



## Depletion of SK1 channel subunits leads to constitutive insulin secretion

Marilou A. Andres<sup>a,\*</sup>, Nicholas C. Baptista<sup>a</sup>, Jimmy T. Efrid<sup>a,b,d</sup>, Kathleen K. Ogata<sup>b,c</sup>, Frederick P. Bellinger<sup>b</sup>, Thomas Zeyda<sup>b</sup>

<sup>a</sup> Pacific Biosciences Research Center, University of Hawaii, 1993 East–West Road, Honolulu, HI 96822, United States

<sup>b</sup> John A. Burns School of Medicine, University of Hawaii, Honolulu, HI 96813, United States

<sup>c</sup> University of Hawaii Kapi'olani Community College, Honolulu, HI 96816, United States

<sup>d</sup> Cincinnati Children's Hospital Medical Center, Cincinnati, OH 45229, United States

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### ABSTRACT

**In the pancreas, the role of the small-conductance, calcium-activated SK channels remains controversial. Here, we show that three SK subtypes are expressed in the rat insulinoma cells. Our findings demonstrate that rat SK1 (rSK1) channels ensure appropriate insulin secretion by establishing the cell's negative resting membrane potential and shortening the duration of the action potential. We also found that the depletion of rSK1 transcripts generated a condition in which beta cells constitutively secrete insulin, even in the absence of a stimulating molecule (such as glucose). Together, these results implicate SK1 subunits as key regulators of excitability and endocrine function in beta cells.**

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### 1. Introduction

Pancreatic beta cells, when stimulated by glucose at concentrations above 7 mM, exhibit a bursting of action potentials that increase intracellular calcium,  $[Ca]_i$ , triggering insulin secretion [1]. Prior to bursting activity, glucose molecules are first metabolized into intermediate molecules that are crucial for generating ATP, the key energy molecules that transduce the external stimulus (glucose) [2]. A conversion of this glucose signal to an electrical response occurs when the ATP molecules block ATP-sensitive  $K^+$  ( $K_{ATP}$ ) channels, which depolarizes the membrane. This membrane depolarization then initiates a cascade of downstream events including the activation of voltage-dependent L-type  $Ca^{2+}$  channels that mediate the calcium influx necessary for triggering insulin secretion.

The pattern of the electrical activity during glucose stimulation is characterized by membrane potential oscillations. These oscillations are tightly linked to transient  $[Ca]_i$  changes and are modulated by different  $K^+$  channels. One of the mechanisms believed to underlie the beta cell bursting pattern involves the activation of calcium-activated  $K^+$  channels ( $K_{Ca}$ ). The large-conductance BK

type  $K_{Ca}$  channels do not appear to play an important role in modulating the electrical activity of beta cells [3]; whereas, the contribution of the small-conductance SK  $K_{Ca}$  channels remains controversial because earlier reports show beta cells to be insensitive to the SK channel blocker apamin [4–6]. Further, the slowly activating and calcium-responsive  $K_{slow}$  currents are also thought to regulate the beta cells' electrical activity; and, although they exhibit certain features attributed to SK channel types, they are insensitive to apamin, thus making it difficult to molecularly identify and isolate the channel responsible for these currents [5,7]. Recently, the expression of the SK channels has been experimentally demonstrated in beta cells; and, while all four SK isoforms (SK1–SK4) have been identified [8], the relationship between  $K_{slow}$  currents and SK channels remains elusive.

In the present study we sought to investigate the role of the SK1 subunits in modulating beta cell excitability and glucose responses. There are functional differences between human and rat SK1 orthologues. In rodents, SK1 isoforms are known to form non-functional or “electrically silent” channels [9–12], however, they could gain channel function by means of specific coassembly with rSK2 subunits to form heteromers (i.e., SK1/SK2) [11]. In contrast, the human SK1 orthologues (hSK1) are capable of coassembling into functional homomeric channels. These channels exhibit different apamin-sensitivities depending upon which heterologous

\* Corresponding author. Fax: +1 808 956 6984.

E-mail address: [marilou@pbrc.hawaii.edu](mailto:marilou@pbrc.hawaii.edu) (M.A. Andres).

expression system is used to express them [10,13,14]. Currently, the *in vivo* physiological role of rSK1 subunits is not clearly defined. To better understand the functional contribution of SK1 to excitability and to the endocrine function of the beta cell, we used a siRNA-based down-regulation approach. We show that SK1 subunits found in the INS-1 beta cells: (1) contribute to the generation of the AHP; (2) establish the negative resting membrane potential; (3) regulate the duration of the action potential; and (4) lead to constitutive insulin secretion when downregulated. These findings suggest a key biological role of a seemingly silent channel subunit in the mechanism that involves maintaining appropriate secretory response in beta cells. These data support the hypothesis that SK1 subunits may be functional in their native environment.

## 2. Materials and methods

### 2.1. Cell Culture

INS-1 cells, derived from rat insulinoma, were obtained from the University of Washington Diabetes Endocrinology Research Center, Seattle. Cells were incubated at 37 °C in a 5% CO<sub>2</sub> environment and grown in RPMI-1640 (Hyclone, Logan, UT) supplemented with 10% (v/v) FBS, 10 mM HEPES, 2 mM L-alanyl-L-glutamine, 50 μM 2-mercaptoethanol, 1 mM sodium pyruvate. Antibiotics were added as necessary.

