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Inhibition of Kv2.1 Voltage-dependent K^+ Channels in Pancreatic β-Cells Enhances Glucose-dependent Insulin Secretion*

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Voltage-dependent (Kv) outward K^+ currents repolarize β-cell action potentials during a glucose stimulus to limit Ca^{2+} entry and insulin secretion. Dominant-negative "knockout" of Kv2 family channels enhances glucose-stimulated insulin secretion. Here we show that a putative Kv2.1 antagonist (C-1) stimulates insulin secretion from MIN6 insulinoma cells in a glucose- and dose-dependent manner while blocking voltage-dependent outward K^+ currents. C-1-blocked recombinant Kv2.1-mediated currents more specifically than currents mediated by Kv1, -3, and -4 family channels (Kv1.4, 3.1, 4.2). Additionally, C-1 had little effect on currents recorded from MIN6 cells expressing a dominant-negative Kv2.1 α-subunit. The insulinotropic effect of acute Kv2.1 inhibition resulted from enhanced membrane depolarization and augmented intracellular Ca^{2+} responses to glucose. Immunohistochemical staining of mouse pancreas sections showed that expression of Kv2.1 correlated highly with insulin-containing β-cells, consistent with the ability of C-1 to block voltage-dependent outward K^+ currents in isolated mouse β-cells. Antagonism of Kv2.1 in an *ex vivo* perfused mouse pancreas model enhanced first- and second-phase insulin secretion, whereas glucagon secretion was unaffected. The present study demonstrates that Kv2.1 is an important component of β-cell stimulus-secretion coupling, and a compound that enhances, but does not initiate, β-cell electrical activity by acting on Kv2.1 would be a useful antidiabetic agent.

Peripheral insulin resistance and defects in insulin secretion from pancreatic β-cells characterize type-2 diabetes mellitus (1). The β-cell defect of type-2 diabetes mellitus is most commonly treated with the sulfonylurea class of compounds (2, 3). Sulfonylureas antagonize ATP-sensitive K^+ (K_{ATP}) channels, depolarizing the membrane, opening voltage-dependent Ca^{2+} channels, and stimulating insulin secretion (4). This essentially mimics the effect of an increased intracellular ATP/ADP ratio resulting from elevated glucose metabolism in the fed state as described in a number of reviews (3, 5–7). Because sulfonylurea drugs act in a glucose-independent manner, hypoglycemic episodes are a major concern (8). Currently, glucose-dependent insulinotropic agents are a major focus of investigation for the development of safer and more appropriate therapeutic agents.

Voltage-dependent outward K^+ currents have been detected in β-cells and are believed to mediate action potential repolarization (5, 9–11), limiting Ca^{2+} influx and insulin secretion. Indeed, previous studies show that the general voltage-dependent K^+ (Kv)[1] and Ca^{2+}-sensitive voltage-dependent K^+ (K_{Ca}) channel antagonist tetraethylammonium (TEA) augments membrane depolarization (12, 13), Ca^{2+} influx (14), and insulin secretion (13, 15) in a glucose-dependent manner. Recently, we have reported that dominant-negative knockout of Kv2 channels reduced outward K^+ currents by 60–70% in rat β-cells and HIT-T15 insulinoma cells and caused a doubling of the insulin secretion response of rat islets to glucose (16). Kv2.1 likely plays this regulatory role because its protein expression is high in insulin-secreting cells (16, 17), and mRNA for Kv2.2, the only other Kv2 family α-subunit known to form functional channels, could not be detected by reverse transcription-PCR (16).

To investigate the functional importance of Kv2.1 and the mechanism by which this channel regulates β-cell stimulus-secretion coupling, we have now examined the effect of acute Kv2.1 inhibition. We report that a small molecule bispidine derivative (termed C-1) related to class III antiarrhythmic agents specifically antagonizes Kv2.1 in insulin-secreting cells, enhances membrane potential and intracellular Ca^{2+} ($[Ca^{2+}]_i$) responses to glucose, and increases glucose-stimulated insulin secretion (GSIS) from insulinoma cells and perfused mouse pancreas. The present study demonstrates an important role for Kv2.1 in ionic stimulus-secretion coupling of insulin secretion and reinforces the view that agents that enhance, but do not initiate, β-cell electrical activity by acting on Kv2.1 would be useful therapeutic agents, stimulating only postprandial insulin secretion.

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[1] The abbreviations used are: Kv, voltage-dependent K^+; K_{Ca}, Ca^{2+}-sensitive voltage-dependent K^+; TEA, tetraethylammonium; RT, room temperature; GSIS, glucose-stimulated insulin secretion; ITX, iberiotoxin; HEK cells, human embryonic kidney cells; EGFP, enhanced green fluorescent protein; CFP, cyan fluorescent protein; ECFP, enhanced CFP; AUC, area under the curve.

EXPERIMENTAL PROCEDURES

Chemical Reagents—TEA chloride was from Sigma-Aldrich. Iberiotoxin (ITX) was from Alomone Labs (Jerusalem, Israel). Glibenclamide (Sigma-Aldrich) was prepared as a 10 mM stock in dimethyl sulfoxide (Me_2SO). The putative Kv2.1 antagonist (4-chloro-benzoic acid 3-(4-benzo[1,3]dioxol-5-yl-butyl)-7-methyl-3,7-diaza-bicyclo[3.3.1]non-9-yl ester), a bispidine derivative termed compound 1 (C-1), was resuspended in Me_2SO at 10 mM from lyophilized stock. Control solutions contained an equal amount of Me_2SO that did not exceed 0.05% of the final solution.

Cell Culture and Islet Isolation—MIN6 insulinoma cells (P29–40) were cultured in high glucose Dulbecco's modified Eagle's medium with 10% fetal bovine serum, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate, and 2 μl/500 ml β-mercaptoethanol at 37 °C and 5% CO_2. The tsA-201 cell line, a HEK-293 derived cell line stably expressing an SV40 T antigen (18, 19), was cultured in the above media without β-mercaptoethanol. Two days before insulin secretion experiments, cells were trypsinized and plated in 12-well plates at 5×10^5 cells/well. For electrophysiology and $[Ca^{2+}]_i$ measurements, cells were trypsinized and plated on glass coverslips in 35-mm dishes 24 h before use. Cell transfection was with LipofectAMINE 2000 (Invitrogen).

Islets were isolated from female CD-1 mice (6 weeks) using a collagenase digestion/histopaque-1077 protocol adapted from that described previously (16). Islets were dispersed to single cells by treatment with 0.015% trypsin (Invitrogen) in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline at 37 °C and 5% CO_2 for 10 min. Islet cells were plated on glass coverslips in 35-mm dishes in RMPI media supplemented with 2.5 mM glucose, 0.25% Hepes, 7.5% fetal bovine serum, 100 units/ml penicillin G sodium, 100 μg/ml streptomycin sulfate and cultured for 1–3 days before electrophysiological recordings.

