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Direct activation of β-cell K_{ATP} channels with a novel xanthine derivative

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Running title: Discovery of a xanthine-based Kir6.2/SUR1 activator

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Non-standard abbreviations: $[Ca^{2+}]_i$: intracellular calcium concentration; CRC: concentration-response curve; DMEM: Dulbecco's Modified Eagle Medium; DMSO: dimethyl sulfoxide; FBS: fetal bovine serum; FCS: fetal calf serum; FDSS: functional drug screening system; HRP: horseradish peroxidase; HTS: high-throughput screening; K_{ATP}: ATP-sensitive potassium channel; KCO: potassium channel opener; Kir: inward rectifier potassium channel; Kv: voltage-

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gated potassium channel; PDE: phosphodiesterase; SUR: sulfonylurea receptor; T-Rex-HEK293: tetracycline inducible human embryonic kidney; Tl^+: thallium; VDCC: L-type voltage-dependent calcium channels; V_m: membrane potential; VU0071063: 7-(4-(*tert*-butyl)benzyl)-1,3-dimethyl-1*H*-purine-2,6(3*H*,7*H*)-dione

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Abstract

ATP-regulated potassium (K_{ATP}) channel complexes of Kir6.2 and SUR1 critically regulate pancreatic islet beta-cell membrane potential, calcium influx, and insulin secretion, and consequently, represent important drug targets for metabolic disorders of glucose homeostasis. The K_{ATP} 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 K_{ATP} channels. Here we report the serendipitous discovery in a high-throughput screen of a novel activator of Kir6.2/SUR1, termed VU0071063. The xanthine derivative rapidly and dose-dependently activates Kir6.2/SUR1 with a half-effective concentration (EC_{50}) 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 Kv2.1. Finally, we show that VU0071063 activates native Kir6.2/SUR1 channels, thereby inhibiting glucose-stimulated calcium entry in isolated mouse pancreatic beta-cells. VU0071063 represents a novel tool/compound for investigating beta-cell physiology, K_{ATP} channel gating, and a new chemical scaffold for developing improved activators with medicinal chemistry.

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Introduction

By integrating cellular metabolism, membrane potential (V_m), and excitability, ATP-sensitive K^+ (K_{ATP}) channels carry out fundamental roles in nerve, muscle, epithelial, and endocrine tissue physiology (Ashcroft, 1988). K_{ATP} channels are octomeric complexes of four pore-forming Kir6.x inward rectifier K^+ (Kir) channel subunits and four regulatory SURx sulfonylurea receptor subunits (Nichols CG, 2006). Kir6.1 (*KCNJ8*), Kir6.2 (*KCNJ11*), and SUR1 (*ABCC8*) 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 K_{ATP} 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, V_m depolarization, Ca^{2+} influx through L-type voltage-dependent Ca^{2+} 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, K_{ATP} channel activators (e.g. diazoxide) are used to treat disorders of severe hypoglycemia, such as congenital hyperinsulinism and insulin-producing pancreatic tumors (Ashcroft, 2007; Nichols et al., 2007). The major K_{ATP} channel subtype in cardiomyocytes consists of Kir6.2 and SUR2A (Nichols et al., 2013), and potassium channel opener (KCO) activation of sarcolemmal K_{ATP} channels and Vm hyperpolarization affords cardioprotection from subsequent ischemia-reperfusion injury (Grover

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and Garlid, 2000). Activation of vascular smooth muscle Kir6.1/SUR2B with pinacidil or diazoxide leads to vasodilation and a reduction in blood pressure (Flagg et al., 2010), but can also result in pathological 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_{ATP} channels, their important physiological roles, and therapeutic as well as pathological potential in various conditions, there is considerable interest in the continued development of pharmacological modulators for targeting specific subtypes of K_{ATP} channels (Hansen, 2006). Here, we report the serendipitous discovery of a novel xanthine derivative that directly activates heterologously expressed Kir6.2/SUR1 channels and native pancreatic β-cell K_{ATP} channels. The activator, termed VU0071063, is more potent and efficacious than diazoxide and is selective for SUR1-containing K_{ATP} channels. VU0071063 represents a new tool compound for interrogating Kir6.2/SUR1 channel physiology and structure-function relationships of K_{ATP} channel gating.

Materials and Methods

Expression vectors

The following vectors were used in this study: pcDNA5/TO-Kir6.2 (NM_010602), pcDNA5/TO-Kir6.1 (NM_004982), pcDNA3.1-SUR1 (L40623.1), pCMV6c-SUR2A (D83598.1), pcDNA3.1-SUR2B (D86038.2), pcDNA5/TO-Kir2.1 (NM_000891.2), pcDNA5/TO-Kir2.2 (NM_021012), pcDNA5/TO-Kir2.3 (NM_152868).

Cell lines and transfections

T-REx-HEK293 cells were transfected with pcDNA5/TO-Kir6.2 using Lipofectamine 2000 (Life Technologies) 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 Kir6.2 expression by Western blot analysis (Supplemental Fig. 1), the cells were co-transfected with pcDNA3.1-SUR1 and grown in G418-containing medium to select cells carrying stably integrated plasmids for both K_{ATP} channel subunits. Monoclonal lines were isolated by limiting dilution, expanded, and tested for tetracycline-inducible thallium (Tl^+) flux, as described below. One cell line exhibiting robust Tl^+ flux was selected for assay development and small-molecule screening. Monoclonal T-REx-HEK293 cell lines expressing other mammalian Kir channels were generated as described previously (Lewis et al., 2009; Raphemot et al., 2011). Monoclonal mGluR8/GIRK/HEK293 cells stably expressing Kir3.1/3.2, the M4 muscarinic receptor and rat mGlu8a were cultured as described previously (Niswender et al., 2008). For transient transfections, HEK293T cells were transfected with 1 µg of Kir6.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 Kir6.2 expression was performed following 24-h induction with tetracycline essentially as described previously (Lewis et al., 2009). Goat polyclonal Kir6.2 antiserum (SC-11226) and donkey anti-goat HRP-conjugated antiserum (SC-2020) were purchased from Santa Cruz.

