

Pharmacological Properties and Functional Role of K_{slow} Current in Mouse Pancreatic β -Cells: SK Channels Contribute to K_{slow} Tail Current and Modulate Insulin Secretion

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The pharmacological properties of slow Ca^{2+} -activated K^+ current (K_{slow}) were investigated in mouse pancreatic β -cells and islets to understand how K_{slow} contributes to the control of islet bursting, $[\text{Ca}^{2+}]_i$ oscillations, and insulin secretion. K_{slow} was insensitive to apamin or the K_{ATP} channel inhibitor tolbutamide, but UCL 1684, a potent and selective nonpeptide SK channel blocker reduced the amplitude of K_{slow} tail current in voltage-clamped mouse β -cells. K_{slow} was also selectively and reversibly inhibited by the class III antiarrhythmic agent azimilide (AZ). In isolated β -cells or islets, pharmacologic inhibition of K_{slow} by UCL 1684 or AZ depolarized β -cell silent phase potential, increased action potential firing, raised $[\text{Ca}^{2+}]_i$, and enhanced glucose-dependent insulin secretion. AZ inhibition of K_{slow} also supported mediation by SK, rather than cardiac-like slow delayed rectifier channels since bath application of AZ to HEK 293 cells expressing SK3 cDNA reduced SK current. Further, AZ-sensitive K_{slow} current was extant in β -cells from *KCNQ1* or *KCNE1* null mice lacking cardiac slow delayed rectifier currents. These results strongly support a functional role for SK channel-mediated K_{slow} current in β -cells, and suggest that drugs that target SK channels may represent a new approach for increasing glucose-dependent insulin secretion. The apamin insensitivity of β -cell SK current suggests that β -cells express a unique SK splice variant or a novel heteromultimer consisting of different SK subunits.

INTRODUCTION

When exposed to glucose concentrations ≥ 7 mM, pancreatic islets of Langerhans exhibit electrical oscillations consisting of bursts of fast Ca^{2+} -dependent action potentials riding upon slower depolarizing plateaus (Dean and Matthews, 1970a; Ashcroft and Rorsman, 1989; Cook et al., 1991; Satin and Smolen, 1994). The period of this bursting typically ranges from tens of seconds to minutes in 11.1 mM glucose, and numerous studies have shown that bursting leads to concomitant oscillations in islet $[\text{Ca}^{2+}]_i$ that drive insulin secretion (Bergsten et al., 1994; Barbosa et al., 1998; Zhang et al., 2003). However, despite extensive investigation, the ionic basis of islet pacemaking is not fully understood. The cyclic activation of a Ca^{2+} -activated K^+ current (K_{Ca}) has been a strong candidate pacemaker (Atwater et al., 1979; Satin and Smolen, 1994; Sherman, 1996), and in support of this, islet bursting is simulated by models incorporating the cyclic activation and deactivation of K_{Ca} channels by bursting-induced elevations in $[\text{Ca}^{2+}]_i$ (Chay and Keizer, 1983). Göpel et al. (1999) presented evidence that K_{slow} , a novel slow Ca^{2+} -activated K^+ current, tracks $[\text{Ca}^{2+}]_i$ as it rises in response to a

voltage clamp command designed to mimic an islet burst. Unlike the fast or “BK type” K_{Ca} channels of β -cells (Kukuljan et al., 1991), K_{slow} was insensitive to charybdotoxin or low concentrations of TEA (Göpel et al., 1999; Hennige et al., 2000), but was regulated by both store and cytoplasmic Ca^{2+} (Goforth et al., 2002). In contrast to their initial description of the current (Göpel et al., 1999), Kanno et al. (2002) suggested that K_{slow} might be a mosaic of K_{ATP} and Ca^{2+} -activated K^+ current. More recently, Tamarina et al. (2003) confirmed that small-conductance calcium-activated K^+ channels (SK type) are expressed in pancreatic islets, and suggested these channels regulate glucose-induced $[\text{Ca}^{2+}]_i$ oscillations in islets by mediating K_{slow} . However, a problem with this hypothesis is that apamin, the canonical SK blocker in other systems, does not affect mouse islet bursting or β -cell K_{slow} current (Lebrun et al., 1983; Ämmälä et al., 1993; Göpel et al., 1999; Goforth et al., 2002). Thus, it is not yet proven that SK channels mediate K_{slow} current in β -cells. The lack of a selective K_{slow} blocker has further hampered progress in determining

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Abbreviations used in this paper: AZ, azimilide; K_{Ca} , Ca^{2+} -activated K^+ current; K_{slow} , slow Ca^{2+} -activated K^+ current.

the function role of K_{slow} current in islet electrophysiology and stimulus–secretion coupling.

We now report that UCL 1684, a highly selective, nonpeptidergic blocker of SK channels (Rosa et al., 1998), or azimilide (NE-10064, (E)-1-[[[5-(4-chlorophenyl)-2-furanyl]methylene]amino]-3-[4-(4-methyl-1-piperazinyl)butyl]-2,4-imidazolidinedione dihydrochloride, AZ), a novel class III antiarrhythmic agent, inhibit K_{slow} in mouse pancreatic β -cells or islets. In cardiac cells, AZ blocks both the slowly (I_{Ks}) and rapidly activating (I_{Kr}) components of delayed rectifier potassium current and as a result prolongs cardiac refractoriness (Busch et al., 1994; Fermini et al., 1995; Salata and Brooks, 1997; Karam et al., 1998). In islets, however, K_{slow} blockade by AZ was due to block of Ca^{2+} -activated SK channels because we found that, on the one hand, AZ was effective in blocking K_{slow} even in β -cells from mice in which I_{Ks} was eliminated by two different global knockouts, and on the other hand, AZ blocked SK3 channels expressed in transfected HEK 293 cells. In terms of function, suppression of K_{slow} by UCL 1684 or AZ resulted in membrane depolarization, increased action potential firing, and a concomitant increase in islet β -cell $[Ca^{2+}]_i$. In islets exhibiting regular $[Ca^{2+}]_i$ oscillations in 11.1 mM glucose (Zhang et al., 2003), UCL or AZ increased $[Ca^{2+}]_i$ as well as oscillation frequency. Furthermore, both K_{slow} blockers significantly enhanced glucose-dependent insulin release, while not affecting basal secretion.

MATERIALS AND METHODS

Culture of Islets and Islet β -Cells

Mouse islets were isolated from the pancreases of Swiss-Webster mice by collagenase digestion (Zhang et al., 2003). Islets were dispersed into single cells by gently shaking the islets in a low-calcium solution. Islets or β -cells were seeded on glass coverslips in 35-mm Petri plates and cultured in RPMI-1640 medium with 11.1 mM glucose, FBS, L-glutamine, and penicillin/streptomycin (Invitrogen). All cultures were kept at 37°C in an air/CO₂ incubator. Cells were fed every 2–3 d, and were kept in vitro for up to 5 d, while islets were typically cultured for 1–2 d.

