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# CLOSURE BY IPTAKALIM, A CARDIOVASCULAR $K_{ATP}$ CHANNEL OPENER, OF RAT PANCREATIC β-CELL $K_{ATP}$ CHANNELS AND ITS MOLECULAR BASIS

Makoto Wakui[1,2], Naoko Misaki[2], Sechiko Suga[3], Xia Mao[4], Yu-Fung Lin[4] and Jie Wu[4]

**Abstract** Diabetes mellitus is a group of diseases characterized by high levels of blood glucose resulting from defects in insulin production, insulin action, or both. Diabetes patients usually have accompanying cardiovascular disorders. Sulfonylureas have been the leading oral antihyperglycemic agents for type 2 diabetes treatment, which currently still constitute the most popular anti-diabetic drugs. Nevertheless, concern has arisen over the side effects of sulfonylureas on the cardiovascular system. Here we report that iptakalim, a novel cardiovascular ATP-sensitive potassium ($K_{ATP}$) channel opener, closed rat pancreatic $\beta$-cell $K_{ATP}$ channels and increased insulin release. Using whole-cell patch-clamp recordings, iptakalim depolarized $\beta$-cells, induced action potential firing and reduced pancreatic $K_{ATP}$ channel currents. Using single-channel recordings, iptakalim reduced $K_{ATP}$ channel open-probability independently of intracellular ATP concentrations. We demonstrated that iptakalim elevated intracellular $Ca^{2+}$ concentrations and increased insulin release as revealed by fluorescence imaging (fura-2) and biochemical measurements, respectively. In addition, iptakalim significantly inhibited the open-probability of recombinant Kir6.2/SUR1 and Kir6.2FL4A (a trafficking mutant of the Kir6.2) channels expressed in transfected human embryonic kidney (HEK) 293 cells. Collectively, iptakalim, a cardiovascular $K_{ATP}$ channel opener, closes rat pancreatic $\beta$-cell $K_{ATP}$ channels, which may result from direct inhibition of the Kir6.2 subunit. Therefore, iptakalim bi-directionally regulates $K_{ATP}$ channels in cardiovascular and pancreatic tissues, and this unique pharmacological property suggests iptakalim could be used as a new therapeutic strategy for the treatment of type 2 diabetes with the potential benefit in alleviating cardiac and/or vascular disorders frequently associated with diabetes.

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**Key words:** iptakalim; $K_{ATP}$ channel; Kir6.2; islet $\beta$-cell; insulin secretion

## Introduction

Type 2 diabetes, characterized by high levels of blood glucose caused by decreased secretion of insulin from pancreatic $\beta$-cells and/or decreased insulin action, is prevalent worldwide and associated with morbidity and mortality secondary to complications such as myocardial infarction, stroke and end-stage renal disease[1]. The importance of tight control of blood glucose levels, in either preventing or delaying the progression of complications, is well-recognized. Although both the control of food intake and regular exercise are thought to benefit diabetes patients, more than 50% of diagnosed type 2 diabetes patients still require medication. Among the choices of anti-diabetic medications used to maintain normal blood glucose levels, the most effective ones reduce insulin resistance (e.g., metformin or glitazones), supply exogenous insulin or increase endogenous insulin release (e.g., sulfonylureas). Sulfonylureas, in particular, have been the leading oral antihyperglycemic agents used

[1] Division of Clinical Research, Hirosaki National Hospital, Hirosaki 036-8545, Japan
[2] Department of Physiology, Hirosaki University Graduate School of Medicine, Hirosaki 036-8562, Japan
[3] Department of Physiology and Membrane Biology, University of California, Davis, School of Medicine, CA 95616, USA
[4] Division of Neurology, Barrow Neurological Institute, AZ 85013-4496, USA