Tl^+ flux assays

Tl^+ flux assays were performed essentially as described previously (Raphemot et al., 2013). Briefly, stably transfected T-Rex-HEK-293 cells expressing Kir6.2/SUR1 channels were cultured overnight in 384-well plates (20,000 cells/20 µL/well black-walled, clear-bottomed PureCoat amine-coated plates; BD, Bedford, MA) with a platting media containing DMEM, 10% dialyzed FBS and 1µg/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 mM HEPES, pH 7.3), 0.01% (w/v) Pluronic F-127 (Life Technologies, Carlsbad, CA), and 1.2 µM of the thallium-sensitive dye Thallos-AM (TEFlabs, Austin, TX). Following 1 hr incubation at room temperature, the dye loading solution was washed from the plates and replaced with 20 µL/well of assay buffer. The plates were transferred to a Hamamatsu Functional Drug Screening System 6000 (FDSS6000; 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 s (excitation 470 ± 20 nm, emission 540 ± 30 nm) followed by addition of the Tl^+ stimulus buffer (10 µL/well) and data collection for an additional 4 min. The Tl^+ stimulus buffer contains in (mM) 125 $NaHCO_3$, 1.8 $CaSO_4$, 1 $MgSO_4$, 5 glucose, 1.8 Tl_2SO_4, 10 HEPES pH 7.4. For Tl^+ flux assay on Kir3.1/3.2 expressing cells, the

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thallium stimulus buffer contains 12 mM Tl_2SO_4 and either an EC_{20} or EC_{80} of glutamate (Sigma-Aldrich, St. Louis, MO).

Test compounds from the Vanderbilt Institute of Chemical Biology (VICB) library 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 2X final concentrations to generate 4- or 11-point 3-fold serial dilution series. The K_{ATP} channel inhibitors glibenclamide and tolbutamide were resuspended in assay buffer containing VU0071063 or diazoxide. Tl^+ flux assays on Kir2.1, Kir2.2, Kir2.3, and Kir3.1/3.2 were performed as described previously (Raphemot et al., 2011).

Tl^+ flux data were analyzed as previously described (Raphemot et al., 2013) using a combination of Excel (Microsoft Corp, Redmond, WA) with XLfit add-in (IDBS, 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 s after Tl^+ addition and ending 15 s after Tl^+ 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.

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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 h 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 $MgCl_2$, 1 EGTA, 10 HEPES, and 3 Na_2ATP, pH 7.3. The standard bath solution contained (in mM): 135 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 glucose, and 10 HEPES, pH 7.4. Whole-cell currents were recorded under voltage-clamp conditions using an Axopatch 200B amplifier (Molecular Devices). The cells were voltage-clamped and stepped every 5 s from a holding potential of -75 to 120 mV for 200 ms, and then ramped at a rate of 2.4 mV/ms from -75 to 120 mV before returning to -75 mV. Electrophysiological 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 Kir6.2, SUR1, and EGFP for 24 hours before patch-clamp analysis. Transfected cells were identified by GFP 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 1 EGTA and 0.5 free Mg^{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.

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Calcium imaging

Islet-cell intracellular calcium ($[Ca^{2+}]_i$) was measured using Ca^{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 h in RPMI-1640 medium supplemented with 10% fetal calf serum (FCS), concentrations of glucose specified, 100 IU ml−1 penicillin, and 100 mg ml−1 streptomycin. Cells were dye-loaded for 20 min at 37°C with 2 μM fura-2-AM in solution containing (in mM): 119 NaCl, 2.5 $CaCl_2 \cdot [(H_2O)_6]$, 4.7 KCl, 10 HEPES, 1.2 $MgS0_4$, 1.2 KH_2PO_4, 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, Japan). The $[Ca^{2+}]_i$ ratios of emitted fluorescence intensities at excitation wavelengths of 340 and 380 nm (*F*340/*F*380) were determined every 5 s with background subtraction. Cells were perifused at 37 °C at a flow of 2 mL/min; the solutions utilized during the experiments are the loading solution with various glucose concentrations and VU0071063, as indicated.

Measurement of complex II activity

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

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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, USA) and diluted in bath solution before use. The final concentration of DMSO used was less than or equal to 0.3% (*v/v*).

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Results

Serendipitous discovery of the Kir6.2/SUR1 activator VU0071063

A Tl^+ flux assay of Kir6.2/SUR1 K_{ATP} channels was developed to assess the specificity of inhibitors of a mosquito Kir channel identified in a high-throughput screen (Raphemot, Denton, unpublished). The assays employ stably transfected T-REx-HEK293 cells expressing Kir6.2 from a tetracycline-inducible promoter and SUR1 constitutively (Supplemental Fig. 1). Kir6.2/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_{ATP} channel opener diazoxide to mediate Tl^+ flux. While testing approximately 300 mosquito Kir1 antagonists for selectivity, diazoxide was inadvertently excluded from one plate, revealing a dose-dependent increase in Tl^+ flux in wells I3, I4, I5, and I6 containing 30, 10, 3, and 1 μM of a mosquito Kir1 antagonist, respectively (Fig. 1B-C). The small-molecule added to these wells, which we termed VU0071063, was re-ordered as a powder, freshly dissolved in DMSO, and characterized in Tl^+ flux and electrophysiological assays.