Electrophysiology

β -cells or islets were superfused with a standard external solution containing (in mM) 115 NaCl, 3 CaCl₂, 5 KCl, 2 MgCl₂, 10 HEPES, 11.1 glucose (pH 7.2). The perforated patch-clamp technique was used to record islet or β -cell membrane potentials or ion currents. Pipettes were pulled from borosilicate glass using a two-stage horizontal puller (P-97, Sutter Instruments). Pipette tips were filled with an internal solution containing (in mM) 28.4 K₂SO₄, 63.7 KCl, 11.8 NaCl, 1 MgCl₂, 20.8 HEPES, 0.5 EGTA (pH 7.2) and then backfilled with internal solution plus 0.1 mg/ml amphotericin B. An Axopatch-200B patch-clamp amplifier (Axon Instruments) was used in the standard tight-seal perforated patch-clamp mode to record membrane potentials or ion currents in current or voltage clamp mode, respectively (Hamill et al., 1981). Pipette resistances ranged from 5 to 8 M Ω using our internal solutions, while seal resistances ranged from 2 to 10 G Ω .

Solutions were applied to β -cells or islets using a gravity-driven perfusion system, which allowed switching between multiple reservoirs and flow rates in excess of 1 ml/min. All experiments were performed at 32°C–35°C using a feedback-controlled temperature regulation system (CellMicro Controls). All drugs were made up fresh daily from frozen stock solutions. Drugs and chemicals were obtained from Sigma-Aldrich with the exception of AZ and HMR1556, which were a gift from G.N. Tseng (VCU, Richmond, VA).

$[Ca^{2+}]_i$ Measurements

Cultured mouse islets were loaded with the Ca^{2+} -sensitive dye, fura-2/AM (Invitrogen). 2 μ mol/l fura-2-AM and 1 μ l of 2.5% pluronic acid were added to cells in 35-mm culture dishes containing 1 ml of medium, and islets were incubated for 30 min at 37°C to load with dye. After loading, islets were washed once and then incubated in standard external solution for 20 min. $[Ca^{2+}]_i$ was measured by placing islets in a small recording chamber mounted on the stage of an Olympus IX-50 inverted epifluorescence microscope (Olympus). Fura-2 was excited at 340/380 nm using a galvanometer-driven mirror that alternated a light beam from a xenon source (“HyperSwitch,” IonOptix Corporation). A photomultiplier and photon counting were used to quantify fura-2 emission at 510 nm (IonOptix Inc.). Fluorescence data were acquired and analyzed using IonWizard software (IonOptix).

$[Ca^{2+}]_i$ values were determined from the fluorescence ratio (R) of Ca^{2+} -bound fura-2 (excited at 340 nm) to unbound fura-2 (excited at 380 nm). Absolute $[Ca^{2+}]_i$ was determined using a standard equation (Grynkiewicz et al., 1985). To convert R to $[Ca^{2+}]_i$ using this equation, R_{max} and R_{min} were obtained by exposing islets to 10 μ M ionomycin plus 3 mM Ca^{2+} or 10 mM EGTA, respectively, at the end of each experiment. The equilibrium constant for Ca^{2+} binding to fura-2 (K_d) was assumed to be 224 nM (Grynkiewicz et al., 1985).

Measurement of Insulin Secretion

Islets were cultured overnight and then washed twice with our standard external saline containing 11.1 mM glucose. Following this, each dish containing 10 islets was incubated for 60 min in 1 ml of standard external solution containing various concentrations of glucose, UCL 1684, or AZ. A portion of the reaction solution was withdrawn at the end of the incubation period and diluted appropriately for insulin assay. Insulin was measured using an ELISA kit for detecting mouse insulin (Mercodia Ultrasensitive Mouse Insulin ELISA Kit; Mercodia), according to the instructions of the manufacturer. Sample absorbance at 450 nm was read using a microplate reader (model 2550, Bio-Rad Laboratories). Data were collected from four mice for UCL 1684 and four mice for AZ, and the experiment was repeated three times. The content of insulin in samples was calculated according to a standard curve.

Functional Expression of the hSK3 Gene in HEK 293

HEK 293 cells were cultured in minimal essential medium containing glutamine and 10% FBS. 1 or 2 d before transfection, cells were transferred to 35-mm Petri dishes and were grown to ~70% confluence. A mixture containing 0.1 μ g of the plasmid DNA encoding EGFP-N1 (BD Biosciences, CLONTECH Laboratories Inc.) and 1 μ g of plasmid DNA encoding human SK3 (Kohler et al., 1996; Chandy et al., 1998) was transfected into HEK 293 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were studied 24–48 h after transfection.

SK3 currents were recorded using whole-cell patch clamp as described in general above. The electrodes used contained 28.4 mM K₂SO₄, 63.7 mM KCl, 10.7 mM NaCl, 20.8 mM HEPES, 1 mM MgCl₂, 1 mM EGTA, 0.9 mM CaCl₂, pH 7.2; the calculated $[Ca^{2+}]_i$ for this solution was ~1 μ M, which is sufficient for nearly

maximal activation of SK₃ channels (Kohler et al., 1996). HEK 293 cells showing green fluorescence in the bath solution (115 mM NaCl, 3 mM CaCl₂, 5 mM KCl, 2 mM MgCl₂, 10 mM HEPES, 11.1 mM glucose, pH 7.35) were selected for voltage clamping. After the formation of a tight seal in cell-attached mode, the external solution was changed to an “SK blocking solution” (71 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 10 mM BaCl₂, 30 mM TEA-Cl, 10 mM HEPES, 11.1 mM glucose, pH 7.35), which contained Ba²⁺ and TEA to prevent SK current activation before establishing whole cell mode to help preserve cell viability. Once whole cell mode was established, SK₃ currents were then initiated by using a high K⁺ external solution (90 mM NaCl, 30 mM KCl, 3 mM CaCl₂, 2 mM MgCl₂, 10 mM HEPES, 11.1 mM glucose, pH 7.35) and were recorded at -80 mV and +10 mV. The effects of UCL 1684 and AZ on SK₃ currents were observed by adding these drugs to the bathing solution.

Data analysis, curve fitting, and graphics were performed using IgorPro Software (Wavemetrics) and statistical analysis and curve fitting of dose-response data was done using GraphPad Prism Pro. Data shown are means ± SEM. Where relevant, paired or unpaired Student's *t* tests were used to test for significance. *P* values <0.05 were considered significant and denoted by * or # in figures; *P* < 0.01 are denoted by ** or ## in figures; and *P* < 0.001 are denoted by *** or ### in figures.