over the past half-century, and continue to be the most popular anti-diabetic drugs prescribed today for type 2 diabetes. The action of sulfonylureas is to trigger insulin release from pancreatic $\beta$-cells. SUR1, the regulatory subunit of pancreatic $\beta$-cell $K_{ATP}$ channels, is capable of binding sulfonylureas, leading to closure of $K_{ATP}$ channels, which in turn causes membrane depolarization, the opening of voltage-gated $Ca^{2+}$ channels and subsequently exocytosis of insulin. Meanwhile, sulfonylureas also interact with $K_{ATP}$ channels in extra-pancreatic tissues, such as cardiac, vascular and smooth muscles, and various brain regions[2]. Side actions caused by the use of sulfonylureas when treating diabetes patients have raised concerns that sulfonylureas may close cardiovascular $K_{ATP}$ channels thereby diminishing endogenously protective responses to ischemia that require $K_{ATP}$ channel activation. Indeed, it has been recognized that type 2 diabetes is associated with increased incidence of cardiovascular disease risk factors including hypertension, dyslipidemia, microalbuminuria and altered hemostasis[3]. Long-term use of sulfonylureas for treatment of type 2 diabetes has been cautiously implicated to be the cause, at least in part, of increased cardiovascular disorders, although experimental evidence remains obscure[4]. Meanwhile, the application of $K_{ATP}$ channel isoform-selective drugs, which close pancreatic $\beta$-cell $K_{ATP}$ channels without clearly affecting $K_{ATP}$ channels in the cardiovascular system, has been strongly recommended[5,6], and there is an urgent need to develop novel, tissue-selective anti-diabetic drugs. Although the major effect of sulfonylureas is to reduce blood glucose levels by closing pancreatic $\beta$-cell $K_{ATP}$ channels, an ideal anti-diabetic compound would be one that selectively closes pancreatic $\beta$-cell while opens cardiovascular $K_{ATP}$ channels, thereby simultaneously controlling glucose homeostasis and improving cardiac and/or vascular disorders. Unfortunately, no such compounds have yet available.

Iptakalim was initially designed and synthesized as an antihypertensive drug and exhibited remarkable antihypertensive effects in a variety of hypertensive animal modelsusing in vivo and in vitro preparations[7-11]. The molecular mechanisms underlying its antihypertensive effect include the opening of cardiovascular $K_{ATP}$ channels[7,8]. For example, iptakalim significantly enhanced $K^+$ currents recorded in whole-cell configurations in vascular smooth muscle cells isolated from pulmonary artery, as well as in isolated rat aorta denuded vascular endothelium[8]. Moreover, Wang et al. also showed that specific binding of the $K_{ATP}$ channel opener [$^3$H]P1075 was displaced by iptakalim in a concentration-dependent manner[8]. These results suggest that iptakalim serves as a $K_{ATP}$ channel opener due to its ability to open cardiovascular $K_{ATP}$ channels. Considering some of its advantages, such as being water-soluble, being able to freely penetrate the blood-brain barrier and exhibiting low-toxic side effects during systemic administration[8,12], iptakalim is a highly promising compound to serve as a useful pharmacological tool for studying $K_{ATP}$ channels as well as a therapeutic agent for antihypertension and cell protection. However, whether iptakalim affects $K_{ATP}$ channel function in pancreatic $\beta$-cells and alters insulin release has hitherto remained unknown.

In the present study, we examined the effects of iptakalim on cell excitability, $K_{ATP}$ channel function, intracellular $Ca^{2+}$ concentrations and insulin release using whole-cell and single-channel patch-clamp recordings, fura-2 fluorescence imaging and biochemical measurements in rat pancreatic $\beta$-cells or islets. To determine the possible molecular basis of iptakalim's action, we also examined its effects on heterologously expressed Kir6.2/SUR1 (a pancreatic $K_{ATP}$ channel isoform) and Kir6.2RKRR368/369/370/371AAAA (i.e.,

Kir6.2FL4A, a Kir6.2 trafficking mutant capable of functional expression without SUR) channels in HEK293 cells. We show that iptakalim failed to open β-cell $K_{ATP}$ channels; instead, it closed these $K_{ATP}$ channels, most likely by acting on the Kir6.2 subunit. By closing pancreatic β-cell $K_{ATP}$ channels, iptakalim depolarized β-cell plasma membrane, activated voltage-gated $Ca^{2+}$ channels and increased intracellular $Ca^{2+}$ concentrations, and in turn led to an increase of insulin release. This novel discovery suggests that iptakalim bi-directionally regulates the function if $K_{ATP}$ channels expressed in the cardiovascular system and pancreatic β-cells, and it may be used to as a new therapeutic strategy to treat hyperglycemia while improving the cardiac and/or vascular function of type 2 diabetes patients.

## Materials and Methods

This study was carried out in accordance with the Guidelines for Animal Experimentation, Hirosaki University, Japan, and USA.