Effects of VU0071063 on pancreatic Kir6.2/SUR1 K_{ATP} channels

The activity of VU0071063 on Kir6.2/SUR1 was compared to diazoxide and the SUR2-preferring opener pinacidil in 11-point CRCs in Tl^+ flux assays. The chemical structures of the three agonists are illustrated in Fig. 2A. Tetracycline-induced T-REx-HEK293-Kir6.2/SUR1 cells were treated with the agonists for 20-min prior to Tl^+ 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 Kir6.2/SUR1-dependent Tl^+ flux than did 250 μM diazoxide. VU0071063 and diazoxide led to a dose-dependent activation of Tl^+ flux, whereas

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pinacidil was predictably inactive against Kir6.2/SUR1 (Fig. 2C). Half-maximal effective concentrations (EC_{50}) derived from logistical fits to CRC data for VU0071063 and diazoxide were 10.3 µM (95% CI: 9.5-11 µM) and greater than 100 µM (EC_{50} ~120 µM), respectively (n = 4-6 independent experiments). To confirm that Tl^+ flux is dependent on Kir6.2/SUR1 channels, and not endogenous Tl^+ flux pathways, the dose-dependent effects of K_{ATP} channel inhibitors glibenclamide and tolbutamide were evaluated. The cells were pre-treated with EC_{80} 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 (IC_{50}) values, the IC_{50} for glibenclamide and tolbutamide in VU0071063-treated cells were 5.60 nM (95% CI: 5-6 nM) and 3.07 µM (95% CI: 2-5 µM), respectively. These values are similar to those of diazoxide-treated cells (glibenclamide IC_{50} = 16.6 nM [95% CI: 15-18 nM]; tolbutamide IC_{50} = 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 Kir6.2/SUR1. Bath application of VU0071063 rapidly (Fig. 3A) and dose-dependently (Fig. 3C) activated Kir6.2/SUR1 currents, with a maximal activation of 1077 ± 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 Kir6.2/SUR1 activation. These data show that at low micromolar concentrations, VU0071063 is a more potent activator of Kir6.2/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 Kir6.2 and SUR1,

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in inside-out membrane patches. In the presence of 0.1 mM MgATP, which inhibits WT channels ~90%, both 10 μM 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 Kir6.2 or Kir6.1 channels containing SUR1 or SUR2A using patch clamp electrophysiology. Diazoxide and pinacidil were used as positive controls for SUR1- and SUR2A-containting channels, respectively. As shown in the representative timecourse experiment in Fig. 4A, and summary data (mean ± SEM; n = 7) in Fig. 4C, bath application of 50 μM VU0071063 rapidly and reversibly activated Kir6.2/SUR1 to a greater extent than an equal concentration of diazoxide. Qualitatively similar results were observed in cells transfected with Kir6.1/SUR1 (Fig. 4D). In striking contrast, VU0071063 had no effect on Kir6.2/SUR2A (Fig 4B, 4E) or Kir6.1/SUR2A (Fig. 4F), whereas pinacidil activated both channel subtypes. Dose-response experiments revealed that VU0071063 had no appreciable effects on Kir6.2/SUR2A at concentrations up to 150 μM (Supplemental Fig. 3, which is 15-fold higher than the IC_{50} for Kir6.2/SUR1.

VU0071063 inhibits glucose-stimulated β-cell Ca^{2+} influx

Glucose-stimulated closure of β-cell K_{ATP} channels results in membrane potential depolarization, activation of voltage-dependent Ca^{2+} channels (VDCC), Ca^{2+} influx, and Ca^{2+}-induced insulin secretion. We therefore tested whether VU0071063 activates native Kir6.2/SUR1

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channels by measuring the effect of the activator on β-cell Ca^{2+} influx during glucose-stimulation. Treatment of β-cells with high (14 mM) glucose induced a significant rise in Ca^{2+} as determined by the fluorescent Ca^{2+} indicator fura-2, which shows an increase in the fluorescent ratio of Ca^{2+} bound to Ca^{2+} unbound dye in response to glucose (red cells, Fig. 5). Activation of β-cell K_{ATP} channels with VU0071063 in the presence of high (14 mM) glucose resulted in inhibition of β-cell Ca^{2+} influx and reduction in Ca^{2+} levels back to those observed in low (2 mM) glucose conditions (green cells, Fig. 5). The reduction in Ca^{2+} influx mediated via K_{ATP} activation is reversible following removal of VU0071063, which results in a return of β-cell Ca^{2+} levels to high (14mM) glucose levels (red cells, Fig. 5). This data indicates that VU0071063 activates native β-cell K_{ATP} channels and thereby reduces VDCC activation and Ca^{2+} influx.

Kir6.2/SUR1 activation by VU0071063 is not mediated by a PDE inhibitory pathway

Vascular smooth muscle K_{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 Kir6.2/SUR1 in Tl^{+} 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 Kir6.2/SUR1-dependent Tl^{+} flux.

