The new antihypertensive drug iptakalim activates ATP-sensitive potassium channels in the endothelium of resistance blood vessels

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Aim: To investigate the mechanisms underlying the activation of ATP-sensitive potassium channels (K_ATP) by iptakalim in cultured rat mesenteric microvascular endothelial cells (MVECs).

Methods: Whole-cell K_ATP currents were recorded in MVECs using automated patch clamp devices. Nucleotides (ATP, ADP and UDP) were added to the internal perfusion system, whereas other drugs were added to the cell suspension on NPC-1 borosilicate glass chips.

Results: Application of iptakalim (10 and 100 μmol/L) significantly increased the whole-cell K_ATP currents, which were prevented by the specific K_ATP blocker glibenclamide (1.0 μmol/L). The opening of K_ATP channels by iptakalim depended upon the intracellular concentrations of ATP or NDPs: iptakalim activated K_ATP channels when the intracellular ATP or NDPs were at 100 or 1000 μmol/L, and was ineffective when the non-hydrolysable ATP analogue ATPγS (1000 μmol/L) was infused into the cells. In contrast, the K_ATP opener pinacidil activated K_ATP channels when the intracellular concentrations of ATP or NDPs ranged from 10 to 5000 μmol/L, and even ATPγS (1000 μmol/L) was infused into the cells.

Conclusion: Iptakalim activates K_ATP channels in the endothelial cells of resistance blood vessels with a low metabolic status, and this activation is dependent on both ATP hydrolysis and ATP ligands.

Keywords: ATP-sensitive potassium channels; microvascular endothelial cells; iptakalim; pinacidil; glibenclamide; nucleotides; ATPγS; automated patch clamp

Introduction

ATP-sensitive potassium channels (K_ATP) link the electrical activity of the cell membrane to cellular metabolism. Because the activation of K_ATP is crucial for modulating the tone of the resistance vasculature, K_ATP channel openers (KCOs) have been used as important antihypertensives and have shown therapeutic efficacy[1-5].

Iptakalim, a new antihypertensive drug, is a KCO with a unique chemical structure that preferentially relaxes resistance blood vessels without affecting capacitance blood vessels[4]. Endothelium-denuded arterioles have a very weak maximal iptakalim-mediated dilatatory response[6]. Therefore, it was reasonable to hypothesize that iptakalim can regulate microvascular tension by activating K_ATP channels in the endothelium of resistance blood vessels. In this study, K_ATP channel opening induced by iptakalim was investigated in microvascular endothelial cells (MVECs).

It has been reported that MVECs are sensitive to metabolic status, which is dependent on K_ATP channel activity. MVEC metabolic disorders have been observed in hypertension, and K_ATP channels can be activated in MVECs with a low metabolic status caused by elevated shear stress[6-11]. Iptakalim strongly lowers blood pressure in hypertensive rodents and humans but has little effect in normotensive rodents and humans. This selective antihypertensive action has not been observed for pinacidil, which is the most commonly studied KCO[4].

A cell internal/external perfusion method was used to investigate the effects of iptakalim on K_ATP channels and the resultant modulation of metabolic status.

Materials and methods

Cell culture

The Local Ethics Committee on Animal Studies and the Academy of Military Medical Sciences (Beijing, China) approved...
this protocol. Eighty-eight Sprague–Dawley rats were used in this study (Experimental Animal Center, Academy of Military Medical Science, Beijing, China). MVECs were isolated from the mesentery of eight 6-day-old rats for each cell culture experiment, as previously described in the literature\cite{12,13}. Briefly, the heart was perfused with phosphate buffered saline (PBS) to flush out the blood cells from the mesenteric vessels after the abdomen was opened. The mesenteric vascular bed was digested with an enzyme solution containing collagenase, papain, dithiothreitol, deoxyribonuclease, and bovine serum albumin for 1.0 h at 37°C. The cells were then centrifuged and plated on a 60-cm\textsuperscript{2} culture plate coated with 0.5% gelatin in medium 199 supplemented with 10% fetal bovine serum (FBS), 1.0% heparin, 1.0% endothelial cell growth factor, and antibiotics. After 2.0 h, nonadherent cells were removed, and adherent endothelial cells were further cultured. Approximately 80% confluency was reached after the cells had been cultured for 4–5 d.