## Pancreatic β-cell isolation

Isolation of rat islets was performed following a previously described protocol[52,53]. In short, adult male Wistar rats were anesthetized with diethyl ether, and 10 ml of Hank's buffered saline (HBSS) containing collagenase (200 U/ml, Wako Chem., Japan) was injected into the common bile duct. The pancreas, swollen with digestion solution, was quickly excised and incubated in a plastic culture bottle for 20 min at 37℃. The suspension obtained by shaking the bottle was filtered through 0.5-mm metal mesh and washed with HBSS, which included 2% bovine serum albumin (BSA). About 100 islets were obtained from one rat using the Histopaque (specific gravity 1.077, Sigma, St. Louis, MO) gradient method. After washing with HBSS, which contained 2% BSA, islets were cultured for 24 h with 5% $CO_2$ in tissue culture medium. Separation of islets was carried out using dispase (1000 U/ml, Godo Shusei, Japan) as previously described[52]. Separated cells were cultured for 1-4 days. Only single cells were chosen for experiments. β-cells were identified by detecting cell responses to 15 mM glucose or 500 μM tolbutamide (Sigma). Furthermore, the percentages of cells isolated from islets (α: 8%, β: 87%, δ: 4%)[54] were determined to determine whether results were to be included.

## Patch-clamp recordings

Cells were kept in a 35-mm Petri dish, and the dish was placed on the stage of an inverted microscope (IMT-2, Olympus, Tokyo, Japan). Membrane potentials and currents were measured using a patch-clamp amplifier (EPC-7, List Electronic, Darmstadt, Germany). The amphotericin B-based perforation patch-clamp method was used to measure the membrane potential and whole-cell currents[55]. The resistance of recording electrodes, when filled with pipette solution, ranged from 2 to 4 MΩ. In order to measure whole-cell membrane current, voltage-ramp pulses from -90 to -50 mV were repeatedly applied using a ramp pulse generator (SET-2100, Nihon Kohden, Tokyo, Japan). The membrane capacitance ranged from 8 to 14 pF. Series resistance below 12 MΩ was accepted. Single-channel current recordings were carried out in the cell-attached and inside-out configurations. All electrophysiological experiments were performed at room temperature (22±1℃). Data of single-channel currents were low-pass filtered at 1 kHz, digitized at 10 kHz and analyzed using a single-channel current analysis program (Clampfit 9.2, Axon Instruments, Foster City, CA). The concentration-inhibition curve created by iptakalim was fitted using Origin 5.0 (Microcal, North Hampton, MA).

For HEK293 cell recordings, the recording electrodes were prepared as described before[14]. The intracellular (bath) solution consisted of (in mM): KCl 110, $MgCl_2$ 1.44, KOH 30, EGTA

10, HEPES 10, pH to 7.2. The extracellular (intrapipette) solution consisted of (in mM): KCl 140, $MgCl_2$ 1.2, $CaCl_2$ 2.6, HEPES 10, pH to 7.4. All salts were obtained from Sigma. The equilibrium potential for potassium ions was around 0 mV as determined from the I-V relationship. Cell-attached single-channel recordings were performed at room temperature 48-72 hr after transfection. All patches were voltage-clamped at -60 mV intracellularly. Single-channel currents were recorded with Axopatch 200B amplifier (Axon Instruments) and were low-pass filtered (3dB, 2 kHz) and digitized at 20 kHz. Single-channel events were detected using Fetchan 6.05 (pCLAMP; Axon) and analyzed with Intrv5 (Dr. Barry S. Pallotta) as described before[14].

## Fura-2 $Ca^{2+}$ imaging

Isolated islets were placed in a glass-bottom culture dish and then loaded with a HEPES buffer solution (in mM: 140 NaCl, 4.7 KCl, 1.2 $MgCl_2$, 1.0 $CaCl_2$, 10 glucose and 10 HEPES) containing 1 $\mu$M fura-2/AM (Dojin, Kumamoto, Japan) for 20 min at room temperature. $Ca^{2+}$ images were captured using an inverted microscope with 40X Plan-Neofluar objectives (Axiovert 135, Zeiss, Oberkochen, Germany), a silicon intensifier target camera and recorded on a fluorescence-imaging system (Argus 50/CA, Hamamatsu Photonics, Hamamatsu, Japan). Excitation wavelengths were 340 nm and 380 nm, selected from a Xenon light source, and the emission wavelength was 510 nm[56]. All microfluorimetric experiments were carried out at room temperature.