Insulin Secretion Experiments—Insulin secretion experiments were performed in Krebs-Ringer bicarbonate buffer containing 115 mM NaCl, 5 mM KCl, 24 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM HEPES, and 0.1% bovine serum albumin as described previously (16). Radioimmunoassay was performed using the rat insulin radioimmunoassay kit from Linco Research Inc. (St. Louis, MO). All MIN6 cell insulin secretion experiments were performed with an $n \geq 9$, and data were normalized to controls. Data were analyzed using Student's unpaired *t* test or a one-way analysis of variance and Dunnett post-test to compare each value *versus* control. A *p* value <0.05 was considered significant. The EC_{50} for dose response of C-1 was determined by fitting to a variable slope sigmoidal curve, $Y = \text{min} + (\text{max} - \text{min})/(1 + 10^{(\log EC_{50} \text{ [minus] } x)n})$, with Origin 3.5 (Microcal Software, Northhampton, MA).

Patch Clamp Experiments—MIN6 cells, tsA-201 cells, or mouse β-cells were patch-clamped in the whole-cell or perforated-patch configuration using an EPC-9 amplifier and PULSE software (HEKA Electronik, Lambrecht, Germany). Patch pipettes, prepared as described previously (16), had typical resistances of 3–5 megaohms when fire-polished. For whole-cell studies extracellular solutions contained 140 mM NaCl, 2 mM $CaCl_2$, 4 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 10 mM HEPES, pH 7.3 with NaOH, and intracellular solutions contained 140 mM KCl, 1 mM $MgCl_2 \cdot 6H_2O$, 1 mM EGTA, 10 mM HEPES, 5 mM MgATP, pH 7.25 with KOH. β-cells were identified by the lack of a transient voltage-dependent inward Na^+ current as described by Gopel *et al.* (20, 21), and results were confirmed in β-cells identified by membrane potential responses to glucose in the perforated-patch configuration. Outward currents were elicited by 500-ms depolarizations from holding potentials of −90 or −50 mV to +30 mV. Alternatively, current-voltage relationship curves were generated by depolarizing cells from a holding potential of −70 mV in 20-mV increments to +70 mV. Sustained outward current was taken as the mean current during the last 25 ms of the depolarizing pulse.

For perforated-patch studies, extracellular solutions contained 140 mM NaCl, 3.6 mM KCl, 2.6 mM $CaCl_2$, 2 mM $NaHCO_3$, 0.5 mM NaH_2PO_4, 0.5 mM $MgSO_4$, 5 mM HEPES, pH 7.4 with NaOH and either 2.5 or 11.1 mM glucose, and intracellular solutions contained 76 mM K_2SO_4, 10 mM KCl, 10 mM NaCl, 1 mM $MgCl_2$, 4 mM HEPES, pH 7.35 with KOH. Membrane potential was recorded in the absence of current injection ($I = 0$). Experiments were discontinued if cells were unresponsive to glucose. The amplifier was occasionally switched to voltage-clamp mode to verify seal resistance and to determine voltage-dependant outward K^+ current amplitude with a single depolarizing pulse from −70 mV to +30 mV for 500 ms.

Electrophysiological recordings were obtained at 32–35 °C unless stated otherwise and normalized to cell capacitance. For pharmacological studies, compounds were applied for at least 5 min before current recording. IC_{50} values of current block were estimated by fitting a dose response to an I/I_{max}-modified Hill function ($I/I_{max} = ((X/IC_{50})^n + c)/(1 + (X/IC_{50})^n - c)$) with Origin 3.5 (Microcal Software). Activation curves were fit to a one-phase exponential association function with Prism 3.03 (Graphpad Software, San Diego) to derive activation time constants. Sustained outward currents were compared with the Student's unpaired *t* test ($p < 0.05$ considered significant). Steady-state current-voltage relationships were compared by one-way analysis of variance with a Tukey post-test to compare currents at individual voltages ($p < 0.05$ considered significant).

Plasmid Vectors—The C-terminal-truncated Kv2.1 subunit (Kv2.1N) has been described previously (16). This construct was expressed with enhanced green fluorescent protein (EGFP) as a marker by insertion into the pIRES-EGFP plasmid (Clontech, Palo Alto, CA) or without EGFP by insertion into the Adlox plasmid. Rat Kv2.1 and 3.1 constructs fused with cyan fluorescent protein (CFP) on the N terminus in the pECFP-C1 plasmid (Clontech) were from O. T. Jones (University of Manchester, Manchester, UK), whereas the wild-type Kv2.1 and Kv3.1 cDNAs were originally from R. H. Joho (University of Texas Southwestern Medical Center, Dallas, TX). The Kv3.1 cDNA was also inserted into the pIRES-EGFP plasmid (Clontech) to allow EGFP co-expression as a marker for transfected cells. Cloned Kv1.4 and 4.2 in the GW1H plasmid were from R. J. Hajjar (Harvard Medical School, Boston, MA). These constructs were co-transfected with empty pIRES-EGFP plasmid (Clontech) to identify transfected cells by EGFP expression. All plasmids express the protein of interest under control of the cytomegalovirus promoter.

Calcium Imaging—Imaging was performed using equipment described previously (22). Cells on glass coverslips were loaded with 4 μM Fura-2-AM (Molecular Probes, Eugene, OR) for 45 min at 37 °C. Solutions were continuously perfused in a heated recording chamber at 3 ml/min at 32–35 °C. Images were obtained with 340- and 380-nm excitation and a 510-nm cutoff emission filter. Exposures lasted 0.2 s, and images were acquired at ~0.2 Hz. Images were analyzed using Merlin software with $[Ca^{2+}]_i$ calculated using the Grynkiewicz equation: $[Ca^{2+}]_i = K_d \times \beta \times (R - R_{min})/(R_{max} - R)$. Fura-2-AM fluorescence was calibrated to Ca^{2+} concentration using 10 μM ionomycin in the presence of 5 mM $CaCl_2$ for maximal fluorescence ratio or with no added Ca^{2+} and 10 mM EGTA for minimal fluorescence ratio. The values obtained were $R_{min} = 0.2$, $R_{max} = 2$, and $\beta = 3$. A value of 224 nM for the apparent K_d of Ca^{2+} binding to Fura-2 was used.

Western Blots and Immunohistochemistry—Immunoblotting of Kv channel proteins was performed as previously described (16). Twenty-five μg of protein from each sample was separated on a 10% polyacrylamide gel and transferred to PVDF-Plus™ membrane (Fisher). Primary antibodies (Kv2.1, Upstate Biotechnology, Lake Placid, NY; Kv1.4 and 4.2, Alomone Labs, Jerusalem, Israel) or antibody-antigen solutions (diluted as per suppliers instructions) were detected with appropriate secondary antibodies (sheep anti-mouse, 1:3000, and donkey anti-rabbit, 1:7500; Amersham Biosciences) for 1 h at RT. Visualization was by chemiluminescence (ECL, Amersham Biosciences) and exposure to Kodak film (Eastman Kodak Co.) for 5 s to 10 min.

Mouse pancreas sections (60 μm) were prepared for immunocytochemistry as described previously (23). Immunostaining was carried out on free-floating serial sections of pancreas permeabilized with 0.04% Triton-X-100 in 0.1 M phosphate-buffered saline for 30 min at RT (24). The primary antibodies were rabbit anti-Kv2.1 (Alomone Labs; 10 μg/ml) and guinea-pig anti-insulin (a gift of R. A. Pederson, University of British Columbia, Vancouver, Canada; 1:1000). Antibodies were applied for 1 h at RT on a rocking platform then overnight at 4 °C. Secondary antibodies were goat anti-rabbit-fluorescein at 1:200 and rabbit anti-guinea-pig horseradish peroxidase, applied for 2 h at RT with rocking. The latter was developed using 3,3′-diaminobenzidine. Immunostained sections were mounted on poly-L-lysine-treated glass slides. Sections with fluorescein secondary antibody were cover-slipped using the Slow Fade Light Antifade Solution from Molecular Probes.