### 2.2. RT-PCR/PCR

Total RNA was isolated from the INS-1 cells using RNeasy Mini Kit (Qiagen, Valencia, CA) per the manufacturer's instructions. Reverse transcription (RT) was carried out using GeneAmp RNA PCR (Applied Biosystems, Foster City, CA). Products were generated from cDNAs by PCR reactions using components of PCR Core System II (PROMEGA, Madison, WI). Control reactions, run in parallel to the experimental reactions, have all the reaction reagents except for the reverse transcriptase enzyme. Reverse transcription (RT) reactions were conducted using MJ Research PTC-200 Peltier Thermal Cycler with settings and primer pairs described in [Supplementary data](#). All PCR products were analyzed in 2% agarose gels stained with ethidium bromide and verified by sequencing.

### 2.3. Quantitative real-time PCR (qPCR)

Amplification of sample material (cDNA) was performed using both the LightCycler 2.0 System (Roche Applied Science, Indianapolis, IN) and the Bio-Rad iCycler iQ Real-Time Detection System (Bio-Rad Laboratories, Hercules, CA) with program settings, qPCR analysis, and primer pairs reported in [Supplementary data](#). Initially, products of target and housekeeping cDNAs were cloned in pDrive (Qiagen) and sequenced to confirm their identity. For subsequent analyses, melting curves for each polymerase chain reaction were used to verify that the correct product had been amplified.

### 2.4. Electrophysiology

Membrane potentials were measured in whole-cell configuration using the current clamp mode of the EPC-9 patch clamp amplifier (HEKA Elektronik, Lambrecht, Germany). The current clamp procedure consisted of current injections of 100 pA for 5 ms performed with zero holding current. Data were acquired using Pulse software (HEKA Elektronik) and were analyzed off-line using PulseFit (HEKA Elektronik) and IgorPro (WaveMetrics, Lake Oswego, OR, USA). Only stable measurements with  $R_s$  of less than 15 MΩ were analyzed. Patch pipettes were pulled from Kimax-51 glass (Kimble Glass, Vineland, NJ, USA) using Flaming/Brown Micropipette

Puller Model P-87 (Sutter Instrument Co., Novato, CA). All experiments were conducted at 32–34 °C. The internal solution for whole-cell recordings consisted of (in mM): potassium-glutamate 110, KCl 10, NaCl 10, MgCl<sub>2</sub> 1, Mg-ATP 3, HEPES 5, EGTA 10, and CaCl<sub>2</sub> 9 to achieve 1 μM free [Ca<sup>2+</sup>] (MaxChelator: <http://www.stanford.edu/~cpatton/maxc.html>); pH was adjusted to 7.2. Standard external solution contained (in mM): NaCl 140, KCl 4, NaHCO<sub>3</sub> 2, NaH<sub>2</sub>PO<sub>4</sub> 1, MgSO<sub>4</sub> 1, HEPES 5, CaCl<sub>2</sub> 2.5, with pH adjusted to 7.4. Where applicable, either glucose was omitted (0 mM glucose) or added (20 mM glucose) to the external recording solution. In the apamin experiments, 100 nM apamin was added to the external solution.

### 2.5. siRNA transfection

Cells were seeded at a density of 1–5 × 10<sup>5</sup> per well/petri-dish and transfected with HP GenomeWide KCNN1 (SK1) siRNA or non-silencing control siRNA (Qiagen) with HiPerfect Transfection Reagent (Qiagen) per the manufacturer's instructions. Note that in the siRNA transfection experiments, transfection reagent alone or in combination with a negative control siRNA did not affect basal expression levels of the target and the housekeeping genes. siRNA directed against SK1 was specific and did not alter the basic expression of the other subunit genes (data not shown).

### 2.6. Western blot analysis

Cultured cells in 100 mm petri-dishes were scraped into 4 ml cold PBS. Supernatant was then discarded following centrifugation at 14000 rpm for 5 min. The resulting cell pellets were then resuspended in 100 μl cold lysis buffer consisting of (in mM): Tris 20 pH 7.5, NaCl 100, 0.5% NP-40 (IGEPAL), EDTA 0.5, fresh PMSF 1, and 1% protease inhibitor cocktail (Sigma, St. Louis, MO). Lysates were then harvested after centrifugation at 14000 rpm for 10 min at 4 °C. Supernatants were stored at –20 °C until further analysis. Protein concentration was determined using the Bradford Assay. Equal volumes of Laemmli buffer/2-Mercaptoethanol were added to each sample, consisting of 40 μg of protein. Samples were then vortexed and incubated at 95 °C for 10 min. Samples were resolved in 4–12% Bis-Tris Criterion™ XT Precast Gel (Bio-Rad Laboratories, Hercules, CA) and electro-transferred onto Immobilon-P polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). Membranes were probed with either anti-SK1 antibodies at 1:200 dilution (Alomone Labs, Israel) and then with goat anti-rabbit IgG HRP at 1:50000 dilution (Amersham Biosciences Co., Piscataway, NJ) or anti-SK1 antibodies at 1:800 dilution (Santa Cruz Biotechnology Inc., Santa Cruz, CA) and then with Purified Recomb Protein G, Peroxidase Conjugated (Thermo Scientific, Rockford, IL) at 1:750 dilution. Signals were then detected with ECL Western Blotting Detection System (Amersham Biosciences Co.) or with SIGMAFAST (Sigma–Aldrich, St. Louis, MO).

