ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 Jul; 23 (7): 601 - 608
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Inhibitory effects of DDPH on two components of delayed rectifier potassium current in guinea pig ventricular cells[1]

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KEY WORDS 1-(2, 6-dimethylphenoxy)-2-(3, 4-dimethoxyphenyl-ethylamino) propane hydrochloride; potassium channels; electrophysiology; patch-clamp techniques; guinea pigs

ABSTRACT

AIM: To study the effects of 1-(2,6-dimethylphenoxy)-2-(3, 4-dimethoxy-phenyl-ethylamino) propane hydrochloride (DDPH) on the rapidly activating component (I_{Kr}), and the slowly activating component (I_{Ks}) of the delayed rectifier potassium current (I_K) in guinea pig ventricular myocytes. **METHODS**: Whole-cell patch clamp recording techniques. **RESULTS**: DDPH (0.1 - 100 μmol/L) blocked the I_{Kr} in a concentration-dependent manner. The IC_{50} (μmol/L) was 6.1 (95 % confidence limits: 2.8 - 13.5). IC_{50} (μmol/L) of DDPH blocking I_{Ks} was 12.5 (95 % confidence limits: 4.8 - 32.2). DDPH (10 μmol/L) did not affect activation time constants and the voltage-dependent activation of both I_{Kr} and I_{Ks}, the half-activation voltage ($V_{1/2}$, mV) and slope factor (k, mV) were I_{Kr}: -23.5 ± 2.4 and 8.1 ± 2.2 [in presence of DDPH, $P > 0.05$, compared with control, $V_{1/2}$ (-21.7 ± 0.8) and k (5.9 ± 0.8)]; I_{Ks}: 27.1 ± 0.7 and 16.6 ± 0.8 [in presence of DDPH, $P > 0.05$, compared with control, $V_{1/2}$ (27.0 ± 0.8) and k (14.9 ± 0.9)]. DDPH slightly increased the deactivation time-constant of I_{Kr} (τ_r) and I_{Ks} (τ_s) at low concentration (< 10 μmol/L). The inactivation of I_{Kr} was significantly accelerated by DDPH. **CONCLUSIONS**: DDPH inhibited both I_{Kr} and I_{Ks}. The blockade was not due to its influence on activation, but the process of deactivation. The blocking of I_{Kr} by DDPH was further associated with its acceleration the channel inactivation.

INTRODUCTION

1-(2, 6-dimethylphenoxy)-2-(3, 4-dimethoxy-phenyl-ethylamino) propane hydrochloride (DDPH) is a synthetic compound with chemical structure similar to verapamil and mexiletine[1]. Our previous studies showed that DDPH could prolong action potential duration (APD) in both isolated ventricular papillary muscles and single ventricular cells in guinea pigs[2,3]. We also reported that DDPH inhibited delayed rectifier potassium current (I_K) in micromolar concentration (IC_{50}: 13.3 μmol/L)[3]. Such effects might contribute to its antiarrhythmic effects in animal models[4].

As a major target for antiarrhythmic drug, I_K consists of two different components: the rapidly activating I_{Kr} and slowly activating I_{Ks}, which could be distinguished by different electrophysiological kinetics, pharmacology and voltage-dependence[5-7]. Class Ⅲ antiarrhythmic agents, such as E-4031, *d*-sotalol, and defetilide, selectively block I_{Kr}, while amidarone and azimilide block both I_{Kr} and I_{Ks} in guinea pig. Numerous studies showed that most class Ⅲ drugs which selectively inhibited I_{Kr} had a reverse rate-dependent activity. Such effects may seriously compromise their antiarrhythmic usefulness and promote proarrhythmia[8]. Therefore it is worth to know the selectivity of DDPH on two components of I_K, it will give out a better understanding of the ionic mechanism underlying the actions of DDPH.

MATERIALS AND METHODS

Preparation of ventricular myocytes Single

[1] Project supported by the National Natural Science Foundation of China, No 38970834.

