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Authors

Hegyi, Bence

Chen-Izu, Ye

Jian, Zhong

et al.

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KN-93 inhibits I_{Kr} in mammalian cardiomyocytes

Bence Hegyi[1], **Ye Chen-Izu**[1,2,3], **Zhong Jian**[1], **Rafael Shimkunas**[1,2], **Leighton T. Izu**[1], and **Tamas Banyasz**[4]

[1]Department of Pharmacology, University of California, Davis, CA, USA

[2]Department of Biomedical Engineering, University of California, Davis, CA USA

[3]Department of Internal Medicine (Cardiology), University of California, Davis, CA USA

[4]Department of Physiology, Faculty of Medicine, University of Debrecen, Debrecen, Hungary

Abstract

Calcium/calmodulin-dependent protein kinase II (CaMKII) inhibitor KN-93 is widely used in multiple fields of cardiac research especially for studying the mechanisms of cardiomyopathy and cardiac arrhythmias. Whereas KN-93 is a potent inhibitor of CaMKII, several off-target effects have also been found in expression cell systems and smooth muscle cells, but there is no information on the KN93 side effects in mammalian ventricular myocytes. In this study we explore the effect of KN-93 on the rapid component of delayed rectifier potassium current (I_{Kr}) in the ventricular myocytes from rabbit and guinea pig hearts. Our data indicate that KN-93 exerts direct inhibitory effect on I_{Kr} that is not mediated via CaMKII. This off-target effect of KN93 should be taken into account when interpreting the data from using KN93 to investigate the role of CaMKII in cardiac function.

Keywords

KN-93; I_{Kr}; CaMKII; ventricular myocytes; off target effect; pharmacology

1 Introduction

Calcium/calmodulin-dependent protein kinase II (CaMKII) is in the focus of research because it is known to modulate various cell functions. Pathologic activation of this enzyme is implicated in different forms of cardiac arrhythmias and CaMKII inhibition is reported to prevent or alleviate development of irregular heartbeats [1-5]. KN-93 (2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine) is widely used as a pharmacological tool to inhibit CaMKII in several

Corresponding Author, Tamas Banyasz, University of Debrecen - Faculty of Medicine - Department of Physiology, H-4012 Debrecen, Nagyerdei krt. 98. PO Box 22. Tel: +36(52)255-575 Fax: +36(52)255-116, banyasz.tamas@med.unideb.hu.

Conflict of interest: None.

studies [1, 2, 6]. However, besides its primary effect, KN-93 has been found to have off-target effects including an open-channel blockade of some voltage-gated potassium channels [7, 8]. Until now the side effects of KN-93 have been explored on several voltage-dependent potassium channels in smooth muscle cells, atrial myocytes and expression cell systems, but no data are available for ventricular myocytes. Furthermore, those studies were performed at room temperature and the KN-93 concentrations used were equal or higher than that needed for complete inhibition of CaMKII [7-9].

In the present work we explore the inhibitory effect of KN-93 on the rapid component of delayed rectifier potassium current (I_{Kr}) in freshly isolated rabbit and guinea pig ventricular myocytes at body temperature. We used both the traditional voltage clamp and action potential voltage clamp (APC) methods [6, 10] to study the effect of KN-93 on altering the amplitude and the time course of I_{Kr} during AP and to determine the dose-response of I_{Kr} to KN93 treatment.

2 Methods

All animal handling and laboratory procedures conform to the approved protocols of the local Institutional Animal Care and Use Committee confirming to the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health (8th edition, 2011). Chemicals and reagents were purchased from Sigma-Aldrich if not specified otherwise. E-4031 and HMR-1556 were from Tocris. All experiments were conducted at 36±0.1 °C.

2.1 Cell isolation

Ventricular myocytes were isolated from 3-4 month old New Zealand White rabbits and 4-6 month old Hartley guinea pigs by a standard enzymatic technique [6] using collagenase type II (Worthington, USA) and protease type XIV (Sigma, USA).

