Both Triggering and Amplifying Pathways Contribute to Fuel-induced Insulin Secretion in the Absence of Sulfonylurea Receptor-1 in Pancreatic β-Cells

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In normal β-cells glucose induces insulin secretion by activating both a triggering pathway (closure of KATP channels, depolarization, and rise in cytosolic [Ca2+]i) and an amplifying pathway (augmentation of Ca2+ efficacy on exocytosis). It is unclear if and how nutrients can regulate insulin secretion by β-cells lacking KATP channels (Sur1 knockout mice). We compared glucose- and amino acid-induced insulin secretion and [Ca2+]i changes in control and Sur1KO islets. In 1 mM glucose (non-stimulatory for controls), the triggering signal [Ca2+]i was high (loss of regulation) and insulin secretion was stimulated in Sur1KO islets. This “basal” secretion was decreased or increased by imposed changes in [Ca2+]i, and was dependent on ATP production, indicating that both triggering and amplifying signals are involved. High glucose stimulated insulin secretion in Sur1KO islets, by an unsuspected, transient increase in [Ca2+]i, and a sustained activation of the amplifying pathway. Unlike controls, Sur1KO islets were insensitive to diazoxide and tolbutamide, which rules out effects of either drug at sites other than KATP channels. Amino acids potently increased insulin secretion by Sur1KO islets through both a further electrogenic rise in [Ca2+]i, and a metabolism-dependent activation of the amplifying pathway. After sulfonylurea blockade of their KATP channels, control islets qualitatively behaved like Sur1KO islets, but their insulin secretion rate was consistently lower for a similar or even higher [Ca2+]i.

In conclusion, fuel secretagogues can control insulin secretion in β-cells without KATP channels, partly by an unsuspected influence on the triggering [Ca2+]i signal and mainly by the modulation of a very effective amplifying pathway.

Because insulin is the only blood glucose-lowering hormone, tight control of its secretion by pancreatic β-cells is critical. Glucose exerts this control through two major signaling pathways leading to the production of triggering and amplifying signals respectively (1). ATP-sensitive K+ (KATP)1 channels are key players in the triggering pathway. They are composed of the high affinity sulfonylurea receptor 1 (SUR1) and the pore-forming Kir6.2 (2, 3) and serve to transduce metabolic changes into biophysical signals through the following sequence of events. The metabolism of glucose by oxidative glycolysis produces a rise in the ATP/ADP ratio in β-cells, which closes KATP channels in the plasma membrane, thereby causing depolarization with subsequent opening of voltage-dependent Ca2+ channels, Ca2+ influx from the extracellular medium, and rise in the concentration of cytoplasmic free Ca2+ ([Ca2+]c) (1–10). This rise in [Ca2+]c is the triggering signal for exocytosis of insulin granules.

Alone, this triggering signal is poorly effective, but amplifying signals, also issued from glucose metabolism, augment its efficacy (1, 10). The experimental paradigms used to study the amplifying pathway require clamping of [Ca2+]c at an elevated level, which is achieved by depolarizing β-cells either with a sulfonylurea that holds KATP channels closed, or by high KCl in the presence of diazoxide that holds KATP channels open (1). Because glucose no longer can modulate KATP channel activity, hence the membrane potential, the amplifying pathway has also been referred to as a KATP channel-independent effect of glucose (8, 10). The nature of the metabolic messengers involved in this pathway is still debated (8, 11–17). A participation of the ATP/ADP ratio is favored by several laboratories (1, 18–20), but the intracellular targets of these putative messengers remain to be identified.

SUR1 belongs to the family of the ATP-binding cassette proteins and possesses classic nucleotide binding folds (21). Besides its localization in KATP channels of the plasma membrane, it is present in large amounts in intracellular membranes (22–24), possibly forming KATP channels in the insulin granule membrane (24). This raises the possibility that intracellular SUR1/KATP channels might be implicated in the amplifying pathway of insulin secretion, whereas SUR1/KATP channels in the plasma membrane are implicated in the triggering pathway.

Two SUR1 knock-out (Sur1KO) mice have been produced, which show no gross abnormalities of glucose homeostasis despite the absence of KATP channels in their β-cells and poor secretion of insulin in response to hyperglycemia (25, 26). In one model, glucose alone was found to exert only minor effects

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The abbreviations used are: KATP, ATP-sensitive K+; SUR1, sulfonylurea receptor 1; Sur1KO, Sur1 knockout mice; Kir6.2, potassium inward rectifier channel; [Ca2+]c, cytoplasmic free Ca2+ concentration; BCH, 2-amino-2-norbornanecarboxylic acid.
on in vitro insulin secretion unless protein kinase A or C pathways were activated (26, 28). These observations prompted the conclusion that the amplifying pathway is defective in β-cells lacking SUR1 (28).

