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Cinnamyl-3,4-dihydroxy-α-cyanocinnamate and nordihydroguaiaretic acid inhibit human Kv1.5 currents independently of lipoxygenase

Ying-Zi Gong [a,b,c], Wei-Guang Ding [a,*], Jie Wu [d], Keiko Tsuji [b], Minoru Horie [b], Hiroshi Matsuura [a]

[a] Department of Physiology, Shiga University of Medical Science, Otsu, Shiga 520-2192, Japan
[b] Department of Cardiovascular and Respiratory Medicine, Shiga University of Medical Science, Otsu, Shiga 520-2192, Japan
[c] Department of Cardiovascular Medicine, Fourth Affiliated Hospital Harbin University, Harbin, 150001, China
[d] Pharmacology Department, Medical School of Xi'an Jiaotong University, Xi'an Shaanxi 710061, China

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ABSTRACT

In humans, Kv1.5 (hKv1.5) channels conduct the ultra-rapid delayed rectifier K^+ current (I_{Kur}) that is important for the repolarization of cardiac action potentials. We aimed at examining the effect of lipoxygenase inhibitors cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC), nordihydroguaiaretic acid (NDGA), and gossypol on hKv1.5 wild-type and mutant channels heterologously expressed in Chinese hamster ovary (CHO) cells, by use of the site-directed mutagenesis and whole-cell patch-clamp method. CDC and NDGA, but not gossypol, a structurally dissimilar inhibitor, reversibly inhibited hKv1.5 current in a concentration-dependent manner with IC_{50} of 5.7 μM and 16.4 μM, respectively. The blockade evoked by both drugs was voltage-dependent between −20 and +10 mV (voltage range of channel opening). Moreover, this blocking action was found to progress with time during depolarizing voltage steps with a more rapid block at higher concentrations. CDC induced slight but significant delay of the deactivation rate. However, NDGA markedly slowed the deactivation time course, resulting in a tail crossover phenomenon. The recovery time constants from current block at repolarizing potentials for CDC and NDGA were 60.9 ms and 129.7 ms, respectively. Mutation of arginine 487 to valine (R487V) in the outer pore region of the channel significantly reduced the CDC action. These results demonstrate for the first time that CDC and NDGA block hKv1.5 channels by binding to the open state of the channels, independently of their effects on lipoxygenase activity. The putative binding site for CDC appears to be related to arginine 487 located in the outer pore region.

1. Introduction

In the cardiac cells, many voltage-gated K^+ (Kv) channels including the transient outward current (I_{to}) and the three (ultra-rapid, rapid, and slow) delayed rectifier potassium currents (I_{Kur}, I_{Kr}, and I_{Ks}, respectively) play an important role in determining the action potential duration. Therefore, these voltage-dependent potassium channels could be potent targets for the action of antiarrhythmic drugs (Knobloch et al., 2002). It is well known that atrial fibrillation is the most frequent cardiac arrhythmia and is associated with significant morbidity and mortality (Chugh et al., 2001). However, the available drugs for the treatment of atrial fibrillation often cause unwanted adverse effects such as the occasional generation of ventricular arrhythmias evoked by excess prolongation of action potential duration (Bril, 2002; Roden, 2001). Because I_{Kur} is expressed in atrial but scarcely in ventricular cells in humans (Li et al., 1996; Stump et al., 2005), I_{Kur} may therefore provide an attractive molecular target for treatment of atrial fibrillation. The molecular component that underlies cardiac I_{Kur} is the Kv1.5 channel (Fedida et al., 1993; Wang et al., 1993b). A line of experiments have been made to identify novel blockers of Kv1.5 (Brendel and Peukert, 2003; Trotter et al., 2006; Varro et al., 2004) and to study their binding site to the channel. Recent studies have shown that several residues that are located near the outer pore region, pore helix as well as in the transmembrane segments (S6) of the human Kv1.5 channels (hKv1.5) are important for the binding of these channel blockers (Decher et al., 2004, 2006; Herrera et al., 2005; Rezazadeh et al., 2006).

