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ORIGINAL ARTICLE

Increasing DHA and EPA Concentrations Prolong Action Potential Durations and Reduce Transient Outward Potassium Currents in Rat Ventricular Myocytes

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Abstract The goal of this study was to determine the mechanisms of n-3 polyunsaturated fatty acids (n-3 PUFA) on anti-arrhythmias and prevention of sudden death. The calcium-tolerant Sprague–Dawley rat ventricular myocytes were isolated by enzyme digestion. Effects of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) on action potentials and transient outward potassium currents (I_{to}) of epicardial ventricular myocytes were investigated using whole-cell patch clamp techniques. Action potential durations (APDs) and I_{to} were observed in different concentrations of DHA and EPA. APD_{25}, APD_{50}, and APD_{90} with 0.1 μmol/L DHA and EPA were prolonged less than 15% and 10%. However, APDs were prolonged in concentration-dependent manners when DHA and EPA were more than 1 μmol/L. APD_{25}, APD_{50}, and APD_{90} were 7.7 ± 2.0, 21.2 ± 3.5, and 100.1 ± 9.8 ms respectively with 10 μmol/L DHA, and 7.2 ± 2.5, 12.8 ± 4.2, and 70.5 ± 10.7 ms respectively with 10 μmol/L EPA. I_{to} currents were gradually reduced with the increased concentrations of DHA and EPA from 1 to 100 μmol/L, and their half-inhibited concentrations were 2.3 ± 0.2 and 3.8 ± 0.6 μmol/L. The results showed APDs were prolonged and I_{to} current densities were gradually reduced with the increased concentrations of DHA and EPA. The anti-arrhythmia mechanisms of n-3 PUFA are complex, however, the effects of n-3 PUFA on action potentials and I_{to} may be one of the important mechanisms.

Keywords n-3 Polyunsaturated fatty acid · Docosahexaenoic acid · Eicosapentaenoic acid · Action potential · Action potential duration · Transient outward potassium current

Abbreviations

PUFA	Polyunsaturated fatty acids
DHA	Docosahexaenoic acid
EPA	Eicosapentaenoic acid
I_{to}	Transient outward potassium currents
APDs	Action potential durations
APD_{25}	Action potential duration of 25% repolarization
APD_{50}	Action potential duration of 50% repolarization
APD_{90}	Action potential duration of 90% repolarization
HP	Holding potential
V_{max}	Maximal velocity of action potential depolarization
APA	Action potential amplitude
OS	Overshoot

Introduction

Fatty acids, especially polyunsaturated fatty acids (PUFA), play an important role in cardiac activities. For example,

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PUFA are essential fuels for mechanical, electrical, and synthetic activities of the heart; and the dietary PUFA are very beneficial to heart functions [1, 2]. Recently, the beneficial effects of PUFA, particularly the n-3 series, have been reported for cardiovascular diseases [3–6]. n-3 PUFA have been shown to reduce cardiovascular mortality, in part, due to their anti-arrhythmic mechanisms that are not fully understood up till now [7, 8].

n-3 PUFA mainly include docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA). The vital importance of the n-3 PUFA, so called because of the location of the first double bond (at carbon no.3, from the methyl end of the carbon chain), is becoming widely recognized in human physiology. Many studies suggest that n-3 PUFA have beneficial effects on human health. Recently, more attentions have been paid to their beneficial effects on cardiovascular diseases, especially in their anti-arrhythmias and prevention of sudden cardiac death [9–13]. Although the underlying mechanisms of which are still not completely known, the effects of n-3 PUFA on action potentials and ionic channels might contribute to this phenomenon [14].

The typical action potentials consist of 5 phases or stages, i.e., 0, 1, 2, 3, and 4. The transient outward potassium current (I_{to}) is a major repolarizing ionic current of action potentials in ventricular myocytes of many mammals. Because of its immediate activation by depolarization and its transient nature, I_{to} plays an important role in the early repolarization of action potentials. It influences the height of the plateau potential and the action potential durations (APDs), especially in the early phase. Accordingly, its inhibition leads to a significant prolongation of APDs. It has been reported that APDs magnitude depends on the origin of the myocytes and is regulated by a number of physiological and pathophysiological signals [15, 16].