RESULTS

K_{slow} Current Is Inhibited by the SK Blocker UCL 1684 in Mouse Single β-Cells

Mouse islet cells and insulin-secreting cell lines express SK channel isoforms, and SK channel modulators alter glucose-dependent [Ca²⁺]_i oscillations in mouse islets (Tamarina et al., 2003). Paradoxically, however, the prototypical SK inhibitor apamin, which modulates [Ca²⁺]_i oscillations in rodent islets (Tamarina et al., 2003), does not block K_{slow} current in mouse β-cells (Göpel et al., 1999; Goforth et al., 2002), and is without effect on islet electrical bursting (Lebrun et al., 1983). The reasons for these discrepancies are not clear. To determine whether K_{slow} is indeed mediated by SK channels, and to determine the role of K_{slow} in islet stimulus-secretion coupling, we more fully characterized the pharmacological properties of K_{slow} current.

To elicit K_{slow} current, we used a standard protocol consisting of a physiological waveform command that resembled an islet burst to isolate a deactivation “tail” of K_{slow} current. This protocol stepped membrane potential from -65 to -40 mV for 5 s, followed by a 5-s train of spike-like pulse depolarizations from -40 to 0 mV, and then a 10-s sojourn back to -40 mV (for more details of the protocol used, see Göpel et al., 1999).

As shown in Fig. 1 A, upon application of the pulse train, an envelope of slow outward current progressively activated, with faster outward currents superposed, and then slowly deactivated once the pulse train was terminated. The peak amplitudes of the deactivating tail currents mediated by K_{slow} are denoted by arrows in the figure, and were used to quantify the degree of K_{slow} activation (Göpel et al., 1999; Goforth et al., 2002).

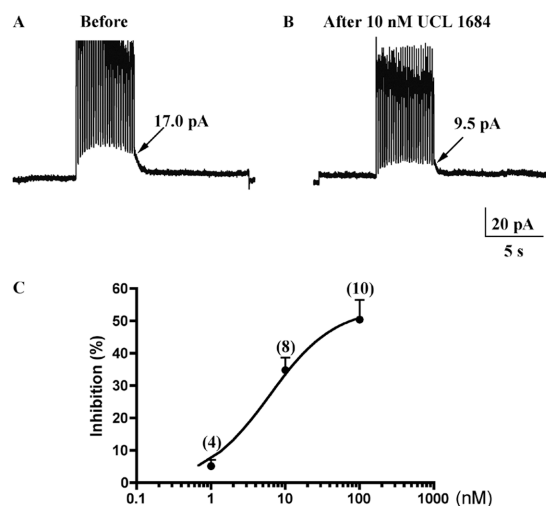


Figure 1. Expression and block of K_{slow} by an SK channel blocker. UCL 1684, a potent and selective nonpeptide SK blocker blocks K_{slow} currents in mouse β-cells. Top traces (A and B) show representative recordings of K_{slow} current. The peak of the K_{slow} tail currents is denoted by arrows. The currents shown were elicited using a burst pulse protocol (see MATERIALS AND METHODS) in voltage clamp. (C) Dose-response curve of UCL 1684 on K_{slow}. UCL 1684 suppressed K_{slow} current with an IC₅₀ of 6.2 nM. Data shown are mean ± SEM, with *n* (number of experiments) indicated in brackets. Curve was fit with single binding site model.

As the bath application of up to 1 μM apamin did not block K_{slow} current (*n* = 10, unpublished data), we tested the potent nonpeptide SK blocker UCL 1684, which is known to selectively target SK 1-3 type K_{Ca} channels (Rosa et al., 1998). As shown in Fig. 1 B, 10 nM UCL 1684 inhibited K_{slow} current. In 17 isolated β-cells, the mean current density was reduced from 1.34 ± 0.14 to 0.83 ± 0.15 pA/pF (*P* < 0.001). K_{slow} block by UCL 1684 was dose responsive, as shown in Fig. 1 C. The data were fit by a single binding site model to yield an IC₅₀ of 6.2 nM and a B_{max} of 54.1%. This IC₅₀ is similar to the sensitivity of SK channels to UCL 1684 in other systems (Dunn, 1999; Malik-Hall et al., 2000; Hosseini et al., 2001). The data are consistent with the hypothesis that SK channels are at least partly involved in the mediation of β-cell K_{slow} current. In a previous study, Kanno et al. (2002) reported that K_{slow} was a composite of K_{Ca} and K_{ATP} channels. In our hands, however, the K_{slow} current of mouse β-cells was insensitive to 200 μM tolbutamide (1.14 ± 0.11 vs. 1.04 ± 0.14 pA/pF, *n* = 16, *P* > 0.05), as initially reported by Göpel et al. (1999). This suggests that K_{slow} current does not appear to be a mosaic of SK and K_{ATP} currents using our standard conditions.

The Class III Antiarrhythmic Drug AZ also Blocks K_{slow} Current in Single Mouse β-Cells

In heart cells, the class III antiarrhythmic agent AZ blocks both I_{Kr} and I_{Ks} delayed rectifying K⁺ currents, the former a fast, voltage-dependent K⁺ channel, and the

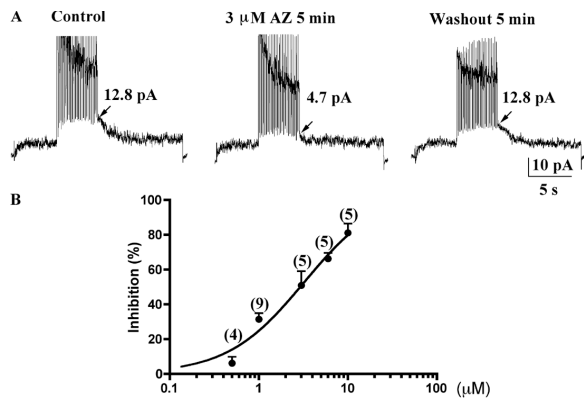


Figure 2. The class III antiarrhythmic drug azimilide also blocked K_{slow} currents in mouse β -cells. (A) Representative recordings showing that AZ reversibly blocks K_{slow} current. (B) Dose-response curve of AZ inhibition of K_{slow} . AZ suppressed K_{slow} current with an IC_{50} of 3.2 μ M. Data shown are mean \pm SEM, with n (number of experiments) indicated in parentheses. Curve was fit with a single binding site model.

latter a slowly activating K^+ channel (Busch et al., 1994; Ohya et al., 2001). HMR 1556 is a selective blocker of I_{Ks} in heart cells (Gögelein et al., 2000). The antiarrhythmic action of AZ is believed to occur due to prolongation of the cardiac action potential and increased relative refractoriness, both secondary to K^+ current blockade (Busch et al., 1994; Fermini et al., 1995; Salata and Brooks, 1997; Karam et al., 1998). As conventional K^+ channel blockers like TEA did not show selectivity for

K_{slow} , and because I_{Ks} in cardiac cells shows a similar slow deactivation characteristic, we decided to test whether two blockers of I_{Ks} affect the β -cell K_{slow} current. The bath application of AZ to β -cells resulted in the reversible and nearly complete suppression of K_{slow} current (Fig. 2 A). The addition of 3 μ M AZ reduced K_{slow} current density from 1.68 ± 0.31 to 0.77 ± 0.15 pA/pF ($n = 5$, $P < 0.05$), and K_{slow} blockade typically reached a steady-state level within 5 min. Fig. 2 B shows the dose-response curve of AZ blockade of K_{slow} , whose fit yielded an IC_{50} of 3.2 μ M and a B_{max} of 100% ($n = 5-8$, $P < 0.05$), as shown in the figure. The IC_{50} for K_{slow} block by HMR 1556 was 127.3 nM (unpublished data). The sensitivity of K_{slow} to AZ and HMR 1556 was generally similar to that reported for cardiac I_{Ks} channels (Busch et al., 1994; Gögelein et al., 2000; Thomas et al., 2003).