## Measurement of insulin release

The amount of insulin released from islets was measured as previously described[57]. Briefly, isolated islets were hand-picked under a microscope, and 20 islets were relocated in a polypropylene syringe filter (0.45-mm filter, Corning Inc., Corning, NY) and continuously perfused with a control solution of HBSS containing 5.5 mM glucose, 10 mM HEPES and 2% BSA at a rate of 1 ml/min. Following pre-incubation for 30 min, the islets were perfused with the control solution for another 12 min, and then were stimulated by a high glucose concentration (22.5 mM), tolbutamide (500 $\mu$M) or iptakalim (100 $\mu$M) for 12 min. Before and during stimulation with glucose, tolbutamide or iptakalim, the perfusate was collected and stored at -20 ℃ until the assay. Insulin was measured by enzyme-linked immunosorbent assay using a microplate reader (Bio-Rad Laboratories, Inc., Hercules, CA). Insulin measurement kits were purchased from Morinaga Seikagaku Institute (Yokohama, Japan).

## Solutions and drugs

The standard external solution contained (in mM): 135 NaCl, 5.6 KCl, 1.2 $MgCl_2$, 1 $CaCl_2$, 5 glucose, 10 HEPES and pH 7.3 adjusted with NaOH. For perforated patch recordings, the pipette solution contained (in mM): 100 K-gluconate, 35 KCl, 5 glucose, 0.5 EGTA, 10 HEPES, 240$\mu$g/ml amphotericin B (Sigma) and pH 7.2. For cell-attached and inside-out single-channel recordings, the pipette solution contained (in mM): 135 KCl, 1.2 $MgCl_2$, 5 glucose, 0.5 EGTA, 10 HEPES and pH 7.3. The ionic composition of the solution inside the membrane (bath solution) during inside-out recordings was the same as the pipette solution, but the pH of this solution was 7.2. For studying ATP sensitivity, ATP (10 $\mu$M) was added to the bath solution when performing inside-out recordings. Iptakalim hydrochloride (N- (1-methylethyl) -1,1,2- trimethyl-propylamine hydrochloride) was kindly provided by Dr. H. Wang (Institute of Pharmacology and Toxicology, Beijing, P.R. China), and both diazoxide and tolbutamide were purchased from Sigma. Pancreatic $\beta$-cells in the bath were continuously exposed to a stream of external solution throughout the experiments.

## Statistics

Data are expressed as mean±SEM of several experiments, and statistical significance was evaluated by the two-tailed paired and unpaired Student's t-tests. P values less than 0.05 were considered to be significant.

## Results

### Iptakalim induced electrical excitation of single rat pancreatic β-cells

Using amphotericin B-based perforated whole-cell recordings in current-clamp mode, the resting membrane potential of rat $\beta$-cells was stable (-52.9±1.1 mV, n=28) and the cells were electrically silent (i.e., no spontaneous action potential firing) with 5.5 mM glucose in the external solution. Bath application of 22.5 mM glucose induced action potential firing following a slowly-developed membrane depolarization, suggesting that the high concentration of glucose increased $\beta$-cell excitability, presumably by increasing intracellular ATP production that led to the subsequent closure of $K_{ATP}$ channels. Under the same experimental conditions, iptakalim (100 $\mu$M; also slowly depolarized $\beta$-cell plasma membrane and elicited action potential firing. Moreover, in the presence of 10 $\mu$M nifedipine, an L-type $Ca^{2+}$ channel blocker that suppressed action potential firing, iptakalim depolarized, but diazoxide (100 $\mu$M, a classic SUR1-selective $K_{ATP}$ channel opener) hyperpolarized, pancreatic $\beta$-cell plasma membrane. These results suggest that iptakalim regulates $\beta$-cell excitability by closing $K_{ATP}$ channels.

### Iptakalim reduced whole-cell currents of $K_{ATP}$ channels in rat pancreatic β-cells

In order to determine whether currents permeating through pancreatic $\beta$-cell $K_{ATP}$ channels were inhibited by iptakalim, membrane currents induced by repetitive ramp pulses from -90 to -50 mV (at 0.2 Hz) were recorded in voltage-clamped whole-cell mode (external glucose=5.5 mM). The classic $K_{ATP}$ channel blocker tolbutamide (500$\mu$M) reversibly suppressed the ramp-evoked currents, suggesting that these currents were $K_{ATP}$ channel-mediated currents. Bath application of iptakalim (100 $\mu$M) reduced whereas diazoxide (100 $\mu$M) increased these currents. The inhibition of $\beta$-cell $K_{ATP}$ channels by iptakalim was concentration-dependent. The $IC_{50}$ and Hill coefficient of iptakalim were 30.4 $\mu$M and 0.8, respectively (n=7). These results suggest iptakalim closes $\beta$-cell $K_{ATP}$ channels in a concentration-dependent manner.

