Styrax blocks inward and outward current of Kir2.1 channel

Shuxi Ren, Chunli Pang, Junwei Li, Yayue Huang, Suhua Zhang, Yong Zhan, and Hailong An

Key Laboratory of Molecular Biophysics, Hebei Province, Institute of Biophysics, School of Sciences, Hebei University of Technology, Tianjin, China

ABSTRACT
Kir2.1 plays key roles in setting rest membrane potential and modulation of cell excitability. Mutations of Kir2.1, such as D172N or E299V, inducing gain-of-function, can cause type 3 short QT syndrome (SQT3) due to the enlarged outward currents. So far, there is no clinical drug target to block the currents of Kir2.1. Here, we identified a novel blocker of Kir2.1, styrax, which is a kind of natural compound selected from traditional Chinese medicine. Our data show that styrax can abolish the inward and outward currents of Kir2.1. The IC50 of styrax for WT, D172N and E299V are 0.0113 ± 0.00075, 0.0204 ± 0.0048 and 0.0122 ± 0.0012 (in volume), respectively. The results indicate that styrax can serve as a novel blocker for Kir2.1.

Keywords: blocker; fluxORTM; ion channel; Kir2.1; patch clamp; SQT3; styrax

Introduction
Kir2.1 channel, mainly expressed in the cardiac muscle and skeletal cells, is a member of inwardly rectifying potassium channel family composing 7 different subfamilies. The typical inward rectifying current of Kir2.1 was first recorded in frog skeletal muscle in 1949. And more than 50 y later, its cDNA was cloned from a mouse macrophage cell line by Kubo et al. This protein has tetrameric structure and each subunit contains 2 transmembrane helix (M1 and M2), the ion-selective P-loop between M1 and M2, and N- and C-terminal domains which are exposed to the cytoplasm. The channel’s current is sensitive to membrane potential and its outward current will disappear because Mg2+ and polyamines block K+ permeation in the intracellular. On the other hand, phosphatidylinositol 4,5-bisphosphate [PIP2] is necessary to sustain the normal function of Kir2.1 channel and the current of Kir2.1 gradually declined to zero as the consumption of PIP2. The functions of Kir2.1 channel are to maintain the rest potential, control excitability and adjust the time of the repolarization of a cell. The abnormal function of Kir2.1 protein can cause the Andersen’s syndrome (LQT type 7) for the loss of function mutation and type 3 short QT syndrome (SQT3) for the gain of function mutation. SQT3 is characterized by abnormally short QT interval (<360 ms) in the electrocardiogram (ECG) with symptoms of atrial fibrillation, cardiac death, ventricular arrhythmias, syncopal event and palpitations. The single point mutation of D172N, E299V, M301K, K346T of Kir2.1 will cause greater outward current (gain of function), and thus accelerate the repolarization process. The patient had to choose to place implantable cardioverter defibrillator (ICD) because there is no effective medicine. But the implant of ICD has serious neurologic damage to people, especially for children. So, clinical inhibitors or blockers of Kir2.1 are needed urgently. People have made great effort to find effective compounds which can be applied in clinical therapy. Chloroethylclonidine inhibit the inward current with an IC50 of 30µM in skeletal muscle at a membrane potential of −50 mV. Zaks-Makhina E et al. discovered an inhibitor named 3-bicyclo[2.2.1]hept-2-yl-benzene-1,2-diol (48F10) (IC50 = 6 µM) from 10,000 small molecules using the method of high-throughput screening in yeast. Celastr (3-hydroxy-24-nor-2-oxo-1(10),3,5,7-friedelatetraen-29-oic acid) can not only inhibit the Kir2.1 channel current, but also reduce the transport rate.
of the channel protein, and thus reduce the membrane expression of Kir2.1 channel.35 The Kir2.1 current inhibited by tamoxifen (IC50 = 0.93 μM) cannot be fully restored. The mechanism of inhibition is not because of pore block but the interference with interaction between the Kir2.1 channel and PIP2.36 Gambogic Acid (IC50 = 27 nM) spend 3 hours to inhibit the current of Kir2.1 channel through changing channel membrane microenvironment.37 Chloroquine (IC50 = 1.2 μM) which binds at the center of the ion permeation vestibule of Kir2.1 can inhibit the Kir2.1 channel current more effectively than quinidine (IC50 = 57 μM).38 Five years ago, Hao-Ran Wang et al. obtained an inhibitor of Kir2.1–ML133 (N-(4-Methoxybenzyl)-1-(naphthalen-1-yl)methanamine) with an IC50 of 1.8 μM utilizing high-throughput Ti⁺ flux screening method.39 However, these inhibitors are chemo synthetic and difficult to evaluate their toxicological and pharmacological actions.