The blockade of K_{slow} by AZ and HMR raised the possibility that I_{Ks} rather than SK channels might mediate or at least contribute to mouse β -cell K_{slow} current. To test this hypothesis, we isolated islets from mice lacking either *KCNQ1* or *KCNE1* genes due to a global deletion (Kupersmidt et al., 1999; Casimiro et al., 2001; Kondo et al., 2003). It has been documented that I_{Ks} current in heart cells is mediated by a heteromultimeric channel consisting of a *KCNQ1* channel subunit and a *KCNE1* ancillary subunit (Robbins, 2001). The presence of *KCNE1* significantly increases the amplitude and activation time constant of I_{Ks} in heterologous expression systems (Seebahn et al., 2001; Melman et al., 2004).

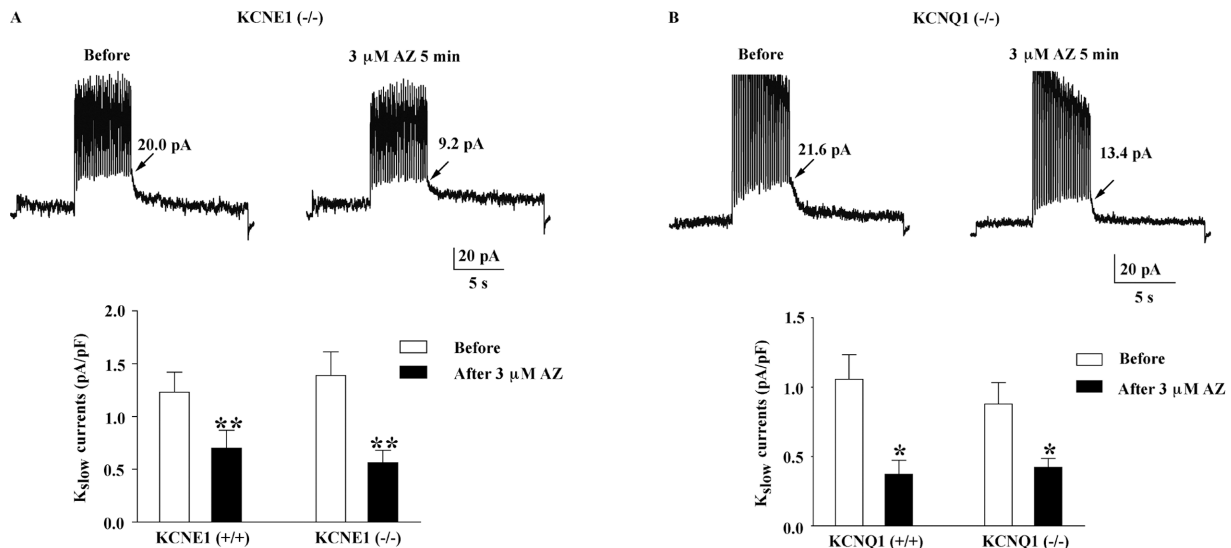


Figure 3. (A) β -cells from *KCNE1* null mice lacking cardiac type I_{Ks} delayed rectifier K^+ channels also express AZ-sensitive K_{slow} currents. Top traces show representative K_{slow} currents from *KCNE1* ($-/-$) mouse β -cells, both β -cells from *KCNE1* ($+/+$) and *KCNE1* ($-/-$) mice are sensitive to AZ. Bottom bar graph shows no difference of mean K_{slow} currents in *KCNE1* ($+/+$) and *KCNE1* ($-/-$) mice ($n = 38-45$, $P > 0.05$), AZ significantly inhibited K_{slow} currents in both group mice (**, $P < 0.01$, $n = 6$). (B) β -cells from *KCNQ1* null mice lacking cardiac type I_{Ks} delayed rectifier K^+ channels still have AZ-sensitive K_{slow} currents. Top traces show representative K_{slow} currents from *KCNQ1* ($-/-$) mouse β -cells. Both β -cells from *KCNQ1* ($+/+$) and *KCNQ1* ($-/-$) mice are sensitive to AZ. Bottom bar graph shows no difference of mean K_{slow} currents in *KCNQ1* ($+/+$) and *KCNQ1* ($-/-$) ($P > 0.05$, $n = 12$), AZ significantly inhibited K_{slow} currents in both groups of mice (*, $P < 0.05$, $n = 5$).