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Ancillary Pharmacology

The selectivity of VU007106 was evaluated in 11-point CRCs in Tl^+ flux assays against Kir2.1, Kir2.2, Kir2.3, and Kir3.1/3.2. VU0071063 was inactive against Kir2.1 and Kir2.2 (IC_{50} > 100 μM) and showed weak inhibitor activity against Kir3.1/3.2 (IC_{50} = 65 μM) and Kir2.3 (IC_{50} = 91 μM)(Supplemental Fig. 5). Patch clamp electrophysiology was used to determine whether VU0071063 acts on the voltage-gated K^+ channel Kv2.1, 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 Kv2.1 current at 40 mV that was fully reversible (Supplemental Fig. 6).

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Discussion

Pancreatic K_{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 Kir6.2/SUR1 channels (de Tullio et al., 2011; Hansen, 2006; Pirotte et al., 2010). Diazoxide is the best known SUR1-preferring 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_{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 Kir6.2/SUR1. Structural modifications to the diazoxide scaffold have led to several new series with sub-micromolar potency and selectivity for pancreatic over smooth muscle K_{ATP} channels (de Tullio et al., 2011; Pirotte et al., 2010). One analog, termed NN414 (Dabrowski et al., 2003), shows favorable activity in obese rats (Alemzadeh et al., 2004; Carr et al., 2003), as well as healthy and type 2 diabetes patients (Zdravkovic et al., 2007; Zdravkovic et al., 2005). Clinical trials were initiated but later suspended due to drug-induced elevations of key liver enzymes (Hansen, 2006). Analogs of the SUR2-preferring openers cromakalim and pinacidil that exhibit selectivity for pancreatic K_{ATP} channels (Florence et al., 2011; Florence et al., 2009; Khelili et al., 2008; Khelili et al., 2006; Sebille et al., 2008; Sebille et al., 2006) have also been developed, showing that is possible to switch SUR preference with chemical modifications to the scaffold. To our knowledge, the only unique pancreatic K_{ATP}

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channel activator chemotypes reported in the last 2 decades were identified in screens of small-molecule libraries. These include the 4-sulfamoylphenylbenzamide and nitropyrazole series of K_{ATP} activators. A 4-sulfamoylphenylbenzamide derivative was shown to activate heterologously expressed Kir6.2/SUR1 channels and inhibit glucose-stimulated insulin secretion from primary rat islets with sub-micromolar efficacy; the activity toward SUR2-containing channels was not reported (Nielsen et al., 2004). One nitropyrazole analog exhibits nanomolar-affinity toward Kir6.2/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 publically disclosed SUR1-preferring 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^+ channels. VU0071063 is slightly more potent than diazoxide, and activates the channel with a faster timecourse. The reason for the discrepancy in IC_{50} values derived from Tl^+ flux and patch clamp experiments is unclear, but likely reflects 1) differences in the behavior of Tl^+ and K^+ in the K_{ATP} channel pore, and 2) the slower kinetics of diazoxide action compared to 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 min before adding Tl^+ stimulus buffer, however we still observed a rightward shift in the EC_{50} value for diazoxide.

VU0071063 is selective for Kir6.2/SUR1 over Kir6.2/SUR2A and Kir6.1/SUR2A, as well as Kir3.1/3.2, Kir2.1, Kir2.2, Kir2.3, and Kv2.1. Kv2.1 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

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the same dose shown to reduce high glucose-induced Ca^{2+} influx in pancreatic β-cells, 10 μM VU0071063 significantly inhibited Kv2.1 currents by approximately 10%. Inhibition of Kv2.1 would be expected to prolong the action potential depolarization and increase, not decrease, Ca^{2+} influx through VDCC, excluding a potential role of Kv2.1 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 CFTR is inhibited at high concentrations (IC_{50} = 250 μM; Sheppard and Welsh, 1992) of diazoxide, future studies should address whether VU0071063 inhibits CFTR 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 (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 (PDE) enzymes. Pharmacological agents that prevent cGMP accumulation predictably suppress KMUP-1-induced dilation. Inhibitors of several different families of K^+ channels, including tetraethylammonium, 4-aminopyridine, iberiotoxin, charybdotoxin, and glibenclamide, blunt the vasodilatory effects of KMUP-1, indicating an important role of the

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membrane potential in its mechanism of action (Dai et al., 2010; Lin et al., 2002; Wu et al., 2001). 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 non-specific 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 contributes to Kir6.2/SUR1 activation. However, theophylline at a concentration of 250 μM had no effect on Kir6.2/SUR1-mediated Tl^+ flux following 20-min incubation. This, together with the observation that VU0071063 directly activates Kir6.2/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/Kir6.2 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

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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 II (Adebiyi et al., 2008; Grimmsmann and Rustenbeck, 1998), 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 II 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 are linked to signaling pathways that can protect a cellular against stress (Wojtovich et al., 2013). While the location of the channel that mediates protection (e.g. canonical surface K_{ATP} vs. 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-preferring chemotype discovered using small-molecule library screening. We anticipate that the Tl^+ flux assay described here will enable the discovery of additional small-molecule modulators of Kir6.2/SUR1 and other K_{ATP} channel subtypes.

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Designed experiments: RR, DS, DJ, PC, SB, CN, JSD

Conducted experiments: RR, DS, DJ, PC, APW, SB

Analyzed Data: RR, DS, DJ, PC, SB, APW, CN, JSD

Wrote paper: RR, DS, DJ, PC, CN, JSD

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Footnotes:

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Figure Legends

Figure 1. Discovery of VU0071063 in a Tl^+ flux assay of Kir6.2/SUR1. A, Plate map used in CRC analyses. DMSO (solvent) and broad-spectrum Kir channel inhibitor VU0573 (Raphemot et al., 2011) were used as controls. 4-point test compound CRCs were distributed horizontally, whereas 11-point VU0573 CRCs were distributed vertically. B, Fluorescence heat map recorded from the assay plate containing 4 doses of VU0071063 (red box). Fluorescence intensity is indicated by the pseudocolored scale (right), with cooler (blue) to hotter (red) colors corresponding to low high Tl^+ flux, respectively. C, Representative time versus normalized (F/Fo) fluorescence intensity in wells containing the indicated concentrations of VU0071063.