2.2 Electrophysiology

Cells were transferred to a temperature-controlled Plexiglas chamber (Cell Microsystems, USA) and continuously superfused with a bicarbonated Tyrode (BTY) solution containing (in mmol/L): NaCl 125, $NaHCO_3$ 25, KCl 4, $CaCl_2$ 1.2, $MgCl_2$ 1, HEPES 10, Glucose 10; pH was set to 7.4 with NaOH. Electrodes were fabricated from borosilicate glass (World Precision Instruments, USA) with tip resistances of 2–2.5 MΩ when filled with internal solution. In experiments aimed to preserve the physiological Ca^{2+} cycling during AP, the internal solution contained (in mmol/L): K-Aspartate 108, KCl 25, NaCl 5, Mg-ATP 3, HEPES 10, cAMP 0.002, phosphocreatine dipotassium salt 10, EGTA 0.01; pH was set to 7.3 with KOH. To study the effect of KN-93 cytosolic calcium concentration below diastolic level, $[Ca^{2+}]_i$ was buffered to nominally zero by using an internal solution containing (in mmol/L): K-Aspartate 100, KCl 25, NaCl 5, Mg-ATP 3, HEPES 10, cAMP 0.002, phosphocreatine dipotassium salt 10, BAPTA 10, with pH = 7.3. The cells were stimulated in current-clamp experiments with supra-threshold depolarizing pulses (2 ms duration) delivered via the patch pipette at 2 Hz pacing rate. By switching the amplifier to voltage-clamp, APC experiments were conducted as described previously [10]. In conventional

voltage-clamp experiments, measuring I_{Kr}, $[Ca^{2+}]_i$ was buffered and BTY was supplemented with 1 μM HMR-1556 plus 10 μM nifedipine. I_{Kr} was measured as the tail current amplitude (which was calculated as the difference between the peak current and the pedestal current after repolarization).

2.3 Statistical analysis

Data are reported as Mean ± SEM. Statistical significance of differences was evaluated using Student's t-test followed by ANOVA. Difference is deemed significant if $p < 0.05$. ANOVA is used for group-wise comparison. Student's t-test is used for pair-wise comparison. The difference in the mean values is deemed significant if $p < 0.05$.

Results

3.1 KN-93 abolishes I_{Kr} recorded with action potential clamp method

First, we recorded I_{Kr} as the E-4031 sensitive current under AP-clamp condition when intracellular Ca^{2+} cycling was preserved (no exogenous Ca^{2+} buffer was used in the pipette solution).

In the first group of experiments E-4031 sensitive current was recorded with the same, pre-recorded (canonical) AP without calcium chelator in the pipette solution (Ca^{2+} cycling). As seen in Fig.1A, I_{Kr} builds up in accelerating manner during AP plateau and reaches a maximum (0.839±0.066 A/F, n=18) during phase 3. Then the current declines rapidly and reduces to zero when resting membrane potential is reached. 1 μM KN-93 pre-treatment abolished E-4031 sensitive current (Fig. 1B). No attempt was made to determine current maximum under these circumstances, because of the lack of a distinguishable peak during the AP. We determined and compared the net charge carried by I_{Kr} (Q_{Kr}) by integrating the current between the AP peak and APD_{95} (AP duration measured at 95% repolarization). The average value of Q_{Kr} under control conditions and in the presence of 1 μM KN-93 was 0.0384±0.0046 C/F and 0.0024±0.0011 C/F respectively when Ca^{2+} was not buffered. The inhibitory effect of KN93 on I_{Kr} was also observed with pipette solution containing 10 mM BAPTA to chelate Ca^{2+}. Hence, cytosolic calcium had no influence on the Q_{Kr}, and I_{Kr} was abolished by 1 μM KN-93 regardless of the Ca^{2+} concentration. Moreover, KN-92, a sister molecule of KN-93 known as “kinase inactive control drug” reduced Q_{Kr} by 45.28% when applied in 1 μM concentration, indicating a non CaMKII mediated effect on I_{Kr} (Fig.1D). To visualize the KN93 effect on the ionic currents during AP, we also recorded KN-93 sensitive current under AP-clamp. The KN-93 sensitive current is a composite current displaying multiple negative peaks along the plateau and terminated after a positive hump during phase three of AP (Fig 1E). The timing and the contour of this late hump closely resembles that of I_{Kr} seen during phase 3, indicating that this phase of KN-93 sensitive current is probably dominated by I_{Kr}. 1 μM KN-93 prolonged AP duration measured at 90% repolarization (APD_{90}) from 323.06±14.36 ms to 400.15±15.09 ms ($p<0.01$) in the presence of 10 mM BAPTA in the pipette solution. Adding 1 μM E-4031 upon 1 μM KN-93 resulted no further lengthening of the APD_{90} (402.81±10.55 ms, $p>0.05$) indicating that I_{Kr} was blocked completely by KN-93 (Fig. 1F).

