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A novel role of the antitumor agent tricyclodecan-9-yl-xanthogenate as an open channel blocker of KCNQ1/KCNE1

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ABSTRACT

Tricyclodecan-9-yl-xanthogenate (D609) is widely known for its antitumor and antiviral properties via the inhibition of phosphatidylcholine-specific phospholipase C and sphingomyelin synthase. Previously, we found that chronic application of D609 suppressed the K^+ channel, KCNQ1/KCNE1, more drastically than expected from its actions on the enzymes, suggesting a direct action of D609 on the channel. Here, we aimed to test this possibility by studying the affinity, specificity, and mechanisms of D609 on KCNQ1/KCNE1. The effect of D609 on KCNQ1/KCNE1 was studied using an *in vitro* expression system and in native cells, using electrophysiological techniques. We found that D609 rapidly and reversibly inhibited KCNQ1/KCNE1 channels expressed in human embryonic kidney 293 T (HEK293T) cells, in a concentration-dependent manner with a high affinity. D609 neither suppressed endogenous K^+ currents in HEK293T cells, nor inhibited the sustained and transient K^+ currents of mouse neostriatal neurons, but blocked a KCNQ1/KCNE1-like current in neostriatal neurons. D609 potently blocked I_{Ks}, the cardiac KCNQ1/KCNE1 channel, in guinea pig cardiac muscle cells. The action of D609 on KCNQ1/KCNE1 depended on the usage of the channel, suggesting that D609 binds to the channel in the open state. We identified D609 as a potent and specific open channel blocker of KCNQ1/KCNE1. Because KCNQ1/KCNE1 is highly expressed in the heart, the inner ear and the pancreas, D609, when used as an antitumor or antiviral drug, may affect the function of a number of organs *in vivo* even when used at low concentrations.

1. Introduction

Tricyclodecan-9-yl-xanthogenate (D609) is an inhibitor of phosphatidylcholine-specific phospholipase C (PC-PLC) and sphingomyelin synthase (SMS) (Amtmann, 1996; Zhou et al., 2001; reviewed in Adibhatla et al., 2012). While PC-PLC plays important roles in metabolism and in cell proliferation, differentiation, senescence, and apoptosis (reviewed in Adibhatla et al., 2012), SMS is involved in cell migration, proliferation, apoptosis, and autophagy (reviewed in Taniguchi and Okazaki, 2014). Thus D609 has a broad range of biological activities, including antitumor, antiviral, and anti-inflammatory activities (reviewed in Adibhatla et al., 2012). D609 has also been extensively used as a valuable reagent for manipulating lipid levels in various types of cells (Kato et al., 2016). We previously found that chronic application of D609 to human embryonic kidney 293 T (HEK293T) cells that express the slowly activating, delayed rectifier potassium channel encoded by KCNQ1 (α subunit, also known as Kv7.1) and KCNE1 (β subunit, also known as Isk or minK) resulted in a much more drastic reduction of current density than the inhibitory effect on the current caused by knockdown of SMS1 (Wu et al., 2016). These observations suggest that D609 may act directly on the channel, in addition to suppressing the enzyme.

KCNQ1/KCNE1 plays important roles in a number of organs including the inner ear, heart, thymus, pancreas, and brain (reviewed in Chabannes et al., 2001; Jespersen et al., 2005; Abbott, 2014; Liin et al., 2015). In the heart, the expression of KCNQ1/KCNE1 has been found in various regions of the organ (reviewed in Jost et al., 2007). Because of its slow activation kinetics, KCNQ1/KCNE1 current may have limited contributions to cardiac repolarization in normal conditions (reviewed in Jost et al., 2007). Nevertheless, loss-of-function mutations of the

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KCNQ1/KCNE1 channel prolong the QT interval as observed in long QT syndrome (Wang et al., 1996), leading to ventricular arrhythmias, ventricular fibrillation, and sudden death; and gain-of-function mutations of the KCNQ1/KCNE1 channel shorten the QT interval, possibly leading to arrhythmia such as in short QT syndrome or atrial fibrillation (Chen et al., 2003; Bellocq et al., 2004). In the pancreas, KCNQ1/KCNE1 channels are expressed in acinar cells and inhibition of this channel on INS-1 cells increased insulin secretion (Ullrich et al., 2005). In the inner ear, KCNQ1/KCNE1 channels are expressed exclusively on the apical surface of marginal cells of the stria vascularis (Sakagami et al. 1991; Hibino et al. 2010) and maintain the endocochlear potential and, thereby, the high sensitivity of the inner ear to sound. These examples demonstrate that KCNQ1/KCNE1 channels can be a therapeutic target for disorders in a number of organs.