Dictionary of Chinese Medicine records the experience of treating diseases with various traditional Chinese medicines, and some can regulate the rhythm of the heart. Here, we focused on the traditional Chinese medicines and found that styrax, a kind of traditional Chinese medicine, can block the current of Kir2.1 channel utilizing high-throughput Ti⁺ flux screening method. For further validation, we performed whole cell and excised patch clamp on both WT and mutant Kir2.1 channels. Our data show that styrax can block the inward and outward currents of Kir2.1. Thus, styrax may serve as a novel blocker of Kir2.1 channels.

Results

The Ti⁺ flux screening methods indicate that styrax can abolish the currents of Kir2.1 channel

FluxOR is a kind of fluorescent probe which has an increased fluorescent intensity after the combination with the Ti⁺ which can flow into the cell through the Kir2.1 channels.40 So, the fluorescent intensity of FluxOR in cell will increase if the Kir2.1 channel is open, and it won’t when the channel is blocked. Here, we selected 25 traditional Chinese medicines from Dictionary of Chinese Medicine as follows: ginsenoside Rb₂, ginsenoside Rb₁, ginsenoside Rb₂, ginsenoside Rd, ginsenoside Rg₁, ginsenoside Rg₂, ginsenoside Rg₃, notoginseng neaf triterpenes, notopterol, liensinine perchlorate, cyclovirobuxine D, osthole, 2’-o-galloylhyperin, puerarin, garlicin, taruine, evening primrose oil, tetradrine, styrax, artemisinin, sinomenine, atrine, oxy-matrine, hyperoside and isoflavones aglycone.

We found that only one of the 25 traditional Chinese medicines, styrax, can block the influx of Ti⁺ from the Kir2.1 channels (Fig. 1). Our data show that 5% (in volume) of styrax completely abolished the increase of the fluorescence of FluxOR by Ti⁺ influx. As negative control, the blank HEK293T cells doesn’t show any change of the fluorescence with or without Ti⁺. The fluorescent increase constants for blank, styrax (−) and styrax (+) are 0.233 ± 0.014 (n = 10), 2.32 ± 0.13 (n = 10) and 0.369 ± 0.017 (n = 10), respectively (Fig. 1C). Thus, styrax can block the currents of Kir2.1 channel effectively.

Patch clamp experiments confirm the blockage of styrax to the Kir2.1 channel

We next verified the blockage of styrax to the Kir2.1 channel using excised patch clamp experiments. As showing in Fig. 2A, the inward currents of the Kir2.1 channel were completely blocked by 5% styrax (in volume) and restored to original level after washing out the styrax. Then, we measured the time courses of styrax washin and washout. As shown in Fig. 3, the time constants for blockage and restore of Kir2.1 currents are 10.12 ± 3.09 s and 25.73 ± 4.14 s, respectively.

Then we measured the dose-response relationship for styrax blockage of the inward currents of Kir2.1. The dose-response curve of styrax at −80 mV was made by different styrax concentrations. The IC50 was 0.01339 ± 0.00075 (n = 6) (Fig. 2B). So, the excised patch clamp results also confirm that styrax is a blocker of Kir2.1 channel.

Styrax can block the Kir2.1 currents from the extracellular side

To confirm if the styrax can block the currents of Kir2.1 from the extracellular side, we performed whole-cell patch clamp experiments. As shown in Fig 4A, styrax abolishes the whole-cell currents of Kir2.1 from the extracellular side. And the IC50 at −80 mV were 0.02840 ± 0.00046 (n = 6) (Fig. 4B). It was more than twice of that on excised patch mode. Thus we think that the binding sites were on the membrane’s inner leaflet and styrax can pass through membrane into the cytoplasm. The time of the penetration was very short because styrax spends the same time to block the current of Kir2.1 on both modes (Fig. 5).
Styrax can block the outward currents of the SQT3 related mutants (D172N and E299V) of Kir2.1