In the present study we compared islets from Sur1KO mice (25) and their wild-type controls, with the specific aim of assessing whether the absence of SUR1 (hence also of KATP channels) causes intrinsic defects in the amplifying pathway of glucose or amino acid-induced insulin secretion. The experiments were thus performed under classic conditions, but also under those conditions permitting dissociation of triggering and amplifying pathways in control β-cells, in particular after blockade of their KATP channels by a sulfonylurea. Because, by definition, the amplifying pathway involves changes in Ca²⁺ action, not in [Ca²⁺]ᵢ, the stability of the latter was consistently verified to obviate pitfalls of KATP channel-independent changes in the triggering signal (1). Our results indicate that both glucose and amino acids are effective stimulators of insulin secretion in the absence of SUR1, and that their effects involve, in variable proportions, a KATP channel-dependent increase in [Ca²⁺]ᵢ, and an activation of the amplifying pathway.

EXPERIMENTAL PROCEDURES

Animals—The generation and characteristics of Sur1KO mice have been described previously (25). Adult females, all females, were sent from Houston to Brussels where they were used at an age of 10–14 months, i.e. older than the males used in previous studies with this model (25, 27). Controls were C57Bl/6 mice obtained from Charles River Laboratories, Brussels, and housed with test mice for use at a similar age. Only females were used for measurements of insulin secretion, whereas both males and females were used for measurements of islet [Ca²⁺]ᵢ. The Sur1KO mice used in the present study were slightly heavier than their female controls (29.7 ± 0.52 g, n = 32 versus 27.0 ± 0.55 g, n = 32, p < 0.001). There was no difference in morning blood glucose between the two groups, but the variability was greater in Sur1KO mice (106 ± 6 mg/dl, range 57–192) than in controls (100 ± 3 mg/dl, range 74–139).

Solutions and Reagents—The control medium was a bicarbonate-buffered solution containing (mM): NaCl, 120; KCl, 4.8; CaCl₂, 2.5; MgCl₂, 1.2; and NaHCO₃, 24. It was maintained under O₂/CO₂ (94:6) to a pH of 7.4, and it contained 10 mM glucose and 1 mg/ml bovine serum albumin. A similar solution was used as test medium after adjustment of stimulation was long (25) or after pretreatment with agents and reagents used in the present study. The control islets were then cultured for about 18 h in RPMI 1640 medium (Invitrogen, Belgium) containing 10 mM glucose, 10 mM HEPES, and 0.1 mM dexamethasone. The number of islets per batch depended on the substance tested in the subsequent experiment. Each batch of islets was then transferred into the chamber of a dynamic perfusion system (30). The islets were then perfused at 37 °C with test solutions described in the figure legends. The effluent fractions were collected at 2-min intervals and saved for insulin assay, using rat insulin as a standard and ethanol to precipitate bound insulin (31). At the end of the experiment, the perfusion was stopped, the chambers were opened, and the islets were recovered and counted before being transferred into Eppendorf tubes. After brief centrifugation, the transfer medium was removed and replaced by 500 μl of an acid ethanol mixture for insulin extraction (32). Fractional insulin secretion rate was then calculated as the percentage of islet insulin content that was secreted per minute. By summing total insulin secretion during the experiment and the final insulin content, the insulin content of the islet at the beginning of the perfusions was obtained. It averaged 115.7 ± 2.6 ng/islet (n = 76) for controls and 99.8 ± 2.8 ng/islet (n = 66) for Sur1KO (p < 0.01). This difference is smaller than the 40% reported previously for islets from younger male mice of the same origin (27).

Measurements of Islet [Ca²⁺]ᵢ—Overt night cultured islets were loaded with the Ca²⁺ indicator fura-PE3 (2 μM, 2 h) in control medium containing 10 mM glucose. Loaded islets were then transferred (three or four at a time) into the perfusion chamber of a spectrofluorimetric system (25) equipped with a camera, and with which [Ca²⁺]ᵢ was measured as described previously (33). Because of technical constraints, the [Ca²⁺]ᵢ experiments are shorter than insulin secretion experiments.

Presentation of Results—All experiments have been performed with islets from four to seven different preparations. Except for a few representative recordings of [Ca²⁺]ᵢ, in individual islets, results are presented as means ± S.E. The statistical significance of differences between Sur1KO and control islets was assessed by unpaired Student’s t test.

RESULTS

Effects of Glucose on Insulin Secretion and Islet [Ca²⁺]ᵢ—When the perfusion medium contained a non-stimulatory concentration of glucose (1 mM), the rate of insulin secretion by control islets was very low and stable (Fig. 1). Increasing the glucose concentration to 15 mM induced a biphasic secretion of insulin, which was completely reversible upon return to the low glucose medium. In contrast, Sur1KO islets already secreted large amounts of insulin in the presence of 1 mM glucose. This high rate of secretion, that was unaffected by 3 mM glucose (not shown), progressively declined with time, but remained higher than in control islets even after 3 h of perfusion in 1 mM glucose (Fig. 1). Stimulation of Sur1KO islets with 15 mM glucose consistently caused an early but transient decrease in insulin secretion followed by an almost 3-fold, monophasic increase above the initial rate. Although this high rate of secretion started to decrease after about 50 min (min 90 of the experiment), the stimulatory effect of glucose remained evident for at least 100 min. Lowering the glucose concentration to 1 mM only partially inhibited insulin secretion. Within 15 min, the secretion rate returned to similar values as those before the stimulation but that remained substantially higher than those in Sur1KO islets maintained in 1 mM glucose from the start (Fig. 1). Because insulin secretion by Sur1KO islets was already high in 1 mM glucose, the relative stimulation by 15 mM glucose was much smaller than in control islets, but the absolute increase over baseline was similar in the two groups: 5.9 ± 0.7% per 100 min in controls and 6.2 ± 0.8% per 100 min in Sur1KO.