The importance of KCNQ1/KCNE1 in physiological and pathological conditions, and the wide use of D609 in research (Kato et al., 2016), call for a test of the possibility that D609 acts directly on KCNQ1/KCNE1 channels, as raised in our previous study (Wu et al., 2016). We have tested this possibility by examining the effect of D609 on the whole-cell KCNQ1/KCNE1 current, recorded from established cells and native cells. We found that D609, working as a specific open channel blocker, rapidly and reversibly blocked KCNQ1/KCNE1 currents with a high affinity. Previously, a few lipophilic compounds have been reported to block KCNQ1/KCNE1 channels (Jost et al., 2007). D609 is the first water soluble KCNQ1/KCNE1 blocker.

2. Materials and methods

All experiments were performed according to the Guidelines for Use of Animals in Experiments of Kumamoto University. The protocol was approved by the Committee of Animal Experiments of Kumamoto University.

2.1. Cell culture and plasmid transfection

The methods for cell culture and plasmid transfection followed protocols in Wu et al. (2016). Briefly, HEK293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% foetal bovine serum (FBS), in 35-mm dishes (1×10^5 cells per dish). We constructed a FLAG-tagged human KCNE1 plasmid by inserting a cDNA encoding human KCNE1, into the pcDNA3 vector (Thermo Fisher Scientific, Waltham, MA, USA) (Takumi et al., 1988; Murai et al., 1989). A GFP-tagged human KCNQ1 cDNA plasmid (RG219869) was purchased from OriGene (Rockville, MD, USA). All plasmid solutions for transfection were prepared using the PureLink™ HiPure Plasmid Maxiprep Kit (Thermo Fisher Scientific). Plasmids were transfected into HEK293T cells using Lipofectamine® 3000 (Thermo Fisher Scientific) according to the manufacturer's protocol. KCNQ1 and KCNE1 plasmids (3 μg of each) and P3000™ Reagent (5 μl) were added to 125 μl Opti-MEM® medium and Lipofectamine® 3000 Reagent (5 μl) was diluted in 125 μl Opti-MEM® medium. The two solutions were mixed, incubated at room temperature for 5 min, and then added to 35-mm dishes with HEK293T cells. The cells were cultured for 48 h before patch-clamp recording. Approximately 5 h before recording, cells were transferred to poly-L-lysine-coated coverslips in DMEM containing 10% FBS.

2.2. Guinea pig ventricular cardiomyocyte isolation

Ventricular myocytes were enzymatically dissociated from the heart of four-week-old female guinea pigs using a procedure similar to that described previously (Yu et al., 2016). In brief, a guinea pig (weight 250–300 g) was anesthetized with a mixture of ketamine (46 mg/kg) and xylazine (24 mg/kg), and the aorta was cannulated in situ under artificial respiration. The dissected heart was mounted on a Langendorff apparatus and perfused with Tyrode solution composed of (in mM) 135 NaCl, 5.4 KCl, 0.33 NaH_2PO_4, 1.0 $MgCl_2$, 5.5 glucose, 1.8 $CaCl_2$, and 10 N-[2-hydroxyethyl] piperazine-N-[2-ethanesulfonic acid] (HEPES)-NaOH buffer (pH 7.4), for 3 min at 37 °C, followed by Ca^{2+}-free Tyrode solution for 5 min, and finally with Ca^{2+}-free Tyrode solution containing collagenase (0.08 mg/ml; Yakult, Tokyo, Japan) for 8–15 min. The heart was then washed with a high-K^+ low-Ca^{2+} solution (storage solution) containing (in mM): 70 KOH, 50 glutamic acid, 40 KCl, 20 KH_2PO_4, 20 taurine, 3 $MgCl_2$, 10 glucose, 10 HEPES, and 0.5 EGTA; pH was adjusted to 7.3 with KOH. The ventricular myocytes were dispersed and filtered through a 100 μm Nylon cell strainer (Falcon™ Cell Strainers). The cells were subsequently incubated with the storage solution containing both DNAse (Cat. No D5025-150KU, Sigma, St. Louis, MO, USA; 1 mg/45 ml) and protease (Cat. No. P5147-100MG, Sigma, St. Louis, MO, USA; 1 mg/45 ml), for 5 min at 37 °C. This treatment facilitates formation of a GΩ seal in patch-clamp recording. Lastly, the myocytes were washed twice with storage solution by centrifugation (100 × g for 3 min) and then stored at 4 °C.