Platelet-endothelial cell adhesion molecule (PECAM/CD31) was identified by indirect immunofluorescence using a rabbit anti-rat CD31 antibody as the primary antibody and a fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G as the secondary antibody. More than 95% of the enriched cells cultured from the third passage were CD31\textsuperscript{+}. Only cells from the third to fifth passages were used for the experiment to prevent cellular alterations as a result of additional passages.

**Electrophysiology**

Optimal results were obtained after splitting the cells every second or third day to avoid confluency. The confluence of MVECs at the time of harvesting was between 50% and 80%. For the automated patch clamp experiment, MVECs were washed twice with PBS (free of Ca\textsuperscript{2+} and Mg\textsuperscript{2+}) and then dissociated with 2.0 mL of warmed 0.125% trypsin for 5.0 min at 37°C. The cell suspension was then transferred to a 14-mL tube containing 9.0 mL FBS-containing medium and centrifuged at 100×g for 2.0 min at room temperature. After discarding the supernatant, the cell pellet was resuspended in an external solution at a final density between 1×10\textsuperscript{5} and 5×10\textsuperscript{6} cells/mL. A symmetrical high K\textsuperscript{+} condition was used to facilitate K\textsubscript{ATP} activation\cite{14}. The external solution for monitoring K\textsubscript{ATP} activity consisted of the following (in mmol/L): 140.0 KCl, 1.0 CaCl\textsubscript{2}, 1.0 MgCl\textsubscript{2}, and 5.0 HEPES (pH 7.4 with KOH).

The internal solution contained the following (in mmol/L): 140.0 KCl, 2.0 MgCl\textsubscript{2}, 5.0 EGTA, and 5.0 HEPES (pH 7.25). Nucleotides were dissolved in the internal solution, which contained 1.4 mmol/L free Mg\textsuperscript{2+} adjusted by MgCl\textsubscript{2}. To evaluate the influence of Mg\textsuperscript{2+} on drug efficacy, Mg\textsuperscript{2+} in the internal solution was omitted, and 5.0 mmol/L EDTA was added instead of EGTA. Nucleotide-containing solutions were freshly prepared on the day of the experiments. The pH of the solutions was readjusted after adding the nucleotide or drug.

Whole-cell patch clamp recordings were performed using a Port-a-Patch system driven by PatchControl software (Nanion Technologies, Munich, Germany) and a HEKA EPC-10 amplifier. Single-use NPC-1 chips with a resistance of 3.0–5.0 MΩ (Nanion Technologies, Munich, Germany) and a ground reference were used. Whole-cell signals were filtered at 2.0 kHz. Currents were recorded using the PatchMaster and FitMaster acquisition and analysis software (HEKA Elektronik, Lambrecht, Germany) on a DELL Vostro 220 computer. The holding potential was -10 mV, which is similar to the resting membrane potential under symmetrical high K\textsuperscript{+} conditions. Currents were elicited by 500-ms depolarizing voltage steps from -100 to +50 mV in 10-mV increments. Whole-cell capacitance and series resistance were adjusted and monitored. A nucleotide-free internal solution was used to form the seal and record baseline currents. Control currents remained unchanged after 30 min (data not shown). In the internal perfusion experiments, stable currents were measured within several minutes of adding intracellular nucleotides to the internal perfusion system, and drugs were added to the cell suspension on the NPC-1 chip at 5.0 min during each recording. The summarized data were measured at -100 mV, and responses to iptakalim and nucleotides were expressed relative to the control currents measured in the external/internal solution without drugs or nucleotides.