Cinnamyl-3,4-dihydroxy-α-cyanocinnamate (CDC) and nordihydroguaiaretic acid (NDGA), are common lipoxygenase inhibitors used in conjunction to study signaling related to biologically active arachidonic acid and its metabolites (Glitsch et al., 2002; Meves, 1994). Arachidonic acid is either intracellularly released or reaches the cell membrane via the circulation. Numerous studies have previously shown the effect of arachidonic acid on various types of ion channels, activating some and blocking others (Meves, 1994). Extracellularly applied arachidonic acid and other polyunsaturated fatty acids are reported to inhibit Kv1.5 channel markedly (Honore et al., 1994). The mechanism related to the arachidonic acid effects varies depending on the different types of ion channels. However, in some cases arachidonic

* Corresponding author. Department of Physiology, Shiga University of Medical Science, Seta Tsukinowa-cho Otsu, Shiga 520-2192, Japan. Tel.: +81 77 548 2152; fax: +81 77 548 2348.
E-mail address: ding@belle.shiga-med.ac.jp (W.-G. Ding).

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acid has direct actions on the ion channel or its lipid environment (Meves, 1994). It is reasonable to consider that CDC and NDGA are structurally close enough to these lipid molecules to affect either the activity of lipoxygenase or the channel activity in a similar manner. Indeed, these lipoxygenase inhibitors could share some actions on the ion channels unrelated to lipoxygenase inhibition. It has been reported that CDC can directly activate background K^+ channels (Danthi et al., 2004), and NDGA can directly enhance Ca^{2+}-dependent K^+ currents in isolated coronary arterial smooth muscle cells (Yamamura et al., 1999). In addition, NDGA was also reported to inhibit voltage-activated Ca^{2+} currents in pituitary cells (Korn and Horn, 1990), fibroblasts (Wang et al., 1993a) and voltage-sensitive K^+ currents in isolated type I carotid body cells (Hatton and Peers, 1997) independently of lipoxygenase inhibition. Therefore, the blocking mechanism or a binding site of the lipoxygenase inhibitors is an important issue with respect to the understanding of the reported fatty acids effects on cardiac function, diseased states and drug development perspective.

In light of these studies, we have investigated the effects of CDC and NDGA on hKv1.5 channels heterologously expressed in CHO cells by using the whole-cell patch-clamp technique. Our findings indicate that CDC or NDGA interacts with hKv1.5 channels in a lipoxygenase-independent manner and directly inhibits hKv1.5 currents as an open channel blocker. The R487 located in the outer pore region is demonstrated as a putative binding site for CDC.

2. Materials and methods

2.1. Cell culture and site-directed mutagenesis

Chinese hamster ovary (CHO) cells were maintained in Dulbecco's modified Eagle's medium/Ham's F-12 (DMEM/F-12) supplemented with 10% fetal bovine serum (GIBCO) and antibiotics (100 IU ml^{-1} penicillin, 100 μg ml^{-1} streptomycin) in an incubator gassed with 5% CO_2 and 95% air at 37 °C. Full-length cDNA of human hKv1.5 was ligated to the mammalian expression vector pcDNA3 (kindly provided by Dr. D. Fedida; University of British Columbia, Canada). Polymerase chain reaction based site-directed mutagenesis was applied to introduce mutations into hKv1.5 cDNA by using Quikchange Kit (Stratagene, La Jolla, CA, USA). All products were fully sequenced (ABI3100x/, Applied Biosystems, Foster City, CA) to ensure the fidelity of the PCR reactions. Wild type hKv1.5 cDNA and hKv1.5 mutants (T462C, H463C, T480A, R487V, A501V, I502A, I508A, L510A and V516A cDNA) were transiently transfected into CHO cells together with green fluorescent protein (GFP) cDNA (wild type or mutants 0.5 μg+GFP 0.5 μg) by using Lipofectamine (Life Technologies, Inc.). In addition, a KCNA5 synonymous polymorphism P532L (kindly provided by Dr. DM. Roden; Vanderbilt University School of Medicine, USA) was also tested. After transfection for 24–48 h, the GFP-positive cell was used for the patch-clamp study.

