Direct Activation of β-Cell KATP Channels with a Novel Xanthine Derivative

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ABSTRACT

ATP-regulated potassium (KATP) channel complexes of inward rectifier potassium channel (Kir) 6.2 and sulfonylurea receptor (SUR) 1 critically regulate pancreatic islet β-cell membrane potential, calcium influx, and insulin secretion, and consequently, represent important drug targets for metabolic disorders of glucose homeostasis. The KATP Channel opener diazoxide is used clinically to treat intractable hypoglycemia caused by excessive insulin secretion, but its use is limited by off-target effects due to lack of potency and selectivity. Some progress has been made in developing improved Kir6.2/SUR1 agonists from existing chemical scaffolds and compound screening, but there are surprisingly few distinct chemotypes that are specific for SUR1-containing KATP channels. Here we report the serendipitous discovery in a high-throughput screen of a novel activator of Kir6.2/SUR1: VU0071063 (7-(4-(tert-butyl)benzyl)-1,3-dimethyl-1H-purine-2,6(3H,7H)-dione). The xanthine derivative rapidly and dose-dependently activates Kir6.2/SUR1 with a half-effective concentration (EC50) of approximately 7 μM, is more efficacious than diazoxide at low micromolar concentrations, directly activates the channel in excised membrane patches, and is selective for SUR1- over SUR2A-containing Kir6.1 or Kir6.2 channels, as well as Kir2.1, Kir2.2, Kir2.3, Kir3.1/3.2, and voltage-gated potassium channel 2.1. Finally, we show that VU0071063 activates native Kir6.2/SUR1 channels, thereby inhibiting glucose-stimulated calcium entry in isolated mouse pancreatic β-cells. VU0071063 represents a novel tool/compound for investigating β-cell physiology, KATP channel gating, and a new chemical scaffold for developing improved activators with medicinal chemistry.

Introduction

By integrating cellular metabolism, membrane potential (Vm), and excitability, ATP-sensitive K+ (KATP) channels carry out fundamental roles in nerve, muscle, epithelial, and endocrine tissue physiology (Ashcroft, 1988). KATP channels are octomeric complexes of four pore-forming inward rectifier K+ (Kir) 6.x channel subunits and four regulatory sulfonylurea receptor (SUR) x subunits (Nichols et al., 2006). Kir6.1 (KCNJ8), Kir6.2 (KCNJ11), and SUR1 (ATP binding cassette [ABC] C8) are encoded by different genes; SUR2A and SUR2B (ABCC9) are splice variants of the same gene. The three major channel subtypes created by different subunit combinations exhibit distinctive biophysical, regulatory, and pharmacological properties, as well as cell type—specific expression (Flagg et al., 2010; Hibino et al., 2010; Nichols et al., 2013).

Pancreatic islet β-cell KATP channels are validated drug targets for type 2 diabetes and severe hypoglycemia resulting from excessive insulin secretion (Denton and Jacobson, 2012). Increases in blood glucose induce MgATP-dependent Kir6.2/SUR1 channel inhibition, Vm depolarization, Ca2+ influx through L-type voltage-dependent Ca2+ channels (VDCC), and secretion of insulin, which in turn acts on a myriad of target tissues to promote glucose uptake and utilization (Ashcroft, 2007). Sulfonylurea drugs (e.g., glibenclamide, tolbutamide) that directly inhibit Kir6.2/SUR1 and stimulate insulin secretion are used clinically to help manage glycemic levels in type 2 diabetic patients. In contrast, KATP channel activators (e.g., diazoxide) are used to treat disorders

ABBREVIATIONS: 95% CI, 95% confidence intervals; ABC, ATP binding cassette; [Ca2+]i, intracellular calcium concentration; CRC, concentration-response curve; DMSO, dimethyl sulfoxide; HEK, human embryonic kidney; KATP, ATP-sensitive potassium channel; Kir, inward rectifier potassium channel; K+, voltage-gated potassium channel; PDE, phosphodiesterase; SUR, sulfonylurea receptor; T1+, thallium; VDCC, L-type voltage-dependent calcium channels; Vm, membrane potential; VU0071063, 7-(4-(tert-butyl)benzyl)-1,3-dimethyl-1H-purine-2,6(3H,7H)-dione.
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of severe hypoglycemia, such as congenital hyperinsulinism and insulin-producing pancreatic tumors (Aschner, 2007; Nichols et al., 2007). The major K<sub>ATP</sub> channel subtype in cardiomyocytes consists of K<sub>6.6</sub> and SUR2A (Nichols et al., 2013), and potassium channel opener activation of sarcolemmal K<sub>ATP</sub> channels and V<sub>m</sub> hyperpolarization afford cardioprotection from subsequent ischemia-reperfusion injury (Grove and Garlid, 2000). Activation of vascular smooth muscle K<sub>6.1</sub>/SUR2B with pinacidil or diazoxide leads to vasodilatation and a reduction in blood pressure (Flagg et al., 2010), but can also result in pathologic edema and other cardiovascular pathologies reminiscent of those found in Cantu syndrome, which results from gain-of-function mutations in SUR2 (Nichols et al., 2013).