During long experiments, apparent islet [Ca²⁺]ᵢ slowly but steadily increases with time (34). This increase in control islets continuously perfused with 1 mM glucose can be seen in Fig. 2A. When these control islets were stimulated by 15 mM glucose, a small decrease in [Ca²⁺]ᵢ, first occurred, followed by a marked and sustained increase for a few minutes, and eventually appearance of large oscillations (33, 35, 36). These oscillations are shown in a representative experiment (Fig. 2C) but, because of their asynchrony between islets, are masked by averaging (Fig. 2A). The stimulatory effect of glucose on [Ca²⁺]ᵢ in control islets was rapidly and completely reversible upon return to a medium containing 1 mM glucose.

In Sur1KO islets, [Ca²⁺]ᵢ, was high already in the presence of 1 mM glucose (Fig. 2A) and it was not affected by an increase in the glucose concentration to 3 mM (not shown). When the results obtained in 28 islets maintained in 1 mM glucose for about 60 min were averaged (Fig. 2B), a time-dependent increase by 1.7 nM per min was measured, which must be taken into ac-
count to evaluate the true effects of glucose. Stimulation of Sur1KO islets with 15 mM glucose initially caused a marked decrease in $\left[\text{Ca}^{2+}\right]_i$ (Fig. 2D), that was so consistent in time not to be distorted by averaging (Fig. 2A and B). Thereafter, $\left[\text{Ca}^{2+}\right]_i$ increased above control values (islets maintained in 1 mM glucose) (Fig. 2B). During the period 30–40 min, average $\left[\text{Ca}^{2+}\right]_i$ was significantly ($p < 0.001$) higher in Sur1KO islets stimulated by 15 mM glucose (200 ± 7 nM, $n = 18$) than in islets maintained in 1 mM glucose (169 ± 4 mM, $n = 28$), although average $\left[\text{Ca}^{2+}\right]_i$ was similar in the two groups during the prestimulatory period (15–25 min). This rebound after the initial drop was, however, transient (Fig. 2A and B); during steady-state stimulation, average $\left[\text{Ca}^{2+}\right]_i$ was again similar in 15 mM glucose (211 ± 7 nM) and in 1 mM glucose (204 ± 5 nM). When, at the end of the experiments, the glucose concentration was lowered from 15 to 1 mM, $\left[\text{Ca}^{2+}\right]_i$ remained high in these Sur1KO islets (Fig. 2A and D). The difference in average $\left[\text{Ca}^{2+}\right]_i$ between stimulated and unstimulated Sur1KO islets usually disappeared after 15 min of glucose application, when $\left[\text{Ca}^{2+}\right]_i$ oscillations appeared (Fig. 2D). It was not our purpose to identify the mechanisms underlying the $\left[\text{Ca}^{2+}\right]_i$ oscillations that Sur1KO islets displayed both in 1 mM glucose (Fig. 2E).
Amplifying Pathway of Insulin Secretion in Sur1KO Islets

Fig. 3. Insulin secretion and \([\text{Ca}^{2+}]_i\), are increased in Sur1KO islets perfused with 1 mM glucose. Control and Sur1KO islets were perfused with a medium containing 1 mM glucose (G) throughout. A second group of control islets was perfused with 1 mM glucose and 500 \(\mu\)M tolbutamide (+ Tolb). A and B, extracellular \(\text{Ca}^{2+}\) was omitted for 10 or 20 min as indicated. C and D, 5 mM azide was added for 10 or 20 min as indicated. Values are means ± S.E. for 11–16 islets from three or four preparations for \([\text{Ca}^{2+}]_i\), measurements and five experiments of insulin secretion.

and in 15 mM glucose (Fig. 2F) nor to investigate the reasons for their variable pattern and generally higher frequency in high glucose (Fig. 2, E and F). However, it is noteworthy that an oscillatory electrical activity is present in Sur1KO islets perfused with low glucose (37, 38) and that these oscillations become shorter and faster in high glucose (37). These similarities with the \([\text{Ca}^{2+}]_i\) oscillations that we measured strongly suggest that \([\text{Ca}^{2+}]_i\) oscillations are controlled by the membrane potential in Sur1KO as in normal \(\beta\)-cells. Whatever the mechanisms of the oscillations, the most important findings in the context of the present study are that \([\text{Ca}^{2+}]_i\) and insulin secretion in Sur1KO islets are both elevated in low glucose and that high glucose markedly increases insulin secretion without further increasing \([\text{Ca}^{2+}]_i\), except during the transient (∼15 min) rebound following the initial decrease.