Figure 2. Characterization of VU0071063 activity against Kir6.2/SUR1 in Tl^+ flux assays. A, Chemical structures of VU0071063 (VU063), diazoxide, and pinacidil. B, Representative Tl^+ flux experiment demonstrating activation of Kir6.2/SUR1 by 30 μM VU0071063 or 250 μM diazoxide, but not the vehicle control DMSO (0.3%). Fluorescence data have been normalized (F/F_0) to baseline values recorded before Tl^+ addition. C, Dose-dependent activation of Kir6.2/SUR1 by VU0071063 and diazoxide, but not pinacidil (n = 4-6 independent experiments, each performed in triplicate). D, Dose-dependent inhibition of VU0071063- and diazoxide-dependent Tl^+ flux by glibenclamide and tolbutamide (n = 3 independent experiments, each performed in triplicate).

Figure 3. Characterization of VU0071063 activity against Kir6.2/SUR1 with patch clamp electrophysiology. A, Transfected cells expressing Kir6.2/SUR1 were voltage clamped at -75 mV and stepped every 5 sec to -120 mV to elicit inward current. Minor current run-up was

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observed following establishment of the whole-cell configuration and dialysis with the pipette solution. Addition of 1 μM or 30 μM VU0071063 led to rapid activation of inward current. B, In contrast, addition of 50 mM diazoxide activated Kir6.2/SUR1 slowly. After achieving steady-state activation, addition of 50 μM VU0071063 led to further activation of Kir6.2/SUR1. Inward currents were blocked with 2 mM Ba^{2+}. C, Mean ± SEM dose-response data fitted with 4-parameter logistic functions to derive EC_{50} values of 7 and 11 for VU0071063 (closed square) and diazoxide (open square), respectively (n = 5 – 10 per concentration). Data are normalized and expressed as percent (%) activation from baseline current in the absence of agonist.

Figure 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 Ba^{2+} on Kir6.2/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 Ba^{2+} on Kir6.2/SUR2A currents. C-F, Mean ± SEM current at -120 mV recorded from cells transfected with Kir6.2/SUR1, Kir6.1/SUR1, Kir6.2/SUR2A, or Kir6.1, SUR2A, respectively (n = 4 – 7).

Figure 5. VU0071063 inhibits glucose-stimulated β-cell calcium entry. (A) Two representative β-cells loaded with FURA-2; displayed as a fluorescent ratio (340/380 nM) in response to 2mM glucose (1), 14 mM glucose (2), 14 mM glucose + 10 μM VU0071063 (3), and 14 mM glucose (4). (B) Relative calcium responses of mouse islet-cells following treatment with 2mM glucose and as indicated by the conditions labeled above (black lines, n=239 islet-cells over 3 days and 13 plates of cells).