### 2.7. Static insulin secretion measurements

INS-1 control and rSK1 siRNA-transfected cells were plated at a density of 5 × 10<sup>5</sup> cells per well (2.3 ml) in 6-well plates 4–6 h prior to the start of stimulation experiments. First, the culture medium was replaced with modified Krebs-Ringer bicarbonate HEPES (KRBH) solution consisting of (in mM): NaCl 135, KCl 3.6, NaHCO<sub>3</sub> 2, NaH<sub>2</sub>PO<sub>4</sub> 0.5, MgCl<sub>2</sub> 0.5, CaCl<sub>2</sub> 1.5, HEPES 10, and 0.25% BSA, pH 7.4. After 1 h of pre-incubation in KRBH solution at 37 °C (5% CO<sub>2</sub> and 90–95% relative humidity), the cells were then exposed to different treatments (KRBH with 20 mM glucose or without glucose) for 1 h to stimulate insulin secretion. The KRBH supernatants were then collected and centrifuged at 14000 rpm for 5 min at 4 °C. Supernatants were then transferred into new

tubes and stored at  $-20^{\circ}\text{C}$  until further analysis. Measurements of insulin concentration were performed using Rat Insulin RIA and Rat/Mouse Insulin ELISA kits (Millipore). At the end of each experiment, cells from each well were recounted using a Neubauer hemocytometer.

### 2.8. Statistical analysis

Differences among group means were statistically compared using one-way analysis of variance (ANOVA). The underlying normality of variables was assessed using bi-variate log-normal plots. When appropriate, a normalizing transformation was applied to the data. *P*-values were adjusted for multiplicity using the Hochberg sequential rejective procedure. Fisher's test was used to obtain exact *P*-values for cross tabulation  $2 \times 2$  analysis. All comparisons were 2-sided. *P*-values  $\leq 0.05$  were considered as statistically significant. Statistical analyses were performed using SAS software (SAS Institute Inc., Cary, NC).

## 3. RESULTS

### 3.1. Three SK subunit isoforms are transcribed (expressed) in pancreatic INS-1 beta cells

To investigate the role of SK1 subunits in beta cells, we first determined the expression of all rSK isoforms by RT/qPCR. We detected rSK1, rSK2, and rSK3 isoforms at approximately equal levels (Fig. 1a and b). Western analyses confirmed the actual expression of these subunits (data not shown). While Tamarina and colleagues (2003) reported SK4 expression in rodent pancreatic islet cells [8], our laboratory was unable to detect expression of this subunit in the INS-1 beta cells in agreement with previous finding [15]. These contrasting results could be explained by the amplification of non-beta cell types that are found in pancreatic islets.

### 3.2. Apamin-sensitive SK channels mediate the AHP in the pancreatic INS-1 beta cell line

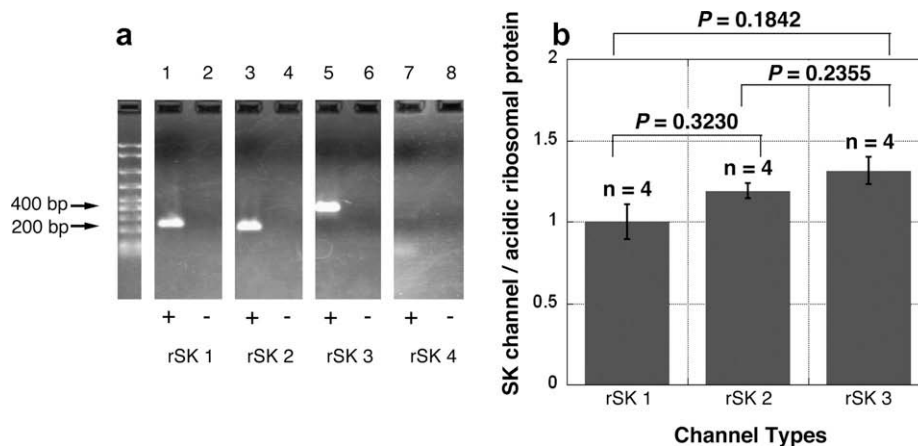
In this study, we used two types of recordings. First, we used whole-cell voltage clamp to measure the functional expression of these SK transcripts in the INS-1 cells. Here, as illustrated in Fig. 2a, apamin (100 nM) reduced the total  $K^{+}$  currents by approx-

imately 30–40%. The remaining  $K^{+}$  currents were further inhibited by subsequently treating the same cell with both an SK blocker (100 nM apamin) and the  $K_{\text{ATP}}$  channel inhibitor tolbutamide (100  $\mu\text{M}$ ) (Fig. 2a). These results demonstrate the functional expression of both the apamin-sensitive SK channels and the  $K_{\text{ATP}}$  channels in the rat insulinoma INS1 cell line. Since the signals between these two  $K^{+}$  channel types, under voltage clamp, are difficult to separate unless we use specific blockers, another form of recording was needed. In whole-cell current clamp recording, a distinction between SK channel activities and  $K_{\text{ATP}}$  channel activities could be made based on the presence of the AHP. To further understand the functional contribution of SK channels, we then measured membrane potential changes using such an approach. Applying a current injection procedure previously described [16], we induced a single action potential by a brief 5 ms injection of current (100 pA) (Fig. 2b). This current injection procedure evoked a rapid membrane depolarization and repolarization followed by an afterhyperpolarization (AHP) lasting over 100 ms (Fig. 2b). To verify that the SK channels underlie this AHP, cells were exposed to 100 nM apamin. As shown (Fig. 2b), the entire AHP component is removed by 100 nM apamin and is restored upon washout. Previous treatment of INS-1 cells with 100  $\mu\text{M}$  tolbutamide did not eliminate AHP (not shown), thus indicating that  $K_{\text{ATP}}$  channels do not contribute to this component [16]. Furthermore, we observed a depolarization of the resting membrane potential in response to apamin (100 nM) (Fig. 2c).