Pancreatic Perfusion—Female CD-1 mice (6 weeks; Charles River Canada, Montreal, Canada) were fasted overnight (15–18 h) and anesthetized with 80 mg/kg intraperitoneal sodium pentobarbital. The surgical procedure for perfusion of the pancreas was similar to that described previously (25, 26). In brief, PE 50 tubing (Intramedic, Parsippany, NJ) was used for blood vessel cannulation, and the perfusate was a modified Krebs-Ringer, 2% bovine serum albumin, glucose, 3% dextran solution. The solution was gassed with 95% O_2, 5% CO_2 to achieve a pH of 7.4. Glucose was switched between 1.4 and 13.4 mM to stimulate insulin secretion. Glucagon secretion was measured in the presence of 5 mM glucose, a concentration intermediate to that required for maximum stimulation or inhibition (27). The infusion pump was a

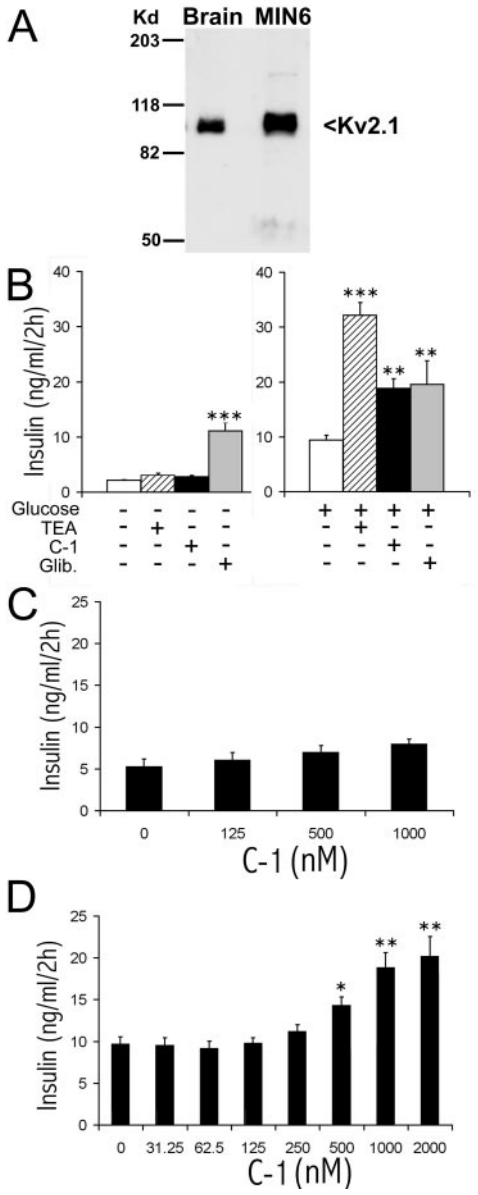

FIG. 1. **Both TEA and the bispidine derivative, C-1, glucose dependently enhance insulin secretion.** In *panel A*, Kv2.1 expression was detected in MIN6 cell protein lysates (25 μg of protein) by Western blot, whereas rat brain lysates were used as a control. In *panel B*, both the general Kv and K_{Ca} channel antagonist TEA (15 mM) and C-1 (1 nM) enhanced GSIS (10 mM glucose) from MIN6 cells compared with controls while having no effect in the absence of glucose ($n = 9$). Antagonism of K_{ATP} channels with glibenclamide (*Glib.*, 500 nM) stimulated insulin secretion both with and without glucose ($n = 12$). The dose response for C-1 is shown in the absence of glucose (*panel C*) and in the presence of 10 mM glucose (*panel D*). *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ compared with controls.

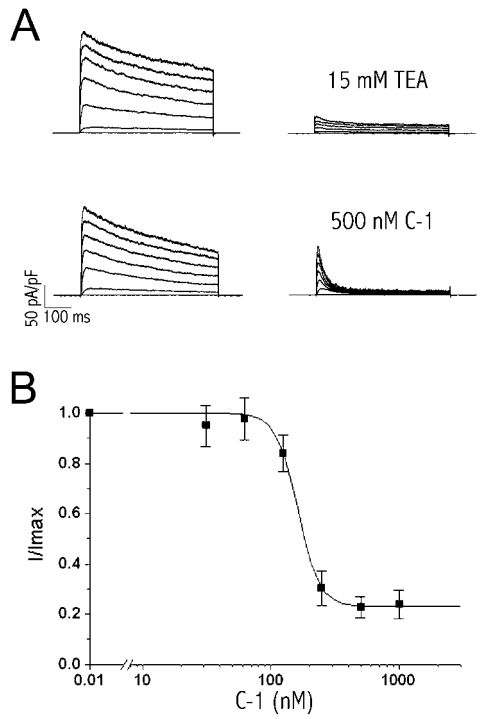

FIG. 2. **Both TEA and C-1 block voltage-dependent outward K^+ currents in MIN6 cells.** Voltage-dependent outward K^+ currents were recorded from MIN6 cells by whole-cell voltage clamp. In *panel A*, outward K^+ currents were blocked by TEA (15 mM, *upper traces*) and C-1 (500 nM, *lower traces*). Representative traces are shown. In *panel B*, C-1 blocked outward currents from MIN6 cells in a dose-dependent manner. The dose-response effect on steady-state currents was best fit with a modified Hill equation (see "Experimental Procedures") with an IC_{50} of 155.7 ± 16.8 nM and a Hill coefficient of 5.2 ± 1.8 ($n = 7–8$). pA/pF, picoamperes/picofarad.

Minipuls 3 (Gilson Inc., Middleton, WI). Data were analyzed by one-way analysis of variance and Bonferroni post-test to compare time points. Area under the curves (AUCs) were compared using the Student's unpaired *t* test. A *p* value of <0.05 was considered significant.

RESULTS

The Bispidine Derivative (C-1) Enhances Glucose-stimulated Insulin Secretion—Kv2.1 is expressed in rat islets, HIT-T15 cells, and βTC cells (5, 16, 17). Western blotting confirmed Kv2.1 expression in MIN6 cell protein lysates (Fig. 1*A*). As reported previously (13, 15, 17, 28), the general Kv/K_{Ca} channel antagonist TEA (15 mM) enhanced only GSIS but had no significant effect in the absence of glucose (Fig. 1*B*). Similar to TEA, the putative small molecule Kv2.1 antagonist C-1 enhanced insulin secretion from MIN6 cells in a glucose- and dose-dependent (EC_{50} = 546.7 ± 1.1 nM, $n = 9–15$) manner (Fig. 1). This is in contrast to the effect of glibenclamide (500 nM), a K_{ATP} channel antagonist, which stimulated insulin secretion even in the absence of glucose (Fig. 1*B*). At the maximum dose of 2 μM, C-1 enhanced GSIS by 209.1 ± 24.7% ($n = 9$, $p < 0.01$) compared with control.