Figure 1

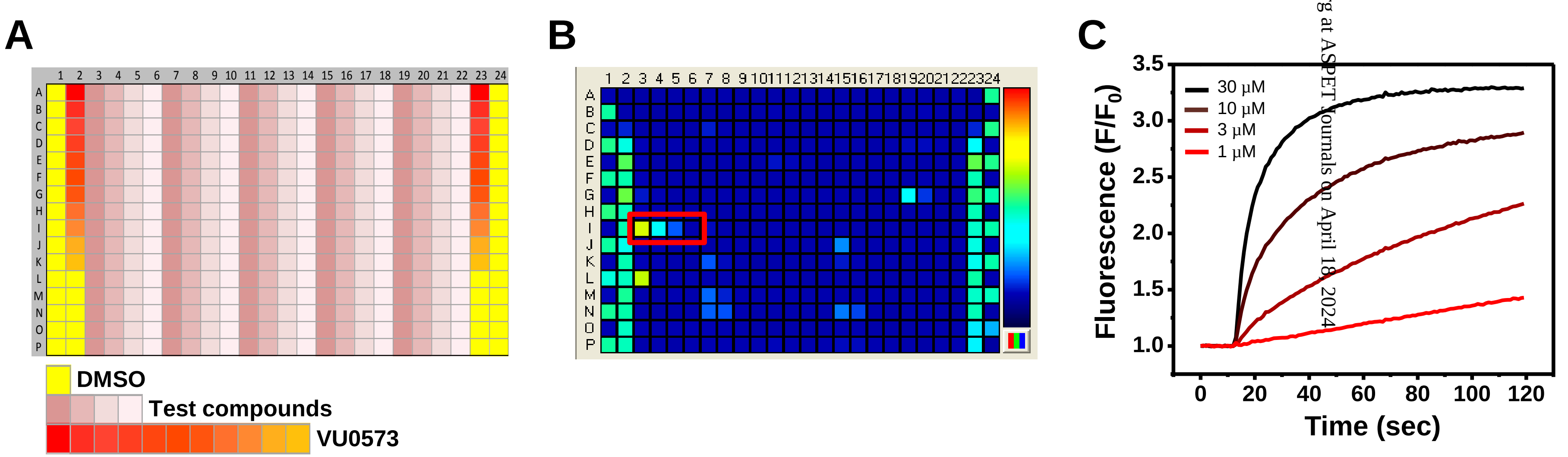
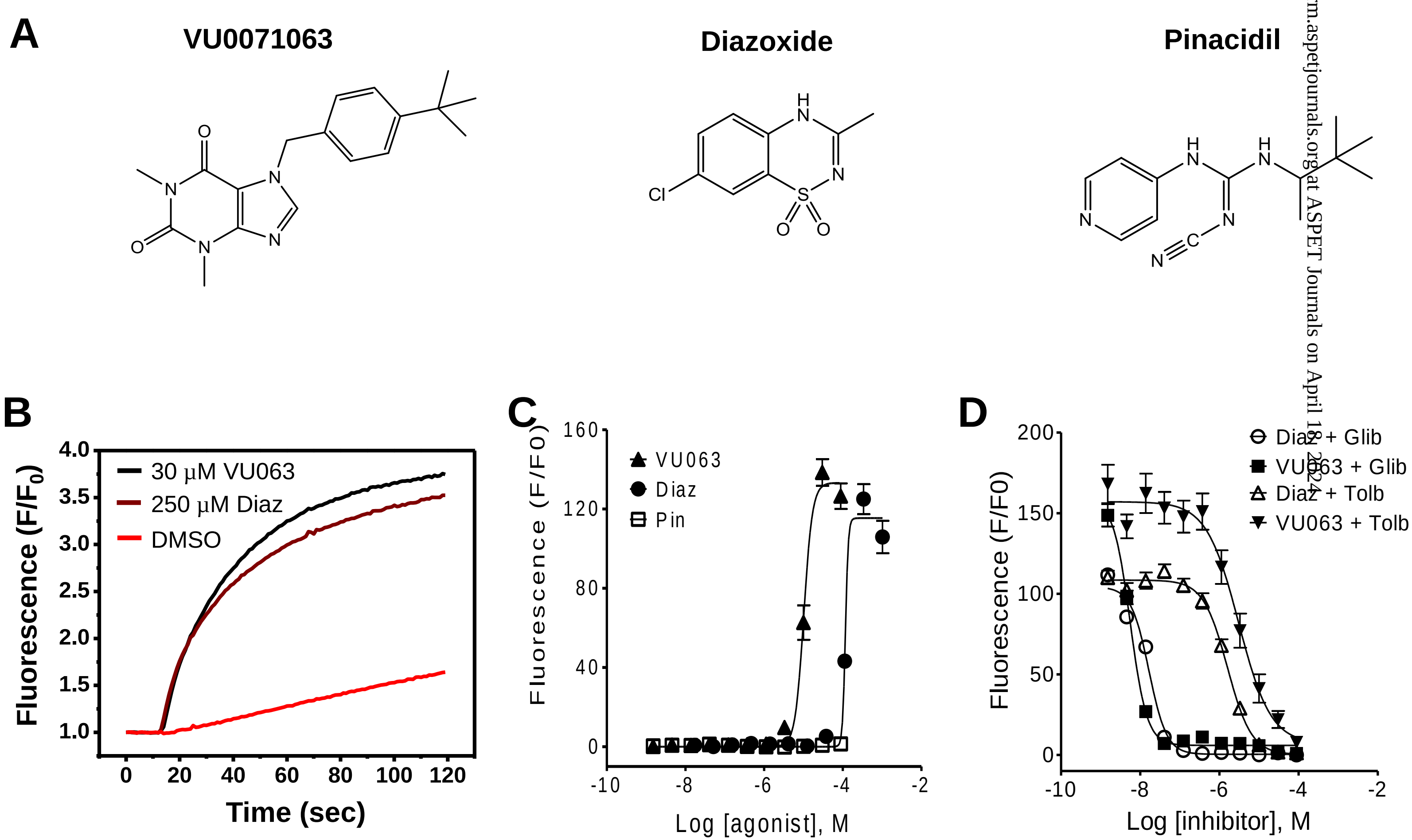

A
DMSO
Test compounds
VU0573
B
C
Fluorescence (F/F0)
Time (sec)
30 µM
10 µM
3 µM
1 µM

Figure 2



A
VU0071063
Diazoxide
Pinacidil
B
30 µM VU063
250 µM Diaz
DMSO
Fluorescence (F/F$_0$)
Time (sec)
C
VU063
Diaz
Pin
Fluorescence (F/F0)
Log [agonist], M
D
Diaz + Glib
VU063 + Glib
Diaz + Tolb
VU063 + Tolb
Fluorescence (F/F0)
Log [inhibitor], M

