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Block of Kir channels by flonicamid disrupts salivary and renal excretion of insect pests

Miaomiao Ren[a], Jianguo Niu[a], Bo Hu[a], Qi Wei[a], Cheng Zheng[a], Xiangrui Tian[a], Congfen Gao[a], Bingjun He[b], Ke Dong[c,**], Jianya Su[a,*]

[a] Key Laboratory of Integrated Management of Crop Diseases and Pests (Ministry of Education), College of Plant Protection, Nanjing Agricultural University, Nanjing, 210095, China
[b] College of Life Sciences, Nankai University, Tianjin, 300071, China
[c] Department of Entomology and Neuroscience Program, Michigan State University, East Lansing, MI, 48824, USA

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ABSTRACT

Flonicamid is a selective insecticide for the control of sap-sucking insects; it exerts toxic effects by inhibiting insect feeding. However, its molecular target remains elusive. In this study, we functionally characterized *NlKir1* channels of the brown planthopper (*Nilaparvata lugens*) in HEK293 cells. Homomeric *NlKir1* channels generated inward-rectifying K^+ currents. Flonicamid inhibited *NlKir1* channels at nanomolar concentrations. Furthermore, flonicamid inhibited honeydew and salivary secretions of planthoppers, and reduced the renal excretion of female mosquitoes in a dose-dependent manner. The inhibitory effect of flonicamid on fluid secretion of isolated Malpighian tubules from *Culex pipiens pullens* was comparable to that of the selective Kir1 inhibitor. The observed physiological alterations by flonicamid are likely mediated by Kir1 channels and could lead to the disruption of feeding behaviors and eventually lethality. Our study establishes the Kir1 channel as the target of flonicamid and provided new insights into the mode of action of flonicamid.

1. Introduction

Flonicamid is a highly selective insecticide belonging to the pyridinecarboxamide group, which was discovered by Ishihara Sangyo Kaisha (ISK) Ltd., and later commercially developed by the joint effort of ISK and FMC (Morita et al., 2014). It is currently marketed under various trade names in over forty countries including the Americas, Asia, Europe and Africa. Flonicamid shows very good to excellent efficacy for controlling hemipterous insects including aphid, whitefly, leafhopper, plant bug, pysllid and planthopper (Kodandaram et al., 2017; Morita et al., 2014). For example, it exhibits strong feeding inhibition and causes mortality of aphids (nymph and adult). According to the mode-of-action classification by Insecticide Resistance Action Committee (IRAC), flonicamid was originally assigned to IRAC group 9 (modulators of chordotonal organs), which includes pymetozine (Sparks and Nauen, 2015). In 2015 the molecular target of pymetrozine was identified as chordotonal organ TRPV channels (Nesterov et al., 2015). However, flonicamid does not activate insect TRPV channel and does not compete with TRPV activator for binding (Kandasamy et al., 2017). Currently, flonicamid is listed as IRAC group 29 with an undefined target site of action. These results suggest that there is a distinct molecular target for flonicamid.

Although flonicamid shares structural similarities with neonicotinoid insecticides, it had no activities on nicotinic acetylcholine receptors from *Periplaneta americana*, *D. melanogaster* or *Heliothis virescens* (Morita et al., 2007). Nevertheless, at 10 μM it has a blocking activity on A-type potassium channel currents in *H. virescens* neurons (Hayashi et al., 2006). Flonicamid was reported to be toxic to female *Anopheles gambiae* and had a blocking effect against voltage-gated K^+ channels, Kv2 channels, with IC_{50} values of about 1 mM (Taylor-Wells et al., 2016). In both studies, the effective concentrations for blocking these K^+ channels were high, suggesting that these K^+ channels may not be the primary target of flonicamid action.