### Iptakalim decreased pancreatic β-cell $K_{ATP}$ channel open-probability in cell-attached and inside-out patches

Single-channel currents in pancreatic $\beta$-cells were recorded in cell-attached patches clamped at a pipette potential (Vp) of 0 mV. Bath-application of tolbutamide (500 $\mu$M) completely abolished, while diazoxide (100 $\mu$M) markedly enhanced, single-channel currents, indicating that the recorded currents were permeating through $K_{ATP}$ channels. Moreover, subsequent application of iptakalim (100 $\mu$M) reduced the single-channel currents. The values of open probability (NPo) before and during application of iptakalim were 0.147±0.014 and 0.067±0.007 (20 patches; $p<0.01$), respectively. From an analysis of total $K_{ATP}$ channel current amplitude distribution before and during application of iptakalim, it was clear that iptakalim reduced numbers of $K_{ATP}$ channel openings without altering single-channel conductance. The mean current amplitudes before and during application of iptakalim were 3.31±0.16 and 3.26±0.14 pA (20 patches; $p>0.05$), respectively. These results suggest that iptakalim suppresses $K_{ATP}$ channel currents in rat pancreatic $\beta$-cells.

In order to examine whether inhibition of $K_{ATP}$ channels by iptakalim was mediated by any effects on intracellular ATP concentrations, inside-out patch experiments were also performed. As shown in Figure 4, in the absence of intracellular ATP the mean open probability values were reduced from 0.120±0.025 (n=7) to 0.048±0.014 (n=7, $p<0.01$) by iptakalim (100 $\mu$M). In the presence of 10$\mu$M ATP, iptakalim (100 $\mu$M) reduced open-time probability from 0.074±0.012 (n=7) to 0.034±0.011 (n=6, $p<0.05$). These results suggest that the block of rat pancreatic $\beta$-cell $K_{ATP}$ channels by iptakalim is likely independent of intracellular ATP concentrations.

## Iptakalim elevated pancreatic β-cell intracellular $Ca^{2+}$ concentrations

To determine whether iptakalim-induced membrane depolarization triggered $Ca^{2+}$ influx (through voltage-gated $Ca^{2+}$ channels), intracellular $Ca^{2+}$ concentrations were measured using fura-2 fluorescence imaging. An increase in glucose concentration from 5.5 to 22.5 mM induced marked elevation of intracellular $Ca^{2+}$ concentrations. The changes were sensitive to diazoxide (100 $\mu$M; 6 cells), suggesting that the increase in intracellular $Ca^{2+}$ concentrations was due to glucose-induced closure of $K_{ATP}$ channels, which subsequently depolarized cell membrane and activated voltage-gated $Ca^{2+}$ channels. The closure of $K_{ATP}$ channels by tolbutamide (500 $\mu$M) caused a similar elevation of intracellular $Ca^{2+}$ concentrations (6 cells). Likewise, bath-applied iptakalim (100 $\mu$M) also induced an increase of intracellular $Ca^{2+}$ concentrations (8 cells), and the increase was sensitive to both nifedipine (1 $\mu$M; 5 cells) and diazoxide (100 $\mu$M; 8 cells). These results indicate that in rat pancreatic $\beta$-cells, iptakalim increases intracellular $Ca^{2+}$ concentrations, presumably by closing $K_{ATP}$ channels, which leads to cellular membrane depolarization and subsequent activation of L-type voltage-gated $Ca^{2+}$ channels.

## Iptakalim increased insulin release from rat pancreatic islets

The effects of iptakalim on insulin release were examined. With 5.5 mM glucose in the external solution, basal insulin secretion was measured. The application of 22.5 mM glucose increased insulin secretion from a basal level of 21.5±0.9 to 59.2±8.6 pg/islet/min ($p<0.01$, 5 cells), while 500 $\mu$M tolbutamide increased insulin secretion from 20.1±1.2 to 43.7±2.1 pg/islet/min ($p<0.01$, 5 cells) and 100 $\mu$M iptakalim increased insulin secretion from 22.5±1.2 to 34.5±1.8 pg/islet/min ($p<0.05$, 7 cells). These data indicate that iptakalim increases insulin secretion, presumably by inhibiting $K_{ATP}$ channels in rat pancreatic $\beta$-cells.