Given the broad tissue distribution of K<sub>ATP</sub> channels, their important physiologic roles, and therapeutic as well as pathologic potential in various conditions, there is considerable interest in the continued development of pharmacological modulators for targeting specific subtypes of K<sub>ATP</sub> channels (Hansen, 2006). Here, we report the serendipitous discovery of a novel xanthine derivative that directly activates heterologously expressed K<sub>6.2</sub>/SUR1 channels and native pancreatic β-cell K<sub>ATP</sub> channels. The activator, VU0071063 (7-(4-(tert-butyl)benzyl)-1,3-dimethyl-1H-purine-2,6(3H,7H)-dione), is more potent and efficacious than diazoxide and is described below. One cell line exhibiting robust Tl-dependent cardiomycocytes consists of Kir6.2 and SUR2A (Nichols et al., 2007), and Western blot analysis (Supplemental Fig. 1), the cells were cotransfected with plasmids expressing Kir2.2 (NM_021012), and pcDNA5/TO-Kir2.3 (NM_152868).

Materials and Methods

Expression Vectors. The following vectors were used in this study: pcDNA5/TO-K<sub>6.2</sub> (NM_010602), pcDNA5/TO-K<sub>6.1</sub> (NM_004982), pcDNA3.1-SUR1 (L40623.1), pCMV6c-SUR2A (D83598.1), pcDNA3.1-SUR2B (D66038.2), pcDNA5/TO-K<sub>2.1</sub> (NM_000891.2), pcDNA5/TO-K<sub>2.2</sub> (NM_021012), and pcDNA5/TO-K<sub>2.3</sub> (NM_152868).

Cell Lines and Transfections. T-REx-human embryonic kidney (HEK) 293 cells were transfected with pcDNA5/TO-K<sub>6.2</sub> using Lipofectamine 2000 (Life Technologies, Carlsbad, CA) and cultured with blasticidin and hygromycin to select stably transfected cells as previously described (Lewis et al., 2009; Raphemot et al., 2011). After confirming they exhibited tetracycline-inducible K<sub>6.2</sub> expression by Western blot analysis (Supplemental Fig. 1), the cells were cotransfected with pcDNA3.1-SUR1 and grown in G418-containing medium to select cells carrying stably integrated plasmids for both K<sub>ATP</sub> channel subunits. Monoclonal lines were isolated by limiting dilution, expanded, and tested for tetracycline-inducible thallium (Tl<sup>+</sup>) flux, as described below. One cell line exhibiting robust Tl<sup>+</sup> flux was selected for assay development and small-molecule screening. Monoclonal T-REx-HEK293 cell lines expressing other mammalian K<sub>6</sub> channels were generated as described previously (Lewis et al., 2009; Raphemot et al., 2011). Monoclonal metabolic glutamate receptor 8G protein–coupled inward rectifier K<sup>+</sup> channel/HEK293 cells stably expressing K<sub>6.3.1.3.2</sub>, the M4 muscarinic receptor, and rat mGlurA were cultured as described previously (Niswender et al., 2008). For transient transfections, HEK293T cells were transfected with 1 μg of K<sub>6.6</sub>X, 2 μg SURX, and 0.5 μg of pcDNA3.1-EGFP (transfection marker) using Lipofectamine LTX Plus according to the manufacturer’s instructions.

Western Blot Analysis. Western blot analysis of K<sub>6.2</sub> expression was performed following 24-hour induction with tetracycline essentially as described previously (Lewis et al., 2009). Goat polyclonal K<sub>6.2</sub> antiserum (SC-11226) and donkey anti-goat horseradish peroxidase–conjugated antisemur (SC-2020) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Tl<sup>+</sup> Flux Assays. Tl<sup>+</sup> flux assays were performed essentially as described previously (Raphemot et al., 2013). Briefly, stably transfected T-REx-HEK-293 cells expressing K<sub>6.2</sub>/SUR1 channels were cultured overnight in 384-well plates (20,000 cells/20 μl per well black-walled, clear-bottomed PureCoat amine-coated plates; BD, Bedford, MA) with a platting media containing Dulbecco’s modified Eagle’s medium, 10% dialyzed fetal bovine serum, and 1 μg/ml tetracycline. On the day of the experiment, the cell culture medium was replaced with dye-loading solution containing assay buffer (Hanks’ balanced salt solution with 20 μM HEPES, pH 7.3), 0.01% (w/v) Phorbol F-127 (Life Technologies), and 1.2 μM of the thallium-sensitive dye Thallos-AM (TEFlabs, Austin, TX). Following 1-hour incubation at room temperature, the dye-loading solution was washed from the plates and replaced with 20 μlwell of assay buffer. The plates were transferred to a Hamamatsu Functional Drug Screening System 6000 (FDS56000; Hamamatsu, Tokyo, Japan) and 20 μl/well of test compounds in assay buffer (as prepared below) was added. After a 20-minute incubation period, a baseline recording was collected at 1 Hz for 10 seconds (excitation 470 ± 20 nm, emission 540 ± 30 nm) followed by addition of the Tl<sup>+</sup> stimulus buffer (20 μl/well) and data collection for an additional 4 minutes. The Tl<sup>+</sup> stimulus buffer contains in (mM) 125 NaHCO<sub>3</sub>, 1.8 CaSO<sub>4</sub>, 1 MgSO<sub>4</sub>, 5 glucose, 1.8 TlCl<sub>4</sub>, and 10 HEPES, pH 7.4. For the Tl<sup>+</sup> flux assay on K<sub>3.1.3.2</sub>-expressing cells, the thallium stimulus buffer contains 12 mM TlCl<sub>4</sub> and either an EC<sub>20</sub> or EC<sub>50</sub> of glutamate (Sigma-Aldrich, St. Louis, MO).