In this study, we evaluated the effects of flonicamid on another type of potassium channels, inward rectifying K^+ channels (Kir), in the brown planthopper (*Nilaparvata lugens*). Kir channels play an important role in the regulation of resting membrane potential, maintenance of K^+ homeostasis, and transduction of cellular metabolism into excitability in mammals and insects (Chen and Swale, 2018; Hibino et al., 2010). Insect Kir channels have been predicted to be promising

* Corresponding author. Department of Pesticide Science, College of Plant Protection, Nanjing Agricultural University, Nanjing, 210095, China. Tel.: +86 25 84395269.
** Corresponding author. Tel. +1 517 432 2034.
E-mail addresses: dongke@msu.edu (K. Dong), sjy@njau.edu.cn (J. Su).

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molecular targets for developing novel insecticides due to their critical roles in insect physiology (Piermarini et al., 2017; Swale et al., 2016). AeKir1 channels from *Aedes aegypti* mosquitoes were reported to regulate transepithelial transport of potassium in Malpighian tubules. The inhibitions of Kir1 channels by selective Kir1 inhibitors led to the disruption of Malpighian tube function in adult female mosquitoes, and render the mosquito flightless or dead in 24 h (Piermarini et al., 2017; Raphemot et al., 2013, 2014b; Rouhier et al., 2014; Swale et al., 2016).

The molecular and functional characterizations show that *NlKir1* encodes a typical Kir channel and flonicamid blocks *NlKir1* channels at nanomolar concentrations. Flonicamid inhibits honeydew and salivary secretions of *N. lugens*. Furthermore, flonicamid inhibits the renal excretion of female adult of *Culex pipiens pullens*. Our study uncovered the Kir channels as the long sought-after target of flonicamid, which paves the way for future resistance monitoring and the development of a new generation of safe insecticides with this new mode of action.

2. Materials and methods

2.1. Insecticides

Fonicamid (97%) was obtained from Sigma, the technical grades of imidacloprid (95%), thiamethoxam (95%), chlorpyrifos (97%), pymetrozine (94%), and buprofezin (98.1%) were provided by Bayer, Syngenta, Nanjing Redsun, Jiangsu Kesheng and Jiangsu Changnong, respectively.

2.2. Insects

The insecticide susceptible strains of *N. lugens* was provided by Zhejiang chemical industry research institute in 2013, and resistant population was collected from rice paddy fields in Jinshan, Shanghai, China in Oct. 9, 2016. Two strains were kept in laboratory without exposure to any insecticides. *N. lugens* was maintained on 10-d old rice seedlings cultured in plastic boxes (12 × 17 × 38 cm) under laboratory conditions at 27 ± 1 °C and 16:8 h light:dark photoperiod (Su et al., 2013).

2.3. Cloning of the Kir channel cDNAs

Total RNA was extracted from nymphs of *N. lugens* using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Next cDNA was synthesized from 1 μg of total RNA using the m-MLV reverse transcriptase and oligo$(dT)_{18}$ (BioTeke, Beijing, China). For 3′ and 5′-end, the SMART RACE cDNA Amplification Kit (Clontech) was used to generate a both 5′- and 3′-rapid amplification of cDNA from total RNA following the procedures described by the manufacturer. All products were stored at −20 °C until use.

Specific primers were designed to amplify the NlKir channel ORF based on the Kir sequences in *N. lugens* genome database (Supplementary Data Table S1). In a 50 μL PCR reaction 2 μL cDNA, 25 μL 2 × Taq Master Mix (Vazyme, Nanjing, China), 1 μL of the specific primers, 21 μL ultrapure water were included. The PCR amplification was performed for 30 cycles as the protocol.

2.4. Protein sequence alignment and phylogenetic analysis

The protein domains of Kir channels were predicted on the THMM server (version 2.0) (http://www.cds.dtu.dk/services/TMHMM/). Multiple sequence alignment was carried out using Clustal W software. The phylogenetic trees were constructed with MEGA5 by using maximum likelihood method (Tamura et al., 2013). Bootstrap analysis was carried out on 1000 replicate data set to evaluate the significance of the nodes.