## Iptakalim inhibited Kir6.2/SUR1 and Kir6.2FL4A $K_{ATP}$ channels heterologously expressed in HEK293 cells

To determine whether the effects of iptakalim on $K_{ATP}$ channels were subunit-dependent, we examined its action on recombinant Kir6.2/SUR1 channels, a pancreatic $K_{ATP}$ channel isoform, in transiently expressed in HEK293 cells. The single-channel currents of Kir6.2/SUR1 channels in cell-attached patches were reduced by iptakalim, and the normalized NPo in the presence of 500 $\mu$M iptakalim was 0.49±0.15 (7 patches, $p<0.05$; control as 1). These results suggest that iptakalim suppresses the Kir6.2/SUR1 channel, supporting the inhibitory effects of iptakalim on native pancreatic $\beta$-cell $K_{ATP}$ channels described above. To determine whether iptakalim exerts its inhibitory action on Kir6.2/SUR1 channels by directly interacting with the pre-forming Kir6.2 subunit, we investigated the effect of iptakalim on the tetrameric Kir6.2FL4A channel in the absence of SUR subunits. The opening pattern of Kir6.2FL4A $K_{ATP}$ channels in cell-attached patches exhibited more brief

openings, a feature also observed in Kir6.2ΔC36 channels, another mutant of Kir6.2 that was capable of functional expression without the SUR subunit[13,14]. Iptakalim (100 and 500 $\mu$M) reduced the single-channel currents of Kir6.2FL4A channels in a concentration-dependent manner, and the normalized NPo values were 0.65±0.04 ($p<0.0001$; 8 patches) and 0.38±0.05 ($p<0.001$, 5 patches), respectively (control as 1). The single-channel conductance was not affected. These results suggest that iptakalim suppresses the function of $\beta$-cell $K_{ATP}$ channels by directly inhibiting the Kir6.2 subunit.

## Discussion

The novel and important finding obtained from the present study is that iptakalim, a cardiovascular $K_{ATP}$ channel opener, closed instead of opened rat pancreatic $\beta$-cell $K_{ATP}$ channels, which in turn increased cell excitability, elevated intracellular $Ca^{2+}$ concentrations and enhanced insulin release. Furthermore, by examining the heterologously expressed Kir6.2/SUR1 and Kir6.2FL4A $K_{ATP}$ channels in HEK293 cells, we provided direct evidence that iptakalim inhibits Kir6.2/SUR1 channels and that the molecular target responsible for interacting with iptakalim is the Kir6.2 subunit, which explained the effects of iptakalim in modulating cell excitability and insulin release in pancreatic $\beta$-cells. The findings that iptakalim closed $\beta$-cell $K_{ATP}$ channels, but opened cardiovascular $K_{ATP}$ channels[7-11], implicate a promising new therapeutic strategy for the treatment of patients afflicted with type 2 diabetes, as iptakalim may be beneficial in relieving symptoms of cardiac and/or vascular disorders or even improve cardiovascular function of diabetes patients.

### Iptakalim did not open β-cell $K_{ATP}$ channels

Iptakalim was initially designed as a new $K_{ATP}$ channel opener with a unique structure and antihypertensive effects; its antihypertensive effects are abolished by the $K_{ATP}$ channel blocker glibenclamide[7-11]. Using smooth muscle cells isolated from pulmonary artery as well as isolated rat aorta denuded vascular endothelium, Wang[8] reported that iptakalim significantly enhances whole-cell $K^+$ currents. Also, specific binding of the labeled $K_{ATP}$ channel opener [$^3$H]P1075 was displaced by iptakalim in a concentration-dependent manner[8]. These results indicate that iptakalim opens vascular $K_{ATP}$ channels, which may account for its antihypertensive effects. In the present study, however, iptakalim failed to open pancreatic $\beta$-cell $K_{ATP}$ channels, while the classic $\beta$-cell $K_{ATP}$ channel opener diazoxide clearly opened $\beta$-cell $K_{ATP}$ channels. Although the precise mechanisms are unclear, the $K_{ATP}$ channel subunits specifically expressed in $\beta$-cells (i.e., Kir6.2 and SUR1) may explain the inability of iptakalim to open $\beta$-cell $K_{ATP}$ channels.