Figure 3

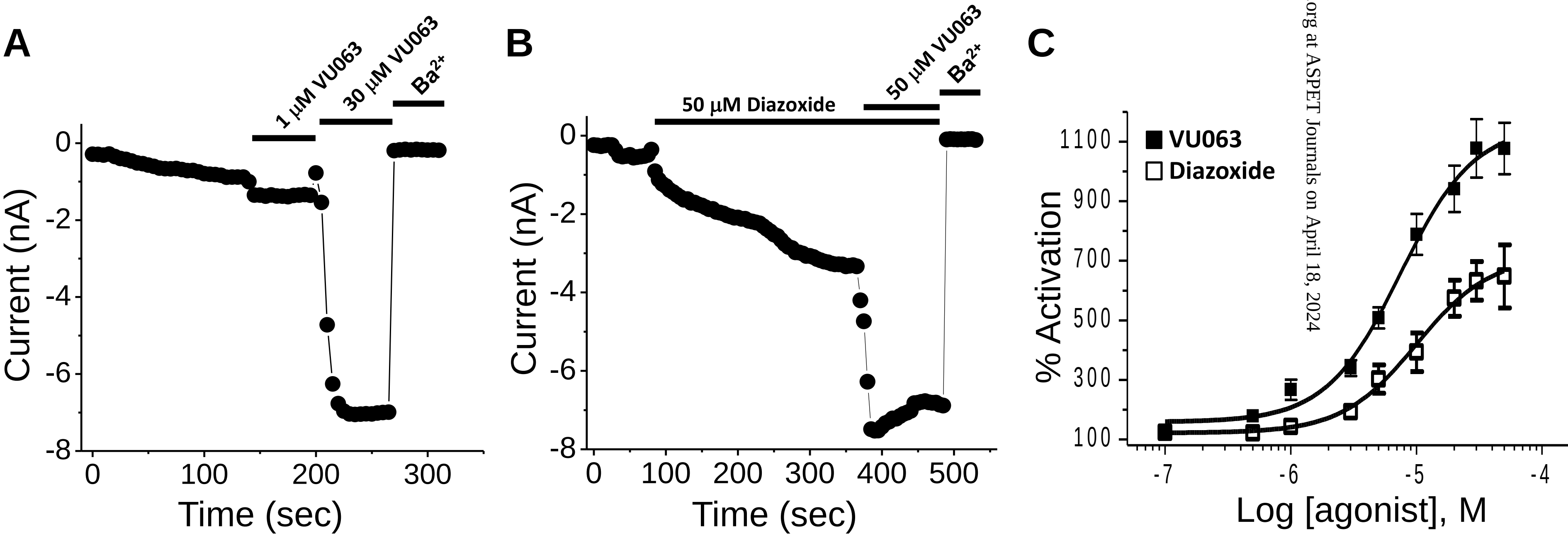

A
1 μM VU063
30 μM VU063
Ba2+
Current (nA)
0
-2
-4
-6
-8
0
100
200
300
Time (sec)
B
50 μM Diazoxide
50 μM VU063
Ba2+
Current (nA)
0
-2
-4
-6
-8
0
100
200
300
400
500
Time (sec)
C
VU063
Diazoxide
% Activation
1100
900
700
500
300
100
-7
-6
-5
-4
Log [agonist], M