Mechanisms of Insulin Secretion by Sur1KO Islets in the Presence of 1 mM Glucose—In this series of experiments we assessed whether the high rate of insulin secretion by Sur1KO islets in 1 mM glucose can simply be ascribed to the elevation of the triggering signal \([\text{Ca}^{2+}]_i\). Control islets were studied in a usual medium or after blockade of \(K_{\text{ATP}}\) channels by a high concentration of tolbutamide (500 \(\mu\)M).

Fig. 3 (A and B) shows again that, in the presence of 1 mM glucose, \([\text{Ca}^{2+}]_i\), was higher and insulin secretion markedly increased in Sur1KO islets as compared with control islets. \([\text{Ca}^{2+}]_i\), was even higher in control islets perfused with 1 mM glucose and 500 \(\mu\)M tolbutamide, but insulin secretion was surprisingly less than in Sur1KO islets.

Despite the major differences in initial \([\text{Ca}^{2+}]_i\), omission of extracellular \(\text{Ca}^{2+}\) was followed by a decrease in \([\text{Ca}^{2+}]_i\), to a similar level in the three groups of islets (Fig. 3A). Simultaneously, basal insulin secretion from control islets marginally, but consistently, increased, whereas the high rates of secretion in Sur1KO and control plus tolbutamide islets decreased, to reach the same rate of secretion as that of control islets (Fig. 3B). All these changes in \([\text{Ca}^{2+}]_i\), and insulin secretion were entirely reversible upon reintroduction of \(\text{CaCl}_2\) into the medium.

To evaluate the role of energy for insulin secretion by Sur1KO islets in 1 mM glucose, azide, a mitochondrial poison, was added to the perfusion medium. In both control and Sur1KO islets, azide caused an increase in \([\text{Ca}^{2+}]_i\), (Fig. 3C), which we ascribe to arrest of \(\text{Ca}^{2+}\) pumping into and release of \(\text{Ca}^{2+}\) from intracellular stores. Basal insulin secretion from control islets was unaffected, but the high secretion rate from Sur1KO islets was progressively inhibited (Fig. 3D). Removal of azide was followed by a decrease in \([\text{Ca}^{2+}]_i\). In Sur1KO islets this decrease was marked and of variable duration, which gives a peculiar, progressive, stepwise pattern to the recovery after averaging (Fig. 3C). Insulin secretion recovered partially but, for unknown reasons, slightly more rapidly than \([\text{Ca}^{2+}]_i\). This inhibition of insulin secretion in low glucose is not specific to azide. Thus, inhibition of mitochondrial metabolism by 10 \(\mu\)M FCCP, 5 \(\mu\)M oligomycin, 10 \(\mu\)M rotenone, or simply lowering the temperature to 22 °C also suppressed (90% or more) insulin secretion by Sur1KO islets incubated in 1 mM glucose.

These experiments establish that the high rate of insulin secretion by Sur1KO islets in low glucose results from a continuous, unregulated, influx of \(\text{Ca}^{2+}\), secondary to the lack of functional \(K_{\text{ATP}}\) channels, and a metabolic regulation of the effectiveness of \(\text{Ca}^{2+}\).

Sur1KO Islets Are Insensitive to Diaxoxide and Tolbutamide—In control islets, \([\text{Ca}^{2+}]_i\), was high during stimulation by 15 mM glucose (Fig. 3A). Addition of 100 \(\mu\)M diazoxide, to open \(K_{\text{ATP}}\) channels, rapidly lowered \([\text{Ca}^{2+}]_i\), an effect that was completely and reversibly counteracted by 100 \(\mu\)M tolbutamide. These changes in \([\text{Ca}^{2+}]_i\), were paralleled by changes in insulin secretion. Addition of diazoxide during sustained stimulation
Amplifying Pathway of Insulin Secretion in Sur1KO Islets

Insulin secretion was measured in glucose-stimulated islets of Sur1KO mice—10-fold (39). In control islets forskolin was without effect on basal insulin secretion, but it potentiated the response to 15 mM glucose 4- to 5-fold and did not alter the inhibitory effect of diazoxide and its reversal by tolbutamide (Fig. 4C). In Sur1KO islets, forskolin increased insulin secretion 4-fold already in the presence of 1 mM glucose. Importantly, the stimulation by 15 mM glucose produced qualitatively and quantitatively similar effects as those in the absence of forskolin: an initial transient decrease and a sustained 2.5-fold amplification. Addition of diazoxide and tolbutamide were again without effect. Under these conditions, the changes in [Ca\(^{2+}\)] were similar to those in the absence of forskolin (data not shown).

In a series of four experiments similar to those shown in Fig. 4B, tolbutamide was not withdrawn at 110 min, but 1 μM forskolin was added. This resulted in a potentiation of insulin secretion by 4-fold in control islets and 3.5-fold in Sur1KO islets. These experiments thus establish that Sur1KO islets are insensitive to the normal inhibitory and stimulatory effects of diazoxide and tolbutamide and that the stimulatory effect of glucose on insulin secretion persists in the presence of elevated cAMP levels.