2.2. Patch-clamp recordings

Whole-cell membrane currents or macroscopic currents were recorded with an EPC-8 patch-clamp amplifier (HEKA, Lambrecht, Germany). Data were low-pass filtered at 1 kHz, acquired at 5 kHz through an LIH-1600 AD/DA interface (HEKA) using Pulse/PulseFit software (HEKA). Patch electrodes were fabricated from glass capillaries (Narishige, Japan) using a Sutter P-97 microelectrode puller (Sutter Instrument Co., USA) and had a resistance of 2.0–3.0 MΩ when filled with the pipette solution. A coverslip with adherent CHO cells was placed on the glass bottom of a recording chamber (0.5 ml in volume) and continuously perfused at a rate of 2 ml min^{-1} with bath solution at 25 °C. The hKv1.5 whole-cell membrane currents were elicited by applying 300-ms depolarizing steps from a holding potential of −80 mV to various levels. Recovery from inhibition at repolarizing potentials was measured by following a double-pulse protocol; the first pre-pulse of a 500-ms depolarizing potential of +40 mV from a holding potential of −80 mV was followed by a 50-ms test pulse of +40 mV after increasing time intervals between 10 and 2000 ms at −80 mV. Every cycle of the double pulse protocol was 30 s. The time course for recovery from block was determined by plotting the normalized peak current (test pulse/pre-pulse) as a function of various interpulse intervals. This time course was fitted with a single exponential function. As previously described (Lagrutta et al., 2006), the derived time constant (τ) for the kinetics of recovery from block in the closed state (repolarized membrane potentials) is related to the off kinetic rate by the expression $1/\tau = k_{off}$. In addition, the macroscopic currents were evoked by 500-ms depolarizing step from a holding potential of −80 mV to +50 mV, recorded from an inside-out membrane patch.

2.3. Solutions and chemicals

The bath solution for whole cell recording contained (in mM): 140 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 0.33 NaH_2PO_4, 5.5 glucose and 5.0 HEPES (pH was adjusted to 7.4 with NaOH). Agents added to the bath solutions included CDC (BIOMOL), NDGA (Sigma) and gossypol (BIOMOL). These compounds were dissolved in dimethyl sulfoxide (DMSO) to make stock solution of 20–50 mM. The final concentration of DMSO used in the present experiments was less than 0.1%, which had no effect on hKv1.5 currents. The pipette solution contained (in mM): 70 potassium aspartate, 40 KCl, 10 KH_2PO_4, 1 $MgSO_4$, 3 Na_2-ATP (Sigma), 0.1 Li_2-GTP (Roche Diagnostics GmbH, Mannheim, Germany), 5 EGTA and 5 HEPES, and pH was adjusted to 7.2 with KOH. For inside-out patches, the electrodes were filled with the following solution (in mM): 130 NaCl, 5.0 KCl, 2.8 sodium acetate, 1.0 $MgCl_2$, pH 7.4 with NaOH. The external recording solution for inside-out patches contained (in mM): 120 potassium aspartate, 20 KCl, 4.0 Na_2ATP, 5.0 HEPES, 1.0 $MgCl_2$, pH 7.2 with KOH.

2.4. Data analysis

The concentration–response relationship for the current inhibition (y) by CDC or NDGA was fitted to the Hill equation:

$$y = E_{max}/\left(1 + (IC_{50}/[drug])^{n_H}\right) \quad (1)$$

where E_{max} is the maximum effect of block expressed as a percentage, IC_{50} is the concentration of CDC or NDGA causing a half-maximal inhibition, and n_H is the Hill coefficient. A first-order blocking scheme was used to describe the drug–channel interaction (Snyders and Yeola, 1995, Yeola et al., 1996). The apparent rate constants for binding (k_{+1}) and unbinding (k_{-1}) were obtained from the following equations:

$$\tau_D = 1/(k_{+1}[D] + k_{-1}) \quad (2a)$$

$$K_d = k_{+1}/k_{-1} \quad (2b)$$

where τ_D is the drug-induced time constant, which was calculated by single exponential fits to the current decay during depolarizing step to +30 mV. The apparent dissociation constant K_d is expressed as $K_d = k_{+1}/k_{-1}$. Data for voltage-dependence of hKv1.5 activation was fitted with a Boltzmann equation:

$$I_{tail} = 1/\left(1 + \exp\left((V_{1/2} - V_m)/k\right)\right) \quad (3)$$

where I_{tail} is the tail current amplitude normalized with reference to the maximum value measured at +50 mV, $V_{1/2}$ is the half-maximal voltage, V_m is the test potential and k is the slope factor. The deactivation kinetics was also determined by a single exponential fit of tail current trace.