3.2 KN-93 inhibits I_{Kr} measured as tail current under conventional voltage-clamp

To verify the above findings observed under AP-clamp, we also recorded I_{Kr} using traditional rectangular voltage-clamp experiments, when I_{Kr} was recorded cytosolic calcium was chelated and I_{Ks} and $I_{Ca,L}$ were blocked with 1 μM HMR-1556 and 10 μM nifedipine, respectively. I_{Kr} was activated by a 3 s long depolarizing pulses to +40 mV from the holding potential of −40 mV on 20 s cycle length. The inhibitory effect of KN-93 on I_{Kr} was measured by the concentration-dependent reduction of tail current amplitude determined following repolarization to holding potential. As shown in Figure 2C, KN-93 reduced the tail current amplitude in concentration-dependent manner. The inhibitory effect of KN93 developed within 1 minute at each drug concentration tested. KN-93 at 1000 nM completely abolished I_{Kr}, as evidence by no E-4031 sensitive current was left after KN93 treatment. The IC_{50} value of KN93 is 102.57±9.28 nM and the Hill coefficient is 0.97±0.12.

The blocking effect of KN-93 was found to be voltage dependent under conventional voltage clamp conditions with buffered cytosolic calcium (Figure 2D). When KN-93 was used in 100 nM concentration (~IC_{50}) the inhibitory effect increased by 65.5±12.4% between −30 and +50 mV (p_{slope}<0.01). To test whether the inhibitory effect of KN93 on I_{Kr} is unique to rabbit or exists also in other mammalian species, we examined the effect of 1 μM KN-93 on I_{Kr} recorded from guinea pig ventricular myocytes. The same pipette and bath solutions were used as previously described in rabbit experiments under conventional voltage clamp conditions, except that the voltage command was adapted to the different kinetics of guinea pig I_{Kr} (see Figure 2 panel E). Like in rabbit cells, 1 μM KN-93 completely abolished the tail current indicating that I_{Kr} in guinea pig is also sensitive to KN-93.

4 Discussion

Our findings indicate that KN-93 exerts direct inhibitory effect on I_{Kr} in mammalian ventricular myocytes and several lines of evidence converge to suggest that this KN93 effect is not mediated through CaMKII. First, the inhibitory effect of KN93 on I_{Kr} develops within 1 minute, a time period too short to be explained by the inhibition of CaMKII [11]. Second, the IC_{50} value for KN93 inhibition of I_{Kr} determined in our experiments (102.57±9.28 nM) is significantly lower than the previously reported IC_{50} value of KN-93 (>300 nM) for inhibiting CaMKII [12, 13]. Third, the inhibitory effect of KN-93 was also present when cytosolic calcium was chelated by 10 mM BAPTA, which should alter the CaMKII activity. Fourth, the inhibitory effect of KN-93 displayed voltage sensitivity. Fifth, KN-92 also inhibited I_{Kr}, even though it does not inhibit CaMKII.

Our present observations are in strong agreement with Rezazadeh et al. who demonstrated in transfected HEK293 cells that intracellular dialysis with 1 μM KN-93 for 5 minutes has no effect on the hERG current. Nevertheless, extracellular application of the same concentration of KN-93 resulted in a rapid inhibition of the current [7]. When KN-93 is used in 1 μM concentration to block CaMKII, I_{Kr} is completely blocked therefore E-4031 cannot prolong AP.