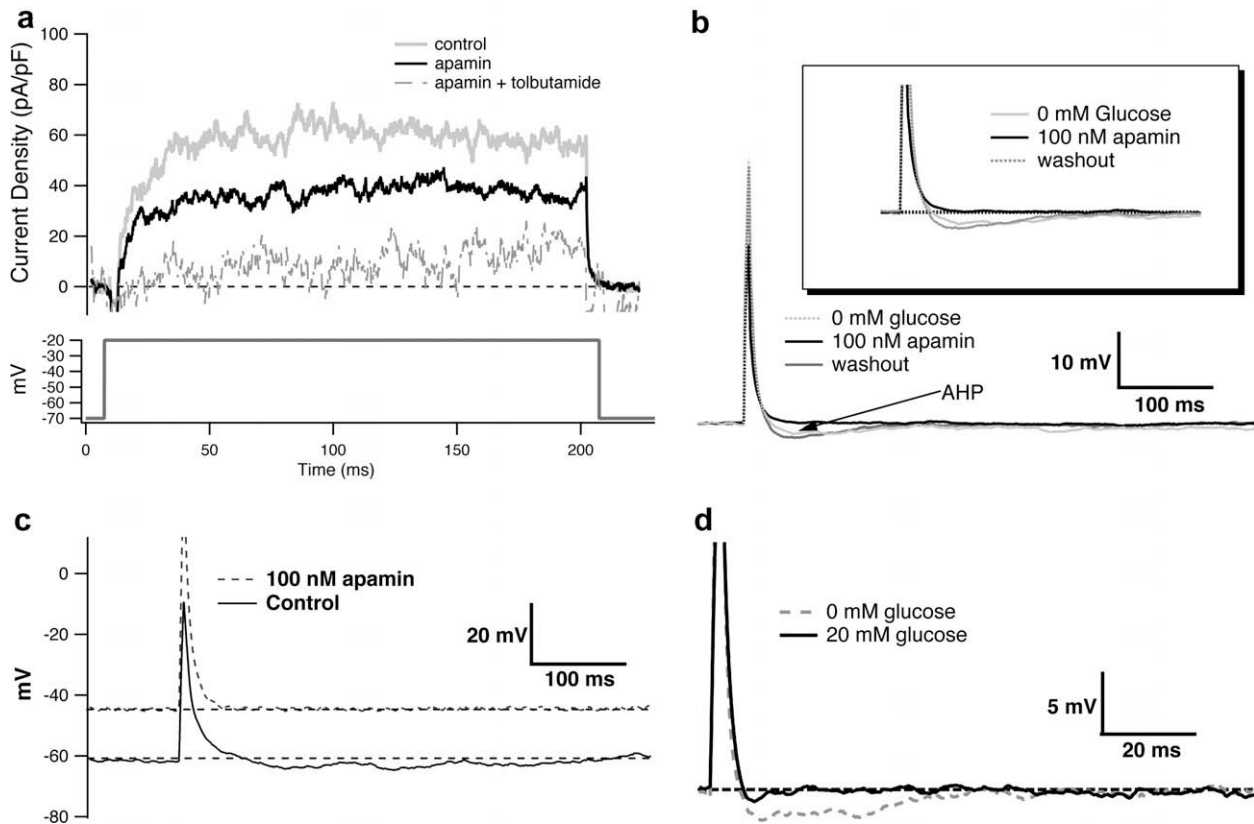
Next, we asked how SK channels would mediate the beta cell response when stimulated with glucose. Our current clamp recordings show that AHP is only present when cells are not exposed to glucose (Fig. 2d). Although this condition is somewhat artificial, it is informative as it shows a principal involvement of SK channels independent of glucose. Further, this implies that SK channels are indeed activated only in zero-glucose conditions; whereas, all components of the AHP (i.e., all contributing SK channel subtypes) are completely “eliminated” when cells are exposed to glucose (20 mM) (Fig. 2d).

### 3.3. rSK1 subunits are involved in the beta cell's AHP response

The fact that rSK1 subunits are as highly transcribed as rSK2 and rSK3 counterparts (Fig. 1b) suggests an important physiological role. Although previous works have shown that heterologously ex-



**Fig. 1.** Three SK channel subtypes are expressed in the rat insulinoma INS-1 cell line. (a) rSK1, rSK2, and rSK3 channels were detected in the INS-1 cell line. SK channel cDNA fragments were PCR-amplified by using primers specific for each SK isoform. On a 2% agarose gel, reactions show products for rSK1 (lane 1), rSK2 (lane 3), and rSK3 (lane 5) but not rSK4 (lane 7). PCR products from excised gel bands were sequenced. Sequencing results confirmed the identity of transcripts for rSK1, rSK2, and rSK3 subunit genes. Reverse transcriptases were omitted in the negative control reactions to probe for genomic DNA background (lanes 2, 4, 6, and 8). (b) qPCR analyses in beta INS-1 cells indicate comparable expression levels (in approximately 1:1:1 ratio) for all three SK isoforms. SK expression level is presented as a ratio of SK channel gene expression to that of the acidic ribosomal protein (ARP-PO) housekeeping gene. Mean expression values ( $\pm$ S.E.M.) for all three SK channel isoforms are as follows: 1.000 ( $\pm 0.108$ ) for rSK1; 1.188 ( $\pm 0.092$ ) for rSK2; 1.313 ( $\pm 0.083$ ) for rSK3. Means values are not significantly different when compared using one-way analysis of variance.