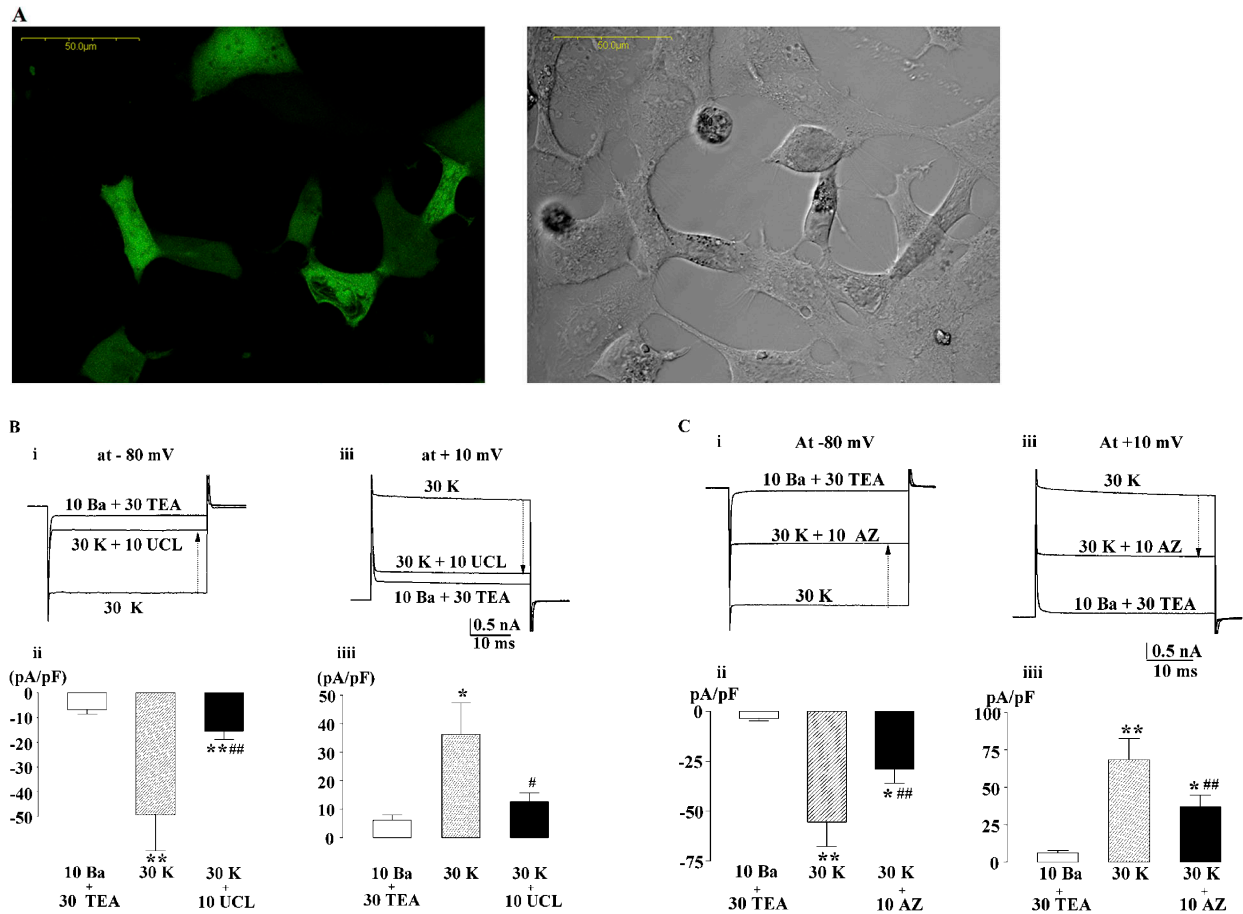


Figure 4. (A) HEK 293 cells coexpressed SK3 and EGFP genes used in the study. Green fluorescent cells were chosen to measure SK3 currents (left), and the figure on the right shows the same cells in bright field. (B) UCL 1684 blocks SK3 channels expressed in HEK 293 cells. i and iii traces show the representative currents recorded at -80 mV (i) or $+10$ mV (iii) from 15 cells. Green fluorescent cells were selected and the SK3 currents were recorded using the whole cell patch clamp configuration. As shown in figure, bath application of an SK blocking solution containing 10 mM BaCl_2 and 30 mM TEA-Cl, resulted in small basal currents. After replacing the external solution with 30 mM KCl, robust SK3 currents are observed (*, $P < 0.05$; **, $P < 0.01$, compared with the basal level). 10 nM UCL 1684 significantly inhibited this current at -80 mV (ii) or $+10$ mV (iii) (#, $P < 0.05$; ##, $P < 0.01$, $n = 6$, bottom bar graph). (C) SK3-mediated currents in HEK 293 cells are also significantly blocked by AZ (##, $P < 0.01$, $n = 7$).

As shown in Fig. 3 (A and B), islet β -cells isolated from global KCNE1 or KCNQ1 knockout mice that lack cardiac I_{Ks} (Kupersmidt et al., 1999) still exhibited slowly activating and deactivating, and AZ-sensitive K_{slow} currents. The mean $t_{1/2}$ of K_{slow} deactivation in wild-type β -cells was 1.9 ± 0.2 s, $n = 16$, and was not significantly different in β -cells from either KCNQ1 $-/-$ or KCNE1 $-/-$ islet β -cells ($n = 16$ or $n = 24$). The bar graphs shown in Fig. 3 (A and B) confirm that K_{slow} current density was not significantly reduced in β -cells from either the KCNQ1 $-/-$ or KCNE1 $-/-$ mice. Furthermore, it is clearly apparent that $3 \mu\text{M}$ AZ retained its ability to inhibit K_{slow} current despite the lack of I_{Ks} . This strongly suggests that AZ does not inhibit K_{slow} by targeting channels composed of KCNQ1/KCNE1, and that, by extension, I_{Ks} is unlikely to mediate K_{slow} current in pancreatic β -cells.

To determine whether AZ also blocked K_{ATP} , we tested its effect on β -cell K_{ATP} current recorded in whole cell patch clamp configuration. We found that whole cell K_{ATP} conductance was unchanged by AZ. Thus, K_{ATP} conductance varied from 3.35 ± 0.36 to 3.30 ± 0.39 nS measured from -80 to -20 mV in the absence or presence of AZ, respectively, $n = 6$). Whole cell K_{ATP} conductance was activated by dialyzing single mouse β -cells with a pipette solution lacking Mg-ATP. Taken together with data showing that K_{slow} current was insensitive to tolbutamide, these findings show that K_{slow} is not mediated by K_{ATP} in our hands. Parenthetically, AZ also had no effect on the amplitude of voltage-dependent Ca^{2+} current in β -cells (unpublished data).

AZ and UCL 1684 Block SK Channels Expressed in HEK Cells
Although AZ is known to interact with a variety of channels and receptors in other systems (for review see

Brooks et al., 2001), a direct interaction with SK channels has not been previously reported. We thus tested the possibility that AZ inhibits K_{slow} by directly inhibiting SK type channels. Cloned SK3 and eGFP genes were cotransfected into HEK 293 cells using standard techniques (see MATERIALS AND METHODS), and cells exhibiting green fluorescence were selected for patching (Fig. 4 A, left). A bright field image of these cells is shown in Fig. 4 (right).

As shown in Fig. 4 B, control recordings made using solutions containing 30 mM external KCl exhibited large inward currents at -80 mV (Fig. 4 B, i and ii, mean value of -46.1 ± 6.9 pA/pF) and outward currents at $+10$ mV (Fig. 4 B, iii and iiiii, mean value of 36.2 ± 11.1 pA/pF). Current increased following establishment of the whole cell mode when pipettes contained $1 \mu\text{M}$ free calcium. To preserve cell viability while recording large SK currents, β -cells were initially bathed in a solution containing the reversible SK blockers Ba^{2+} and TEA to inhibit osmotic stress due to SK activation and resulting large K^+ fluxes. Following break in, Ba^{2+} and TEA were then washed off the cells and 30 mM KCl added to the bath solution to measure SK current, and to test the sensitivity of the current to UCL 1684 or AZ (Wittekindt et al., 2004). The current measured in HEK 293 cells dialyzed with $1 \mu\text{M}$ $[\text{Ca}^{2+}]_i$ reversed at -70 mV, near the calculated potassium equilibrium potential (-80 mV, Shah and Haylett, 2000), and showed a near-Nernstian shift when $[\text{K}]_o$ was increased from 5 to 30 mM (38 mV shift vs. 45 mV predicted).