Insulin Secretion under Conditions Testing the Amplification Pathway in Control Islets—Two experimental approaches are classically used to study the amplifying pathway of glucose-induced insulin secretion: to hold K\(_{ATP}\) channels closed by a sulfonylurea or to hold them open by diazoxide and depolarize β-cells with KCl before addition of glucose (1).

In control islets, perfused with 1 mM glucose, 500 μM tolbutamide induced a rapid and sustained increase in [Ca\(^{2+}\)] (Fig. 5A). Subsequent stimulation with 15 mM glucose caused a rapid, transient decrease in [Ca\(^{2+}\)], followed by a return slightly above initial values (34), whereas the final switch to 1 mM glucose was accompanied by a small increase in [Ca\(^{2+}\)] (Fig. 5A). Under similar conditions, tolbutamide induced a rapid increase in insulin secretion followed by a lower, sustained second phase, which was markedly and reversibly increased by the subsequent rise in the glucose concentration (Fig. 5B). No initial drop in insulin secretion is visible after averaging, because the phenomenon was not consistent.

In Sur1KO islets, [Ca\(^{2+}\)] was high in 1 mM glucose and unaffected by tolbutamide, whereas 15 mM glucose induced a transient decrease in [Ca\(^{2+}\)], followed by a return slightly above initial values (Fig. 5A). Simultaneously, the high rate of insulin secretion in 1 mM glucose was not significantly affected by tolbutamide, whereas a transient drop, further increased by 15 mM glucose (Fig. 5B). The final return to 1 mM glucose inhibited insulin secretion without lowering [Ca\(^{2+}\)].

Stimulation of control islets with 30 mM KCl in the presence of diazoxide and 1 mM glucose rapidly increased [Ca\(^{2+}\)], and insulin secretion (Fig. 5, C and D). Subsequently raising the glucose concentration to 15 mM caused a partial lowering of [Ca\(^{2+}\)], followed by a return to similar values as those in 1 mM glucose, and simultaneously increased insulin secretion. The final return to 1 mM glucose led to a decrease in insulin secretion, although [Ca\(^{2+}\)] did not decrease (Fig. 5, C and D).

In Sur1KO islets perfused with 1 mM glucose, [Ca\(^{2+}\)], and insulin secretion were high despite the presence of diazoxide, and both were further increased by 30 mM KCl (Fig. 5, C and D). Subsequent stimulation with 15 mM glucose and arrest of the stimulation affected [Ca\(^{2+}\)], and insulin secretion in a similar way as in control islets.

These experiments confirm that tolbutamide is ineffective in Sur1KO islets and show that a further increase in insulin secretion can be elicited in these islets if [Ca\(^{2+}\)] is further increased (e.g. by KCl). They also establish that glucose similarly amplifies insulin secretion (i.e. increases secretion with-

Fig. 4. Influence of diazoxide and tolbutamide on [Ca\(^{2+}\)], and insulin secretion in glucose-stimulated islets. A, [Ca\(^{2+}\)], in perfused islets. Control and Sur1KO islets were perfused with a medium containing 15 mM glucose throughout. Diazoxide (Dz, 100 μM) and tolbutamide (Tolb, 100 μM) were added as indicated. Values are means ± S.E. for 11 or 14 islets from three or four preparations. B and C, insulin secretion from perfused islets. Control and Sur1KO islets were stimulated with 15 mM glucose (G) from 40 to 130 min. Diazoxide and tolbutamide were added as indicated. In C, the experiments were performed in the presence of 1 mM forskolin (Fk) throughout. Values are means ± S.E. for six (B) or five (C) experiments.

with glucose virtually abrogated insulin secretion, which was reversibly restored by addition of tolbutamide on top of diazoxide (Fig. 4B).

The behavior of Sur1KO islets was strikingly different. Their [Ca\(^{2+}\)], in the presence of 15 mM glucose alone was similar to that in control islets (Fig. 4A), but the insulin secretion rate was again higher (Fig. 4B). Neither diazoxide alone nor its subsequent combination with tolbutamide affected [Ca\(^{2+}\)], or insulin secretion. Note that the apparent, paradoxical, decrease in insulin secretion occurring in the presence of tolbutamide around 100 min of the experiments (Fig. 4B) coincides with the onset of a spontaneous decrease in the effect of glucose alone (Fig. 1).

Insulin secretion was also measured in the presence of 1 μM forskolin, which is known to increase the cAMP content of...
out further elevating \([\text{Ca}^{2+}]_i\) in Sur1KO and control islets.

The Responses of Sur1KO Islets to Amino Acids—Nutrients other than glucose can also increase insulin secretion through the amplifying pathway (17, 18, 40, 41) in normal islets. We therefore investigated the effect of selected amino acids in Sur1KO islets.