To investigate the underlying mechanisms of n-3 PUFA in anti-arrhythmias and prevention of sudden cardiac death, the magnitude of I_{to} and APDs were studied in rat ventricular myocytes of endocardial, mid-myocardial and epicardial origin using whole-cell patch clamp recordings. The aim of this study was to test the hypothesis that a differential distribution of I_{to} is the most important cause of different action potential waveforms in endocardial, mid-myocardial and epicardial myocytes, and the effects of n-3 PUFA on action potentials and I_{to} may be one of the important mechanisms of anti-arrhythmias. Since I_{to} may not be uniformly expressed in the ventricle [17], we have only investigated the epicardial cardiomyocytes in order to avoid experiment errors. These results may provide some experimental evidences for rational applications of n-3 PUFA to prevent and treat arrhythmias in clinical practice.

Experimental Procedures

Major Experimental Instruments

The instruments used were: MultiClamp 700B patch clamp amplifier (Axon Instruments, USA), D/A and A/D converter (DigiData 1322, Axon Instruments, USA), Pclamp 9.0 pulse software (Axon Instruments, USA), MP-285 motorized micromanipulator (Sutter Instruments, USA), IX71 inverted microscope (Olympus, Japan), SA-OLY/2 and DH-35 culture dish heater (Warner Instruments, USA), P-97 micropipette puller (Sutter Instruments, USA).

Reagents, Solutions and Drugs

The reagents, solutions and drugs used were: DHA (Sigma, USA), molecular weight 328.5, and EPA (Sigma, USA), molecular weight 302.45; 100 mmol/L stock solutions were prepared respectively by being dissolved in absolute ethanol and protected from light in a refrigerator at −20 °C. The experimental concentrations of DHA and EPA were obtained by dilution of stock solutions before each experiment. For the recording of action potentials, the internal solution (in mmol/L) contained KCl 120, $CaCl_2$ 1, $MgCl_2$ 5, Na_2ATP 5, EGTA 11, HEPES 10, glucose 11, pH 7.3 adjusted with KOH. The external solution was Tyrode's solution [18]. For the recording of I_{to}, the external solution (in mmol/L) contained NaCl 140, KCl 4, $CaCl_2$ 1.5, $MgCl_2$ 1, $CdCl_2$ 0.5, HEPES 5, glucose 10, pH 7.4 adjusted with NaOH. The internal solution (in mmol/L) contained KCl 140,$MgCl_2$ 1,K_2ATP 5, EGTA 5, HEPES 10, pH 7.4 adjusted with KOH. KB solution (in mmol/L) contained L-glutamic acid 50, KCl 40, KH_2PO_4 20, Taurine 20, $MgCl_2$ 3, KOH 70, EGTA 0.5, HEPES 10, glucose 10, pH 7.4 adjusted with KOH.

Cell Isolation

The investigation was approved by our institute ethics committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23, revised 1996). Healthy Sprague–Dawley rats of both sexes, aged 8–12 weeks and weighing approximately 200 g, were provided by the Experimental Animal Center of Soochow University (Suzhou, China). Animals were anesthetized with pentobarbital sodium intraperitoneally (i.p.), Hearts were removed and retrograde perfusion through the aorta was performed as described previously [19]. After retrograde perfusion, epicardial, mid-myocardial, and endocardial ventricular myocardium were obtained respectively by cutting with eye scissors and plyers. Isolated cells were kept at room temperature in KB solution and used within 6 h; only relaxed, striated, and rod-shaped cells were used.

Recordings of Action Potentials and I_{to} with and Without DHA and EPA

Currents in whole-cell voltage clamp configuration were recorded following the method of Hamill et al. [20]. Cardiomyocytes were transferred to a 1-ml chamber (DH-35 culture dish heater, Warner Instruments, USA) with external solution on the stage of an inverted microscope. The chamber was continuously perfused at a rate of 1–2 ml/min with external solution. Electrodes were prepared from borosilicate glass (Clark Instruments, UK) using a P-97 micropipette puller with resistances typically between 2 and 4 MΩ when filled with internal solution. Whole-cell voltage-clamp experiments were performed with a MultiClamp 700B amplifier. Whole-cell capacitance and series resistance were compensated by 60–80%. Experiments were performed at 36–37 °C. Voltage clamp pulses were generated via an IBM-compatible computer connected to Digidata 1322. Data acquisition and analyses were performed using pCLAMP software. To obtain action potentials, a 5-ms depolarizing pulse with 900pA, 1 Hz in current-clamp configuration was applied. DHA and EPA at 0.01, 0.1 , 1, 10, and 100 μmol/L were perfused for 10 min respectively to observe the effects on APDs. To obtain I_{to}, 600-ms depolarizing pulses in the range −40 mV to +70 mV were applied to the ventricular myocytes every 5 s in +10 mV increments from −40 mV holding potential (HP). Recordings of action potentials and I_{to} were performed in the physiological temperature range (36–37 °C). DHA and EPA at various concentrations were applied to investigate the effects on I_{to}.