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Received 2001-08-31 Accepted 2002-04-03

ventricular myocytes from guinea pigs (♂, 270 g ± 35 g, Grade Ⅱ, Certification № 19 – 050, provided by Shanghai Experimental Animal Center, Chinese Academy of Sciences) were prepared by enzymatic dissociation. The heart was dissected and rinsed in a cold oxygenated Ca^{2+}-free Tyrode's solution and then perfused on a Langendorff apparatus at 37 ℃ ± 0.5 ℃. A perfusion with Ca^{2+}-free Tyrode's solution for 5 min was followed by low Ca^{2+} (0.1 mmol/L) Tyrode's solution containing 0.03 % collagenase and 1 % bovine serum albumin (BSA) for 5 min. Then the ventricles were dissected, minced, incubated, and stirred mechanically in collagenase-free Tyrode's solution containing 1 % BSA with low-Ca^{2+} (0.1 mmol/L) at 37 ℃ ± 0.5 ℃ for 10 min. The cells were filtered through 200 μm nylon mesh, and resuspended in the Tyrode's solution in which the Ca^{2+} concentration was gradually increased to 1.0 mmol/L. The cells (30 % – 50 %) rod-shaped striated ventricular myocytes were harvested in Tyrode's solution (Ca^{2+} 1.0 mmol/L) and stored at room temperature (22 – 25 ℃) until use.

Electrophysiological recording Myocytes were placed in a 500 μL chamber on the stage of an inverted microscope (Olympus CK2, Japan). The chamber was continuously superfused (2 $mL \cdot min^{-1}$) with extracellular solution. Membrane currents were recorded using the whole-cell configuration of the patch-clamp techniques with a patch-clamp amplifier (CEZ 2300, Nihon Kohden, Japan). Patch electrodes were pulled with a vertical puller (PB-7, Narishige, Tokyo, Japan) and had a resistance of 2 – 3 MΩ when filled with electrode internal solution. Voltage-clamp command was generated by a 12-bit D/A converter (TL-1, Axon Instruments Inc, USA) controlled by a pClamp software. Experimental protocols, data acquisition, and storage were accomplished with pClamp 5.55 (Axon Instrument, USA) running on a PC computer. All experiments were conducted at 22 – 25 ℃.

Protocol Only the cells with rod shape, clear cross striation, and no spontaneous activity were used in our experiments. Resting membrane potentials were around from – 6 to – 70 mV, and could maintain at such level for 30 min during experiments. Holding potential was set at – 40 mV to inactivate Na^+ (I_{Na}) and T-type Ca^{2+} ($I_{Ca\text{-}T}$) current. The external solution contained nifedipine (2 μmol/L) to block L-type Ca^{2+} current ($I_{Ca\text{-}L}$). E-4031 (5 μmol/L, block I_{Kr}) was perfused the cells when measuring I_{Ks}. After the assessment of membrane currents, control data was recorded without bath of drugs, then perfusate was changed to solution containing various concentration of DDPH. Washout of DDPH was achieved by restoring perfusion of normal extracellular solution.

Chemicals and solutions DDPH (obtained from China Pharmaceutical University), was white powder (mp 167 – 168 ℃) of > 99 % purity and dissolved in distilled water. BSA, collagenase type Ⅱ, taurine, HEPES, egtazic acid, and K_2ATP were products of Sigma (USA), E-4031 from Calbiochem (USA), and 3-(*N*-morpholino)-propanesulfonic acid (MOPS) from Shanghai SIBAS Biotech Co.

Standard Tyrode's solution composed of (in mmol/L): NaCl 137.0, KCl 5.4, $MgCl_2$ 1.0, $CaCl_2$ 1.8, HEPES 10.0, glucose 20.0; pH adjusted to 7.4 with KOH.

The composition of the Ca^{2+}-free Tyrode's solution was (in mmol/L): NaCl 100, KCl 10, NaH_2PO_4 1.2, $MgSO_4$ 5.0, glucose 20, taurine 10, MOPS 10; pH was adjusted with KOH to 7.2.

Extracellular solution for recording K^+ current (in mmol/L): choline chloride 137, KCl 5.4, $MgCl_2$ 1.0, HEPES 10, glucose 10; pH was adjusted to 7.4 with KOH. The electrode solution for recording K^+ current contained (in mmol/L): KCl 140, $MgCl_2$ 0.5, egtazic acid 10, HEPES 10, K_2ATP 5.0; pH was adjusted to 7.4 with KOH.

Data analysis All membrane current data were analyzed off-line by ClampFit 6.01 (Axon Instrument, USA).