Test compounds from the library of Vanderbilt Institute of Chemical Biology were dispensed into in polypropylene 384-well plates (Greiner Bio-One, Monroe, NC) using an Echo555 liquid handler (Labcyte, Sunnyvale, CA) diluted in assay buffer to 2× final concentrations to generate 4-11 point 5-fold serial dilution series. The K<sub>ATP</sub> channel inhibitors glibenclamide and tolbutamide were resuspended in assay buffer containing VU0071063 or diazoxide. Tl<sup>+</sup> flux assays on K<sub>6.2</sub>, K<sub>2.2</sub>, K<sub>2.3</sub>, and K<sub>3.1.3.2</sub> were performed as described previously (Raphemot et al., 2011).

Tl<sup>+</sup> flux data were analyzed as previously described (Raphemot et al., 2013) using a combination of Excel (Microsoft Corp, Redmond, WA) with XLfit add-in (IBDS, Guildford, Surrey, UK) and OriginPro (OriginLab, Northampton, MA) software. Each data point in a given trace was divided by the first data point from that trace (static ratio) followed by subtraction of data points from control traces generated in presence of vehicle controls. The slope of the fluorescence increase beginning 5 seconds after Tl<sup>+</sup> addition and ending 15 seconds after Tl<sup>+</sup> addition was calculated. The data were then plotted in Prism software (GraphPad Software, San Diego, CA) to generate concentration–response curves (CRCs). Potencies were calculated from fits to CRC data using a four-parameter logistic equation.

Patch-Clamp Electrophysiology. Transfected cells were dissociated with trypsin, plated on poly(L-lysine)-coated glass coverslips, and allowed to recover for at least 1 hour before experiments. Coverslips were transferred to a small-volume perfusion chamber and mounted on the stage of an inverted microscope. Patch electrodes were pulled from 1.5-mm outer diameter glass capillaries and had resistances ranging from 3–5 MΩ when filled with the following intracellular solution (in mM): 135 KCl, 2 MgCl<sub>2</sub>, 1 EGTA, 10 HEPES, and 2 Na<sub>2</sub>ATP, pH 7.3. The standard bath solution contained (in mM): 135 NaCl, 5 KCl, 2 CaCl<sub>2</sub>, 1 MgCl<sub>2</sub>, 5 glucose, and 10 HEPES, pH 7.4. Whole-cell currents were recorded under voltage-clamp conditions using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). The cells were voltage-clamped and stepped every 5 seconds from a holding potential of −75 to 120 mV for 200 milliseconds, and then ramped at a rate of 2.4 mV/ms from −75 to 120 mV before returning to −75 mV. Electrophysiologic data were collected at 5 kHz and filtered at 2 kHz. Data acquisition and analysis were performed using pClamp 9.2 software (Molecular Devices).
For excised patch-clamp measurements, COSm6 cells were transiently transfected with K\textsubscript{\text{r}},6.2, SUR1, and EGFP for 24 hours before patch-clamp analysis. Transfected cells were identified by green fluorescent protein fluorescence and membrane patches were voltage-clamped. The pipette (resistance 1–2.5 MΩ) and bath solutions were (in mM): 140 KCl, 10 HEPES, and 0.5 free Mg\textsuperscript{2+}, pH 7.35. After sealing, the membrane patch was excised to the inside-out configuration. Currents were recorded at a membrane potential of −50 mV using pClamp 8.2 software.

**Calciuim Imaging.** Islet cell intracellular calcium ([Ca\textsuperscript{2+}]\textsubscript{i}) was measured using Ca\textsuperscript{2+} sensitive dye fura-2 (Life Technologies) as previously described (Jacobson et al., 2007). Briefly, mouse islets were dissociated in 0.005% trypsin, plated on glass coverslips, and cultured for 16 hours in RPMI 1640 medium supplemented with 10% fetal calf serum, concentrations of glucose specified, 100 IU ml\textsuperscript{-1} penicillin, and 100 mg ml\textsuperscript{-1} streptomycin. Cells were dye-loaded for 20 minutes at 37°C with 2 μM fura-2-AM in solution containing (in mM): 119 NaCl, 2.5 CaCl\textsubscript{2}·(H\textsubscript{2}O)\textsubscript{6}, 4.7 KCl, 10 HEPES, 1.2 MgSO\textsubscript{4}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, and 2 glucose, pH 7.35. Fluorescence imaging was performed using a Nikon Eclipse TE2000-U microscope equipped with an epifluorescent illuminator (Sutter instruments, Novato, CA), a CoolSNAP HQ2 camera (Photometrics, Tucson, AZ), and Nikon Elements software (Nikon, Tokyo, Japan). The [Ca\textsuperscript{2+}]\textsubscript{i} ratios of emitted fluorescence intensities at excitation wavelengths of 340 and 380 nm (F340/F380) were determined every 5 seconds with background subtraction. Cells were perfused at 37°C at a flow of 2 ml/min; the solutions used during the experiments were the loading solution with various glucose concentrations and VU0071063, as indicated.

**Measurement of Complex 2 Activity.** Mitochondria were isolated from four mouse hearts using differential centrifugation in sucrose-based buffer as previously described (Wojtovich and Brookes, 2008; Wojtovich et al., 2011). Complex 2 enzymatic activity was measured spectrophotometrically at 600 nm as previously described (Wojtovich and Brookes, 2008; Wojtovich and Brookes, 2009).

**Chemicals.** VU0071063 was purchased from AldrichCPR (Sigma-Aldrich, LLC, Milwaukee, WI). Diazoxide, glibenclamide, and tolbutamide were purchased from Sigma-Aldrich. All Compounds were dissolved in anhydrous dimethyl sulfoxide (DMSO) (Fisher Scientific, Pittsburgh, PA) and diluted in bath solution before use. The final concentration of DMSO used was less than or equal to 0.3% (v/v).