The inhibitory effects of KN-93 on I_{Kr} in mammalian ventricular myocytes were first analyzed in this study. Our most important finding is that KN-93 exerts a direct inhibitory effect on I_{Kr} in concentrations substantially lower than that required to inhibit CaMKII. This inhibitory potential must be taken into consideration when KN-93 (or KN-92) is used in cardiac research, especially in arrhythmia studies when I_{Kr} contributes significantly to shaping the cardiac action potential.

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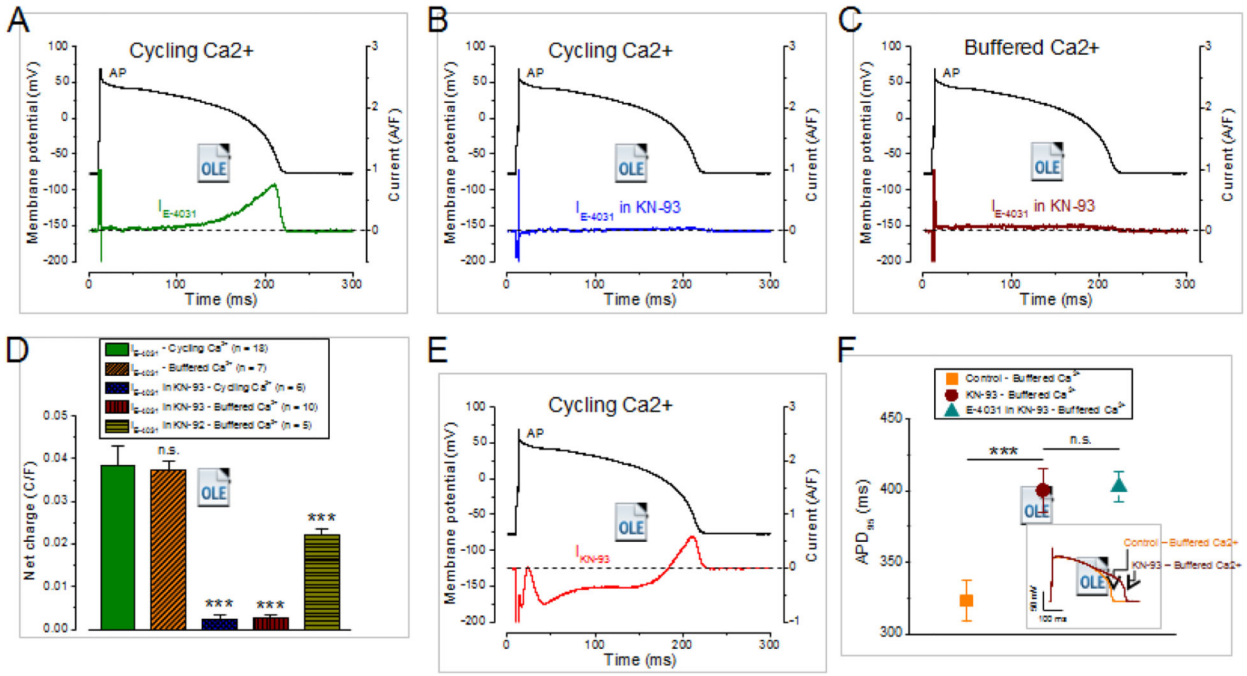

Figure 1. I_{Kr} is abolished by 1 μM KN-93 in rabbit ventricular myocyte

Panels A and B show representative traces of I_{Kr} as E-4031 sensitive currents recorded under AP clamp method in the absence (A) and presence (B) of 1 μM KN-93 without buffering cytosolic calcium. E-4031 sensitive current was abolished by 1 μM KN-93 when cytosolic Ca^{2+} was buffered by 10 mM BABTA in pipette solution too (C). Panel D displays the net charge carried by E-4031 sensitive current, calculated by integration of the current profile during the AP. 1 μM KN-93 reduced net charge profoundly regardless the presence or absence of cytosolic calcium buffer. Panel E displays representative traces of KN-93 sensitive current measured under AP clamp condition. The positive hump present during phase 3 of AP resembles the late phase of E-4031 sensitive current seen on Panel A. Panel F shows the AP prolonging effect of 1 μM KN-93 with buffered cytosolic calcium. When 1 μM E-4031 was added upon KN-93, no further prolongation was observed.
Asterisks indicate significant differences from control (*:p<0.05, **:p<0.02, ***:p<0.001).