All data and curve fit were achieved *via* software SigmaPlot 2000 (SPSS Inc, USA) and ClampFit 6.01 (Axon Instrument, USA). The concentration-response curves were fitted to a modified Hill equation, $Y = (a - d)/[1 + (X/c)^b] + d$, using Marquardt-Levenberg methods of nonlinear regression analysis.

To study the kinetics of deactivation of the drug-sensitive currents, both tail currents of $I_{Kr\text{-}tail}$ and $I_{Ks\text{-}tail}$ were fitted by a single exponential function according to the following equation: $I(t) = A_0 + A_1 \exp(-t/\tau)$. Also the kinetics of deactivation of the total delayed rectifier potassium current (I_K) was determined by fits of tail current ($I_{K\text{-}tail}$) with a two exponential function: $I(t) = A_1 \exp(-t/\tau_1) + A_2 \exp(-t/\tau_2)$. A is the amplitude (pA), τ is the time constant of decay (ms), and t is time (ms). The activation kinetics of I_{Kr}, I_{Ks}, and inactivation kinetics of I_{Kr} were fitted by a single

exponential function.

Data of voltage-dependent activation of the I_{Kr} and I_{Ks} were fitted with a Boltzman function: $I = I_{max}/\{1 + \exp[(V_m - V_{1/2})/k]\}$, where I is the tail currents, I_{max} is the maximum tail current, V_m is the test potential, $V_{1/2}$ and k is half-activation voltage and the slope factor, respectively.

Data were expressed as $\bar{x} \pm s$ and statistic significance was determined by Student's t-test.

RESULTS

Inhibition of I_{Ks} In the presence of E-4031 (5 μmol/L), I_{Ks} was obtained by a depolarizing step pulse from the holding potential of −40 mV to +50 mV at the frequency of 0.1 Hz. The step pulse duration was 5000 ms (Fig 1). DDPH (0.1 − 100 μmol/L) produced concentration-dependent inhibition of I_{Ks} and tail current ($I_{Ks-tail}$). The IC_{50}(μmol/L) for blocking I_{Ks}-tail was 12.5 (95 % confidence limits: 4.8 − 32.2, Fig 2). The effect of DDPH was reversible after 30 min wash out (Fig 1).

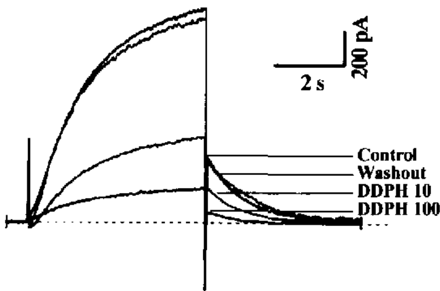

Fig 1. Effects of different concentration (μmol/L) of DDPH on a representative trace recording of I_{Ks} and $I_{Ks-tail}$ in guinea pig ventricular cells.

Inhibition of I_{Kr} We employed a short test-pulse protocol, which is sufficient to activate I_{Kr} and minimize any contamination of I_{Ks} activation. I_{Kr} was elicited by applying a 100 ms depolarization pulse from a holding potential of −40 to 0 mV, followed by repolarizing to −50 mV (Fig 3). The purity of I_{Kr} was also confirmed by E-4031, a selective I_{Kr} blocker. The amplitude of tail current, $I_{Kr-tail}$, was markedly reduced by 85 % by 5 μmol/L E-4031 (Fig 3A, from control; 22 pA ± 7 pA to 3.7 pA ± 1.2 pA, $P < 0.05$; $n = 3$ cells from 2 animals). DDPH (10 μmol/L) blocked I_{Kr} to the similar extend as E-4031 (Fig 3B). The IC_{50}(μmol/L) of blocking $I_{Kr-tail}$ was 6.1 (95 % confidence limits: 2.8 − 13.5, $n = 6$ cells from 4 animals, Fig 2).