Statistical Analysis

Continuous variables were expressed as means ± standard error ($\bar{x}$ ± SE). SPSS11.5 SPSS Inc, Chicago, IL, USA) was used for statistical analysis. Comparisons among groups were performed by repeated measurement analysis of variance (ANOVA) and least-significant difference contrast. Control and drug data for individual groups were compared by a Paired t test. $P \leq 0.05$ was considered significant. OriginPro 7.5 software (OriginLab, USA) was utilized to calculate the half-inhibited concentration (IC_{50}).

Results

Characteristics of Action Potentials and I_{to} of Rat Ventricular Myocytes

Action potentials of epicardial, mid-myocardial and endocardial ventricular myocytes were recorded respectively with action potential stimulus protocol. The action potential configurations were different in epicardial, mid-myocardial and endocardial ventricular myocytes (Fig. 1). APD_{25}, APD_{50}, and APD_{90} were gradually prolonged from epicardial to endocardial ventricular myocytes. APD_{25}, APD_{50}, and APD_{90} were 3.6 ± 1.2, 10.3 ± 2.1, and 46.3 ± 4.8 ms in epicardial ventricular myocytes ($n = 50$); 6.4 ± 1.8, 14.7 ± 2.4, and 69.4 ± 8.3 ms in mid-myocardial ventricular myocytes ($n = 58$) and 13.8 ± 2.1, 45.3 ± 10.2, and 152.1 ± 33.4 ms respectively in epicardial ventricular myocytes ($n = 62$). There were statistical significance of APDs' variations in cardiomyocytes of different layers ($P < 0.05$); however, there were no remarkable changes in the maximal velocity of action potential depolarization (V_{max}), amplitude (APA), and overshoot (OS) in epicardial, mid-myocardial and endocardial ventricular myocytes. V_{max} were 228.3 ± 14.5 V/s ($n = 71$), 10.3 ± 2.1 V/s ($n = 63$), and 46.3 ± 4.8 V/s ($n = 70$) in epicardial, mid-myocardial and endocardial ventricular myocytes ($P > 0.05$). APA were 110.7 ± 10.1 mV ($n = 71$),

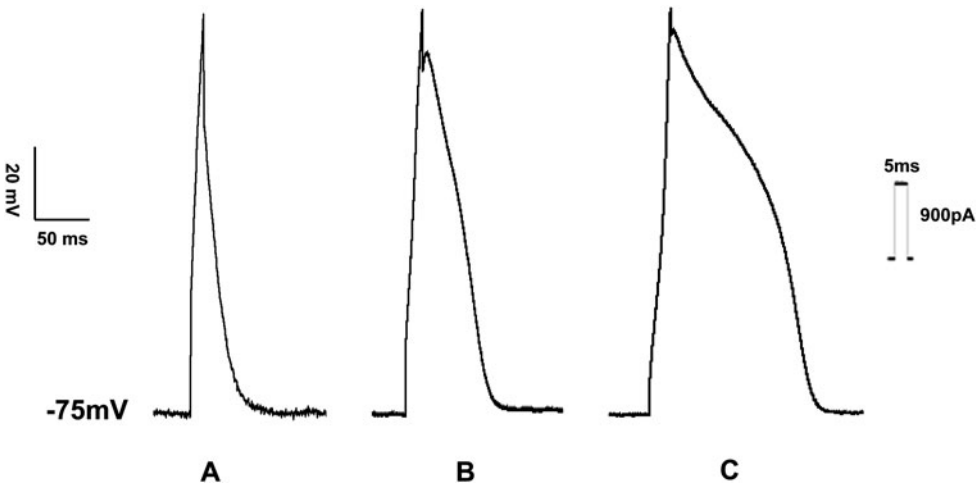

Fig. 1 Action potential configurations of rat ventricular myocytes. *A*, *B* and *C* were action potentials in epicardial, mid-myocardial and endocardial ventricular myocytes, respectively. Action potential durations were gradually increased from epicardial ventricular myocytes to endocardial ventricular myocytes