The islets were stimulated with 10 mM leucine plus 10 mM glutamine in the presence of 1 mM glucose. This combination of amino acids provides mitochondria with both acetyl-CoA and \(\alpha\)-ketoglutarate (anaplerosis via glutamate dehydrogenase) (41). In control islets, leucine plus glutamine induced a modest increase in \([\text{Ca}^{2+}]_i\) and insulin secretion (Fig. 6). In Sur1KO islets, they consistently produced a rapid further increase in \([\text{Ca}^{2+}]_i\), followed by a longer, marked drop and a final return slightly above initial values (Fig. 6A) while insulin secretion was rapidly and strongly increased (Fig. 6B). After blockade of K<sub>ATP</sub> channels by tolbutamide in control islets, the association of leucine plus glutamine produced qualitatively similar changes in \([\text{Ca}^{2+}]_i\) and insulin secretion as in Sur1KO islets (Fig. 6, A and B). Note, however, that the insulin secretion rate was higher or similar in Sur1KO islets despite lower \([\text{Ca}^{2+}]_i\) than in control islets treated with tolbutamide.

The immediate increase in \([\text{Ca}^{2+}]_i\), produced by leucine plus glutamine indicates that these amino acids influence the triggering signal and that their effects on insulin secretion cannot entirely be attributed to the amplifying pathway. To separate the two effects, we applied glutamine and BCH sequentially. BCH was used because it activates glutamate dehydrogenase like leucine but, unlike the latter, is not metabolized (41–43).

In Sur1KO islets perifused with 1 mM glucose, 2 mM glutamine produced a rapid but modest increase in \([\text{Ca}^{2+}]_i\), (Fig. 7A) and a small increase in insulin secretion (Fig. 7B); in contrast to the continuous decrease in secretion observed in 1 mM glucose alone (Fig. 1), a slight, consistent \((p < 0.01)\) increase followed glutamine addition. Much more spectacular was the...

Fig. 5. Influence of glucose on islet \([\text{Ca}^{2+}]_i\) and insulin secretion in the presence of a high concentration of tolbutamide or KCl. Control and Sur1KO islets were initially perifused with a medium containing 1 mM glucose (G). A and B, tolbutamide (Tolb 500 \(\mu\)M) was then added, and the glucose concentration was increased to 15 mM as indicated. Values are means \pm S.E. for 11 islets from 3 preparations (A) and for 5 experiments (B). C and D, the medium contained 100 \(\mu\)M diazoxide (Dz) throughout. The concentrations of KCl and glucose were increased to 30 and 15 mM as indicated. Values are means \pm S.E. for 15 islets from 4 preparations (C) and for 6 experiments (D).

Fig. 6. Influence of leucine and glutamine on \([\text{Ca}^{2+}]_i\) and insulin secretion in control and Sur1KO islets. A, \([\text{Ca}^{2+}]_i\), in control islets, Sur1KO islets and control islets with 500 \(\mu\)M tolbutamide (+ Tolb). The concentration of glucose (G) was 1 mM throughout, and leucine plus glutamine, 10 mM each, were added as indicated. Values are means \pm S.E. for 15 to 16 islets from 4 preparations. B, insulin secretion by control and Sur1KO islets, and control islets with 500 \(\mu\)M tolbutamide, stimulated by leucine plus glutamine in the presence of 1 mM glucose as in A. Values are means \pm S.E. for five experiments.
response to 5 mM BCH, which produced glucose-like effects (compare Fig. 7 with Figs. 1 and 2). A transient fall in $[\text{Ca}^{2+}]_i$, and insulin secretion initially occurred, followed by a return of $[\text{Ca}^{2+}]_i$ to initial values and a 3-fold increase in insulin secretion (Fig. 7). Subsequent addition of tolbutamide was without effect on $[\text{Ca}^{2+}]_i$, and insulin, whereas removal of the amino acids, tolbutamide remaining present, resulted in a decrease in insulin secretion with only a short-lived drop in $[\text{Ca}^{2+}]_i$. Qualitatively similar changes in $[\text{Ca}^{2+}]_i$ and insulin secretion were observed when BCH was added without prior application of glutamine (data not shown).

The responses of control islets were strikingly different (Fig. 7). Alone, 2 mM glutamine had no effect, and the subsequent addition of BCH only produced a small, transient increase in $[\text{Ca}^{2+}]_i$ and insulin secretion initially occurred, followed by a return of $[\text{Ca}^{2+}]_i$, to initial values and a 3-fold increase in insulin secretion (Fig. 7). Subsequent addition of tolbutamide was without effect on $[\text{Ca}^{2+}]_i$, and insulin, whereas removal of the amino acids, tolbutamide remaining present, resulted in a decrease in insulin secretion with only a short-lived drop in $[\text{Ca}^{2+}]_i$. Qualitatively similar changes in $[\text{Ca}^{2+}]_i$ and insulin secretion were observed when BCH was added without prior application of glutamine (data not shown).

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We finally compared the responses of the two types of islets to 5 mM (twice the total concentration of amino acids in the plasma of fasting mice) (44) of a mixture of four amino acids (1 mM alanine, 1 mM leucine, 1.5 mM lysine, and 1.5 mM glutamine) chosen as representative of the major classes of amino acids in plasma. The amino acid mix was tested in the presence of 1 mM glucose. It caused minimal effects in control islets but triggered a biphasic reversible increase in $[\text{Ca}^{2+}]_i$, and insulin secretion in Sur1KO islets (Fig. 8). Similar responses were evoked in control islets in the presence of 500 mM tolbutamide.