Voltage-dependent Outward K^+ Currents Are Blocked by C-1—Outward K^+ currents were elicited from MIN6 cells voltage-clamped in the whole-cell configuration at 32–35 °C (Fig. 2*A*). Current amplitudes were similar to those reported previously from rat islet cells at RT (16) but inactivated to a greater extent (~50% greater than 500 ms). Inclusion of 1 mM EGTA and 5 mM MgATP resulted in a minimal contribution from K_{Ca} or K_{ATP} channels. Therefore the outward currents observed reflect the opening of Kv channels. Sustained outward K^+ currents were 85.2 ± 1.8% ($n = 4$, $p < 0.001$) inhibited by 15 mM TEA (Fig. 2*A*). C-1 (500 nM) inhibited sustained outward K^+ current from MIN6 cells specifically (77.2 ± 4.3%, $n = 8$, $p < 0.001$) while having little effect on an inactivating component (Fig. 2*A* and 3*C*). The inhibitory effect of C-1 on sustained currents was dose-dependent, with an IC_{50} of 155.7 ± 16.8 nM and a Hill coefficient of 5.2 ± 1.8 ($n = 7–8$), suggesting multiple binding sites or co-operative binding of the compound (Fig. 2*B*). The inactivating portion of MIN6 outward K^+ currents could be selectively abolished by holding at more positive potentials (−50 mV), allowing separation of inactivating and non-inactivating currents (Fig. 3*C*). These inactivating currents could conceivably result from expression of the TEA-insensitive Kv1.4 and/or Kv4.2 channels (29, 30) (detected by Western blot, not shown). However, the sensitivity of these currents to TEA

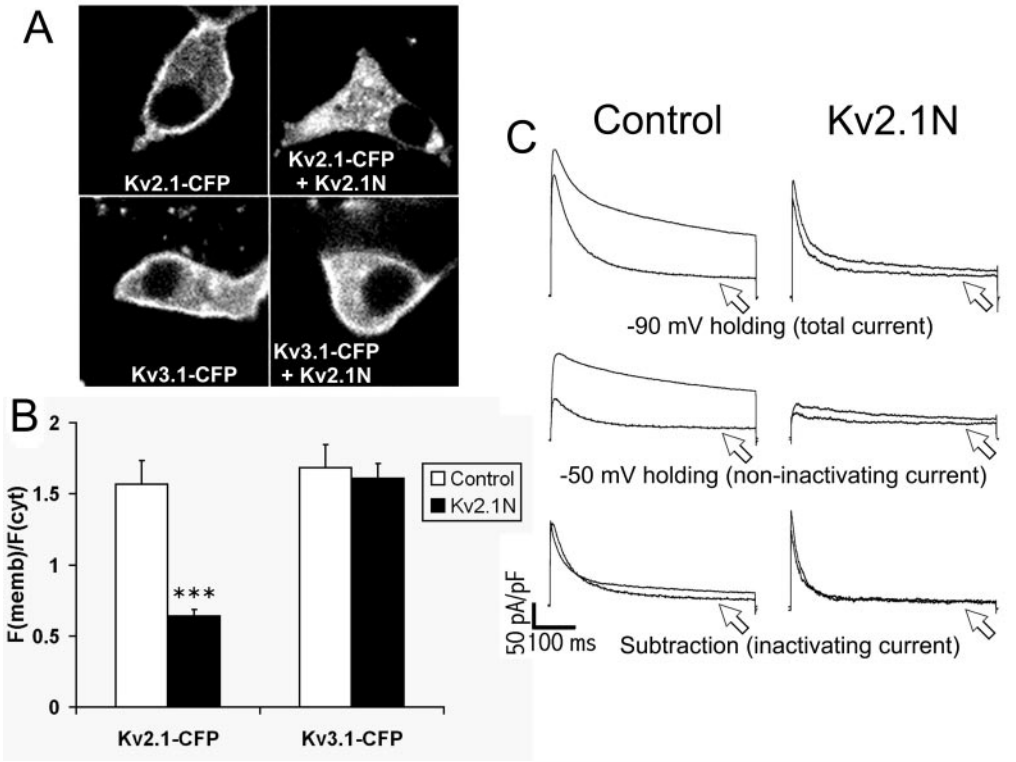

FIG. 3. **C-1 inhibits Kv2.1 currents in MIN6 cells.** In *panel A*, co-expression of Kv2.1N prevented membrane targeting of an ECFP-tagged Kv2.1 construct (Kv2.1-CFP) but did not affect membrane expression of an ECFP-tagged Kv3.1 construct (Kv3.1-CFP). Images were obtained using a laser-scanning confocal microscope with a 63× oil immersion objective. The membrane/cytoplasm fluorescence intensity ratio (*F*(memb)/*F*(cyt) was quantified in *panel B*. Co-expression of Kv2.1N prevented membrane targeting of Kv2.1-CFP compared with controls (n = 8 and 11) but did not affect targeting of Kv3.1-CFP (n = 12 and 10). In *panel C*, outward K^+ currents were recorded from control (*left panels*) or Kv2.1N-expressing (*right panels*) MIN6 cells. The inactivating current component was derived by subtracting currents elicited from −50 mV from currents elicited from −90 mV. In control cells, C-1 (500 nM, *arrows*) largely blocked the non-inactivating current, whereas inactivating current was unaffected. In Kv2.1N-expressing cells, C-1 (500 nM, *arrows*) did not effect either non-inactivating or inactivating currents. The traces shown are averages from 7 (control) and 5 (Kv2.1N) cells.

suggest they may be mediated by the TEA-sensitive Kv3.4 channel detected in mouse β-cells (20). These inactivating currents are consistent with the previous detection of small inactivating outward K^+ currents in rodent β-cells at RT (16, 20, 31) and with the inactivating current that remains when Kv2.1 channel function is disrupted (see below and Fig. 3).

C-1 Specifically Blocks Kv2.1 in MIN6 Cells—To investigate the specificity of the bispidine derivative C-1 for Kv2.1, we first studied the effect of this compound in MIN6 cells expressing a dominant-negative Kv2.1 channel subunit (Kv2.1N) previously used to reduce Kv2 currents in HIT-T15 cells and rat β-cells (16). Laser-scanning confocal microscopy and quantification of the membrane/cytoplasmic fluorescence intensity ratio (*F*(memb)/*F*(cyt)) confirmed that Kv2.1N expression inhibits plasma membrane targeting of an enhanced cyan fluorescent protein (ECFP)-Kv2.1 fusion protein ($p < 0.001$, n = 8) while not affecting a related ECFP-tagged full-length Kv3.1 subunit (n = 12) (Fig. 3). Similar results were obtained using an EGFP-tagged Kv2.1N construct.[2]

In MIN6 cells expressing Kv2.1N, non-inactivating outward K^+ currents were reduced (by 63.6 ± 4.7%, n = 5, $p < 0.001$), whereas inactivating currents were unaffected (Fig. 3*C*, *right panels*). The remaining outward K^+ currents in these cells were only marginally inhibited by C-1 (500 nM, Fig. 3*C*, *right panels*), which reduced outward K^+ currents from control MIN6 cells by 77.2 ± 4.3% ($p < 0.001$, n = 8, Fig. 3*C*, *left panels*, indicated by *arrows*). C-1 did not inhibit inactivating currents (Fig. 3*C*, *bottom panels*) and blocked non-inactivating currents only slightly in cells expressing Kv2.1N (9.3 ± 3.9% compared with controls) (Fig. 3*C*, *middle right panel*). The small effect of C-1 on non-inactivating currents in Kv2.1N-expressing cells may be attributed to residual Kv2.1 channels at the membrane or an inhibitory effect on non-Kv2.1 channels.