111.9 ± 9.3 mV ($n = 63$), and 109.8 ± 8.9 mV ($n = 70$) in epicardial, mid-myocardial and endocardial ventricular myocytes ($P > 0.05$). OS were 31.5 ± 5.4 mV/s ($n = 71$), 32.4 ± 6.3 mV ($n = 63$), and 30.8 ± 4.8 mV ($n = 70$) in epicardial, mid-myocardial and endocardial ventricular myocytes ($P > 0.05$). I_{to} current tracings at various test potentials were elicited by 600 ms depolarization in the range of −40 mV to +70 mV pulses applied to the ventricular myocytes every 5 s in +10 mV increments from −40 mV HP. The current densities of epicardial, mid-myocardial and endocardial ventricular myocytes at +70 mV were 59.5 ± 16.0 , 29.2 ± 5.5, and 12.3 ± 3.6 pA/pF, respectively.

The current–voltage curves of I_{to} were plotted with current densities at each test potential. The threshold potential of I_{to} channel opening was −30.3 ± 2.8 mV, i.e., I_{to} channel began to activate at more than −30 mV. I_{to} currents were gradually enhanced with the increase of test potentials. The activation of I_{to} channel was very rapid, and only needed about 10 ms, nonetheless, its inactivation was relatively slow. The time constants of epicardial, mid-myocardial, and endocardial ventricular myocytes were almost the same at each test potential. They were 31.8 ± 1.7, 32.9 ± 2.4, and 33.2 ± 2.9 ms, respectively, at +70 mV test potential ($P > 0.05$).

Effects of DHA and EPA on Action Potentials

DHA and EPA at 0.01, 0.1, 1, 10, and 100 μmol/L were applied to epicardial ventricular myocytes, respectively. The results showed that: ① APDs were gradually prolonged with the increase of DHA and EPA concentrations, whereas APD changes were not significant at low concentrations of DHA and EPA (<1 μmol/L). The prolongation of APD_{25}, APD_{50}, and APD_{90} was less than 15% compared with the control (0 min) when 0.1 μmol/L DHA was applied, and less than 10% with 0.1 μmol/L EPA application (Fig. 2). ② APDs were prolonged in concentration-dependent manners when DHA and EPA were more than 1 μmol/L. APD_{25}, APD_{50}, and APD_{90} were 7.7 ± 2.0, 21.2 ± 3.5, and 100.1 ± 9.8 ms respectively after 10 μmol/L DHA was used for 5 min (Fig. 3C), and 7.2 ± 2.5, 12.8 ± 4.2, and 70.5 ± 10.7 ms respectively with 10 μmol/L EPA application for 5 min (Fig. 3C1). APD_{25}, APD_{50}, and APD_{90} were 15.2 ± 4.0, 45.7 ± 6.8, and 215.6 ± 15.7 ms respectively when 100 μmol/L DHA was utilized for 5 min (Fig. 3D), and 13.1 ± 5.4, 48.2 ± 9.1, and 132.3 ± 21.2 ms respectively with 100 μmol/L EPA for 5 min (Fig. 3D1), which were significantly prolonged compared with those without addition of DHA and EPA ($P < 0.05$).