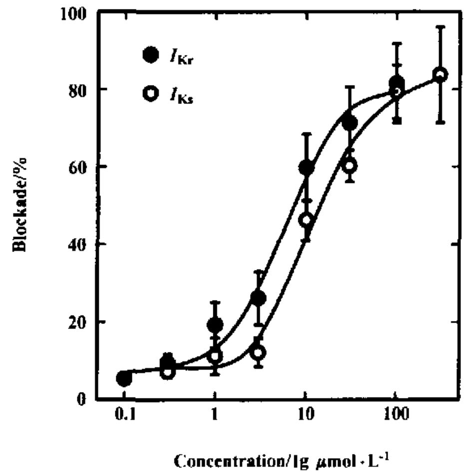

Fig 2. Concentration-response curve of inhibition of I_{Ks}(○, $n = 8$ cells from 5 animals) and I_{Kr}(●, $n = 6$ cells from 4 animals) by DDPH. $\bar{x} \pm s$. I_{Ks} and I_{Kr} were measured as $I_{Ks-tail}$ and $I_{Kr-tail}$, respectively.

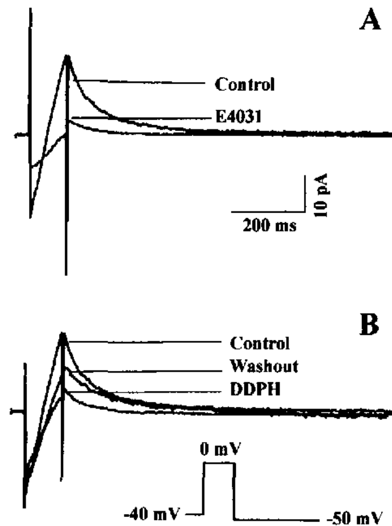

Fig 3. Inhibition of I_{Kr} and $I_{Kr-tail}$ by E-4031 (5 μmol/L, A) and DDPH (10 μmol/L, B) in guinea pig myocytes.

The effects of DDPH on I_{Kr} were further examined by an envelope of tails test on I_K, which is another way to distinguish two different component of I_K. Depolarizing voltage steps from −40 mV to a test potential of +50 mV were applied with stepwise increase in the pulse

ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 Jul; 23 (7)
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duration from 100 to 7000 ms. It showed a representative current trace before (Fig 4A) and after (Fig 4B) applying 10 μmol/L DDPH. The ratio of tail current over time-dependent current (I_{Ktail}/I_K) indicated two components of I_K, of which it was much larger for short pulses (1.0 ± 0.1, $n = 5$ cells from 4 animals, < 500 ms, I_{Kr} component) than that for long pulses (0.31 ± 0.02, > 1000 ms, I_{Ks} component). In the presence of DDPH (10 μmol/L), I_{Ktail}/I_K for short pulse was significantly depressed (0.33 ± 0.05, $P < 0.05$, compared with control), while the ratio for long pulses was unaffected (0.32 ± 0.04, $P > 0.05$, compared with control). It suggested that I_{Kr} component was blocked (Fig 4C).

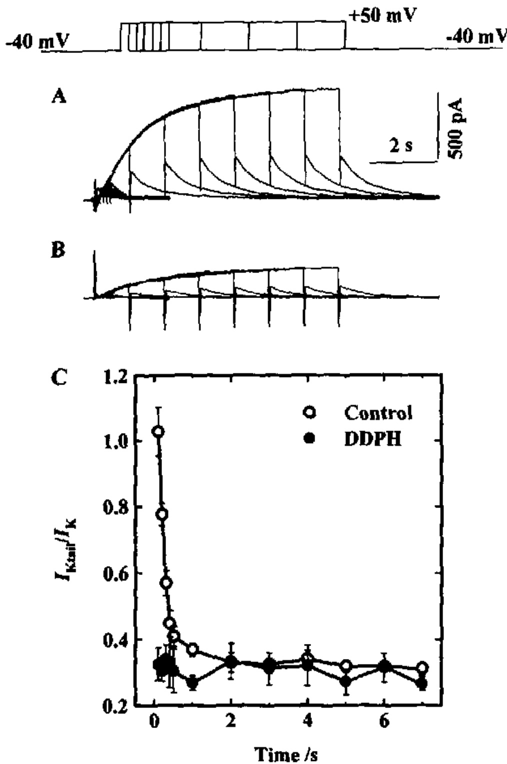

Fig 4. Envelope of tails test for delayed rectifier K⁺ current (I_K) in the absence (A) and presence (B) of DDPH (10 μmol/L). $n = 5$ cells from 4 animals. $\bar{x} \pm s$. The ratio of tail current over time-dependent current (I_{Ktail}/I_K) was plotted (C).