The specificity of C-1 for Kv2.1 over related K^+ channels was investigated further in cells overexpressing wild-type Kv channels. Kv2.1 channels expressed in tsA-201 cells at RT were blocked by C-1 with a similar IC_{50} (164.0 ± 6.1 nM, Hill coefficient = 1.3, n = 6) as native MIN6 currents. Currents mediated by Kv1.4 were blocked with an IC_{50} of 2205 ± 198.2 nM (Hill coefficient = 1.2, n = 6), whereas Kv3.1 (IC_{50} = 3627 ± 509.0 nM, Hill coefficient = 0.87, n = 6) and Kv4.2 (IC_{50} = 3429 ± 78.2 nM, Hill coefficient = 0.97, n = 6) were even less sensitive to block by C-1. Additionally, we investigated the effect of C-1 on Kv channels overexpressed in MIN6 cells at 32–35 °C. Results were similar to those obtained in tsA-201 cells in that Kv2.1 (IC_{50} = 247.5 ± 45.8 nM, Hill coefficient = 1.7, n = 6) was blocked more potently than Kv1.4 (IC_{50} = 2964 ± 32.3 nM, Hill coefficient = 1.0, n = 6) or Kv3.1 (IC_{50} = 914.0 ± 87.5 nM, Hill coefficient = 0.97, n = 6).

Kv2.1 Inhibition Enhances Membrane Potential and Intracellular Ca^{2+} Responses to Glucose—To confirm that the effect of Kv2.1 inhibition is downstream of K_{ATP} channel inhibition, we used diazoxide to prevent K_{ATP} channel closure and membrane depolarization. Diazoxide (250 μM) caused a 56.8 ± 8.4% decrease in GSIS ($p < 0.001$, n = 9; Fig. 4*A*). In the presence of diazoxide (250 μM), the effect of C-1 (500 nM) was not significant (n = 9, Fig. 4*A*). Similarly, prevention of Ca^{2+} entry by antagonism of voltage-dependent Ca^{2+} channels with vera-

[2] P. E. MacDonald and M. B. Wheeler, unpublished data.

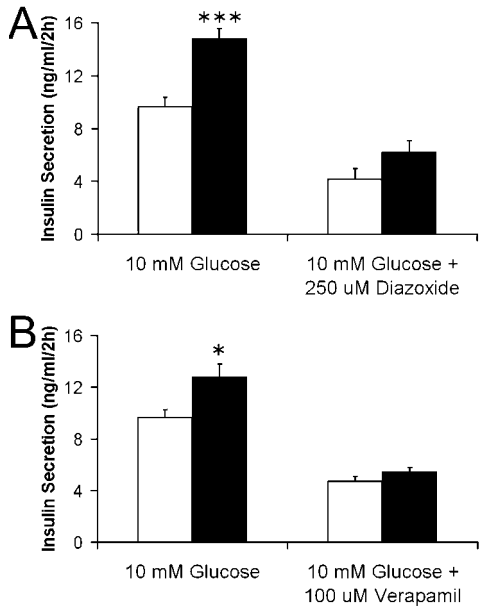

FIG. 4. **The insulinotropic effect of Kv2.1 inhibition in MIN6 cells is abolished by diazoxide or verapamil.** In *panel A*, Kv2.1 inhibition (500 nM C-1, *black bars*) was unable to significantly stimulate insulin secretion compared with controls (*white bars*) when glucose-induced depolarization was prevented with 250 μM diazoxide ($n = 9$). In *panel B*, when glucose-induced Ca^{2+} entry into MIN6 cells was prevented by closing voltage-dependent Ca^{2+} channels with 100 μM verapamil, Kv2.1 inhibition (500 nM C-1, *black bars*) was unable to significantly stimulate insulin secretion compared with controls (*white bars*, $n = 9$). *, $p < 0.05$; ***, $p < 0.001$ compared with controls.

pamil (100 μM) reduced GSIS by 51.1 ± 4.1% ($p < 0.001$, $n = 9$) and prevented the insulinotropic effect of C-1 (500 nM, $n = 9$, Fig. 4*B*).

Intracellular Ca^{2+} and membrane potential responses to glucose were measured in MIN6 cells. Of 471 cells in 19 separate experiments, 326 responded to 10 mM glucose with an increase in $[Ca^{2+}]_i$. TEA (15 mM) and C-1 (500 nM) augmented $[Ca^{2+}]_i$ responses in 86.3 ± 3.8 and 76.0 ± 5.8% of glucose-responsive cells, respectively (Fig. 5, *C* and *D*). Importantly, C-1 neither directly augmented Ca^{2+} currents elicited by step depolarizations (1 μM, not shown) nor blocked K_{ATP} currents in MIN6 cells and rat β-cells as measured using a voltage ramp protocol (500 nM, not shown). Interestingly, TEA produced oscillations in $[Ca^{2+}]_i$ (in 77.6 ± 13.3% of responsive cells), whereas Kv2.1 inhibition with C-1 did not (only 35.0 ± 6.2% of responsive cells showed very weak oscillations, $p < 0.01$, compared with TEA) (Fig. 5). Also of note, the addition of TEA or C-1 to the cells that did not initially respond to glucose enabled $[Ca^{2+}]_i$ responses in 42.8 ± 16.1 and 64.6 ± 9.7% of these non-glucose-responsive cells, respectively. The cells exposed to C-1 were still responsive to stimulation as demonstrated by perfusion with a 30 mM KCl solution at the end of the experiment (not shown). Similar to the effect on $[Ca^{2+}]_i$, TEA (15 mM) enhanced glucose-stimulated (11.1 mM) membrane depolarization and resulted in an oscillatory pattern of electrical activity (Fig. 5*A*; $n = 6$). The addition of C-1 (500 nM) enhanced the electrical response of MIN6 cells to glucose (Fig. 5*B*); however, this effect was never oscillatory in nature ($n = 6$).