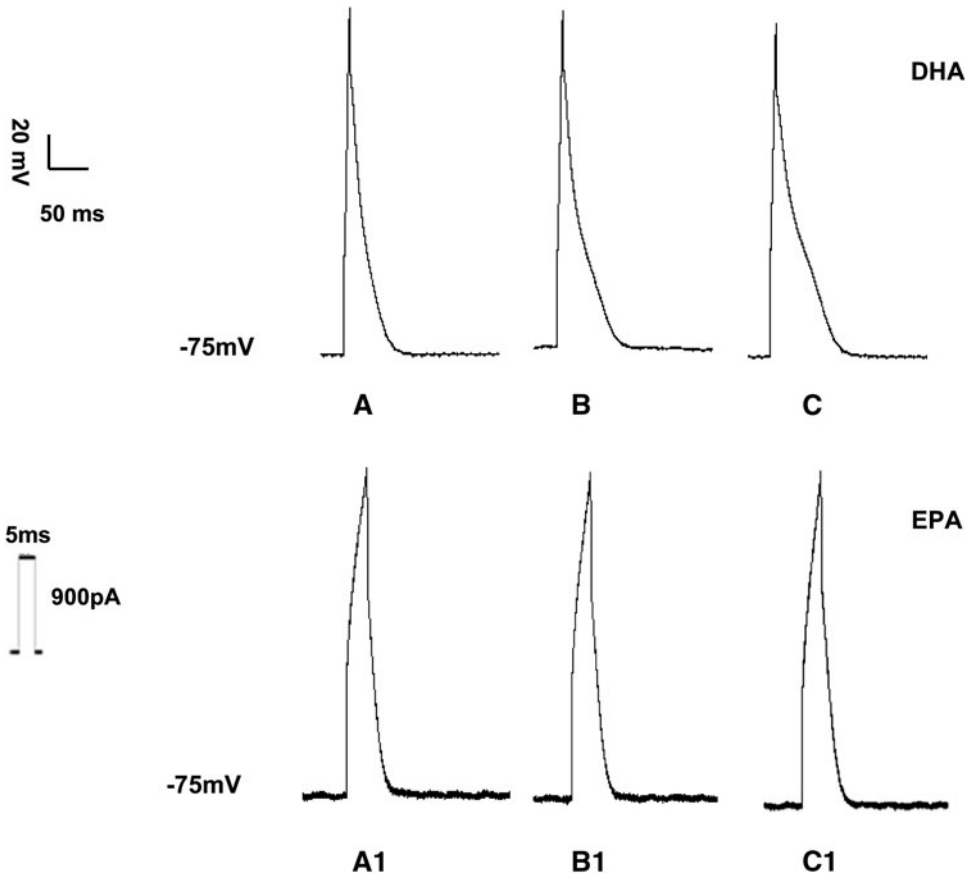

Fig. 2 Action potential changes of rat ventricular myocytes at 0.1 μmol/L DHA and EPA. *A*, *B*, and *C* were configurations of action potential when 0.1 μmol/L DHA was applied at 0, 1, and 5 min, respectively. The action potential duration was increased; however, compared with the control (0 min), the prolongation of action potential duration was less than 15%. *A1*, *B1*, and *C1* were configurations of action potential when 0.1 μmol/L EPA was applied at 0, 1, and 5 min, respectively. The action potential duration was increased; however, compared with the control (0 min), the prolongation of action potential duration was less than 10%

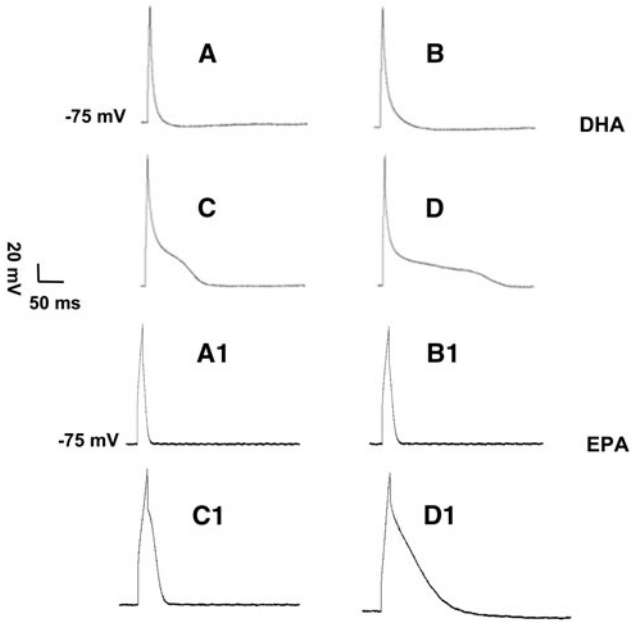

Fig. 3 Action potential changes of rat ventricular myocytes at different concentrations of DHA and EPA. *A*, *B*, and *C* were the applications of 10 μmol/L DHA at 0, 1, and 5 min. *D* was DHA at concentration of 100 μmol/L for 5 min. Action potential durations were significantly prolonged in concentration-dependent manners when DHA concentrations were more than 10 μmol/L. *A1*, *B1*, and *C1* were the applications of 10 μmol/L EPA at 0, 1, and 5 min. *D* was EPA at concentration of 100 μmol/L for 5 min. Action potential durations were significantly prolonged in a concentration-dependent manner when EPA concentrations were more than 10 μmol/L