These results show that amino acids can indeed stimulate insulin secretion from Sur1KO islets when they are virtually inactive in control islets, but this effect is often accompanied, and thus partly caused by, an increase in $[\text{Ca}^{2+}]_i$. A strong effect on insulin secretion can also be mediated by the amplifying pathway when glutamate dehydrogenase is activated.

DISCUSSION

This study shows that, in vitro, $\beta$-cells without SUR1, hence without $K_{ATP}$ channels, already secrete substantial amounts of insulin in the presence of 1–3 mM glucose, which can be explained by a persistent elevation of the triggering signal, $[\text{Ca}^{2+}]_i$, and an already very efficient amplifying pathway. It also shows that an increase in the glucose concentration or the addition of amino acids markedly increases insulin secretion sometimes by augmenting the triggering signal but more often without such a change, i.e. through the amplifying pathway. It is thus unlikely that SUR1 plays a role other than the regulation of $K_{ATP}$ channels in the plasma membrane during nutrient-induced insulin secretion.

As expected from the known structure and function of $K_{ATP}$ channels, Sur1KO $\beta$-cells do not contain $K_{ATP}$ channels (25) and, therefore, are depolarized (25, 37, 45) and have an elevated $[\text{Ca}^{2+}]_i$ (26, 28) even when the ambient glucose concentration is non-stimulatory for normal $\beta$-cells. A similar $\beta$-cell phenotype has been observed in $K_{ATP}$KO mice (46) and can be
experimentally reproduced by closure of $K_{\text{ATP}}$ channels with a sulfonylurea (34, 47). By extrapolation of the effects observed in the latter model (47), it was therefore anticipated that, at least in vitro, Sur1KO islets would secrete excessive amounts of insulin in the presence of low glucose and increase their insulin secretion rate, via the amplifying pathway, when challenged by high glucose. Surprisingly, in all but one (48) of the previous studies “basal” insulin secretion was not increased and, with one exception (49), the response to high glucose alone was virtually absent or exceedingly small (25–28).

Our results first establish that the triggering capacity of $Ca^{2+}$ is fully retained in $\beta$-cells lacking SUR1, although these are subjected to a chronic elevation of $[Ca^{2+}]_i$. Thus, under stable metabolic conditions (1 mM glucose), imposing rapid decreases and increases in $[Ca^{2+}]_i$, by removal and reintroduction of extracellular $Ca^{2+}$ resulted in parallel changes in insulin secretion. Moreover, a further increase in $[Ca^{2+}]_i$ by KCl immediately triggered a larger secretion of insulin. Although persistent elevation of $[Ca^{2+}]_i$, in normal islets has been reported to down-regulate calmodulin-dependent protein kinase II (50), an effect of $Ca^{2+}$-induced exocytosis (51), Sur1KO $\beta$-cells remain exquisitely sensitive to changes in $[Ca^{2+}]_i$. This conclusion is borne out by patch-clamp experiments that measured capacitance changes at different intracellular $[Ca^{2+}]_i$, to estimate exocytosis in Sur1KO $\beta$-cells (26). There is no doubt that the triggering signal is efficient in Sur1KO $\beta$-cells. What is lost with $K_{\text{ATP}}$ channels is the ability of glucose to control generation and magnitude of the triggering signal $[Ca^{2+}]_i$, via the physiological triggering pathway. This does not mean, however, that glucose is without influence on $[Ca^{2+}]_i$. In control islets, $Ca^{2+}$-induced exocytosis (52) results in rapid secretion of insulin that accompanies the rapid $[Ca^{2+}]_i$ increase that this pool is affected by the abnormally high rate of insulin secretion in Sur1KO islets depolarized with sulfonylureas (47, this study). It is possible that this pool is affected by the abnormally high rate of insulin secretion in low glucose, but it is not empty as shown by the rapid secretion of insulin that accompanies the rapid $[Ca^{2+}]_i$ rise produced in Sur1KO islets by KCl or the mixture of amino acids.

The lack of effects of tolbutamide and diazoxide on insulin secretion by Sur1KO islets is attributed to the inability of the drugs to increase and decrease $[Ca^{2+}]_i$, in these $\beta$-cells. This interpretation is classic, but deserves additional comments in the light of the observation that intracellularly applied tolbutamide paradoxically remained able to increase cell capacitance (exocytosis) in Sur1KO $\beta$-cells (49). This implies that the intracellular target of sulfonylureas is not SUR1, but a related protein (53). It is plausible that this protein participates in insulin secretion, but we still find it irrelevant for the therapeutic effects of sulfonylureas (54). Thus, there is consensus that sulfonylureas do not increase insulin secretion from islets without $K_{\text{ATP}}$ channels, be they Sur1KO (25, 49, this study) or Kir6.2KO (46), and this in low or high glucose.