The Oscillatory Response to TEA Is Replicated by Blocking Kv2.1 and Large Conductance K_{Ca} (BK_{Ca}) Channels—Because TEA is expected to non-specifically block both Kv and K_{Ca} channels (32–34), we investigated the effect of the large conductance K_{Ca} (BK_{Ca}) channel antagonist ITX together with C-1. In 5 separate experiments, 195 of 267 glucose pretreated (10 mM for 20–25 min) cells showed enhanced $[Ca^{2+}]_i$ responses, and 5 of 6 cells showed enhanced depolarization responses to C-1 (500 nM). ITX (30 nM) further enhanced the $[Ca^{2+}]_i$ (63.3 ± 12.6%) and membrane potential (83.3%, 5 of 6) responses, and 41.0 ± 8.3 and 40% of these responses, respectively, were strongly oscillatory (Fig. 6*C*). ITX alone did not produce oscillatory membrane potential responses to glucose (not shown). Currents measured during brief switches to voltage-clamp mode (*i*, *ii*, and *iii* in Fig. 6*C*) represent the additive result of Kv and K_{Ca} channel activation since $[Ca^{2+}]_i$ was not chelated to inactivate K_{Ca} channels as in the previous experiments. C-1 (500 nM) reduced outward currents by 52.7 ± 7.9% ($n = 6$, $p < 0.01$) compared with controls, and further treatment with ITX (30 nM) reduced currents by an additional 18.2 ± 8.3% ($n = 5$, $p < 0.05$ compared with C-1 alone). These results suggest that the ability of TEA to produce oscillatory increases in both membrane potential and $[Ca^{2+}]_i$ in MIN6 cells results at least in part from its ability to block BK_{Ca} channels in addition to Kv2.1.

The Effect of Kv2.1 Inhibition on Mouse β-Cells and Perfused Mouse Pancreas—Immunohistochemistry shows that Kv2.1 expression is highly correlated to that of insulin-containing β-cells in mouse pancreas, although the present staining cannot rule out expression in α- or δ-cells (Fig. 7*A*). Consistent with this, C-1 blocked voltage-dependent outward K^+ currents from mouse β-cells (Fig. 7*B*). This effect was dose-dependent (IC_{50} = 20.6 ± 1.9 nM, Hill coefficient = 1.2, $n = 6$) with a maximum block of 86.1 ± 7.7% ($n = 6$, $p < 0.001$) at 500 nM C-1. Current subtraction gives the C-1-sensitive component that activates at potentials positive to −30 mV, with a half-maximal activation at 21.4 ± 7.3 mV ($n = 6$) (Fig. 7*B*). Activation of the C-1-sensitive current component occurred with a single time constant of 8.81 ± 0.88 ms ($n = 6$) upon depolarization to +70 mV. To determine whether Kv2.1 antagonism enhances insulin secretion from whole pancreas, we used an *ex vivo* perfused mouse pancreas model. Antagonism of Kv2.1 channels at the beginning of the 13.4 mM glucose pulse enhanced first-phase secretion, defined as the AUC for the first 5 min of stimulation (Fig. 7*C*, $n = 6$, $p < 0.05$). Second-phase insulin secretion was also enhanced by antagonism of Kv2.1 (Fig. 7*D*, $n = 7$), also shown as an increased AUC ($p < 0.05$). Importantly, Kv2.1 inhibition stimulated neither insulin ($n = 4$) nor glucagon ($n = 5$) secretion in the presence of 5 mM glucose (Fig. 7*E*). These data support the results of experiments in MIN6 cells showing the glucose dependence of the insulinotropic effect of Kv2.1 inhibition.

DISCUSSION

Dominant-negative functional knockout of Kv2 family channels enhances the insulin response of rat islets to glucose, likely the result of an ~60% reduction of voltage-dependent outward K^+ currents in β-cells (16). Drawbacks of the dominant-negative strategy include the inability to block specific Kv2 channel family members, inability to express the dominant-negative construct in all β-cells, and the unknown effects of a chronic down-regulation of channel activity. Therefore, acute inhibition of Kv2.1 would provide valuable new information. We have now extended our previous studies using a novel non-peptide compound (the bispidine derivative, C-1) related to class III antiarrhythmic agents such as tedisamil (35) that block delayed-rectifier K^+ channels in the heart (36), of which Kv2.1 is a major component (37).

Three lines of evidence suggest that the bispidine derivative C-1 antagonizes Kv2.1 selectively. First, MIN6 voltage-dependent outward K^+ currents were reduced by the dominant-negative Kv2.1N subunit, and further addition of C-1 had little effect. Although the Kv2.1N construct is expected to be a dominant-negative inhibitor of all Kv2 family channels, Kv2.1 pro-

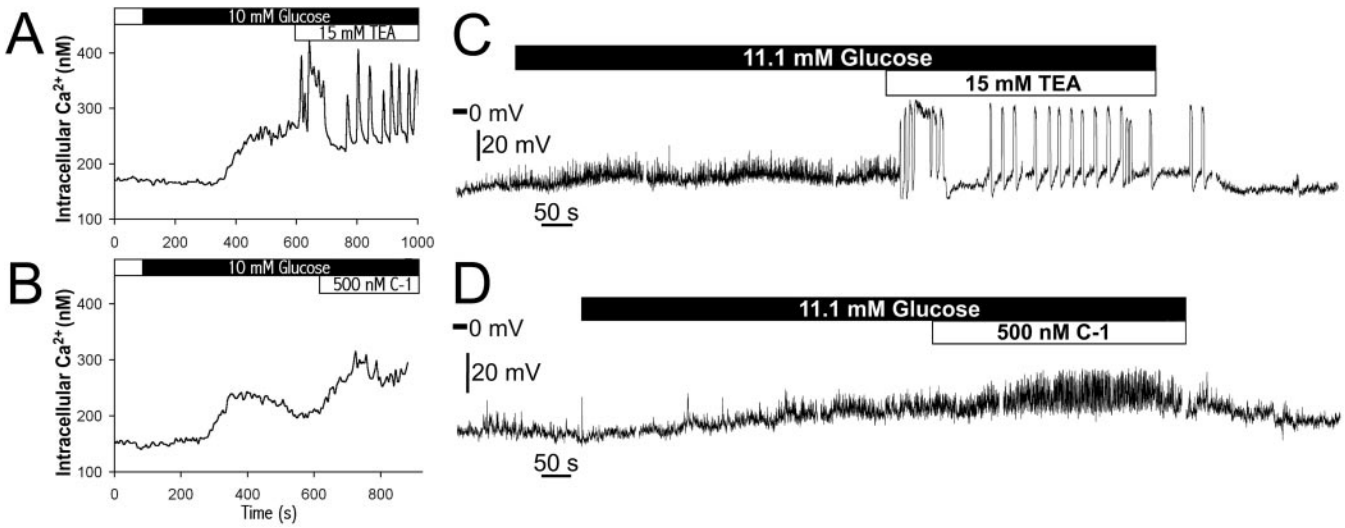

FIG. 5. **Kv2.1 inhibition enhances glucose-stimulated $[Ca^{2+}]_i$ and membrane potential responses.** Intracellular Ca^{2+} in MIN6 cells was monitored by Fura-2 fluorescence. In *panel A*, 15 mM TEA was applied in the presence of 10 mM glucose as indicated and further increased $[Ca^{2+}]_i$. Similarly, in *panel B*, inhibition of Kv2.1 channels (500 nM C-1) in the presence of 10 mM glucose also increased $[Ca^{2+}]_i$ responses. The above $[Ca^{2+}]_i$ traces are representative of 86 glucose-responsive cells treated with TEA and 240 glucose-responsive cells treated with C-1. Membrane potential was measured in MIN6 cells current-clamped in the perforated-patch configuration. In *panels C* and *D* membrane potential responses to TEA (15 mM) and C-1 (500 nM) in the presence of 11.1 mM glucose are shown. These traces are representative of six glucose-responsive cells each.