Effects on tail current kinetics of I_{Kr} and I_{Ks} The deactivation time constant was examined either by two exponential fit for tail current of total current (I_K), or by single exponential fit for $I_{Kr\text{-}tail}$ or $I_{Ks\text{-}tail}$ (Fig 5).

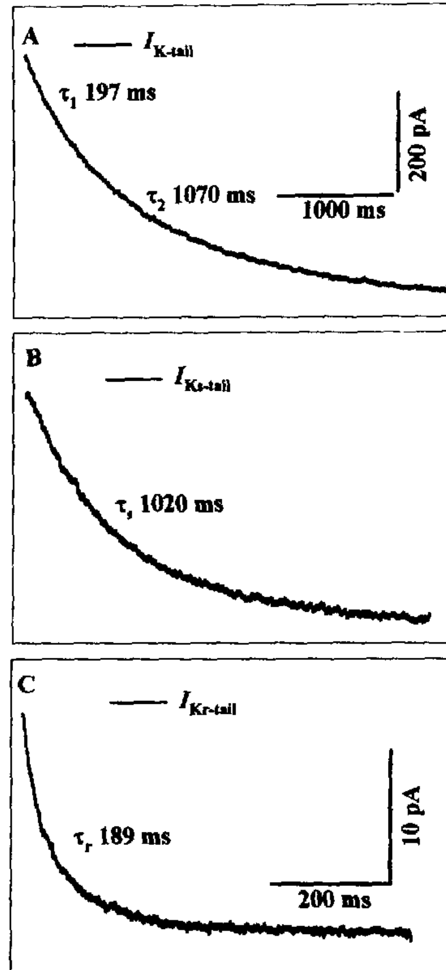

Fig 5. Representative curve fitting of tail currents of I_K(A), I_{Kr}(B), and I_{Ks}(C).

The two exponential of tail current of I_K (recorded on repolarization to holding potential −40 mV after a 5000 ms pulse to +50 mV) revealed two time constants: the fast (τ_1) and slow (τ_2) time constant. τ_1 (197 ms ± 32 ms, $n = 8$ cells from 7 animals) and τ_2 (1072 ms ± 210 ms, $n = 8$ cells from 7 animals) was similar to the time constant of $I_{Kr\text{-}tail}$ (τ_r, 188 ms ± 27 ms, $n = 6$ cells from 5 animals) and $I_{Ks\text{-}tail}$ (τ_s, 1019 ms ± 124 ms, $n = 8$ from 5 animals). It was another evidence confirmed two different components, I_{Kr} and I_{Ks}, existing. At low concentration (< 10 μmol/L), DDPH caused a slightly increases in both τ_r and τ_s, but high concentration (100 μmol/L) DDPH depressed the time constant (Tab 1), which might be due to non-specific effects since DDPH could affect multiple ion channels at high concentrations

Tab 1. Effect of DDPH on kinetics of tail current in guinea pig ventricular myocytes. n = 6 cells from 4 animals for measuring I_{Ks}. $\bar{x} \pm s$. $^bP<0.05$, $^cP<0.01$ *vs* control.

Group		I_{Kr}		I_{Ks}	
		τ_r/ms	$I_{Kr\text{-tail}}$/pA	τ_s/ms	$I_{Ks\text{-tail}}$/pA
Control		188±27	25±7	1019±124	336±35
DDPH/μmol·L^{-1}	1	203±38	19.4±2.6^b	1136±196	298±34^b
	10	171±24	9.77±1.9^c	1002±92	141±32^c
	100	58±13^c	4.8±1.3^c	736±141^c	71±11^c
Washout		183±63	21±4	979±242	304±29

from our previous observations.

