets from *ob/ob* mice (Sandström, 1993). The drug was without effect on [Ca²⁺]_c and insulin secretion in both normal and *Sur1KO* islets (Table 2, line 8).

Catecholamines. Activation of α₂-adrenoceptors by adrenaline or selective agonists such as clonidine is known to inhibit insulin secretion via multiple mechanisms, including a hyperpolarization of the beta cell membrane, which has been attributed to opening of potassium channels (Drews *et al.*, 1990; Sieg *et al.*, 2004). In glucose-stimulated control islets, clonidine rapidly lowered [Ca²⁺]_c to basal values, but oscillations, sometimes of large amplitude, resumed in almost 50% (10/22) of the islets (Figure 3D). In *Sur1KO* islets, the initial inhibition was similar, but resumption of [Ca²⁺]_c oscillations was more frequent (19/24 islets), so that the steady-state decrease in [Ca²⁺]_c was smaller than in controls (Table 2, line 9). This, however, did not influence the degree of inhibition of insulin secretion that was virtually complete in both types of islets. These observations therefore support previous evidence that adrenoceptor-mediated inhibition of insulin secretion is mainly achieved by interference with distal steps of stimulus-secretion coupling (Ullrich and Wollheim, 1985; Jones *et al.*, 1987). The rapid decrease in [Ca²⁺]_c produced by clonidine in *Sur1KO* islets is also in keeping with the report that adrenaline rapidly hyperpolarized *Sur1KO* beta cells (Sieg

et al., 2004). Although this and the present studies support the conclusion that the hyperpolarization involves channels other than K_{ATP} channels, some contribution of the latter cannot be completely ruled out in view of the greater inhibition of [Ca²⁺]_c in control than *Sur1KO* islets.

Activation of dopamine D₂ receptors by dopamine has been reported to lower [Ca²⁺]_c in INS1 cells and to inhibit insulin secretion in the cell line and in rodent islets (Rubí *et al.*, 2005). We found the effects of dopamine on [Ca²⁺]_c to be qualitatively similar to those of clonidine, and again quantitatively larger in control than *Sur1KO* islets (Table 2, lines 9, 10). Insulin secretion was more inhibited than expected for the decrease in [Ca²⁺]_c, which suggests that, like α₂-adrenoceptors, dopamine D₂ receptors interfere with stimulus-secretion coupling at steps distal to the [Ca²⁺]_c increase.

Both clonidine and dopamine also very effectively inhibited insulin secretion by *Sur1KO* islets in low glucose (Table 2, lines 9, 10). Thus, targeting these receptors might theoretically be interesting to counter congenital hyperinsulinism due to inactivation of K_{ATP} channels, but the usefulness and tolerance of such treatments remain to be evaluated.

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Conflicts of interest

The authors declare that there is no conflict of interest associated with this manuscript.

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