**Results**

**Serenidipitous Discovery of the K\textsubscript{\text{r}},6.2/SUR1 Activator VU0071063.** A T\textsuperscript{1+} flux assay of K\textsubscript{\text{r}},6.2/SUR1 K\textsubscript{ATP} channels was developed to assess the specificity of inhibitors of a mosquito K\textsubscript{r} channel identified in a high-throughput screen (R. Raphemot and J. S. Denton, unpublished data). The assays employ stably transfected T-REx-HEK293 cells expressing K\textsubscript{\text{r}},6.2 from a tetracycline-inducible promoter and SUR1 constitutively (Supplemental Fig. 1). K\textsubscript{\text{r}},6.2/SUR1 is inhibited in T-REx-HEK293 cells under control conditions and must be activated by metabolic poisoning (data not shown) or the SUR1-prefering K\textsubscript{ATP} channel opener diazoxide to mediate T\textsuperscript{1+} flux. While testing approximately 300 mosquito K\textsubscript{r},1 antagonists for selectivity, diazoxide was inadvertently excluded from one plate, revealing a dose-dependent increase in T\textsuperscript{1+} flux in wells 13, 14, 15, and 16 containing 30, 10, 3, and 1 μM of a mosquito K\textsubscript{r},1 antagonist, respectively (Fig. 1, B and C). The small-molecule added to these wells, which we termed VU0071063, was reordered as a powder, freshly dissolved in DMSO, and characterized in T\textsuperscript{1+} flux and electrophysiologic assays.

**Effects of VU0071063 on Pancreatic K\textsubscript{\text{r}},6.2/SUR1 K\textsubscript{ATP} Channels.** The activity of VU0071063 on K\textsubscript{\text{r}},6.2/SUR1 was compared with diazoxide and the SUR2-prefering opener pinacidil in 11-point CRCs in T\textsuperscript{1+} flux assays. The chemical structures of the three agonists are illustrated in Fig. 2A. Tetracycline-induced T-REx-HEK293-K\textsubscript{\text{r}},6.2/SUR1 cells were treated with the agonists for 20 minutes prior to T\textsuperscript{1+} addition to allow full activation of the channel. As shown in the representative fluorescence traces in Fig. 2B, 30 μM VU0071063 led to a slightly greater steady-state activation of K\textsubscript{\text{r}},6.2/SUR1-dependent T\textsuperscript{1+} flux than did 250 μM diazoxide. VU0071063 and diazoxide led to a dose-dependent activation of T\textsuperscript{1+} flux, whereas pinacidil was predictably inactive against K\textsubscript{\text{r}},6.2/SUR1 (Fig. 2C). Half-maximal effective concentrations (EC\textsubscript{50}) derived from logistical fits to CRC data for VU0071063 and diazoxide were 10.3 μM (95% confidence intervals [95% CI]: 9.5–11 μM) and greater than 100 μM (EC\textsubscript{50} ~120 μM), respectively (n = 4–6 independent experiments). To confirm that T\textsuperscript{1+} flux is dependent on K\textsubscript{\text{r}},6.2/SUR1 channels and not endogenous T\textsuperscript{1+} flux pathways, the dose-dependent effects of K\textsubscript{ATP} channel inhibitors glibenclamide and tolbutamide were evaluated. The cells were pretreated with EC\textsubscript{50} doses of VU0071063 (20 μM) or diazoxide (250 μM) and 3-fold dilutions of glibenclamide or tolbutamide ranging from 2 nM to 90 μM. Similar to
published half-maximal inhibitory concentration values, the IC50 values for glibenclamide and tolbutamide in VU0071063-treated cells were 5.60 nM (95% CI: 5–6 nM) and 3.07 mM (95% CI: 2–5 mM), respectively. These values are similar to those of diazoxide-treated cells (glibenclamide IC50 = 16.6 nM [95% CI: 15–18 nM]; tolbutamide IC50 = 1.60 μM [95% CI: 1.4–2 μM]).

Whole-cell patch-clamp electrophysiology was used to further characterize the effects of VU0071063 and diazoxide on Kε.6.2/SUR1. Bath application of VU0071063 rapidly (Fig. 3A) and dose-dependently (Fig. 3C) activated Kir6.2/SUR1 currents, with a maximal activation of 1,077 ± 87% at a dose of 50 μM. In contrast, diazoxide activated Kir6.2/SUR1 more slowly (Fig. 3B) and with significantly (t test P = 0.01) lower
efficacy (maximal activation 580 ± 105% at 50 μM) than VU0071063. As shown in Fig. 3B, following steady-state activation with 50 μM diazoxide, bath addition of 50 μM VU0071063 led to further Kₖ,6.2/SUR1 activation. These data show that at low micromolar concentrations, VU0071063 is a more potent activator of Kₖ,6.2/SUR1 than diazoxide.

To exclude the possibility that VU0071063 might be activating channels in intact cells by altering cell metabolism, currents were recorded from COSm6 cells expressing Kₖ,6.2 and SUR1, in inside-out membrane patches. In the presence of 0.1 mM MgATP, which inhibits wild-type channels ~90%, both 10 and 20 μM of VU0071063 markedly increased the patch current (Supplemental Fig. 2).