As shown in Fig. 4 B (ii and iiiii), SK3-mediated current was reduced $\sim 60\%$ by the addition of 10 nM UCL 1684 (from -46.1 ± 6.9 to -18.0 ± 2.0 pA/pF at -80 mV, or from 36.2 ± 11.1 to 12.6 ± 3.2 pA/pF at $+10$ mV, $P < 0.05$, $n = 6$). As shown in Fig. 4 C, the application of $10 \mu\text{M}$ AZ also significantly inhibited SK3 current $\sim 46\%$ (from -55.5 ± 12.2 to -29.0 ± 6.9 pA/pF at -80 mV or from 68.5 ± 14.0 to 36.9 ± 7.9 pA/pF at $+10$ mV, $P < 0.01$, $n = 7$). The direct blockade of cloned SK3 current by either AZ or UCL 1684 supports the hypothesis that both UCL1684 and AZ block K_{slow} in single β -cells by inhibiting an SK type K_{Ca} current, although one that is insensitive to apamin (see DISCUSSION). We believe this is the first report that AZ blocks SK-type K^+ channels.

Functional Role of K_{slow} in Mouse β -Cells and Islets

To elucidate the role of K_{slow} current in mouse islets, we used UCL and AZ as probes for K_{slow} participation in the physiological regulation of glucose-induced islet electrical activity, $[\text{Ca}^{2+}]_i$, and insulin secretion from β -cells or islets.

As shown in Fig. 5, the application of 10 nM UCL 1684 or $3 \mu\text{M}$ AZ to single mouse β -cells that were firing rapid action potentials in 11.1 mM glucose resulted

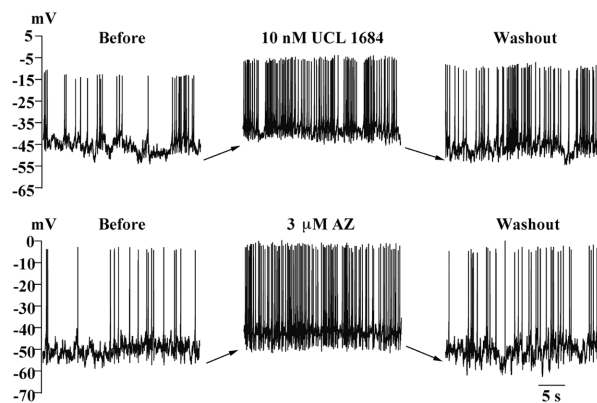


Figure 5. Bath application of K_{slow} blockers in 11.1 mM glucose depolarize mouse isolated β -cells and increase the frequency of action potential firing. The perforated patch clamp technique was used to record the membrane potential of single mouse β -cells. Cell 1 (top trace) shows a representative sample from 13 recordings before and after bath addition of 10 nM UCL 1684. Cell 2 (bottom trace) shows a representative sample from eight recordings before and after bath application of $3 \mu\text{M}$ AZ.

in a reversible membrane depolarization and significantly increased action potential firing. In the β -cells, sporadic action potentials or fast bursting were observed under control conditions (for detail of single β -cells firing properties, see Kinard et al., 1999; Zhang et al., 2003). The addition of UCL 1684 depolarized the cells and increased the frequency of the spikes (Fig. 5, top). In 13 β -cells, 10 nM UCL 1684 depolarized β -cell silent potential from -50.2 ± 2.5 mV to -44.4 ± 2.4 mV ($P < 0.01$), and increased the spike frequency from $58.8 \pm 18.9 \text{ min}^{-1}$ to $133.7 \pm 31.4 \text{ min}^{-1}$ ($P < 0.01$). Similar results were obtained using micromolar concentrations of AZ, as shown in the bottom trace of Fig. 5. In eight cells, the addition of $3 \mu\text{M}$ AZ depolarized β -cell potential from -48.3 ± 3.7 mV to -41.3 ± 2.5 mV ($P < 0.01$) and increased spike frequency from $76.4 \pm 25.0 \text{ min}^{-1}$ to $146.6 \pm 44.5 \text{ min}^{-1}$ ($P < 0.05$). These data suggest that K_{slow} helps maintain the resting membrane potential of single β -cells, and acts as a brake to limit cell firing, as in other systems (Wolfart et al., 2001). Decreased repolarization would also be expected if UCL 1684 inhibited rapid delayed rectifier outward K^+ current (K_V) in β -cells, but the application of 10 nM UCL 1684 had no effect on whole cell K_V current in parallel studies (unpublished data). The data are also in accord with our previous observation that β -cells exhibiting larger K_{slow} currents tended to be more hyperpolarized (Goforth et al., 2002). This would be expected if K_{slow} contributed to the maintenance of the hyperpolarized silent phase potential of β -cells, along with K_{ATP} .

In mouse islets, the addition of 11.1 mM glucose results in the appearance of electrical bursting (Ashcroft and Rorsman, 1989; Zhang et al., 2003). As shown in