Effects on voltage-dependent activation and activation kinetics of I_{Kr} and I_{Ks} The voltage dependence of I_{Kr} or I_{Ks} activation was determined by measuring the amplitude of steady-state currents after 250 ms or 5000 ms step depolarization pulses to test potentials between −40 to +30 mV (I_{Kr}) or −40 to +50 mV (I_{Ks}) from holding potential of −40 mV, respectively. The current-voltage (I-V) relation of I_{Kr} was different from that of I_{Ks}. I_{Kr} showed a bell-shaped curve with a maximum around 0 to +10 mV, which exhibited a prominent inward rectification at positive membrane potential. I_{Ks} exhibited a slightly outward rectification (Fig 6A). This finding was in consistent with well

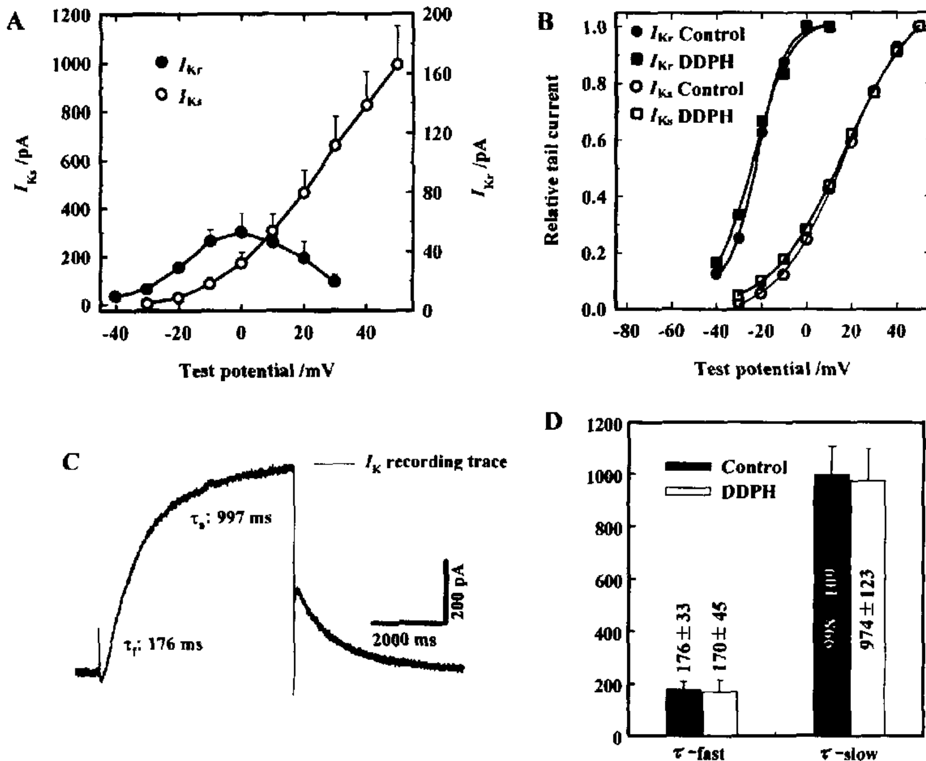

Fig 6. Current-voltage (I-V) relation for I_{Kr} and I_{Ks}, and voltage-dependent activation of I_{Kr} and I_{Ks} in guinea pig ventricular cells. I-V relation of I_{Ks} and I_{Kr} (A, n = 4 cells from 3 animals). Effects of DDPH (10 μmol/L) on voltage-dependent activation of I_{Kr} (closed symbol) and I_{Ks} (opened symbol) (B, n = 5 cells from 4 animals). A representative fitting curve of activation current of I_K (C). Activation time constants before and after applying DDPH 10 μmol/L (D, n = 6 cells from 5 animals). $\bar{x} \pm s$.

ISSN 1671-4083 Acta Pharmacol Sin 中国药理学报 2002 Jul; 23 (7)
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defined activation kinetics of I_{Kr} and I_{Ks}.

The currents were normalized relative to peak tail current and plotted against test potential. DDPH (10 μmol/L) did not affect the activation curve of both I_{Kr} and I_{Ks} (Fig 6B). The half-activation voltage ($V_{1/2}$, mV) and slope factor (k, mV) were determined as: I_{Kr}: -23.5 ± 2.4 and 8.1 ± 2.2 (in presence of DDPH, $P>0.05$, compared with control, $V_{1/2}$: -21.7 ± 0.8 and k: 5.9 ± 0.8); I_{Ks}: 27.1 ± 0.7 and 16.6 ± 0.8 (in presence of DDPH 10 μmol/L, $P>0.05$, compared with control, $V_{1/2}$: 27.0 ± 0.8 and k: 14.9 ± 0.9). The activation time course was described by the double exponential function on the I_K currents evoked at testing potential of +50 mV (lasting 5000 ms to fully activated both I_{Kr} and I_{Ks}) from a holding potential of −40 mV (Fig 6C). Two time constants were obtained, fast time constant (τ_f) and slow time constant (τ_s) represented activation process of I_{Kr} and I_{Ks}, respectively. DDPH did not show any significant changes in activation time constants (Fig 6D).