VU0071063 Is Selective SUR1-Containing K_ATP Channels. The pharmacological selectivity of known K_ATP channel agonists is achieved through interactions with the SUR subunit. To determine if VU0071063 activity is also dependent on the SUR, we tested its effects on Kₖ,6.2 or Kₖ,6.1 channels containing SUR1 or SUR2A using patch-clamp electrophysiology. Diazoxide and pinacidil were used as positive controls for SUR1- and SUR2A-containing channels, respectively. As shown in the representative time-course experiment in Fig. 4A, and summary data (mean ± S.E.M.; n = 7) in Fig. 4C, bath application of 50 μM VU0071063 rapidly and reversibly activated Kₖ,6.2/SUR1 to a greater extent than an equal concentration of diazoxide. Qualitatively similar results were observed in cells transfected with Kₖ,6.1/SUR1 (Fig. 4D). In striking contrast, VU0071063 had no effect on Kₖ,6.2/SUR2A (Fig. 4, B and E) or Kₖ,6.1/SUR2A (Fig. 4F), whereas pinacidil activated both channel subtypes. Dose-response experiments revealed that VU0071063 had no appreciable effects on Kₖ,6.2/SUR2A at concentrations up to 150 μM (Supplemental Fig. 3), which is 15-fold higher than the IC₅₀ for Kₖ,6.2/SUR1.

VU0071063 Inhibits Glucose-Stimulated β-Cell Ca²⁺ Influx. Glucose-stimulated closure of β-cell K_ATP channels results in membrane potential depolarization, activation of voltage-dependent Ca²⁺ channels (VDCC), Ca²⁺ influx, and Ca²⁺-induced insulin secretion. We therefore tested whether VU0071063 activates native Kₖ,6.2/SUR1 channels by measuring the effect of the activator on β-cell Ca²⁺ influx during glucose-stimulation. Treatment of β cells with high (14 mM) glucose induced a significant rise in Ca²⁺ as determined by the fluorescent Ca²⁺ indicator fura-2, which shows an

**Fig. 4.** VU0071063 is selective for SUR1-containing K_ATP channels. (A) Representative whole-cell patch-clamp experiment showing the effects of 50 μM VU0071063, 50 μM diazoxide, and 2 mM Ba²⁺ on Kₖ,6.2/SUR1 current at −120 mV. Note the differences in the kinetics of activation of VU0071063 and diazoxide. (B) Representative recording showing effects of 50 μM VU0071063, 50 μM pinacidil, and 2 mM Ba²⁺ on Kₖ,6.2/SUR2A currents. (C–F) Mean ± S.E.M. current at −120 mV recorded from cells transfected with Kₖ,6.2/SUR1, Kₖ,6.1/SUR1, Kₖ,6.2/SUR2A, or Kₖ,6.1, SUR2A, respectively (n = 4–7).
increase in the fluorescent ratio of Ca\textsuperscript{2+} bound to Ca\textsuperscript{2+}-
unbound dye in response to glucose (red cells, Fig. 5). Activation of β-cell K\textsubscript{ATP} channels with VU0071063 in the presence of high (14 mM) glucose resulted in inhibition of β-cell Ca\textsuperscript{2+} influx and reduction in Ca\textsuperscript{2+} levels back to those observed in low (2 mM) glucose conditions (green cells, Fig. 5). The reduction in Ca\textsuperscript{2+} influx mediated via K\textsubscript{ATP} activation is reversible following removal of VU0071063, which results in a return of β-cell Ca\textsuperscript{2+} levels to high (14 mM) glucose levels (red cells, Fig. 5). These data indicate that VU0071063 activates native β-cell K\textsubscript{ATP} channels and thereby reduces VDCC activation and Ca\textsuperscript{2+} influx.

K\textsubscript{6.2}/SUR1 Activation by VU0071063 Is Not Mediated by a Phosphodiesterase Inhibitory Pathway. Vascular smooth muscle K\textsubscript{ATP} channels are activated by cAMP/PKA- and cGMP/PKG-dependent pathways following phosphodiesterase (PDE) inhibition with theophylline (see Discussion). Because VU0071063 contains a theophylline moiety (Supplemental Fig. 4A), we tested whether theophylline could activate K\textsubscript{6.2}/SUR1 in Ti\textsuperscript{1+} flux assays under conditions identical to those used to discover VU0071063. However, as shown in Supplemental Fig. 4B, theophylline at a concentration of 250 μM had no effect on K\textsubscript{6.2}/SUR1-dependent Ti\textsuperscript{1+} flux. Ancillary Pharmacology. The selectivity of VU0071063 was evaluated in 11-point CRCs in Ti\textsuperscript{1+} flux assays against K\textsubscript{g,2.1}, K\textsubscript{g,2.2}, K\textsubscript{g,2.3}, and K\textsubscript{g,3.1/3.2}. VU0071063 was inactive against K\textsubscript{g,2.1} and K\textsubscript{g,2.2} (IC\textsubscript{50} > 100 μM) and showed weak inhibitor activity against K\textsubscript{g,3.1/3.2} (IC\textsubscript{50} = 65 μM) and K\textsubscript{g,2.3} (IC\textsubscript{50} = 91 μM) (Supplemental Fig. 5). Patch-clamp electrophysiology was used to determine whether VU0071063 acts on the voltage-gated K\textsuperscript{+} channel (K\textsubscript{g})\textsubscript{2.1}, which contributes to action repolarization in pancreatic β cells (Philipson et al., 1994; Roe et al., 1996). Cells were voltage-clamped at a holding potential of −75 mV and stepped to +50 mV every 5 seconds. Bath application of 10 μM VU0071063 led to a 7.8 ± 0.9% (n = 4) reduction in outward K\textsubscript{,2.1} current at 40 mV that was fully reversible (Supplemental Fig. 6).