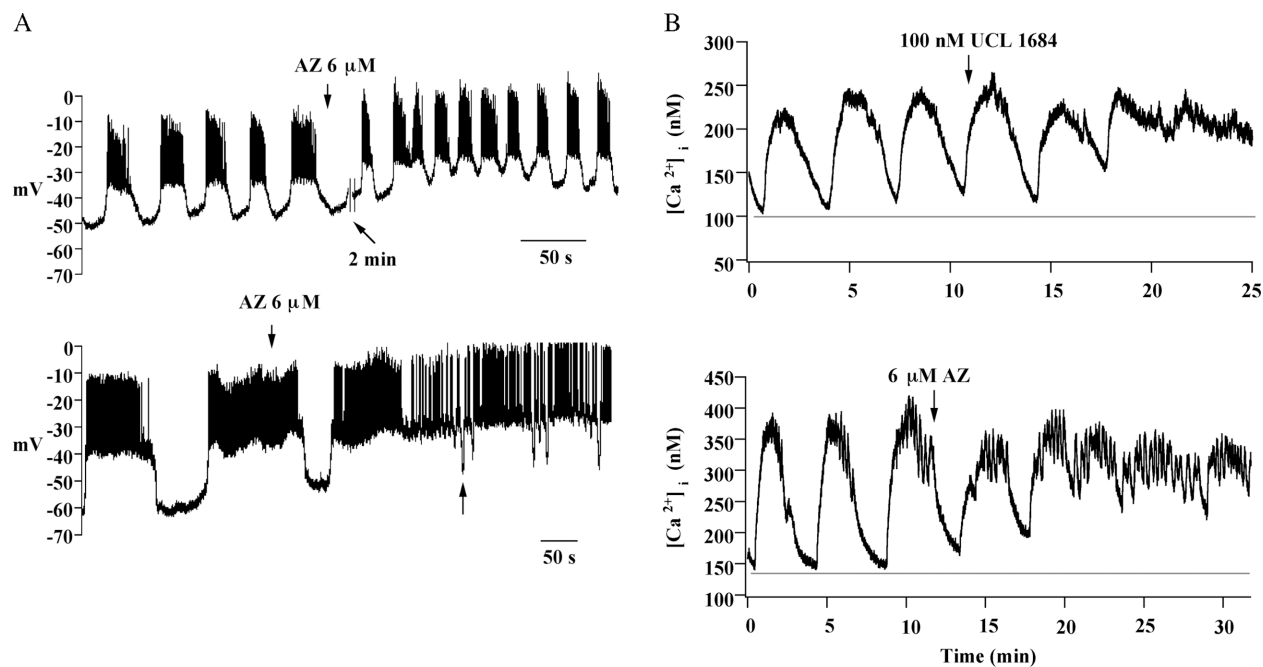


Figure 6. (A) Islets exposed to 6 μM AZ in 11.1 mM glucose exhibited depolarized silent phase potential and increased bursting. Top, response of a medium bursting islet to AZ; bottom, response of a slow bursting islet to AZ. Traces are representative of 14 islet recordings. (B) UCL 1684 and AZ increase mean $[\text{Ca}^{2+}]_i$ in mouse islets. The acute application of 100 nM UCL 1684 or 6 μM AZ to islets bathed in 11.1 mM glucose increased silent phase $[\text{Ca}^{2+}]_i$, while the peak levels appeared to be unchanged. The traces shown are representative samples of 10 islets from UCL 1684 group and seven islets from AZ group. Dotted lines indicate the silent phase levels of $[\text{Ca}^{2+}]_i$.

Fig. 6 A, medium (top) as well as slow bursting islets (bottom) were similarly affected by K_{slow} blockade. Thus, the application of 6 μM AZ to islets bathed in 11.1 mM glucose reversibly depolarized islets and increased action potential frequency. While some fast membrane repolarization could still be observed in the presence of AZ (arrow; Fig. 6 A, bottom), these repolarizing events appeared to be much shorter than in the absence of drug, and the mean silent phase potential was significantly depolarized by AZ (from -54.4 ± 2.3 mV vs. -43.9 ± 2.7 mV, $n = 13$, $P < 0.001$).

UCL 1684 or AZ addition also reproducibly modulated the $[\text{Ca}^{2+}]_i$ responses of islets exposed to 11.1 mM glucose. Thus, UCL 1684 increased the $[\text{Ca}^{2+}]_i$ level corresponding to the membrane silent phase (solid line), and increased the frequency of islet $[\text{Ca}^{2+}]_i$ oscillations (Fig. 6 B, representative of 10 islets). Mean $[\text{Ca}^{2+}]_i$ increased from 269.4 ± 18.8 nM to 328.6 ± 16.2 nM after treatment with 100 nM UCL ($P < 0.05$). The application of 6 μM AZ to islets increased the frequency of islet $[\text{Ca}^{2+}]_i$ oscillations and the mean $[\text{Ca}^{2+}]_i$ level from 288.4 ± 18.3 to 322.6 ± 23.1 nM ($n = 7$, $P < 0.05$).

Blocking K_{slow} Potentiates Mouse Islet Insulin Secretion in a Glucose-dependent Manner

The increase in $[\text{Ca}^{2+}]_i$ we observed following K_{slow} blockade suggested that glucose-dependent insulin se-

cretion might also be increased by UCL 1684 or AZ. While 10 and 100 nM UCL 1684 had no effect on basal insulin secretion (measured in 2.8 mM glucose ($P > 0.05$)), these concentrations increased glucose-stimulated insulin secretion from 722.3 ± 42.7 to 919.4 ± 54.2 or 1096.0 ± 54.2 $\text{pg} \cdot \text{l}^{-1} \cdot \text{islet}^{-1} \cdot \text{h}^{-1}$, respectively (Fig. 7 A, $P < 0.01$, $n = 8$).

AZ also potentiated glucose-dependent insulin secretion as shown in Fig. 7 B. Thus, basal insulin release in 2.8 mM glucose was 264.7 ± 19.7 $\text{pg} \cdot \text{l}^{-1} \cdot \text{islet}^{-1} \cdot \text{h}^{-1}$. When glucose concentration was increased to 11.1 mM, insulin release increased to 722.3 ± 42.7 $\text{pg} \cdot \text{l}^{-1} \cdot \text{islet}^{-1} \cdot \text{h}^{-1}$. The addition of 3.0 or 10.0 μM AZ significantly enhanced islet insulin release by 34.8% and 68.4%, respectively (Fig. 7 B, $P < 0.01$, $n = 8$).

The similar potentiating effects of these two structurally unrelated K_{slow} blockers suggest that targeting this channel may represent a new pharmacological approach for increasing insulin release from the pancreas without also producing hypoglycemia (MacLeod, 2004). Thus, blockers of K_{slow} such as AZ and UCL 1684 could work in a similar manner to the K_{ATP} channel-blocking sulfonylureas, as both drug types block a resting K^+ conductance, resulting in cell membrane depolarization, increased Ca^{2+} uptake, and increased insulin exocytosis (Ferner and Neil, 1988; Aguilar-Bryan et al., 1995; Philipson and Steiner, 1995). However, in the case of K_{slow} blockers, insulin secretion would only be

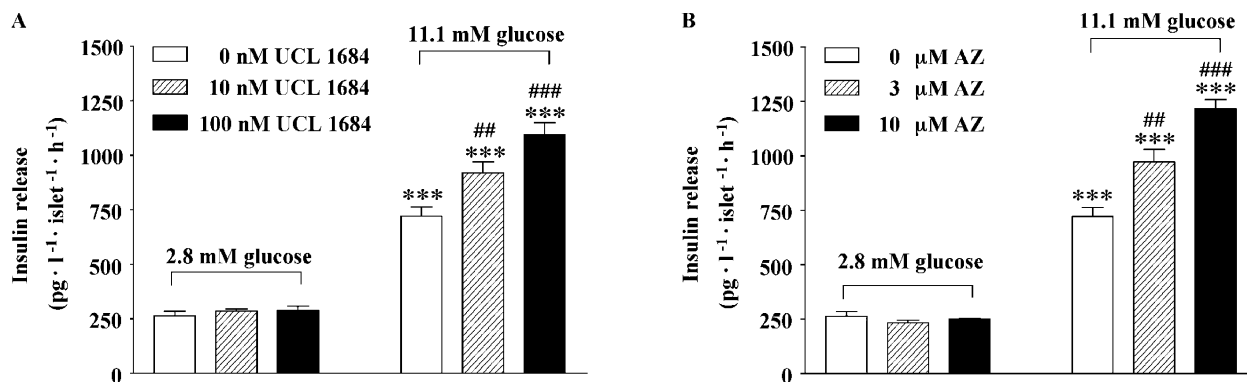


Figure 7. (A) UCL 1684 enhanced glucose-dependent insulin secretion from mouse islets. Islets were treated with different concentrations of UCL 1684 for 1 h using either basal (2.8 mM) or high (11.1 mM) glucose. Data shown were collected from four mice. ***, $P < 0.001$ vs. 2.8 mM glucose group. ##, $P < 0.01$; ###, $P < 0.001$ vs. untreated 11.1 mM glucose group. (B) AZ also increased insulin release from islets in 11.1 mM glucose. Data shown were obtained using islets isolated from four different mice. ***, $P < 0.001$ vs. 2.8 mM glucose group. ##, $P < 0.01$; ###, $P < 0.001$ vs. untreated 11.1 mM glucose group.

amplified when glucose was already elevated, reducing the possibility of hypoglycemia, a drawback of the sulfonyleureas (Ferner and Neil, 1988). K_{slow} blockade is expected to require elevated glucose for it to be effective, since β -cell depolarization due to K_{ATP} closure by glucose must first occur for $[Ca^{2+}]_i$ to rise sufficiently to activate K_{slow} (Göpel et al., 1999; Goforth et al., 2002). Under basal condition, $[Ca^{2+}]_i$ would be too low to sufficiently activate K_{slow} .