**Fig. 2.** SK channels mediate the afterhyperpolarization in the rat insulinoma INS-1 cell line. (a) This is a typical whole-cell voltage clamp recording from a representative INS-1 cell. The same cell was first exposed to physiological saline solution (control), then to 100 nM apamin and subsequently to both 100 nM apamin and 100  $\mu$ M tolbutamide in zero-glucose saline solution. In each condition, the cell was depolarized to  $-20$  mV for 200 ms from a holding potential of  $-70$  mV. Pipette solution contained 1  $\mu$ M free calcium to activate the SK channels. (b) The afterhyperpolarizing potential (AHP) is mediated by apamin-sensitive channels in the absence of glucose in saline solution. Records were acquired consecutively from the same beta cell under different conditions: control; 100 nM apamin; and washout. Traces were superimposed at the baseline for better visual comparison. (c) Membrane potential changes in response to 5 ms 100 pA current injection were measured in zero-current clamp mode from the same cell. Note the depolarization of membrane potential upon application of 100 nM apamin. (d) The AHP is only present when there is no glucose present. Shown are representative recordings from the same cell first exposed to zero-glucose recording solution (*broken line*) and subsequently to 20 mM glucose (*heavy line*).

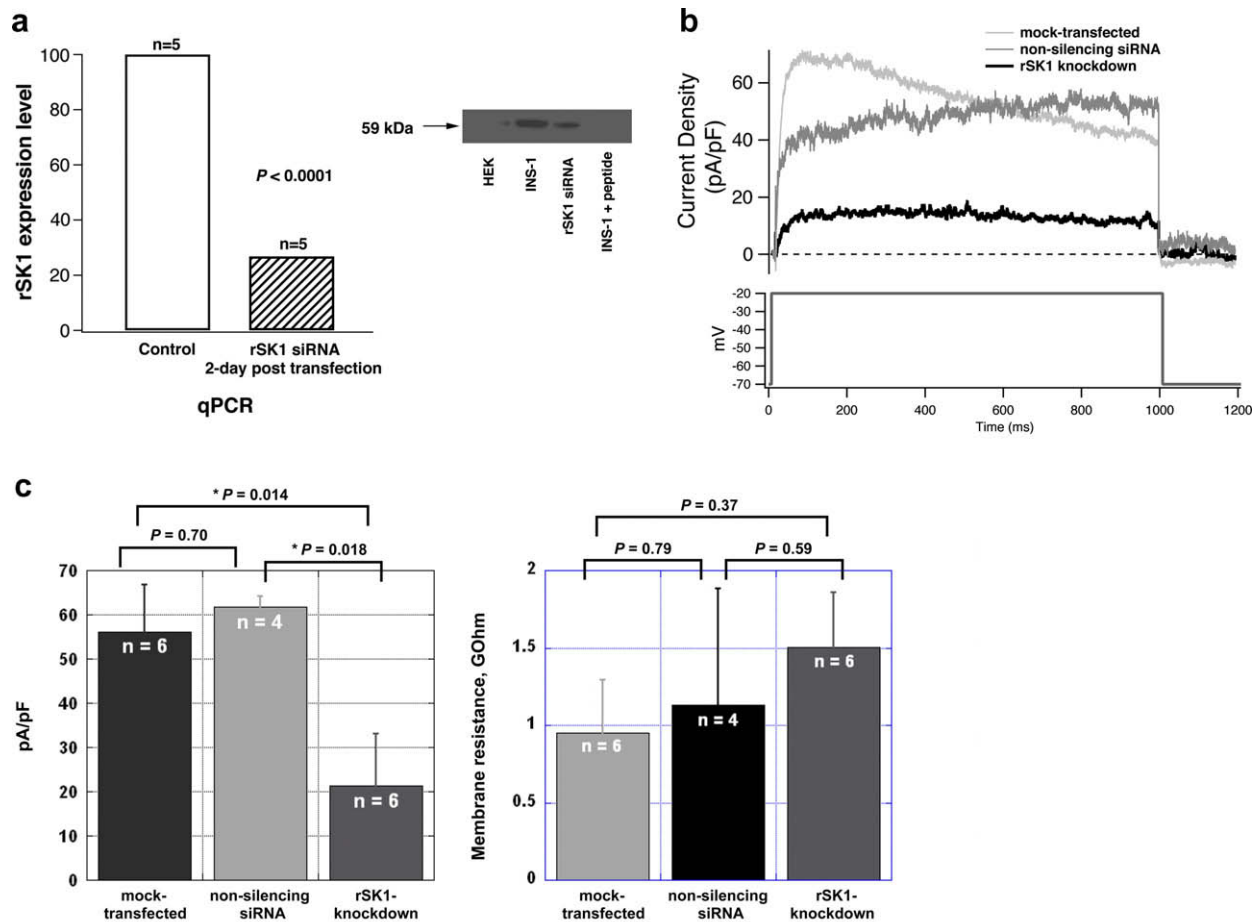
pressed rSK1 subunits form non-functional channels [9–12], it may well be that these channels are functional when natively expressed. To determine whether rSK1 is functional, we conducted siRNA knockdown experiments targeting rSK1 transcripts, thus reducing the formation of all possible channel forms consisting of rSK1 subunits. We reasoned that if rSK1 subunits are downregulated and the AHP is reduced or abolished, there must be a significant level of rSK1 channel population mediating the AHP in the absence of glucose (shown in Fig. 2d). We achieved  $\sim 73\%$  downregulation of SK1 transcripts within 2 days after transfection (Fig. 3a). This result is further supported by the Western blot analyses where we observed a reduction of rSK1 protein levels following knockdown (Fig. 3a, right panel). Confirming these results, whole-cell voltage clamp recordings also demonstrate that current density is significantly reduced in rSK1 siRNA-transfected cells when compared to both the mock-transfected and the non-silencing siRNA-transfected control cells (Fig. 3b and c). Targeting with siRNA (Fig. 3b and c) is more effective in reducing SK currents than blocking the channels with apamin (Fig. 2a). Further, siRNA treatment did not damage the cells, as one of the properties of dead cells is leaky membranes. Using electrical membrane resistance to gauge cell viability, we found no evidence that siRNA is toxic to these cells (Fig. 3c).