Inactivation kinetics of I_{Kr} I_{Kr} displayed a prominent and very rapid inactivation during depolarization, while there was no time-independent inactivation process for I_{Ks}. The voltage-dependent inhibition of DDPH on I_{Kr} was first observed since the inward rectification of I_{Kr} was determined by voltage-gated fast inactivation of channel. As shown in Fig 7A, the activation curve in the presence of DDPH began to decline when the membrane potential positive to 0 mV. This resulted in voltage-dependent inhibition of I_{Kr} after the channel had reach full activation (>0 mV). It suggested that DDPH affected the inactivation process. Direct evidence was obtained by analyzing the inactivating kinetics of I_{Kr}. A three-pulse protocol was applied. The holding potential was −85 mV, an initial pulse to +50 mV (1500 ms) was used to activate and/or inactivate all channels. A second hyperpolarizing pulse to −100 mV (25 ms) was sufficient for I_{Kr} recovery from inactivation without significant deactivation. A third testing pulse to 0 mV (200 ms) was to re-inactivate the channel. The elicited outward current reflected the opening of I_{Kr}, which again inactivated rapidly. Therefore the decaying tail current represented the I_{Kr} inactivation, and the time course for the onset of inactivation was determined by fitting a single exponential function to the tail current (Fig 7B). The inactivation time constant was 28.5 ms ± 4.6 ms before and 19.1 ms ± 3.8 ms ($P<0.05$) after bath perfusing DDPH (10 μmol/L, $n=4$ cells from 3 animals).

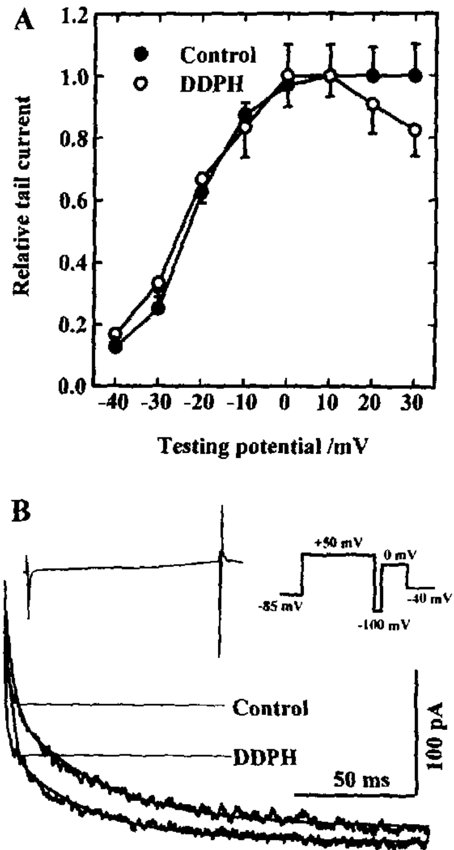

Fig 7. Effects of DDPH (10 μmol/L) on inactivation kinetics of I_{Kr}. Voltage-dependent inhibition of I_{Kr} by DDPH, and the currents were normalized to peak tail currents (A). n = 4 cells from 3 animals. $\bar{x}\pm s$. DDPH accelerated I_{Kr} inactivation (B), the inset showed a sample trace and experiment protocol to elicit inactivation curve of I_{Kr}.