The amplifying action of glucose on insulin secretion can be mimicked by metabolized amino acids in normal $\beta$-cells (17, 18, 41). Our study shows that this holds true for Sur1KO $\beta$-cells. However, some amino acids also influence $[Ca^{2+}]_i$, by electrogenic mechanisms independent of $K_{\text{ATP}}$ channels (40). For example, 2 mM glutamine alone and the amino acid mixture increased $[Ca^{2+}]_i$, in Sur1KO $\beta$-cells presumably because their entry into the cell with Na$^+$ (55) or in cationized form (56) further depolarized the membrane. Unmasking of this effect, as that of arginine (56), by closure of $K_{\text{ATP}}$ channels in control islets (increasing electrical membrane resistance) supports this interpretation. The ability of glutamine to increase insulin secretion in Sur1KO islets, while being inactive in control islets, has recently been reported by others (48), who hypothesized that the amino acid might play a signaling role in stimulus-secretion coupling. In our hands, glutamine alone never increased insulin secretion under conditions where it did not increase $[Ca^{2+}]_i$, (17, this study). Our interpretation, therefore, is that the small effect of 2 mM glutamine alone is mediated by a change in the triggering signal, not the amplifying signals. In contrast, the large increase in insulin secretion produced by addition of BCH (an activator of glutamate dehydrogenase) to Sur1KO islets pretreated (or not) with glutamine occurred without a further increase in $[Ca^{2+}]_i$, and can thus be attributed to the amplifying pathway (17, 41). Interestingly, this combination of glutamine and BCH did not produce a triggering signal in control islets unless $K_{\text{ATP}}$, channels were closed with tolbutamide. Yet, even though the increase in $[Ca^{2+}]_i$, was larger under these conditions than in Sur1KO islets, insulin secretion was less. A similar situation was observed during stimulation with high concentrations of glutamine and leucine. Altogether this indicates that the amplifying pathway is more efficient in Sur1KO than control islets not only when it is activated by glucose (see above) but also when it is activated by a stimulation of glutamate dehydrogenase. The large stimulation of insulin secretion produced by the amino acid mixture in Sur1KO islets and in normal islets, depolarized with tolbutamide, is ascribed to an increase of both $[Ca^{2+}]_i$, and the amplifying pathway.

These experiments permit the following conclusions. At low...
glucose, amino acids are more potent insulin secretagogues in Sur1KO than control islets for two reasons. First, electrogenuically transported amino acids further increase \( [Ca^{2+}] \) in Sur1KO \( \beta \)-cells but cannot influence the triggering signal in control \( \beta \)-cells whose membrane electrical resistance is too low (K\(_{\text{ATP}}\) channels are open). Second, metabolized amino acids produce amplification signals that augment the efficacy of elevated \( [Ca^{2+}] \), on exocytosis, whereas the same signals remain silent in control \( \beta \)-cells until \( [Ca^{2+}] \), is increased. Importantly, the responsiveness of Sur1KO \( \beta \)-cells to amino acids should not be mistaken for a hypersensitivity specifically caused by the lack of SUR1. This responsiveness is conferred by the absence of functional K\(_{\text{ATP}}\) channels. A similar responsiveness is conferred on normal \( \beta \)-cells by blockade of K\(_{\text{ATP}}\) channels with a sulfonylurea. We do not exclude the possibility that the acute or chronic elevation of \( [Ca^{2+}] \), also influences the metabolism of amino acids.

Our findings that Sur1KO \( \beta \)-cells are not intrinsically abnormal in terms of efficacy of the triggering signal or generation and efficacy of the amplifying signals deepen the paradox of their behavior in \textit{vivo}. Why are Sur1KO mice not secreting inappropriately high amounts of insulin during fasting and why do they not secrete more insulin during imposed hyperglycemia? We suggest that an inhibitory signal of unknown nature restrains excessive insulin secretion in \textit{vivo}.

In conclusion, both the triggering and amplifying signals of insulin secretion are efficient in islets from Sur1KO mice. Because \( \beta \)-cell \([Ca^{2+}]\), can no longer be regulated by controlling K\(_{\text{ATP}}\) channels, the triggering signal remains high in the presence of low glucose and, in association with an already operative amplifying signal, causes insulin secretion. High glucose markedly increases insulin secretion by two mechanisms: a previously unsuspected, K\(_{\text{ATP}}\) channel-independent increase in \([Ca^{2+}]\), during 5–20 min, and the amplifying pathway. Amino acids also increase insulin secretion by a dual mechanism in Sur1KO islets. First, their ability to increase the triggering signal via their electrogeneric transport (an intrinsically K\(_{\text{ATP}}\), channel-independent phenomenon) is facilitated by the absence of K\(_{\text{ATP}}\) channels. Second, their metabolism, like that of glucose, fuels the amplifying pathway. Because they found glucose unable to increase insulin secretion from Sur1KO islets, the authors of a recent paper (28) conclude that the concept of a dual control of insulin secretion by triggering and amplifying signals (40) needs modifications. In sharp contrast, the present study validates the concept and provides additional support for the proposal that the terms K\(_{\text{ATP}}\) channel-dependent and -independent pathways are sometimes misnomers and should be replaced by triggering and amplifying pathways (1).
Both Triggering and Amplifying Pathways Contribute to Fuel-induced Insulin Secretion in the Absence of Sulfonylurea Receptor-1 in Pancreatic β-Cells
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