We then investigated the AHP response as a measure of SK activity when rSK1 message and protein were downregulated. As

initially demonstrated, when control cells were exposed to glucose-omitted recording solution, SK channels were activated as evident by the presence of the AHP following depolarization-induced action potentials (Figs. 2d and 4a). Under the same conditions (no glucose), however, cells following SK1 knockdown were completely devoid of any AHP component (Fig. 4), suggesting that channels containing rSK1 are the predominant channels responsible for the AHPs. These data suggest then that rSK1 appears to be functional and mediate the AHP under zero-glucose condition.

### 3.4. rSK1 subunits ensure appropriate insulin secretion

Changes in the AHP and SK channel composition could significantly alter beta cell physiology. We thus investigated how a reduction in rSK1 expression influences beta cell function in view of the tight-coupling between excitability and secretion. We found that following rSK1-knockdown, beta cells secreted insulin at an increased rate compared with control cells (Fig. 4b). In fact, even when exposed for 2 hours in zero-glucose, the secretion rate in rSK1-deficient cells was comparable with control cells stimulated by 20 mM glucose ( $n = 7$ ). Moreover, stimulation of these rSK1 siRNA-treated cells with glucose (20 mM) further increased insulin release by 2-fold ( $n = 8$ ), indicating that rSK1 normally contributes to a mechanism involved in dampening insulin secretion under zero or possibly low glucose conditions.



**Fig. 3.** Targeting rSK1 with siRNA reduces SK1 transcript and protein expression levels. (a) We achieved 73% mRNA knockdown efficiency by Day 2 post-transfection. Bar graphs show the percentage of cells exhibiting rSK1 expression following transfection with rSK1 siRNA (left panel). qPCR analysis (left panel) shows ~73% knockdown at Day 2 based on reduced SK1 expression level. Western blot analyses of knocked-down cells (right panel) also show reduction of the ~59 kDa rSK1 protein [24] at Day 2 post-siRNA transfection. HEK cells (lane 1, right panel), which lack SK1 channels, served as controls. Also, anti-SK1 antibodies when preincubated with antigenic peptides prevented the staining of SK1 proteins in the INS-1 lysates, thus demonstrating specificity for the SK1 proteins (lane 4, right panel). (b) A representative rSK1-knockdown cell show significantly reduced  $K^+$  currents when compared to a control mock-transfected INS-1 cell and a non-silencing siRNA-transfected cell. Whole-cell voltage clamp recordings were conducted under physiological saline solution and pipette solution containing of 1  $\mu$ M free calcium concentration. Each cell was depolarized at  $-20$  mV for 1 s from a holding potential of  $-70$  mV. (c) Comparison between the current density of mock-transfected control ( $n = 6$ ), non-silencing siRNA-transfected control ( $n = 4$ ), and rSK1-knockdown ( $n = 6$ ) cells. Bar graph represents the mean current density expressed as pA/pF  $\pm$  S.E.M. High membrane resistance (in the G $\Omega$  range) demonstrates that the mock-transfected control and non-silencing siRNA-transfected control cells do not exhibit leaky membranes, a reliable indicator of damaged and dying cells. Mean membrane resistance values ( $\pm$ S.E.M.) are shown.

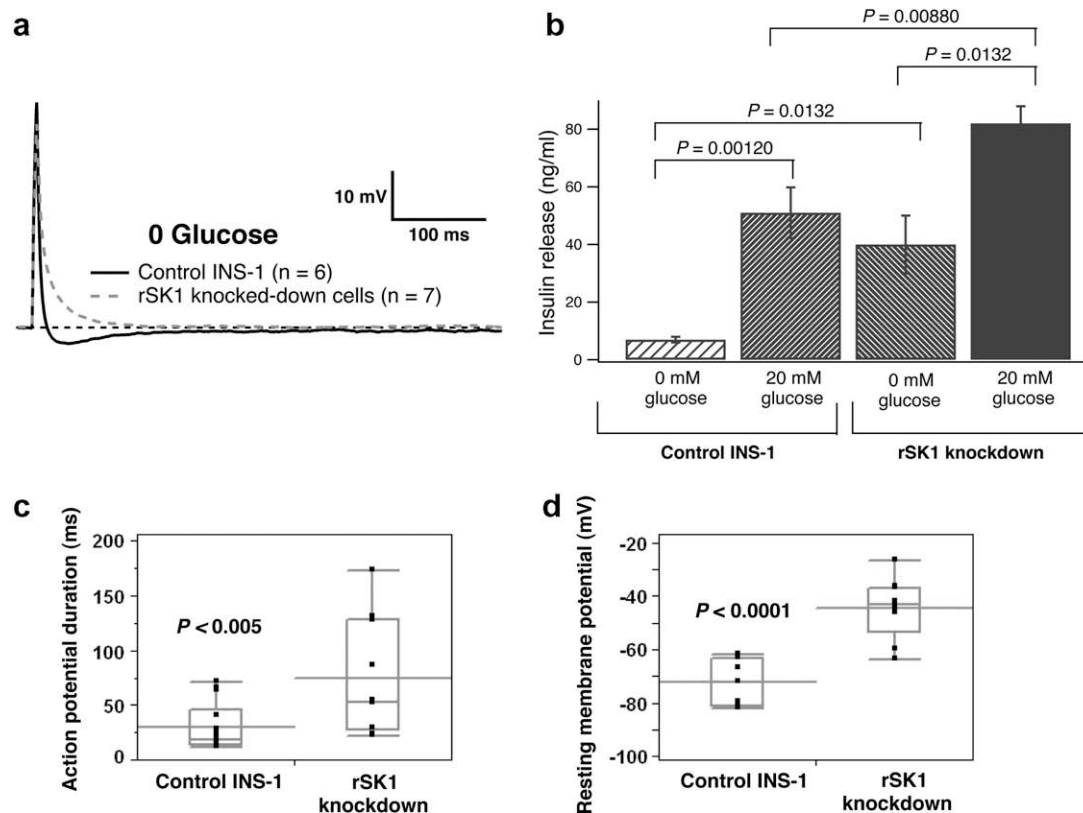
### 3.5. rSK1 subunits determine the shape and duration of action potentials as well as the resting membrane potential