The mutants of D172N and E299V (Fig. 6A) weaken the rectification of Kir2.1 and permeate the outward currents (gain of function) (Fig. 6B and C) which accelerates the repolarization process and last produces a shorter QT interval (<360 ms) on an electrocardiogram (ECG). So, the blockage to the outward currents of the Kir2.1 mutants is more significant. Then we moved to verify if styrax can block the outward currents permeated by Kir2.1 mutants, D172N or E299V. As shown in Fig. 6, styrax can block the inward and outward currents of WT and SQT3 related mutants in a reversible way. The IC50 at 30 mV were 0.0204 ± 0.0048 (n = 4) for D172N and 0.0122 ± 0.0012 (n = 8) for E299V (Fig. 6D).

Discussion

Kir2.1 mainly distributes in cardiac and skeletal muscle cells and modulates the excitability of muscle cells. Abnormal function of Kir2.1 will induce several diseases, such as Andersen syndrome and SQT3 syndrome. It has been identified that the gain function mutants, D172N and E299V, of Kir2.1 can cause SQT3 syndrome by enhancing the outward currents. However, there is no any clinical drug for SQT3 targeting at Kir2.1.

We started to screen 25 Chinese herbal which are thought to relief the arrhythmia by the Tl+ flux screening methods. Among these Chinese herbal, styrax was identified to block both the inward and outward currents of WT and SQT3 related mutants of Kir2.1. To our knowledge, it is the first blocker for Kir2.1 and its mutants from Chinese herbal.

As we know that traditional Chinese medicine (TCM) has been developed for more than 2000 y and widely used in China and also in west. However, the TCM is group of effective compounds. It is hardly to say which compound plays a key role. Here, we identified that styrax may serve as a targeted drug to SQT3 syndrome. In fact, styrax is a mixture which composes of hundreds of monomers. We should go further...
to confirm which monomer plays a critical role in block Kir2.1 channel and understand the mechanism of blockage to Kir2.1 by the monomer in the future.

**Methods**

**Cell line, transfection reagent, and chemicals**

HEK293T cells were cultured in DMEM supplemented with 10% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO₂. One day before transfection, cells were seeded on coverslips (0.13 – 0.17 mm thick) in 24 - well plates. Twelve hours later, transient transfection was performed before experiment using XtremeGENE HP DNA Transfection Reagent (Roche, Mannheim, Germany). There were 500 ng DNA, 1.5 μL X-tremeGENE HP and 50 μL Opti-MEM® I (Life, USA) for every well. At last, cells were transferred to incubator at 37°C in a humidified atmosphere containing 5% CO₂.
Figure 4. Styrax can also block the currents of Kir2.1 on whole-cell patch clamp mode. (A) Currents recorded using whole-cell patch clamp with HEK293T cells expressing Kir2.1. The voltage protocol was a 1-s ramp from $-100$ mV to $+100$ mV. The three curves were attained from one seal in the perfusion with bath solution with or without styrax in turn. The volume ratio of styrax and bath solution was 0.05. (B) Dose-response relationship for styrax blockage. Styrax concentrations ($V_{\text{styrax}}/V_{\text{bath solution}}$) were 0.017, 0.021, 0.026, 0.033, 0.041, and 0.05. The current was measured at $-80$ mV. Data points represent mean $\pm$ SE ($n = 6$). IC$_{50}$ = 0.02840 $\pm$ 0.00046.

Figure 5. The time course of blockage of styrax to Kir2.1 on whole-cell patch clamp mode. (A) The representative time courses (black) of blockage (blocked by styrax) and open (washing out styrax) at $-80$ mV on whole-cell mode. The red lines represent a single exponential best fit. (B) Bars are the time constants (mean $\pm$ SE $n > 9$) corresponding to A. The time constants for block and open times were 12.63 $\pm$ 3.14 s and 13.70 $\pm$ 3.18 s.
containing 5% CO₂ for 24—36 hours before recording.

Twenty-five kinds of traditional medicines (ginsenoside Rb0, ginsenoside Rb1, ginsenoside Rb2, ginsenoside Rd, ginsenoside Rg1, ginsenoside Rg2, ginsenoside Rg3, notoginseng neaf triterpenes, notopterol, liensinine perchlorate, cycloviroxbuxine D, osthole, Z-α-galloylhyperin, puerarin, garcinic, taruine, evening primrose oil, tetradrine, styra, artemisinin, sinomenine, atrine, oxmatrine, hyperoside, isoflavoues aglycone) for potential modulators of Kir2.1 channel come from the National Institutes for Food and Drug Control of China (Beijing, China). All other chemicals were purchased from Sigma-Aldrich (Shanghai, China) in addition to being annotated.