DISCUSSION

SK Channels Mediate K_{slow} Current in Mouse β -Cells

While there is agreement in the field that K_{slow} is mediated at least in part by a Ca^{2+} -activated K^+ channel in pancreatic β -cells, the specific channel involved has been controversial. Thus, while the insensitivity of K_{slow} to charybdotoxin, which blocks the large conductance BK type K_{Ca} channels of β -cells (Kukuljan et al., 1991; Li et al., 1999), specifically rules out mediation by BK channels, SK mediation has been unclear because apamin, a bee venom peptide that selectively blocks SK but not BK channels, has yielded disparate results, with one group reporting effects of the toxin on islet Ca^{2+} oscillations (Tamarina et al., 2003), while two other groups reporting that K_{slow} current was insensitive to apamin (Göpel et al., 1999; Goforth et al., 2002).

We thus considered other pharmacological agents in an attempt to further clarify matters. The sensitivity of K_{slow} current to the nonpeptide SK inhibitor UCL 1684, and its insensitivity to the $IK_{(Ca)}$ (or SK4) inhibitor chlortrimazole (0.94 ± 0.07 vs. 0.94 ± 0.08 pA/pF, $n = 10$, $P > 0.05$) suggests that K_{slow} current is mediated at least in part by SK channels similar to SK1, 2, or 3, although the β -cell isoform involved must be apamin insensitive.

Further evidence in favor of K_{slow} mediation by SK channels was obtained with AZ, which readily blocked K_{slow} current and which up to now has been discussed as a selective inhibitor of the slow delayed rectifier current in heart, I_{Ks} (Busch et al., 1994). We ruled out that islet K_{slow} current included a contribution from a cardiac-like I_{Ks} , as AZ-blockable K_{slow} current persisted in islets obtained from $KCNQ1$ or $KCNE1$ null mice lacking I_{Ks} , which in cardiac cells is formed by the coexpression of $KCNQ1$ and $KCNE1$ subunits (Barhanin et al., 1996). Moreover, showing that AZ can directly block heterologously expressed hSK3 channels in HEK cells suggests SK mediation of K_{slow} current. However, the apamin sensitivity of hSK3 suggests it is unlikely that SK3 is the isoform that mediates K_{slow} in β -cells (Tamarina et al., 2003). The fact that the extended current-voltage curves we observed in some native β -cells had the pronounced inward rectification expected of SK currents (unpublished data; Soh and Park, 2001) further supports the hypothesis that SK channels mediate K_{slow} in β -cells.

We also briefly considered the possibility that apamin-insensitive K_{Ca} channels such as those that mediate the slow afterhyperpolarization of CNS neurons (sAHP; Vogalis et al., 2003; Stocker et al., 2004) might be involved in mediating β -cell K_{slow} current. However, this seems to be unlikely given that the neuronal sAHP, which is believed to be mediated by a non-SK type isoform is insensitive to the UCL compounds (Shah et al., 2001; Bond et al., 2004). Furthermore, the kinetic properties of sAHP would appear to be slower than for β -cell K_{slow} current (Vogalis et al., 2003).

What SK Isoforms Mediate K_{slow} ?

While it might appear paradoxical that K_{slow} could be mediated by an apamin-insensitive SK isoform, recent studies have revealed that alternative splicing of the SK

gene can yield splice variants that are relatively insensitive to apamin, as we found for K_{slow} (e.g., SK3_ex4; Wittekindt et al., 2004), and that combining different SK subunits in a heteromultimeric assembly can modify SK channel pharmacology (Benton et al., 2003; Monaghan et al., 2004; D'hoedt et al., 2004). While we cannot at present select among these possibilities, further work is required to clone and sequence the β -cell-specific SK isoforms. A recent paper reported that SK1–4 are all expressed in islet tissue and β -cell lines, although whether these isoforms are β -cell specific was not determined (Tamarina et al., 2003).

The Effects of Selective K_{slow} Blockers Suggests that K_{slow} Participates in Islet Bursting

We found that either UCL 1684 or AZ inhibited K_{slow} current in a dose-dependent manner in mouse β -cells, leading to increased membrane depolarization, action potential firing, and, concomitantly, elevated $[Ca^{2+}]_i$ and insulin secretion. These findings broadly support the hypothesis that K_{slow} plays an important role in the genesis of islet electrical activity, as has been proposed (Göpel et al., 1999; Goforth et al., 2002) but not previously demonstrated experimentally. We found that K_{slow} activation contributes to β -cell membrane potential during the silent phase of bursting, and may play a role in terminating the cyclic bursts of Ca^{2+} -dependent action potentials that drive Ca^{2+} influx and insulin secretion in mouse islets. Similar findings were observed in neurons following inhibition of slow Ca^{2+} -activated currents like sAHP (El Manira et al., 1994; Ghamari-Langroudi and Bourque, 2004). While the activation and deactivation kinetics of K_{slow} are relatively brief given the more prolonged nature of electrical bursting observed in islets, modeling studies have previously shown that fast K_{slow} may interact with slower process in the β -cells (e.g., K_{ATP} via ATP/ADP changes; ER Ca^{2+}) to mediate slower modes of electrical activity (Bertram et al., 2000; Bertram and Sherman, 2004a,b). K_{slow} may also display slower deactivation kinetics during more prolonged phases of Ca^{2+} influx than provided experimentally here. Alternatively, we cannot rule out that in situ the kinetics of K_{slow} current are slower than we observed in the isolated β -cells.

The Development of Novel Drugs To Block K_{slow} Could Represent a New Way To Increase Glucose-dependent Insulin Secretion in Diabetic Patients without also Causing Hypoglycemia

There is currently much interest in the islet field in investigating novel targets for drugs that might increase insulin secretion in a glucose-dependent manner (MacLeod, 2004). Our work suggests that inhibition of K_{slow} could be useful clinically, as K_{slow} appears to help control Ca^{2+} influx into the β -cell when glucose is elevated

and that its inhibitors significantly enhance insulin secretion. Targeting K_{slow} pharmacologically may thus provide a novel, glucose-sensitive target for a new generation of antidiabetic agents.

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