**Drug and solution**

Iptakalim was synthesized by Thadweik Academy of Medicine, Beijing, China. All other chemicals were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA). Glibenclamide and pinacidil were stored as a 10 mmol/L stock solution in dimethyl sulfoxide. Other stock solutions were prepared in distilled water. Stock solutions were frozen at -20°C. Prior to usage, they were diluted in the internal or external solution to produce the desired concentration.

**Statistical analysis**

Data are expressed as the mean±standard deviation (SD), and n indicates the number of cells under each experimental condition (each column). Significance was assessed using a two-tailed Student’s t-test to compare different groups. The statistical analyses were performed using SPSS 13.0 (SPSS Inc, Chicago, IL, USA), and P<0.05 was considered statistically significant.

**Results**

The opening effects of iptakalim

The K\textsubscript{ATP} currents of MVECs were recorded by whole-cell patch clamp with a membrane potential of -100 mV (Figure 1). Whole-cell currents were suppressed by application of 1000 μmol/L intracellular ATP. These currents were enhanced with 1000 μmol/L intracellular ADP or UDP. This result indicates that the toggled channels were in fact K\textsubscript{ATP} channels. In the presence of 1000 μmol/L of ATP, ADP, or UDP, 1.0–100 μmol/L iptakalim increases the whole-cell currents. This can be prevented by application of 1.0 μmol/L glibenclamide, a specific K\textsubscript{ATP} blocker. The currents of the control groups, in the presence of only 1000 μmol/L of ATP, ADP, or UDP, also decreased with application of 1.0 μmol/L glibenclamide.
The currents decreased by a similar amount to that in groups treated with 100 μmol/L iptakalim, which suggests that the iptakalim-induced currents were completely blocked by 1.0 μmol/L glibenclamide.

**The modulatory effects of ATP**

The modulatory effects of intracellular ATP on the channel-opening ability of 100 μmol/L iptakalim or 100 μmol/L pinacidil were investigated. Intracellular ATP was added at 10, 100, 1000, 3000, or 5000 μmol/L, and the resultant current amplitudes were 92.42±1.48%, 100.67±3.87%, 83.1%±0.55%, 74.08%±2.69% and 67.3%±2.02% of the control amplitude, respectively. These results suggest that MVEC whole-cell currents can be regulated by intracellular ATP. The channel-opening effects of iptakalim were observed in the presence of 10 or 100 μmol/L ATP (Figure 2A). Under the same experimental conditions, the opening effects of pinacidil were observed in the presence of 10 to 5000 μmol/L ATP (Figure 2B, P<0.01).

The mechanism by which ATP modulates the K<sub>ATP</sub> opening effects of iptakalim was also investigated. The opening effects of iptakalim and pinacidil were compared in the presence of ATP with Mg<sup>2+</sup>, ATP without Mg<sup>2+</sup>, and ATPγS (a nonhydrolysable ATP analogue) with Mg<sup>2+</sup>. Pinacidil activated K<sub>ATP</sub> in the presence of ATPγS, but iptakalim did not. Both iptakalim and pinacidil were Mg<sup>2+</sup> dependent. These results suggest that ATP hydrolysis was necessary for iptakalim to activate K<sub>ATP</sub> channels and that ATP ligands are involved in K<sub>ATP</sub> activation by pinacidil (Figure 3).

**The modulatory effects of NDPS**

As shown in Figure 2C and E, the opening effects of iptakalim...
were induced by NDPs, such as ADP and UDP, at concentrations of 100 and 1000 μmol/L but not at 10, 30, or 3000 μmol/L. This was not the case for pinacidil. The channel-opening effects of pinacidil were induced by ADP or UDP at all tested concentrations (10–3000 μmol/L), which is a larger range of activation than that of iptakalim (Figures 2D, F).

**Discussion**

In our previous research, multiple pharmacological inhibitors were used to explore the signaling pathways underlying the action of iptakalim on mesenteric arterioles. Iptakalim-induced vasodilation was not affected by the K⁺ channel blockers Ba²⁺, 5-HD, TEA, and 4-AP, but it was inhibited by glibenclamide (1.0–1000 μmol/L) in a dose-dependent fashion (unpublished data). The electrophysiology method was used to confirm the direct activation of K<sub>ATP</sub> channels in mesenteric endothelium in this study.