What mechanism underlies the increased basal secretion rates of rSK1-knockdown cells in the absence of stimulating molecules? One of the marked differences between control and rSK1-knockdown cells is the duration of the action potential (Fig. 4a and c). We found that the action potential duration of rSK1-deficient cells ( $n = 9$ ) was increased 4-fold compared to control beta cells ( $n = 9$ ; Fig. 4c). The decay of the action potential to the baseline level varied (i.e., duration of AP) from 22 to 130 ms in duration. Additionally, we observed that rSK1-downregulated cells, when recorded in the absence of glucose, exhibited a more depolarized resting membrane potential (Fig. 4d) at a mean of  $-44.6 \pm 3.8$  mV compared to control beta cells at  $-72.4 \pm 3.3$  mV. This control value is close to that reported by others for beta cells [17–20]. Interestingly, the resting membrane potential of rSK1-knockdown cells is more depolarized than the voltage range ( $\sim -50$  mV) in which bursting commences in beta cells of intact pancreatic islets [18,21]. Since dispersed or dissociated beta cells in culture rarely

exhibit bursting activities, it unlikely that we would observe bursting activities in these cell line [22]. However it is very likely that at this depolarized voltage range, even at rest, the rSK1-downregulated beta cells are more prone to “unstimulated” secretion. Therefore, by mediating AHP only in the absence of glucose, rSK1 contributes to maintaining a hyperpolarized resting membrane potential and prevents bursting of action potentials in the absence of stimulation. Thus, rSK1 ensures the dampening of insulin secretion under conditions when glucose in the extracellular beta cell environment falls below the normoglycemic point. These findings suggest that the physiological role of rSK1 subunits is to shorten the duration of action potentials and restore a hyperpolarized resting membrane potential in the absence of glucose, thus ensuring controlled insulin secretion.

## 4. Discussion

In this study, we investigated a specific role of the SK1 subunit in the INS-1 insulinoma cell line. Our data demonstrate a functional role for rSK1 in the control of insulin secretion. We showed



**Fig. 4.** Depletion of rSK1 subunits eliminates the AHP. (a) Averaged current clamp recordings from control INS-1 cells ( $n = 6$ ) exhibit an AHP; whereas, reduction of rSK1 subunits results in cells without any AHP ( $n = 7$ ). Shown are average traces from the number of indicated experiments. (b) Depletion of rSK1 modifies the cells' basal and glucose-induced insulin secretion. Depletion of rSK1 in INS-1 cells results in constitutive secretion despite the absence of glucose. Control cells showed a low basal level of secretion in the zero-glucose condition ( $n = 7$ ). Control cells significantly increased insulin secretion in response to 20 mM glucose ( $n = 7$ ). rSK1 knocked-down cells secreted insulin despite the absence of glucose during the 1-h incubation period plus the 1-h pre-incubation time ( $n = 10$ ). Insulin secretion was further increased when knocked-down cells were stimulated with 20 mM glucose ( $n = 8$ ). Mean insulin release ( $\pm$ S.E.M.) is shown. (c) rSK1-knockdown cells exhibit a prolonged action potential. rSK1 knocked-down cells have prolonged depolarization. Shown is the box plot for the distribution of action potential durations for both control and rSK1-knockdown cells in the absence of glucose. As indicated, compared to the control cells ( $14.7 \pm 1.1$  ms,  $n = 9$ ), depletion of rSK1 subunits results in cells ( $n = 9$ ) exhibiting more variable durations that had longer mean action potential duration of  $63.8 \pm 13.9$  ms. (d) Reducing rSK1 subunits in rat beta cells results in a more depolarized resting membrane potential. Without glucose, the average resting membrane potential of control INS-1 beta cells is  $-72.4 \pm 3.3$  mV ( $n = 7$ ) compared to  $-44.6 \pm 3.8$  mV ( $n = 9$ ) for the rSK1-knockdown cells as described in the box plot.

that under reduced SK1 expression, unregulated insulin secretion occurs in beta cells, primarily because normally SK1 ensures a more negative resting membrane potential and regulates the duration of action potentials. Reducing SK1 expression results in a more depolarized resting membrane potential at a voltage range in which insulin secretion is easily triggered, and, interestingly, at the voltage range in which bursting commences in intact pancreatic beta cells [18,21].

#### 4.1. Native expression of SK1 subunits and channel composition

Like other members of the  $K^+$  channel family, SK channels are formed by the assembly of 4 subunits from the same subunit type (homotetramers or homomers) or from different subunit types (heterotetramers or heteromers) [9,11,23]. In the many regions of the brain there are overlapping expression patterns of SK1, SK2 and SK3 subunits. Unlike a previous finding by Sailer et al. [24], Strassmaier et al. have detected native heteromeric SK2/SK3 channel assembly [25].