Effects of DHA and EPA on I_{to}

DHA and EPA at 0.01, 0.1, 1, 10, and 100 μmol/L were applied, respectively. I_{to} currents were blocked by DHA and EPA in concentration-dependent manners. Current densities were gradually decreased with the increases of DHA and EPA concentrations. The I_{to} current densities at +70 mV in different concentrations of DHA and EPA were illustrated in Table 1. The representative current tracings blocked by DHA and EPA at 100 μmol/L were shown in Fig. 4. IC_{50} of DHA and EPA on I_{to} were fitted with Hill function and calculated by OriginPro 7.5 software, which were 2.3 ± 0.2 and 3.8 ± 0.6 μmol/L (Fig. 5).

Discussion

The typical action potentials consist of 5 phases or stages, i.e., 0, 1, 2, 3, and 4. The present study has showed that there are no typical action potential configurations of rat ventricular myocytes. APDs are the shortest in epicardial ventricular myocytes, and repolarization rapidly appears after depolarization, demonstrating no platform phase [21]. The phenomenon of "spike and dome" [22] sometimes can

Table 1 I_{to} current density changes in different concentrations of DHA and EPA

Concentrations (μmol/L)	I_{to} current densities (pA/pF)	
	DHA	EPA
0	59.5 ± 16.0	59.5 ± 16.0
0.01	58.4 ± 15.2	59.1 ± 14.3
0.1	57.1 ± 14.1	59.0 ± 13.2
1	49.3 ± 10.2	52.3 ± 10.5
10	35.4 ± 8.0	40.0 ± 9.2
100	30.1 ± 7.2	32.7 ± 8.1

be seen, however, this phenomenon does not appear in endocardial ventricular myocytes. Action potential repolarization in endocardial ventricular myocytes is slow, showing a relative standard action potential profile. The action potential configurations of mid-myocardial ventricular myocytes are between epicardial and endocardial ventricular myocytes [23, 24], but action potential repolarization is still rapid, and has the tendency of the platform phase, compared with the epicardial ventricular myocytes. The reasons why these alterations appear are that there are regional differences of I_{to} in epicardial, mid-myocardial and endocardial ventricular myocytes. The I_{to} channels are the most abundant in rat epicardial ventricular myocytes and then by turns in mid-myocardial and endocardial ventricular myocytes. The I_{to} current densities in epicardial, mid-myocardial and endocardial ventricular myocytes were different in this study, which further illustrates that I_{to} channels of rat ventricular myocytes have regional differences. I_{to} channels in epicardial myocytes are extremely abundant, and therefore, I_{to} currents are the largest, which makes action potential repolarization rapid, calcium inflow time and APDs short. In contrast, I_{to} channels in endocardial myocytes are few or absent, and therefore, APDs prolong. V_{max}, APA, and OS are formed mainly by 0 phase depolarization. Because regional differences of I_{to} channels do not affect depolarization of ventricular myocytes, and thus, there are no significant differences of V_{max}, APA, and OS in epicardial, mid-myocardial and endocardial myocytes [25].

The Ca^{2+}-insensitive but 4-aminopyridine-sensitive I_{to} currents play a major role in modulating cardiac electrical activity [26]. It underlies phase 1 repolarization, and thus, by setting the voltage of the early plateau phase, it influences activation and inactivation of other plateau currents that affect repolarization. It has also been reported in several studies that I_{to} channels are potentially important targets for both neuromodulatory control [27] and antiarrhythmic drug actions [28]. This current has been suggested to contribute significantly to the regional electrophysiological heterogeneity within the ventricular wall,