The K<sub>ATP</sub> channel activities were tested on MVECs using whole-cell recordings. In recordings using a nucleotide-free internal solution, some portion of K<sub>ATP</sub> was activated. This
Figure 3. Modulation of the opening effects of iptakalim and pinacidil in microvascular endothelial cells (MVECs) by ATP. The opening effects of 100 μmol/L iptakalim (Ipt, A) or pinacidil (Pin, B) were tested in the presence of MgATP, MgATPγS, or Mg-free ATP (1 mmol/L). The K<sub>ATP</sub> currents were recorded in MVECs with a membrane potential of -100 mV. *P<0.01 vs control. (C) Typical recordings showed the modulatory effects of ATP on iptakalim-mediated activation.

**Figure 3:***

**A**

| Normalized amplitude (%) | MgATP | MgATPγS | Mg-free ATP |
|--------------------------|-------|---------|-------------|
| Control                  | 100   | 100     | 100         |
| Ipt 100 μmol/L           | 90    | 90      | 90          |

**B**

| Normalized amplitude (%) | MgATP | MgATPγS | Mg-free ATP |
|--------------------------|-------|---------|-------------|
| Control                  | 100   | 100     | 100         |
| Pin 100 μmol/L           | 90    | 90      | 90          |

**C**

| (Nucleotide-free) | ATP 1 mmol/L | ATP 1 mmol/L + Ipt 100 μmol/L | ATPγS 1 mmol/L | ATPγS 1 mmol/L + Ipt 100 μmol/L | Mg-free ATP 1 mmol/L | Mg-free ATP 1 mmol/L + Ipt 100 μmol/L |
|-------------------|--------------|-------------------------------|---------------|---------------------------------|-----------------------|----------------------------------------|
| Control           | 100          | 100                           | 100           | 100                             | 100                   | 100                                    |

Partially activated state was observed at two stages of the experiment—suppression after the addition of intracellular ATP, and enhancement after the addition of NDPs. Neither iptakalim nor pinacidil had an effect on cells in the partially activated state under nucleotide-free conditions (data not shown); therefore, it was reasonable to speculate that the K<sub>ATP</sub> opening effects of iptakalim and pinacidil are dependent on the presence of intracellular nucleotides. As expected, both drugs caused a significant increase in membrane conductance in the presence of nucleotides. When intracellular nucleotides (ATP, ADP, or UDP) were infused into the MVECs at a concentration of 1000 μmol/L, iptakalim increased whole-cell currents in a dose-dependent fashion. That result was reversed with the addition of 1.0 μmol/L glibenclamide (Figure 1). This finding provides evidence that iptakalim can activate K<sub>ATP</sub> channels in MVECs.

The modulatory effects of intracellular nucleotides were further tested in this study. The required nucleotide concentration for iptakalim was found to be different from that observed for pinacidil. Iptakalim activated K<sub>ATP</sub> channels only at low concentrations of ATP or NDPs (100–1000 μmol/L), but pinacidil was effective at all tested ATP or NDP concentrations, even those <100 μmol/L or >1000 μmol/L (Figure 2). Several studies have shown that a complicated interaction exists between the functional sites of K<sub>ATP</sub>[15]. ATP and NDPS are major allosteric modulators for K<sub>ATP</sub> channels and affect drug binding differently[16-22]. Allosteric regulation by nucleotides makes the K<sub>ATP</sub> opener binding site accessible to iptakalim or pinacidil. Iptakalim attaches to the binding site in a different way than pinacidil does because of differences in chemical structure[1], and iptakalim shows a higher selectivity than pinacidil for nucleotide supplements of MVECs.