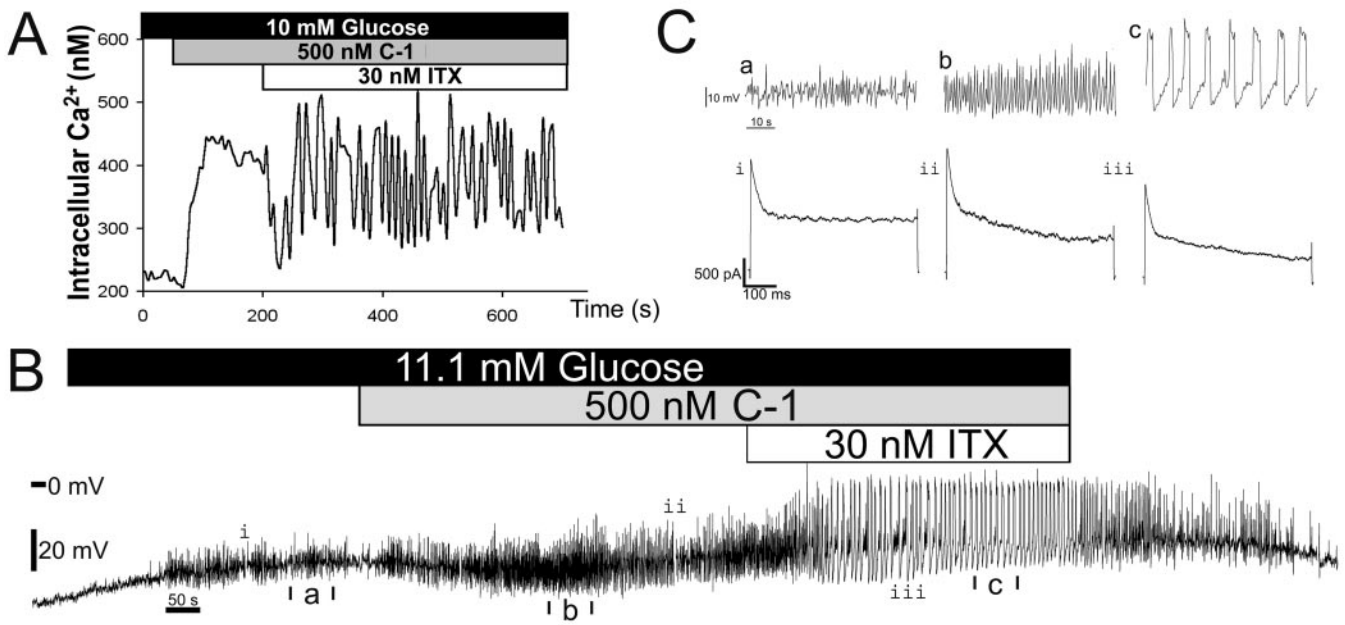

FIG. 6. **Inhibition of Kv2.1 and BK_{Ca} channels can replicate the oscillatory effects of TEA.** In *panel A*, the $[Ca^{2+}]_i$ response in MIN6 cells pre-treated with 10 mM glucose (20–25 min) was enhanced by C-1 (500 nM), and subsequent addition of the BK_{Ca} channel blocker ITX (30 nM) resulted in regular oscillations of $[Ca^{2+}]_i$. The trace shown is representative of the 40% (78 of 195 cells in 5 experiments) of C-1-responsive cells that responded to ITX with oscillations in $[Ca^{2+}]_i$. In *panel B*, the membrane potential response to glucose was enhanced by C-1 (500 nM), and subsequent addition of ITX (30 nM) further augmented the response and resulted in regular membrane potential oscillations. The trace shown is representative of the 40% of cells in which the membrane potential showed regular oscillations in response to C-1 and ITX. The segments denoted as *a*, *b*, and *c* are shown in an expanded time scale in *panel C*. Also in *panel C*, voltage-dependent outward K^+ currents were monitored during a brief switch into voltage-clamp mode at the time points marked *i*, *ii*, and *iii* in *panel B*. These currents result from the activation of both Kv and K_{Ca} channels since $[Ca^{2+}]_i$ was not chelated in this experiment.

tein is highly expressed and easily detectable in insulin-secreting cell lines and islets (16, 17). Kv2.2 on the other hand, the only other known Kv2 channel family member to produce functional channels, could not be detected at the mRNA level in islets by reverse transcription-PCR (16). In a second approach, C-1 blocked related channels (Kv1.4, 3.1, 4.2) expressed in tsA-201 or MIN6 cells with IC_{50} values that ranged from ~6- to 23-fold greater than blockade of Kv2.1 or endogenous MIN6 currents. Third, using the perforated patch clamp method without chelating $[Ca^{2+}]_i$ we recorded outward K^+ currents mediated by both Kv and K_{Ca} channels. C-1 alone blocked ~50% of these currents, whereas the BK_{Ca} channel antagonist ITX blocked an additional 20%, providing evidence that C-1 does not block BK_{Ca} channels.

Compounds related to C-1 are open channel blockers of voltage-dependent outward K^+ currents that increase the inactivation time constant of transient outward K^+ currents (38, 39). More potent effects of these compounds, however, have been reported on a slowly inactivating outward K^+ current, where tedisamil reduced the current amplitude (40). The only known peptide