Discussion

Pancreatic K\textsubscript{ATP} channels are validated drug targets for intractable hypoglycemia due to insulinoma and congenital hyperinsulinism, and therefore considerable efforts have been made to develop specific activators of K\textsubscript{6.2}/SUR1 channels (Hansen, 2006; Pirotte et al., 2010; de Tullio et al., 2011). Diazoxide is the best-known SUR1-prefering opener and has been used clinically for more than 50 years. However, its use has been limited by a lack of potency and selectivity, leading to undesirable side effects, such as low blood pressure, blurred vision, reduced urination, fluid retention, and hirsutism, mimicking the effects of Cantu syndrome, which results from gain-of-function in the cardiovascular SUR2 isoform (Nichols et al., 2013), and reflecting enhanced opening of vascular smooth muscle K\textsubscript{ATP} channels and potentially effects on mitochondrial respiration (Coetzee, 2013). In an effort to develop openers with fewer side effects, several groups have synthesized analogs from existing lead compounds that show improved potency and selectivity toward K\textsubscript{6.2}/SUR1. Structural modifications to the diazoxide scaffold have led to several new series with submicromolar potency and selectivity for pancreatic over smooth muscle K\textsubscript{ATP} channels (Pirotte et al., 2010; de Tullio et al., 2011). One analog, termed NN414 (Dabrowski et al., 2003), shows favorable activity in obese rats (Carr et al., 2003; Alemzadeh et al., 2004), as well as healthy and type 2 diabetes patients (Zdravkovic et al., 2005, 2007). Clinical trials were initiated but later suspended due to drug-induced elevations of key liver enzymes (Hansen, 2006). Analogs of the SUR2-prefering openers cromakalim and pinacidil that exhibit selectivity for pancreatic K\textsubscript{ATP} channels (Khelili et al., 2006, 2008; Sebille et al., 2006, 2008; Florence et al., 2009, 2011) have also been developed, showing that it is possible to switch SUR preference with chemical modifications to the scaffold. To our knowledge, the only unique pancreatic K\textsubscript{ATP} channel activator chemotypes reported in the last 2 decades were identified in screens of small-molecule libraries. These include the 4-sulfamoylphenylbenzamide and nitropryazole series of K\textsubscript{ATP} activators. A 4-sulfamoylphenylbenzamide derivative was shown to activate heterologously expressed K\textsubscript{g,6.2}/SUR1 channels and inhibit glucose-stimulated insulin secretion from primary rat islets with submicromolar efficacy; the activity toward SUR2-containing channels was not reported (Nielsen et al., 2004). One nitropryazole analog exhibits nanomolar-affinity toward K\textsubscript{g,6.2}/SUR1 and at least 15-fold selectivity over SUR2A and SUR2B-containing channels (Peat et al., 2004). No in vivo efficacy of either series has been published.

To our knowledge, VU0071063 is only the third publicly disclosed SUR1-prefering chemotype identified with compound screening and therefore provides an important starting point for the development of new channel openers. The discovery of VU0071063 underscores the value of mining focused libraries from primary screens for modulators of diverse inward rectifier K\textsuperscript{+} channels. VU0071063 is slightly
more potent than diazoxide, and activates the channel with a faster time-course. The reason for the discrepancy in IC_{50} values derived from Ti^{+} flux and patch-clamp experiments is unclear, but clearly reflects 1) differences in the behavior of Ti^{+} and K^{+} in the K_ATP channel pore, and 2) the slower kinetics of diazoxide action compared with that of VU0071063 (e.g., Fig. 4). In an effort to avoid the latter issue, T-REx-HEK293-Kir6.2/SUR1 cells were incubated with compounds for 20 minutes before adding Ti^{+} stimulus buffer; however, we still observed a rightward shift in the EC_{50} value for diazoxide.

VU0071063 is selective for K_{b}.6.2/SUR1 over K_{b}.6.2/SUR2A and K_{b}.6.1/SUR2A, as well as Kir3.1/3.2, Kir2.1, Kir2.2, Kir2.3, and Kir2.1. Kir2.1 was tested for VU0071063 sensitivity because it plays important roles in the electrophysiology of pancreatic β cells by modulating action potential repolarization, Ca^{2+} influx through VDCC, and insulin secretion. At the same dose shown to reduce high glucose-induced Ca^{2+} influx in pancreatic β cells, 10 μM VU0071063 significantly inhibited Kir2.1 currents by approximately 10%. Inhibition of Kir2.1 would be expected to prolong the action potential depolarization and increase, not decrease, Ca^{2+} influx through VDCC, excluding a potential role of Kir2.1 in the VU0071063 mechanism of action. Specific effects of VU0071063 on the electrical excitability and underlying ion channels in β cells will be examined in future studies. Furthermore, the selectivity of VU0071063 suggests the binding site is located within SUR1, which belongs to the ABC superfamily of transporters. Considering that the ABC transporter family member cystic fibrosis transmembrane conductance regulator is inhibited at high concentrations (IC_{50} = 250 μM; Sheppard and Welsh, 1992) of diazoxide, future studies should address whether VU0071063 inhibits cystic fibrosis transmembrane conductance regulator or other members of this superfamily. Finally, although VU0071063 does not activate SUR2A-containing channels, potential effects on SUR2B-containing channels and hence vascular smooth muscle tone should be considered before using VU0071063 as an in vivo probe of SUR1-containing K_ATP channels. Despite several attempts and different experimental conditions, we were unable to measure the activity of SUR2B-containing K_ATP channels in our expression system and could not determine the effect of VU0071063 on these channels (D. R. Swale and J. S. Denton, unpublished observations).