It is well known that $K_{ATP}$ channels are expressed in a variety of tissues with different SUR subunits[15-17]. For instance, cardiovascular $K_{ATP}$ channels are formed by Kir6.2/6.1 and SUR2A/2B[18,19], pancreatic $\beta$-cell $K_{ATP}$ channels are formed by Kir6.2 and SUR1[20], while neuronal $K_{ATP}$ channel isoforms are Kir6.2/SUR1[21-24] and/or Kir6.2/SUR2B[24,25]. This tissue-specific expression of SUR renders different sensibilities of $K_{ATP}$ channels to metabolic stress, sulfonylureas and $K_{ATP}$ channel openers. It has also been reported that in the presence of ATP, pinacidil effectively opens Kir6.2/SUR2A, but fails to open Kir6.2/SUR1, $K_{ATP}$ channels in Xenopus oocytes[26,27]. Scuvee-Moreau et al.[28] compared the efficacy of $K_{ATP}$ channel openers in two types of central neurons and found that hippocampal CA1 pyramidal neurons were sensitive to the $K_{ATP}$ channel openers diazoxide, pinacidil and lemakalim, whereas A10 dopaminergic neurons were only sensitive to diazoxide. The presence of SUR1 also explains the activation of tolbutamide-

and iptakalim-sensitive $K_{ATP}$ channels by diazoxide in pancreatic β-cells in the present study, as diazoxide has been reported to bind to both SUR1 and SUR2 and opens Kir6.2/SUR1 and Kir6.2/SUR2B $K_{ATP}$ channels when MgATP is present[29-31]. In addition, our data obtained from recombinant Kir6.2/SUR1 channels in transfected HEK293 cells demonstrated that iptakalim failed to open, although diazoxide clearly opened, these channels[32]. Therefore, it is likely that the SUR2A and/or SUR2B, rather than the SUR1, subunit of $K_{ATP}$ channels may be the target of iptakalim that mediates $K_{ATP}$ channel activation.

## Iptakalim closed pancreatic β-cell $K_{ATP}$ channels

Iptakalim was unable to open pancreatic β-cell $K_{ATP}$ channels, perhaps due to the presence of the SUR1, but not the SUR2, subunit in these cells. Moreover, an intriguing finding revealed here was that iptakalim in fact closed pancreatic β-cell $K_{ATP}$ channels. It has been reported that PNU-99963, a non-sulfonylurea $K_{ATP}$ channel inhibitor, is structurally similar to the $K_{ATP}$ channel opener pinacidil[33]. Structurally, iptakalim is similar to the core portion of pinacidil, and it may be possible that $K_{ATP}$ channel opener analogues with such structure can inhibit $K_{ATP}$ channels. Would this be the possible structural basis for iptakalim's block of pancreatic β-cell $K_{ATP}$ channels? Several possible mechanisms may underlie the iptakalim-induced β-cell $K_{ATP}$ channel closure. First, iptakalim may bind to glibenclamide sites of the SUR1 subunit, thereby altering SUR subunit conformation, which in turn reduce β-cell $K_{ATP}$ channel opening. Emerging evidence has demonstrated that iptakalim-induced pharmacological effects in the cardiovascular and central nervous systems can be prevented by pretreatment with glibenclamide[9,10,12,34-36], suggesting that iptakalim and glibenclamide may compete for similar ligand binding sites on SUR2A/B subunits. In rat pancreatic β-cells, however, this mechanism appears to be absent since β-cell $K_{ATP}$ channels do not express SUR2A/B subunits. Second, iptakalim may suppress β-cell $K_{ATP}$ channel activity by increasing either ATP production or sensitivity of $K_{ATP}$ channels to intracellular ATP. We recently reported that systemic administration of either iptakalim or diazoxide protects rats against metabolic stress in a 6-OHDA-induced Parkinson's disease model, and that this protection is abolished by the relatively selective mitochondrial $K_{ATP}$ channel blocker 5-hydroxydecanoate[37], suggesting that iptakalim may open mitochondrial $K_{ATP}$ channels expressed in rat midbrain cells. Based on these results, it seemed possible that iptakalim would be able to open mitochondrial $K_{ATP}$ channels expressed in rat β-cells, depolarize mitochondrial membrane and alter ATP production. In addition, we previously found that some $K_{ATP}$ channel modulators regulate $K_{ATP}$ channel activity by altering $K_{ATP}$ channel sensitivity to intracellular ATP[38]. However, the results that iptakalim showed equal inhibition in the inside-out patch recordings with and without intracellular ATP appears to exclude this possibility. Finally, iptakalim may directly block β-cell $K_{ATP}$ channels by acting on the Kir6.2 subunit. It is well known that some $K_{ATP}$ channel modulators, such as nicorandil, pinacidil or glibenclamide, regulate $K_{ATP}$ channel activity by targeting the regulating subunit SUR[27,39], whereas others (e.g., phentolamine and cibenzoline) directly inhibit the pore-forming subunit Kir6.2[40,41]. Moreover, tolbutamide has been shown to act on both SUR1 and Kir6.2[29]. As previously discussed, since iptakalim-induced inhibition of pancreatic β-cell $K_{ATP}$ channels does not appear to result from interacting with SUR1 or altering β-cell $K_{ATP}$ channel sensitivity to intracellular ATP, another possible target of iptakalim may be the Kir6.2 subunit. The best model to test this