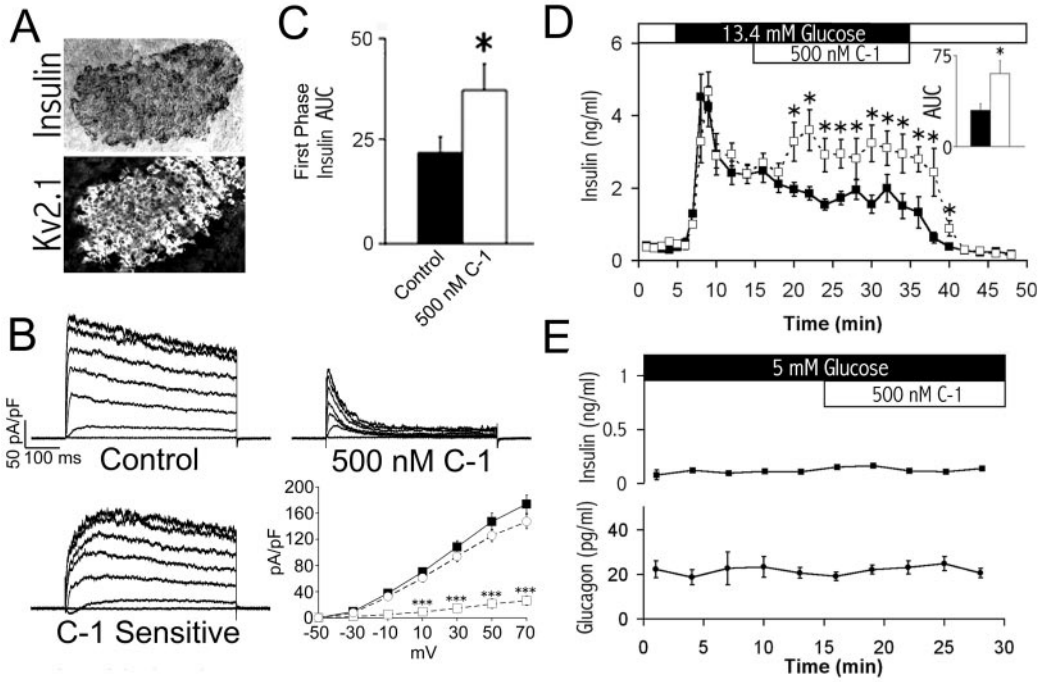

FIG. 7. **Inhibition of Kv2.1 enhances first- and second-phase insulin secretion from perfused mouse pancreas.** In *panel A*, Kv2.1 expression in mouse islets is demonstrated by immunohistochemistry. In *panel B*, voltage-dependent outward K^+ currents elicited from mouse β-cells voltage-clamped in the whole-cell configuration were largely blocked by the Kv2.1 antagonist C-1 (500 nM). Subtraction reveals the C-1-sensitive component (*bottom left panel*). Current-voltage relationships of the steady-state currents for the control (*black squares*), 500 nM C-1 (*white squares*), and subtracted (*white circles*) currents are shown (*bottom right panel*). pA/pF, picoamperes/picofarad. In *panel C*, Kv2.1 inhibition (*white*, $n = 6$) enhanced the first-phase peak of insulin secretion, defined as the AUC for the first 5 min of glucose (13.4 mM) stimulation compared with controls (black, $n = 6$). In *panel D*, inhibition of Kv2.1 channels (*white*, $n = 7$) also increased second-phase secretion compared with controls (*black*, $n = 6$). The AUC for the 16–35-min time period is shown in the *inset*. In *panel E*, Kv2.1 inhibition altered neither insulin ($n = 4$) nor glucagon ($n = 5$) secretion in the presence of a non-stimulatory concentration of glucose (5 mM). *, $p < 0.05$; ***, $p < 0.001$ compared with controls.

blocker of Kv2.1, hanatoxin, blocks channels by a mechanism involving a rightward shift in the voltage dependence of activation resulting from modification of channel gating rather than pore occlusion (41). In contrast to hanatoxin, C-1 does not shift the voltage dependence of activation but rather decreases the amplitude of the steady-state current at all depolarization voltages (Fig. 7*B* and not shown), possibly through an open channel mechanism,[2] while having no effect on the fast-inactivating K^+ currents. Because C-1 may act through an open channel mechanism, the C-1-sensitive currents shown by subtraction in Fig. 7*B* may underestimate the contribution of Kv2.1 during the first 50 ms of the depolarizing pulse. Accordingly, the current-voltage relationship shown was calculated from the steady-state currents.

Kv2.1 inhibition dose-dependently enhanced GSIS (200% at 2 μM C-1) and blocked voltage-dependent outward K^+ currents (80% at 1 μM C-1) in MIN6 cells, confirming previous results using Kv2.1N (16). Although the EC_{50} for the insulinotropic effect of C-1 is somewhat higher than the IC_{50} for endogenous current inhibition (550 *versus* 150 nM), it must be noted that the relationship between current block and insulin secretion is not necessarily linear. Importantly, glucose-stimulated membrane depolarization was necessary to allow the insulinotropic action of Kv2.1 inhibition, since the effect was prevented by the K_{ATP} channel agonist diazoxide. This is expected, because Kv2.1 would not be active in the absence of membrane depolarization.

Similar to previous studies (14, 17, 42), we observed an increase in glucose-stimulated membrane potential and $[Ca^{2+}]_i$ responses in MIN6 cells when repolarizing currents were blocked by TEA (Fig. 5*A*). Both of these parameters were also enhanced by inhibition of Kv2.1 channels with C-1 (Fig. 5*B*). The effect of C-1 on membrane potential and $[Ca^{2+}]_i$ could not be attributed to a direct effect of C-1 on K_{ATP} or Ca^{2+} channels. Diazoxide, a K_{ATP} channel opener, and verapamil, a commonly used antagonist of voltage-dependent Ca^{2+} channels, prevented the insulinotropic effect of Kv2.1 inhibition (Fig. 4), indicating that membrane depolarization and entry of extracellular Ca^{2+} is required for the C-1 insulinotropic effect.

The kinetics of the membrane potential and $[Ca^{2+}]_i$ responses to TEA and C-1 were markedly different. In most responding cells TEA elicited oscillatory spikes, whereas Kv2.1 inhibition had non-oscillatory effects on both membrane potential and $[Ca^{2+}]_i$ (Fig. 5). TEA blocks numerous Kv channels other than Kv2.1 that may be expressed in insulin-secreting cells (5, 11, 16, 17, 32–34). Additionally, TEA blocks K_{Ca} currents, which are present in insulin-secreting cells (33, 34, 43–48) and can partially block K_{ATP} channels (34, 49). The ability of C-1 and ITX together to replicate the oscillatory effects of TEA in a portion of cells strongly suggests that the response to TEA results in part from blockade of both Kv2.1 and BK_{Ca} channels. Additional effects of TEA on other K^+ channels cannot be completely ruled out since, compared with the effect of TEA, a lower proportion of cells responded with oscillations to C-1 and ITX, and the oscillations were often of higher frequency.

In the present study we show that Kv2.1 channels are expressed in a pattern consistent with insulin-containing β-cells of mouse pancreas (Fig. 7*A*). Importantly, and similar to results obtained in MIN6 cells, the Kv2.1 antagonist C-1 blocks voltage-dependent outward K^+ currents in primary mouse β-cells. Inhibition of Kv2.1 channels increased both the first-phase peak and second phase of insulin secretion from perfused mouse pancreas. Similar to MIN6 cells, Kv2.1 inhibition did not affect basal insulin secretion. These results confirm a role for Kv2.1 in regulating whole pancreas insulin secretion and indicate

that the channel is active during both the first and second phases of secretion. Immunohistochemical analysis does not rule out Kv2.1 expression in glucagon-secreting α-cells. However, the inability of Kv2.1 antagonism to alter glucagon secretion suggests little role for this channel in α-cells under the present conditions (5 mM glucose), designed to be neither maximally stimulatory nor inhibitory to allow detection of either an increase or decrease in glucagon secretion.

Here we have shown that a bispidine derivative, C-1, specifically blocks Kv2.1 currents in insulin-secreting cells, leading to enhanced insulin secretion in the presence of glucose. The present data support the hypothesis that, distal to K_{ATP} channel closure and membrane depolarization induced by glucose, opening of Kv2.1 channels repolarizes β-cell action potentials, limiting Ca^{2+} entry and, thus, limiting insulin secretion. Compounds that enhance but do not initiate β-cell electrical activity by acting on Kv2.1 augment only glucose-stimulated (*i.e.* postprandial) insulin secretion and, therefore, are potentially useful antidiabetic agents. Because its activity is dependent upon glucose-stimulated membrane depolarization, Kv2.1 is a potential candidate for the development of such agents. However, because Kv2.1 is expressed in a number of extrapancreatic tissues, the issue of tissue specificity of any potential therapeutic agent must be addressed. It is possible that investigation of the tissue-specific processing and regulatory interactions of Kv2.1 may allow the development of antagonists with sufficient tissue specificity for the treatment of type-2 diabetes mellitus.

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Inhibition of Kv2.1 Voltage-dependent K^+Channels in Pancreatic β-Cells Enhances Glucose-dependent Insulin Secretion

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