VU0071063 is structurally related to the xanthine derivative KMUP-1, which induces smooth muscle relaxation and vasodilation through activation of the cGMP and cAMP pathways. KMUP-1 induces the accumulation of cGMP and cAMP in part by inhibiting their degradation by phosphodiesterase enzymes. Pharmacologic agents that prevent cGMP accumulation predictably suppress KMUP-1-induced dilation. Inhibitors of several different families of K^{+} channels, including tetraethylammonium, 4-aminopyridine, iberiotoxin, charybdoxin, and glibenclamide, blunt the vasodilatory effects of KMUP-1, indicating an important role of the membrane potential in its mechanism of action (Wu et al., 2004; Adebiyi et al., 2008). However, this does not appear to involve direct K^{+} channel activation. For example, (Wu et al., 2004) found that KMUP-1 activates large-conductance Ca^{2+}-activated K^{+} (BK) currents in cerebral smooth muscle cells, but this was dependent on cGMP generation. Although the effects of KMUP-1 on K_ATP currents have not been reported, cGMP is known to activate vascular K_ATP channels (Kubo et al., 1994). The PDE-inhibitory activity of KMUP-1 is likely mediated through the theophylline moiety, since the addition of theophylline, a nonspecific PDE inhibitor, recapitulates the effects of KMUP-1 on cGMP levels and vascular tone (Wu et al., 2004). As noted earlier, VU0071063 also contains a theophylline group, raising the possibility that PDE inhibition and cGMP or cAMP accumulation contribute to K_{b}.6.2/SUR1 activation. However, theophylline at a concentration of 250 μM had no effect on K_{b}.6.2/SUR1-mediated Ti^{+} flux following 20-minute incubation. This, together with the observation that VU0071063 directly activates K_{b}.6.2/SUR1 in excised membrane patches (Supplemental Fig. 2), suggests that PDE inhibition and cyclic nucleotides are not essential components of its mechanism of action.

There are several important questions regarding VU0071063 and its mechanism of action remaining to be answered. KMUP-1 and VU0071063 differ only in the structure of their side-chains that project off a common theophylline moiety, yet only VU0071063 appears to be a direct SUR1/K_{b}.6.2 channel activator. Determination of VU0071063 structure-activity relationships with medicinal chemistry will inform a deeper understanding of pharmacophore requirements for activation of SUR1- and SUR2-containing K_ATP channels and may lead to the development of improved xanthine-based activators. Do VU0071063 and diazoxide activate Kir6.2/SUR1 through common molecular mechanisms? For instance, do they share the same receptor binding site in SUR1, and does VU0071063 require ATP hydrolysis for channel activation like diazoxide (Larsson et al., 1993)? It is well established that diazoxide has direct effects on mitochondrial respiration, although the underlying mechanisms are a matter of ongoing debate (Coetzee, 2013). At least some of the effects of diazoxide in cardiac and smooth muscle cells are mediated through inhibition of mitochondrial complex 2 (Grimsmann and Rustenbeck, 1998; Adebiyi et al., 2008), which has made it difficult to ascribe beneficial and undesirable effects of the drug to K_ATP channel–mediated effects or other mechanisms. Importantly, VU0071063 (<100 μM) had no effect on complex 2 activity (Supplemental Table 1). It will be important to determine whether VU0071063 action is limited to plasma membrane SUR1-containing channels or also has off-target effects on mitochondrial respiration and potentially other signaling pathways. The activation of K_ATP channels is linked to signaling pathways that can protect against cellular stress (Wojtovich et al., 2013). While the location of the channel that mediates protection (e.g., canonical surface K_{b}.TP versus mitochondrial K_ATP channels) remains elusive (Sato et al., 2000; Suzuki et al., 2002; Wojtovich et al., 2013), VU0071063 may prove to be a valuable tool to investigate the role of K_ATP channels in stress responses.

In conclusion, VU0071063 is a novel xanthine derivative that directly and selectively activates K_ATP channels containing SUR1. Despite K_ATP channels being validated drug targets for numerous diseases, VU0071063 is only the third SUR1-prefering chemotype discovered using small-molecule library screening. We anticipate that the Ti^{+} flux assay described here will enable the discovery of additional small-molecule modulators of K_{b}.6.2/SUR1 and other K_ATP channel subtypes.

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Authorship Contributions

Participated in research design: Raphemot, Swale, Jacobson, Cooper, Banerjee, Nichols, Denton.

Conducted experiments: Raphemot, Swale, Dadi, Jacobson, Cooper, Winters, Raphemot, Swale, Denton.

Performed data analysis: Raphemot, Swale, Jacobson, Cooper, Banerjee, Wojtovich, Nichols, Denton.

Wrote or contributed to the writing of the manuscript: Raphemot, Swale, Jacobson, Cooper, Nichols, Denton.