Site-directed mutagenesis

Human Kir2.1 pcDNA3.1 was generously provided by Prof. Hailin Zhang (Department of Pharmacology, Hebei Medical University, Shijiazhuang, China). The single point mutants were made with a QuickChange II site-directed mutagenesis Kit (Agilent Technologies, USA) and confirmed by DNA sequencing (Sangon Biotech, Shanghai, China).

Thallium-based fluorescence assay

The thallium-based fluorescence assay was performed using FluxOR™ Potassium Ion Channel Assay (Life, USA, Cat no. F10016). Loading buffer, assay buffer and

Figure 6. Styrax can block the outward current of the mutants (D172N and E299V) of Kir2.1. (A) The positions of S165 (red) and E299 (yellow). (B) D172N and E299V currents recorded from transfected HEK293T cells under inside-out patch clamp. The voltage protocol was a 1- s ramp from -100 mV to +100 mV. (C) D172N and E299V dose-response relationships for styra blockage. Styra concentrations (Vstyra/Vbath solution) were 0.002, 0.004, 0.008, 0.017, 0.03 and 0.05. The current was measured at + 30 mV. Data points represent mean ± SE (n = 4 for D172N and n = 8 for E299V). IC₅₀ of D172N and E299V were 0.0204 ± 0.0048 and 0.0122 ± 0.0012 respectively.
stimulus buffer were prepared according to the manufacturer’s protocol. There were no coverslips in 24-well plates in this experiment. Briefly, remove media from cells and add 400 μL of loading buffer to each well; incubated at room temperature for 60 minutes in dark; the loading buffer was replaced by 400 μL assay buffer for each well; test compounds were added to the assay buffer with final concentration of 1 mM; 10 minutes later, 24-well plates was moved to Confocal Laser Scanning Microscope (Leica SP5, Germany); set the excitation wavelengths to 488 nm, the emission wavelengths to 530–540 nm, the scan speed to 400 Hz, the time of every frame to 1.38 s, the time between 2 adjacent frames to 0 s; the number of frames is 100.

Electrophysiological recordings

The electrodes were pulled from borosilicate glass capillaries (Vital Sense Scientific Instruments Co., Ltd. Wuhan, China) on a Sutter P-97 puller (Sutter Instrument CO. USA) with pipette resistance around 1.5 – 3 MΩ. The pipette solution and the bath solution for inside-out mode and whole-cell mode both contained (mM): KCl, 116.88; EDTA, 2; KH₂PO₄, 2.83; K₂HPO₄, 7.17 (adjusted to pH 7.4 with KOH; adjusted to 295 mOsm/L for pipette solution and to 300 mOsm/L for bath solution with sucrose). The current of Kir2.1 channel was recorded with EPC10 amplifier (HEKA, Lambrecht, Germany) and controlled by the software of Pulse (v8.78, HEKA, Germany). Data were filtered at 2.9 kHz and digitized at 3.33 kHz with a Digi LIH1600 interface. The current of Kir2.1 channel was recorded with the following voltage protocol: hold cells at −100 mV for 20.1 ms, than a ramp from −100 mV to +100 mV in 800.1 ms followed by a holding potential of V-membrane for 20.1 ms.

Statistics

Patch clamp data was analyzed using Clampfit 9.0 (Axon Instruments, CA) and OriginPro 8.0 (Origin-Lab Corporation, Northampton, MA). The fluorescence data of the thallium-based flux assay were analyzed by LAS AF v2.2.0 (Leica Microsystems CMS GmbH, Germany) and OriginPro 8.0.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

Acknowledgments

We thank Jing Du coming from School of Foreign Languages of HeBei University of Technology with the help of English usage.

Funding

This work was supported by the Natural Science Fund for Distinguished Young Scholars of the Hebei Province of China (Grant No. C2015202340 to HA, C2013202244 to YC), the Fund for Outstanding Talents of Hebei Province of China (Grant No. C201400305 to HA), the National Natural Science Fund of China (Grant No. 11247010 to HA, 11175055 and 11475053 to YZ, 11347017 to SZ, 31400711 to YC), the Fund for the Science and Technology Program of Higher Education Institutions of Hebei Province, China (Grant No. QN2016113 to JL) and the Scientific Innovation Grant for Excellent Young Scientists of Hebei University of Technology, China (Grant No. 20150510 to JL).