2.5. Whole-cell patch clamp electrophysiology

HEK293 cell line was purchased from the Cell Bank of the Chinese Academic of Sciences (China) and cultured in DMEM (Life technologies, Carlsbad, CA) containing 10% FBS, 50 U/mL penicillin and 50 μg/mL streptomycin in a humidified environment at 37 °C with 5% CO_2. For Whole-cell patch-clamp experiments, *NlKir1* channel cDNA in pEGFP-Nl plasmid (Clontech, Palo Alto, CA) was transfected in HEK293 cells using Lipofectamine LTX (Life technologies, Carlsbad, CA) according to the manufacturer's instructions. Empty plasmid was transfected as control. After transfection for 48 h, the *NlKir1* cells were dissociated by 0.25% Trypsin containing 1 mM EDTA, plated on poly-L-lysine-coated round glass coverslips and followed to recover at 37 °C in a 5% CO_2 incubator for at least 1 h before electrophysiological recording. Then the coverslips were transformed to a small-volume perfusion chamber (Warner Instrument, Hamden, CT). Patch electrodes were pulled from silanized 1.5-mm outer diameter borosilicate microhematocrit tubes. Electrode resistance ranged from 2.5 to 3.5 MΩ when filled with following intracellular solution (in mM): 135 KCl, 2 $MgCl_2$, 1 EGTA, 10 HEPES and 2 Na_2ATP, adjusted pH to 7.3 by KOH,275 mOsmol/kg with sucrose. The bath solution contained (in mM): 90 NaCl, 50 KCl, 2 $CaCl_2$, 1 $MgCl_2$, 5 Glucose, 10 HEPES-free acid, pH 7.4, 275 mOsmol/kg. Whole-cell current were recorded under voltage-clamp conditions using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Electrical connections to the amplifier were made using Ag/AgCl wires and 3 M KCl/agar bridges. Flonicamid purchased from Sigma-Aldrich (St. Louis, MO) was dissolved in anhydrous dimethyl sulfoxide (DMSO) and diluted into serial concentrations before use. The final concentration of DMSO used was less than or equal to 0.05%. After recording the stable whole-cell currents, the chemicals were applied intermittently or continuously for 4–10 min. and the application of a known blocker (i.e., 2 mM barium chloride) followed as a positive control. Data acquisition and analysis were performed using Clampfit 10 software (Axon Instruments Inc, USA). All recordings were made at room temperature (20–23 °C).

2.6. Thallium flux assay

Thallium flux assay was made with FluOR thallium assay kit (Life Technologies Corporation, USA). The open-reading frame of *NlKir1* was sub-cloned into pcDNA3 (Carlsbad, CA, USA) using the PCR primers in Supporting Information Table S3. The stable cell lines used for Tl^+ flux assay were generated as described previously (Lewis et al., 2009; Raphemot et al., 2014b). Briefly, HEK293 cells were transfected using Lipofectamine LTX (Life technologies, Carlsbad, CA) according to the manufacturer's instructions and cultured in geneticin (antibiotic)-containing medium to select individual stably transfected cells. Monoclonal cell lines were isolated through limiting dilution in 96-well plates. The control cell line was generated from cells transfected with the vector alone and selected with G418. Then the control cell line, *NlKir1* cell line was placed in 96-well black-walled, clear-bottomed, poly-D-lysine coated plate (BD Biosciences, Bedford, MA) at the density of 40,000 cells/well and cultured overnight. The following day, the G418-containing culture medium was replaced with FluxOR dye loading buffer, prepared according to the manufacturer's instructions to each well and protected samples from light and incubate at 37 °C for 60 min. FluxOR assay solution contained 2.5 mM probenecid and adjusted pH to 7.4 by addition of NaOH. After removing dye loading buffer manually, the cells were treated with the assay solution 100 μL in the absence or presence of the test compounds and incubated for 20 min. At the end of 20-min incubation period, the plate was transferred to Synergy H1 microplate reader (BioTek, Winooski, VT) and background fluorescence (F_0) was measured every 10s (excitation 470 ± 20 nm, emission 520 ± 40 nm) before addition of Tl^+ stimulus buffer. Immediately after adding of Tl^+ stimulus buffer (20 μL/well), fluorescence data (F) were recorded for an additional 5 min. The kinetic fluorescence values