References


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Direct activation of β-cell K$_{ATP}$ channels with a novel xanthine derivative. Raphemot, R., Swale, D.R., Dadi, P.K., Jacobson, D.A., Cooper, P., Wojtovich, A.P., Banerjee, S., Nichols, C., Denton, J.S., *Molecular Pharmacology*. T-REx-HEK293-Kir6.2/SUR1 cell line characterization. A, Cells were cultured overnight with the indicated concentration of tetracycline and subjected to Western blot analysis of Kir6.2 expression in whole-cell lysates. Membranes were stripped and re-probed for b-actin as a loading control. B, Representative Tl$^+$ flux experiment in cells cultured overnight with (red) or without (black) tetracycline and then pre-treated with 250 µM diazoxide for 20 min before Tl$^+$ (12 mM Tl$_2$SO$_4$) addition.
Direct activation of b-cell $K_{\text{ATP}}$ channels with a novel xanthine derivative. Raphemot, R., Swale, D.R., Dadi, P.K., Jacobson, D.A., Cooper, P., Wojtovich, A.P., Banerjee, S., Nichols, C., Denton, J.S., *Molecular Pharmacology*. VU0071063 activates Kir6.2/SUR1 in excised inside-out patches. A, Representative inside-out membrane patch of COS cells transiently expressing Kir6.2 and SUR1. Membrane potential held at -50mV. Patches were excised in ATP-free K-internal (150mM-KCl, 10mM-HEPES, 1mM-EGTA) solution and then exposed to 0.1 mM-MgATP followed by 0.1mM-Mg-ATP + VU0071063 (10 µM or 20 µM) solutions as indicated by solid lines above the representative trace. (B) The relative current in 0.1 mM Mg-ATP + VU0071063 (10 µM or 20 µM) with respect to current in ATP-free solution averaged from all recordings (n=4).
Direct activation of b-cell $K_{ATP}$ channels with a novel xanthine derivative. Raphemot, R., Swale, D.R., Dadi, P.K., Jacobson, D.A., Cooper, P., Wojtovich, A.P., Banerjee, S., Nichols, C., Denton, J.S., Molecular Pharmacology. Lack of effect of VU0071063 on Kir6.2/SUR2A channels. A, Whole-cell Kir6.2/SUR2A currents were measured every 5 sec at -120 mV before and after addition of 5, 15, or 50 µM VU007106 or 50 µM Pinacidil (positive control), followed by application of 2 mM Ba$^{2+}$. B, Single-dose exposure to 150 µM VU0071063 has only a modest effect on Kir6.2/SUR2A channel activity. C, VU0071063 CRC data normalized to 50 µM pinacidil response. Data are means ± SEM (n = 4 at each dose).
Direct activation of b-cell K\textsubscript{ATP} channels with a novel xanthine derivative. Raphemot, R., Swale, D.R., Dadi, P.K., Jacobson, D.A., Cooper, P., Wojtovich, A.P., Banerjee, S., Nichols, C., Denton, J.S., Molecular Pharmacology. Lack of effect of theophylline on Kir6.2/SUR1-dependent Tl\textsuperscript{+} flux. A, Chemical structure of theophylline. B, Mean ± SEM slope fluorescence recorded from wells treated with 30 µM VU0071063, 250 µM diazoxide, or 250 µM theophylline for 20 min before Tl\textsuperscript{+} addition. Data are from 2 independent experiments performed in triplicate. Significance *P = 0.03, ** P = 0.003, unpaired Students t-test.
Direct activation of b-cell $K_{\text{ATP}}$ channels with a novel xanthine derivative. Raphemot, R., Swale, D.R., Dadi, P.K., Jacobson, D.A., Cooper, P., Wojtovich, A.P., Banerjee, S., Nichols, C., Denton, J.S., *Molecular Pharmacology*. Selectivity of VU0071063 among members of the Kir channel family. 11-point CRC of VU0071063 were established for Kir2.1, Kir2.2, Kir2.3, and Kir3.1/3.2 expressing cell lines ($n = 2$ independent experiments performed in triplicate per cell line).
Direct activation of b-cell \( K_{\text{ATP}} \) channels with a novel xanthine derivative. Raphemot, R., Swale, D.R., Dadi, P.K., Jacobson, D.A., Cooper, P., Wojtovich, A.P., Banerjee, S., Nichols, C., Denton, J.S., *Molecular Pharmacology*. Effects of VU0071063 on heterologously expressed Kv2.1. A, Time versus current amplitude plot illustrating the rapid and reversible inhibition of Kv2.1 current at 40 mV by 10 \( \mu \text{M} \) VU0071063. B, Representative current traces recorded at 40 mV before and during bath perfusion with 10 \( \mu \text{M} \) VU0071063. C, Mean SEM current amplitude at 40 mV in the absence (control) or presence of 10 \( \mu \text{M} \) VU0071063. *P = 0.01, paired Students t-test.
<table>
<thead>
<tr>
<th>[Compound] µM</th>
<th>VU0071063</th>
<th>Diazoxide</th>
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<tbody>
<tr>
<td>1</td>
<td>1.5 ± 3.5</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>-2.2 ± 4.5</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>2.4 ± 2.7</td>
<td>7.6 ± 2.5</td>
</tr>
<tr>
<td>30</td>
<td>0.0 ± 4.3</td>
<td>14.9 ± 6.0</td>
</tr>
<tr>
<td>100</td>
<td>4.9 ± 2.9</td>
<td>24.2 ± 4.3</td>
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<tr>
<td>300</td>
<td>-</td>
<td>36.0 ± 3.5</td>
</tr>
<tr>
<td>600</td>
<td>-</td>
<td>41.6 ± 0.7</td>
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Direct activation of b-cell $K_{ATP}$ channels with a novel xanthine derivative. Raphemot, R., Swale, D.R., Dadi, P.K., Jacobson, D.A., Cooper, P., Wojtovich, A.P., Banerjee, S., Nichols, C., Denton, J.S., *Molecular Pharmacology*. Percent inhibition of Complex II activity in response to VU0071063 and diazoxide, underwent three cycles of freeze-thaw and subject to the complex II enzymatic activity assay. Complex II activity was measured in mitochondria isolated from mouse hearts using an enzymatic activity. Data are mean ± S.E.M. percent inhibition (n=4 independent mitochondria preparations). Control complex II activity was 88.0 ± 3.1 nmol/min/mg protein.