hypothesis is to use $K_{ATP}$ channels composed of the heterologously expressed truncated or mutant Kir6.2 subunit in the absence of the SUR subunit[41]. Our data obtained from the tetrameric Kir6.2 (i.e. Kir6.2FL4A) channels expressed in the absence of the SUR subunit suggest that the Kir6.2 subunit likely mediates iptakalim-induced pancreatic $\beta$-cell $K_{ATP}$ channel inhibition.

## Therapeutic implications

In pancreatic $\beta$-cells, $K_{ATP}$ channels play a pivotal role in maintaining plasma membrane potential and regulating cell excitability. The closure of these $K_{ATP}$ channels causes $\beta$-cells to depolarize, in turn activates voltage-sensitive $Ca^{2+}$ channels and increases cytosolic $Ca^{2+}$ concentrations, thereby leading to insulin release. Therefore, the pancreatic $\beta$-cell $K_{ATP}$ channel is thought to be a key target for the treatment of type 2 diabetes[6,15]. Indeed, many $K_{ATP}$ channel blockers, including tolbutamide, glyburide, gliclazide, nateglinide, repaglinide and glibenclamide have been used for many years for the treatment of type 2 diabetes[42-44]. On the other hand, $K_{ATP}$ channels are also widely expressed in a variety of different tissues, including heart, smooth muscle, skeletal muscle and the nervous system[15]. Blockade of $K_{ATP}$ channels in non-pancreatic tissues due to the treatment of type 2 diabetes using $K_{ATP}$ channel blockers may cause some severe side effects[45]. For example, it is believed that in the heart, $K_{ATP}$ channels play an important role in the intrinsic mechanisms that protect cardiac muscle during hypoxia/ischemia[46]. In arterial smooth muscle, $K_{ATP}$ channels help maintain contractile tone, in turn controlling blood pressure and blood flow[47]. In patients with type 2 diabetes that have been treated with $K_{ATP}$ channel blockers, the major cause of death is cardiovascular disease, which has been argued that could, at least in part, be related to the side effects of sulfonylureas in blocking cardiovascular $K_{ATP}$ channels[48-50]. Therefore, there is a substantial need to develop novel types of pancreatic $\beta$-cell $K_{ATP}$ channel blockers that exhibit little blocking effects on, or even open, cardiovascular $K_{ATP}$ channels. Unfortunately, there are no such optimal drugs that meet these specifications thus far. Although tolbutamide and gliclazide are reported to produce high-affinity closure of $\beta$-cell (Kir6.2/SUR1) but not cardiac (Kir6.2/SUR2A) or smooth muscle (Kir6.1/SUR2B), $K_{ATP}$ channels[5,27,51], they exhibit insignificant (or no) opening effects on cardiovascular $K_{ATP}$ channels. In the present investigation, we found, for the first time, that iptakalim closed $\beta$-cell $K_{ATP}$ channels, depolarized $\beta$-cells, elevated $\beta$-cell intracellular $Ca^{2+}$ concentrations and increased insulin release. The finding that iptakalim, a cardiovascular $K^{ATP}$ channel opener, blocked pancreatic $\beta$-cell $K^{ATP}$ channels indicates that iptakalim is a highly promising compound to satisfy therapeutic demands. Evidence has indicated that iptakalim exerts remarkable protective effects against cardiovascular disorders, especially hypertension, in a variety of animal models that use in vivo and in vitro preparations[7,8,11], and clinical trials to treat hypertension are currently being conducted[8]. The unique property of bi-directional regulation of pancreatic $\beta$-cell and cardiovascular $K_{ATP}$ channels suggests that iptakalim exhibits great potential to serve as, and stimulate the development of, a new generation of anti-diabetic (type 2 diabetes) drugs, and is particularly desirable for the treatment of type 2 diabetes in patients that have accompanying cardiac and/or vascular disorders. Considering its physical and pharmacological properties, such as being a small molecule, water-soluble, able to cross the blood-brain barrier, with few side effects after long-term systemic administration, iptakalim is a promising agent for the treatment of type 2 diabetes and may even benefit the preservation of normal functioning of the cardiac and/or vascular systems.

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