In the present study, cells with downregulated rSK1 subunits exhibit constitutive insulin secretion (Fig. 4b) and exhibit action potentials devoid of any AHP components (Fig. 4a). We also observed a reduction in  $K^+$  current in knocked-down cells when compared to the controls (Fig. 3b and c). These effects could only be possible if rSK1 channels are indeed functional. To date, it is known

that heterologously expressed rSK1 homomers and rSK1/rSK3 heteromers result in channel proteins that are sequestered intracellularly and prevented from being expressed in the plasma membrane [11,12,23]. Therefore, the only known mechanism by which rSK1 subunits could gain a function is through coassembly with rSK2 subunits, by translocating to the plasma membrane as functional heteromeric channels (rSK1/rSK2). These rSK1/rSK2 heteromers have reduced apamin-sensitivity and exhibit biophysical properties different from homomeric rSK2s [11].

It is tempting to think that since all the rSK subunits are equally expressed in the INS-1 cells (Fig. 1a and b), rSK2 and rSK3 subunits may exist in a coassembled state with rSK1. The presence of rSK1 subunits could keep both rSK1 homomers and rSK1/rSK3 heteromers "intracellularly trapped", rendering these subunits both non-functional and leaving only rSK1/rSK2 heteromers to mediate the AHP under the zero-glucose condition (Fig. 2d). Heteromeric channels have reduced apamin-sensitivity [11,23,26] and our data shows that inhibition by 100 nM apamin (as shown in Fig. 2a) is much less reduced ( $\sim 30$ – $40\%$ ) compared to heterologously expressed homomeric SK2 and SK3 channels ( $\sim 80$ – $90\%$ ) [11,23,26]; these observations altogether suggest that heteromeric SK channels may be natively expressed in these insulinomas. Based on our initial findings, we propose that rSK1 may be natively expressed as functional heteromeric channels (rSK1/rSK2). However, it might also be that rSK1 activity depends on an as yet undiscovered

ered accessory protein (or subunit) that allows both the rSK1 homomers and the rSK1/rSK3 heteromers to escape to the plasma membrane as functional channels. However, these hypotheses await further evidence from co-immunoprecipitation experiments that would convincingly demonstrate interaction between these different channel (or accessory) subunits.

#### 4.2. SK channel activation at basal intracellular $Ca^{2+}$ levels

The AHP that appears under zero-glucose conditions (Fig. 2d) is also intriguing considering that under these conditions the voltage-gated  $Ca^{2+}$  channels are closed, and the SK channels, presumably, would not be activated at basal intracellular calcium levels (~50–100 nM) [27]. However, since all three SK subtypes (*hSK1*, SK2, and SK3) exhibit roughly similar half-maximal activation ( $K_{0.5}$ ) at ~0.3  $\mu$ M  $Ca^{2+}$  [28], it seems unlikely that a significant number of channels would be activated at resting levels. The small magnitude of the AHP shown in Fig. 2d suggests then that only a small population of SK channels is recruited. This population of SK channels could be those that heteromerize as functional channels and may be more sensitive to nanomolar resting calcium levels than their homomeric counterparts. This, however, needs to be further investigated. Also, we did not observe more channel activation (and higher magnitude of the AHP component) under high glucose conditions (Fig. 2d), suggesting that this SK channel population is in an inactivated or inhibited state. This could be the case if: (1) glucose-induced depolarization inactivates the channels or (2) glucose directly inhibits the SK channels. These two possibilities await further study, preferably under whole-cell voltage clamp of heterologously expressed SK channels in different glucose conditions.

#### 4.3. Regulatory role of SK1 subunits

A crucial feature of rSK1 subunits is their seeming ability to act as a suppressor of their own subunit (rSK1) and of rSK3 subunits by inhibiting the formation of functional homomeric [9–12] and heteromeric channels [23], respectively. Unfortunately, the existence of SK1 splice variants further complicates this picture, as they possibly contribute to the generation of SK1 heteromers, especially because the SK1 gene could have as many as 20–32 spliced variants [29] whose protein products could interact with the long and short isoforms generated from the SK2 gene [25] or among each other. Although these splice variants were detected in mice, the high degree of homology between the mouse and the rat genome makes it likely that the mouse variants also exist in the rat. In the current study, we did not investigate rSK1 splice variants; however, the siRNA used for this study targeted the sequences located in the conserved region of the channel, away from the C-terminus region where truncation occurs in the reported variants. Our observation supports the hypothesis that rSK1 variants exist and may explain the significant differences in the AHP kinetics when rSK1 is targeted by siRNA. As noted in Fig. 4c, the duration of the action potential ranged from 22 to 130 ms in the rSK1-knockdown cells. We postulate that such a wide range of action potential duration may reflect the different rSK1 complexes or the different stoichiometry of SK subunits that possibly exist in the INS-1 beta cells.

#### 4.4. Implications to hyperinsulinemia

The current findings may imply that specifically targeting the SK1 channel expression may be a plausible mechanistic approach for enhancing insulin secretion in failing pancreatic beta cells. Alternatively, expression of the SK1 subunit may be the key to maintaining healthy, responsive pancreatic beta cells. The results of our study provide substantive evidence that changes of SK sub-

unit patterns have an important impact on cellular function. Thus, further investigation is warranted in this area, especially because age-related changes in AHP [30] and in SK channel expression have been documented in the brain [31]. If such changes occur in the pancreas, proper functioning of beta cells will undoubtedly be altered over time. Therefore, studying the function of the SK1 human orthologue in human beta cells will provide further insights as to whether these subunits play a role in the development of diabetes and may hold the key to better understanding of the progression of hyperinsulinemia.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2008.12.024.

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