References

[1] Kurachi Y. Voltage-dependent activation of the inward-rectifier potassium channel in the ventricular cell membrane of guinea-pig heart. J Physiol 1985; 366:365-85; PMID:2414434; http://dx.doi.org/10.1113/jphysiol.1985.sp015803
[2] Katz B. Les constantes electriques de la membrane du muscle. Arch Physiol 1949; 15.
[3] Kubo Y, Baldwin TJ, Jan YN, Jan LY. Primary structure and functional expression of a mouse inward rectifier potassium channel. Nature 1993; 362:127-33; PMID:7680768; http://dx.doi.org/10.1038/362127a0
[4] Pegan S, Arrabit C, Zhou W, Kwiatkowski W, Collins A, Slesinger PA, Choe S. Cytoplasmic domain structures of Kir2.1 and Kir3.1 show sites for modulating gating and rectification. Nature Neurosci 2005; 8:279-87; PMID:15723059; http://dx.doi.org/10.1038/nn1411
[5] Lu Z, MacKinnon R. Electrostatic tuning of Mg²⁺ affinity in an inward-rectifier K⁺ channel. Nature 1994; 371:243-6; PMID:7915826; http://dx.doi.org/10.1038/371243a0
[6] Stanfield PR, Davies NW, Shelton PA, Sutcliffe MJ, Khan IA, Brammar WJ, Conley EC. A single aspartate residue is involved in both intrinsic gating and blockage by Mg²⁺ of the inward rectifier, IRK1. J Physiol 1994; 478(Pt 1):1-6; PMID:7965824
[7] Ficker E, Taglialetela M, Wible BA, Henley CM, Brown AM. Spermine and spermidine as gating molecules for inward rectifier K⁺ channels. Science 1994; 266:1068-72; PMID:7973666; http://dx.doi.org/10.1126/science.7973666
[8] Fakler B, Brandle U, Glowatzki E, Weidemann S, Zenner HP, Ruppersberg JP. Strong voltage-dependent inward rectification of inward rectifier K⁺ channels is caused by intracellular spermine. Cell 1995; 80:149-54; PMID:7813010; http://dx.doi.org/10.1016/0092-8674(95)90459-X
[9] Kurata HT, Akrouh A, Li JB, Marton LJ, Nichols CG. Scanning the topography of polyamine blocker binding in an inwardly rectifying potassium channel. J Biol Chem
Huang CL, Fung S, Hilgemann DW. Direct activation of inward rectifier potassium channels by PtdIns(4,5)P$_2$ and its stabilization by Gbetagamma. Nature 1998; 391:803-6; PMID:9486652; http://dx.doi.org/10.1038/35882

[10] Lopes CM, Zhang H, Rohacs T, Jin T, Yang J, Logothetis DE. Alterations in conserved Kir channel-PtdIns(4,5)P$_2$ interactions underlie channelopathies. Neuron 2002; 34:933-44; PMID:12086641; http://dx.doi.org/10.1016/S0896-6273(02)00725-0

[11] Donaldson MR, Jensen JL, Tristani-Firouzi M, Tawil R, Bendahhou S, Tsunoda A, Donaldson MR, Iannaccone ST, Brunt E, Barohn R, et al. Mutations in Kir2.1 cause the Andersen syndrome. J Mol Cell Cardiol 2003; 35:933-44; PMID:12086641; http://dx.doi.org/10.1016/S0022-5193(02)00725-0

[12] Huang CL, Feng S, Hilgemann DW. Direct activation of inward rectifier potassium channels by PtdIns(4,5)P$_2$ and its stabilization by Gbetagamma. Nature 1998; 391:803-6; PMID:9486652; http://dx.doi.org/10.1038/35882

[13] Lopes CM, Zhang H, Rohacs T, Jin T, Yang J, Logothetis DE. Alterations in conserved Kir channel-PtdIns(4,5)P$_2$ interactions underlie channelopathies. Neuron 2002; 34:933-44; PMID:12086641; http://dx.doi.org/10.1016/S0896-6273(02)00725-0