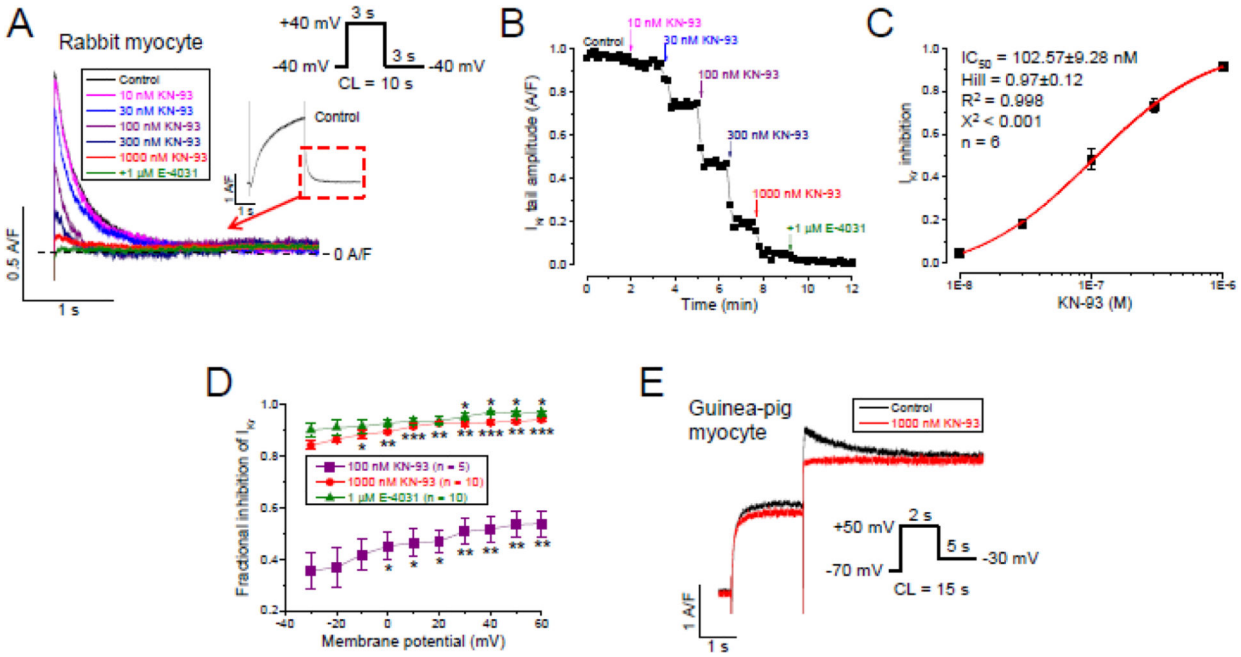

Figure 2. KN-93 inhibits I_{Kr} in mammalian ventricular myocytes

Panel A: Concentration-dependent inhibition of I_{Kr} in rabbit ventricular myocytes measured as tail current under conventional voltage clamp conditions with 10 mM BAPTA in the pipette solution. Panel B: Time course of the development of the effect of KN-93 on I_{Kr} recorded in a representative cell. Note that the inhibitory effect develops within 1 minute and KN-93 abolished completely the E-4031 sensitive current. Panel C: Dose response curve obtained from 6 ventricular cells. Panel D: KN-93 inhibits I_{Kr} at all membrane potentials but the inhibition is more effective at positive voltages. Asterisks indicate significant differences from fractional inhibition measured at −30 mV (*:p<0.05, **:p<0.02, ***:p<0.001). Panel E: Representative superimposed current traces recorded before and after the administration of 1 μM KN-93 in guinea pig ventricular myocyte with the voltage command shown in the inset. KN-93 abolished the tail current indicating complete inhibition of I_{Kr}.