DISCUSSION

Most class Ⅲ drugs selectively block I_{Kr}, and express reverse rate-dependent effect. Such effect limits efficacy in terminating rapid arrhythmias, thus increase the risk of *Torsades de Pointes* arrhythmias. It has been hypothesized that an increased contribution of I_{Ks} at fast heart rate could be involved in the reverse rate-dependent APD prolongation caused by I_{Kr}-blocking agents. Selective I_{Ks} block is speculated to increase APD and refractoriness in a frequency-independent manner[8]. Amidarone, by blocking I_{Kr} and I_{Ks}, prolonged APD and refractoriness with less reverse rate-dependence[9,10]. In guinea pig right ventricular papillary muscle, DDPH

showed a frequent-dependent alteration on APD[2], and our present results showed that DDPH effectively blocked both I_{Kr} and I_{Ks} with similar potency. Such effects were distinct from selective I_{Kr} blocker such as dofetilide, and also different from verapamil and mexiletine. Mexiletine blocked I_K in rabbit atrioventricular node (AVN) but showed no effects on both I_{Kr} and I_{Ks} in guinea pig ventricular myocytes[11,12]. Jones *et al* reported that verapamil was a potent I_{Kr} inhibitor (IC_{50}: 3 μmol/L) but not I_{Ks} blocker (IC_{50}: 280 μmol/L)[13].

DDPH did not affect the voltage-activation of these two components, while showed influences on deactivation kinetics of the channel. DDPH, at low concentration (< 10 μmol/L) slightly prolonged the deactivation. The inhibitory effects of DDPH were similar to amidarone[9]. It reflects a state-dependent block of K^+ channels. The inactivation process of I_{Kr} is a very important determinant in repolarization of action potential (I_{Ks} activated very slowly and no inactivation process was observed during repolarization phase)[5,14,15], therefore we presented evidence of DDPH interacting with the inactivated state of the I_{Kr} channel. The time course of inactivation was accelerated by DDPH and inhibition of I_{Kr} by DDPH was voltage-dependent (greater inhibition at more depolarized membrane potential at which the channel has turned to inactivated state[16]). The inactivation block of I_{Kr} might be unique ionic characteristics of DDPH action, and this may contribute to its antiarrhythmic mechanisms.

The electrophysiological actions of DDPH are complicated and it also inhibited $I_{Ca\text{-}L}$ and I_{Na}[17]. The net effects of DDPH on action potential should be determined by the sum of the lengthening influence related to blockade of I_K and the shortening influence due to inhibition of $I_{Ca\text{-}L}$. It is now suggested that antiarrhythmic drugs affecting multiple ion channels in a diseased myocardium may be useful for treating arrhythmia and hopefully reduce the incidence of proarrhythmias[18].

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DDPH 对豚鼠心室肌细胞延迟整流钾电流两种成分的抑制作用[1]

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关键词 1-(2,6-二甲基苯氧基)-2-(3,4-二甲氧基苯乙氨基)丙烷盐酸盐; 钾通道; 电生理学; 膜片箝技术; 豚鼠

目的: 研究 1-(2, 6-二甲基苯氧基)-2-(3,4-二甲氧基苯乙氨基) 丙烷盐酸盐(DDPH)对豚鼠心室肌细胞快激活(I_{Kr})和慢激活(I_{Ks})延迟整流钾电流的作用. **方法**: 全细胞膜片箝技术. **结果**: DDPH 0.1 - 100 μmol/L 浓度依赖性抑制 I_{Kr}, $I_{kr\text{-}tail}$[IC_{50}(μmol/L) 为 6.1, 95 %可信限为(2.8 - 13.5)]. DDPH 同时浓度依赖性抑制 I_{Ks}, $I_{Ks\text{-}tail}$[IC_{50}(μmol/L)为 12.5, 95 %可信限为(4.8 - 32.2)]. DDPH (10 μmol/L)不影响 I_{Kr}和 I_{Ks}的电压依赖性激活过程, 给药前 I_{Kr}的半激活电压($V_{1/2}$, mV) 和斜率因子(k, mV)分别为(-21.7±0.8)和(5.9±0.8), 给药后分别为(-23.5±2.4)和(8.1±2.2), 无统计学意义($P>0.05$). 用药前后 I_{Ks}的半激活电压和斜率因子的差异亦无统计学意义($P>0.05$), 用药前分别为(27.0±0.8)和(14.9±0.9), 用药后分别为(27.1±0.7)和(16.6±0.8). DDPH (<10 μmol/L)可抑制 I_{Kr}和 I_{Ks}的去激活过程, 并且加快 I_{Kr}的失活. **结论**: DDPH 抑制 I_{Kr}和 I_{Ks}无选择性. 且主要作用于其去激活过程, 而非激活过程. DDPH 进一步通过加速其失活过程抑制 I_{Kr}.

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