Figure 4

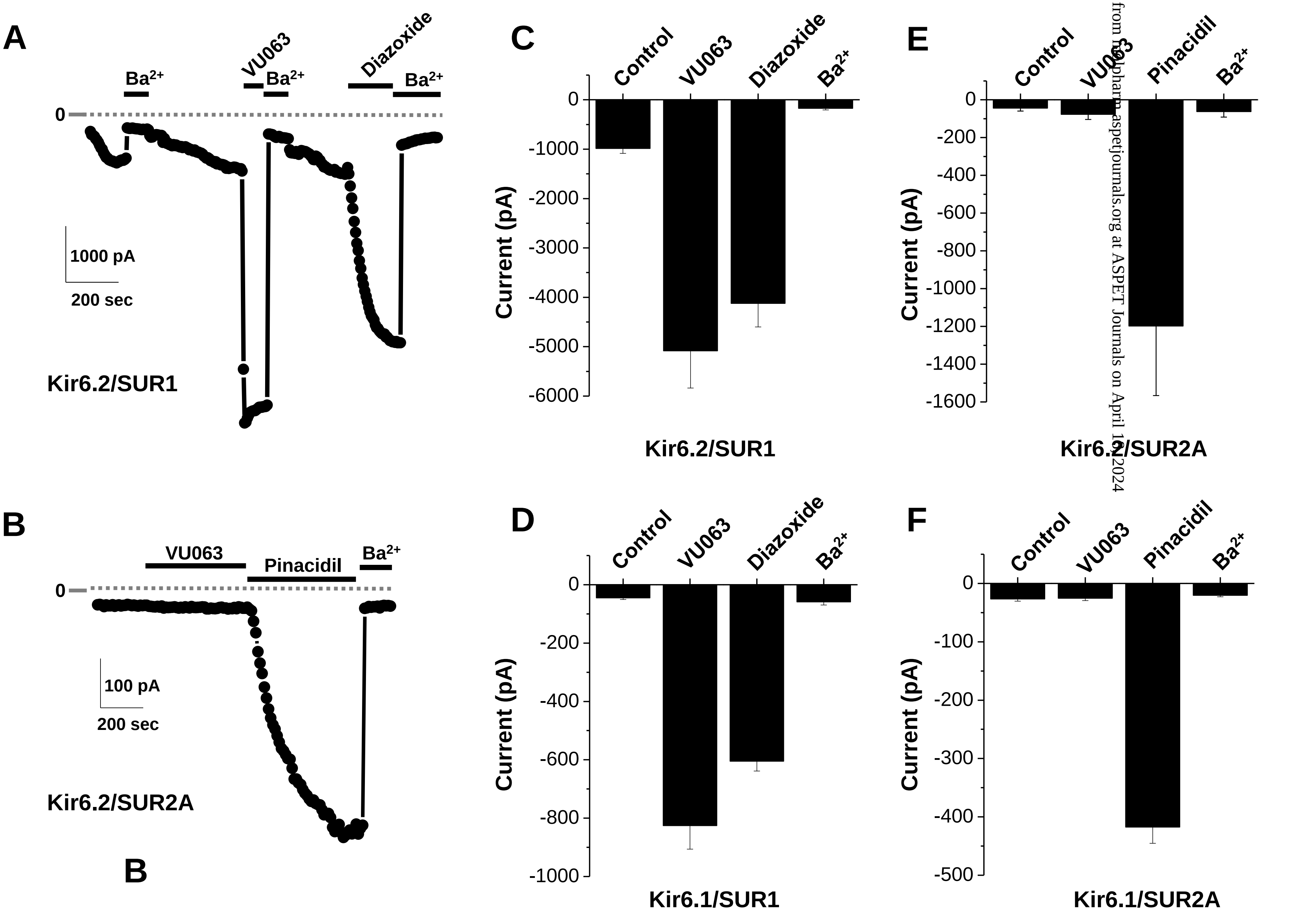
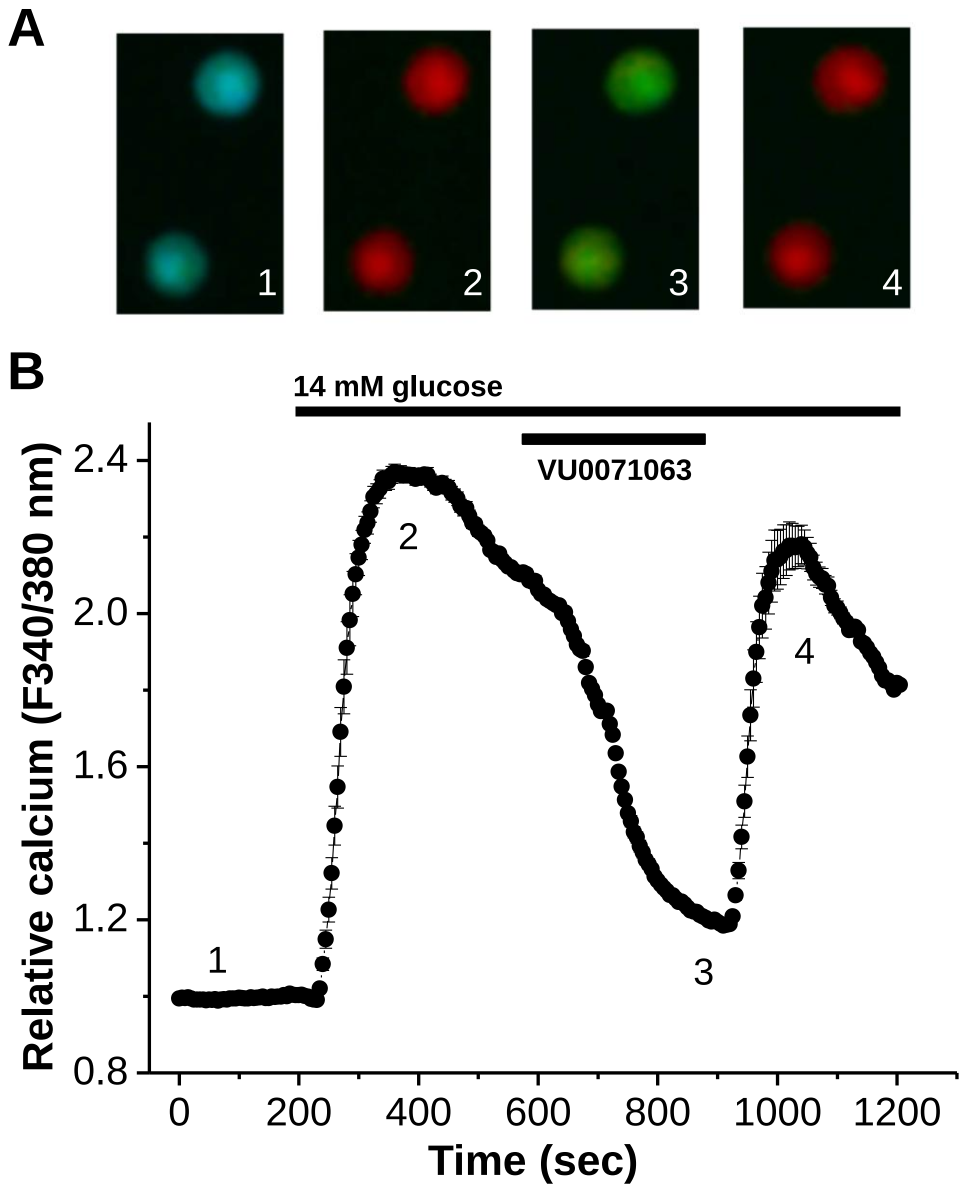

A
Ba^{2+}
VU063
Ba^{2+}
Diazoxide
Ba^{2+}
0
1000 pA
200 sec
Kir6.2/SUR1
B
VU063
Pinacidil
Ba^{2+}
0
100 pA
200 sec
Kir6.2/SUR2A
B
C
Control
VU063
Diazoxide
Ba^{2+}
Current (pA)
0
-1000
-2000
-3000
-4000
-5000
-6000
Kir6.2/SUR1
D
Control
VU063
Diazoxide
Ba^{2+}
Current (pA)
0
-200
-400
-600
-800
-1000
Kir6.1/SUR1
E
Control
VU063
Pinacidil
Ba^{2+}
Current (pA)
0
-200
-400
-600
-800
-1000
-1200
-1400
-1600
Kir6.2/SUR2A
F
Control
VU063
Pinacidil
Ba^{2+}
Current (pA)
0
-100
-200
-300
-400
-500
Kir6.1/SUR2A

Figure 5


A
1
2
3
4
B
14 mM glucose
VU0071063
Relative calcium (F340/380 nm)
2.4
2.0
1.6
1.2
0.8
0
200
400
600
800
1000
1200
Time (sec)