The differences between iptakalim and pinacidil in terms of the modulatory effects of ATP were further compared in this experiment (Figure 3). Iptakalim’s ineffectiveness in the presence of ATPγS suggests that ATP hydrolysis is required to activate K<sub>ATP</sub> channels in MVECs. The opening effects of pinacidil are more potent in the presence of ATPγS than in the presence of ATP (120.45±4.36 vs 100.61±4.17, P<0.05, Figure 3B), which suggests that ATP ligands are required for pinacidil activation. ATP binding alone appears to be sufficient for pinacidil to activate K<sub>ATP</sub> channels; however, for iptakalim, ATP binding with subsequent hydrolysis is necessary.

K<sub>ATP</sub> channel participates in metabolism-related vascular responses[23, 24]. In studies involving endothelial cells, it was shown that one of its main physiological functions is to serve as a metabolic barrier. The physiological concentration of ATP ranges from 3.0 to 5.0 mmol/L in rats. Intracellular NDPS
play a critical role in the regulation of cellular energy status\(^{[25]}\). When MVECs were internally perfused with ATP at physiological or extremely low concentrations, pinacidil, but not iptakalim, affected the \(K_{\text{ATP}}\) channels. This finding indicates that iptakalim might preferentially activate \(K_{\text{ATP}}\) in cells with a specific pathological metabolic status. Endothelial cells and \(K_{\text{ATP}}\) functions are altered by hypertension\(^{[26-27]}\), and pathological changes in hypertension result in an energy metabolism disorder in endothelial cells\(^{[5-10]}\). This selectivity of cell energy status by iptakalim might be responsible for its targeted hypertension therapeutic effect. Pinacidil, but not iptakalim, activates \(K_{\text{ATP}}\) in the presence of NDPs at high concentrations (3.0 mmol/L), which indicates that iptakalim might be more suitable for mild-to-moderate hypertension. Pinacidil was shown to be non-selective for nucleotide supplements in our research. It was effective under both physiological and pathological conditions, which might be the underlying cause of its side effects\(^{[28]}\). Our results offer a new perspective: activation by iptakalim might link functional changes in EC to the perturbation of normal metabolic supplies and the consequent changes in the intracellular concentration of nucleotides. It is reasonable to presume that because of its unique effects, iptakalim might have an advantage in endothelial metabolism-related cardiovascular diseases, such as hypertension and pulmonary hypertension.

Seal formation was particularly difficult in whole-cell recordings of MVECs because of their small size and membrane properties. The internal perfusion method using NPC-1 borosilicate glass chips with a 1.0 µm aperture provided electrophysiological access not only to facilitate seal formation but also to maximize the duration of high-quality recordings. In recent years, studies using patch clamp chips have stressed the advantages of enhanced resolution\(^{[29]}\). Because of its stable and steady nature, the method can be used successfully in whole-cell recordings of MVECs.

**Perspectives**

The functions of endothelial \(K_{\text{ATP}}\) in resistance blood vessels can be altered by exposure to elevated shear stress caused by hypertension. The pharmacological characteristics of iptakalim are related to endothelial cell metabolic status, which explains its highly selective antihypertensive activity. The clinical evaluation of iptakalim in hypertension and pulmonary hypertension is ongoing, and iptakalim could become a new generation antihypertensive drug.

**Abbreviations**

4-AP, 4-aminopyridine; 5-HD, 5-hydroxydecanote; ADP, adenosine diphosphate; ATP, adenosine triphosphate; ATP\(\gamma\)S, adenosine 5'-O-(3-thiotriphosphate); EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol tetraacetic acid; FBS, fetal bovine serum; Gli, glibenclamide; Ipt, iptakalim; \(K_{\text{ATP}}\), ATP-sensitive potassium channel; MVEC, microvascular endothelial cell; PBS, phosphate buffered saline; PECAM/CD31, platelet-endothelial cell adhesion molecule; Pin, pinacidil; TEA, tetraethylammonium; UDP, uridine diphosphate.

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**Author contribution**

Hai WANG designed the study; Su-yang WANG performed the experiments; Su-yang WANG and Wen-yu CUI analyzed the data and wrote the paper.

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