2.3. Acute-dissociation of mouse neostriatal neurons

Acute-dissociation was performed according to procedures previously employed in our laboratory (Song et al., 1998; Hattori et al., 2003). Briefly, C57BL/6 J mice aged postnatal day 30 were anesthetized with ethyl-ether and decapitated; brains were quickly removed, iced and then blocked for slicing. The blocked forebrain region was cut in the frontal plane into 400 μm slices with a Microslicer (Dosaka, Kyoto, Japan) while bathed in a low Ca^{2+} (100 μM), HEPES buffered salt solution (in mM: 140 Na isethionate, 2 KCl, 4 $MgCl_2$, 0.1 $CaCl_2$, 23 glucose, 15 HEPES, pH = 7.4, 300–305 mOsm/l). Slices were then incubated for 1–6 h at room temperature in a $NaHCO_3$ buffered saline bubbled with 95% O_2, 5% CO_2 (in mM: 126 NaCl, 2.5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 26 $NaHCO_3$ 1.25 NaH_2PO_4 ,1 pyruvic acid, 0.2 ascorbic acid, 0.1 NG-nitro-L-arginine, 1 kynurenic acid, 10 glucose, pH = 7.4 with NaOH, 300–305 mOsm/l). All reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Slices were then removed into the low Ca^{2+} buffer and, with the aid of a dissecting microscope, regions of the dorsal striatum were dissected with a pair of thin tungsten needles and placed in an oxygenated beaker containing pronase (1–3 mg/ml) in HEPES-buffered Hank's balanced salt solution (HBSS, Sigma Chemical Co.) at 35 °C. After 30–35 min of enzyme digestion, tissue was rinsed three times in the low Ca^{2+}, HEPES-buffered saline solution and mechanically dissociated with a graded series of fire-polished Pasteur pipettes. The cell suspension was then plated into a 35 mm Lux Petri dish mounted on the stage of an inverted microscope containing HEPES-buffered HBSS saline (in mM: 140 NaCl, 2 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 15 HEPES, pH = 7.4 with NaOH, 300–305 mOsm/l).

2.4. Electrophysiology

Whole-cell recordings of K^+ currents from HEK293T cells were performed according to our previously published methods (Song et al., 1998; Hattori et al., 2003; Wu et al., 2016). Transfected (GFP-labelled) cells were randomly selected for recording. The recording chamber was perfused with a background solution containing (in mM) 140 NaCl, 2 KCl, 1 $CaCl_2$, 2 $MgCl_2$, 10 glucose, 15 HEPES; pH adjusted to 7.4 with NaOH; osmolarity adjusted to 295–300 mOsm/l. The external solution consisted of (in mM) 140 NaCl, 2 KCl, 2 $MgCl_2$, 10 glucose, 10 HEPES; pH = 7.4 with NaOH; 295–300 mOsm/l. Ca^{2+} was excluded from the external solution to eliminate Ca^{2+} currents. During recording, the extracellular solution was applied to the cell under study via a gravity-fed capillary perfusion array positioned about 2 mm away from the cell. D609 (Sigma, St. Louis, MO, USA) was included in the external solution at various concentrations. The internal solution consisted of (in mM) 120 potassium gluconate, 3 $MgCl_2$, 10 HEPES, 10 EGTA, 2 ATP, 12 phosphocreatine, 0.2 GTP; pH = 7.2–7.3 with KOH; 275–285 mOsm/l.

For whole-cell recording of guinea pig cardiac ventricular myocytes, the recording chamber was perfused with a normal Tyrode solution as a