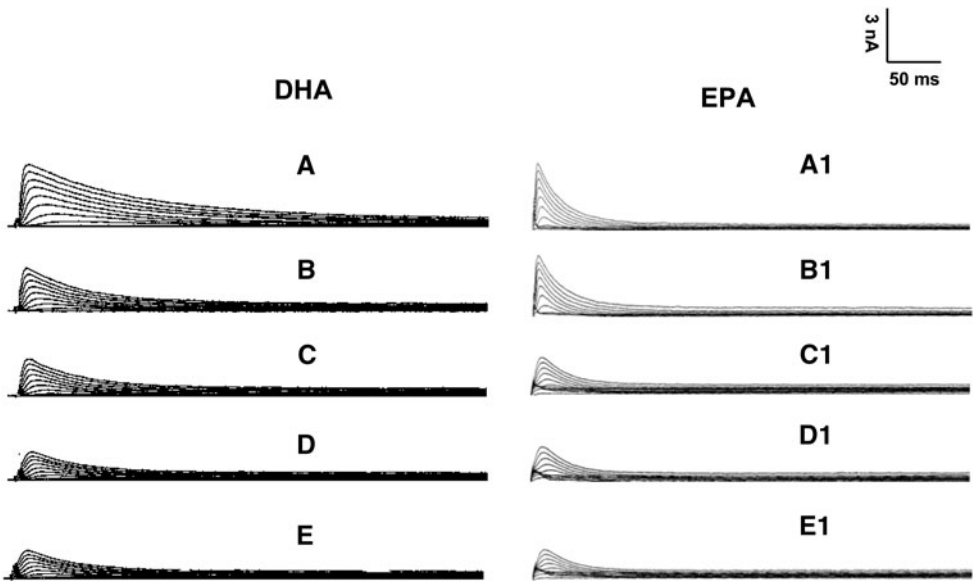

Fig. 4 Alterations of transient outward potassium currents after DHA and EPA at 100 μmol/L were applied. *A*, *B*, *C*, *D*, and *E* were representative transient outward potassium current tracings with DHA at 0, 1, 5, 10, and 15 min, respectively. Transient outward potassium currents were remarkably blocked by DHA. *A1*, *B1*, *C1*, *D1*, and *E1* were representative transient outward potassium current tracings with EPA at 0, 1, 5, 10, and 15 min, respectively. Transient outward potassium currents were significantly blocked by EPA

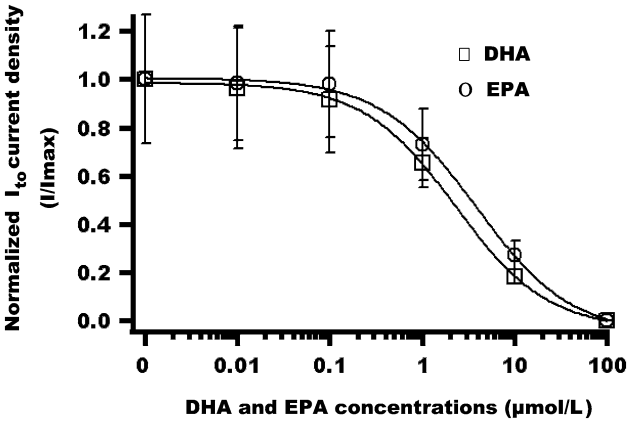

Fig. 5 Half-inhibited concentrations of DHA and EPA on transient outward potassium currents of rat ventricular myocytes. Half-inhibited concentrations of DHA and EPA on transient outward potassium currents were fitted with Hill function, which were 2.3 ± 0.2 and 3.8 ± 0.6 μmol/L

a fact considered to be responsible for T-wave polarity. The heterogeneous distribution of I_{to} thus appears to be essential in causing the transmural electrical gradients necessary for proper repolarization of cardiac action potentials. It is expected that changes in I_{to} distribution and availability can be expressed in the ECG by typical J-wave and T-wave alterations and may lead to cardiac arrhythmias during evolving heart diseases.

The present study results have shown that there are regional differences of action potentials and I_{to} amplitude and configuration of rat epicardial, mid-myocardial and endocardial myocytes. Regional differences of action potentials and I_{to} should be considered when rats are chosen as the experimental animal. We should try to obtain same regional ventricular myocytes to avoid experiment errors when we perform cellular electrophysiological studies. Consequently, in this study, we only chose epicardial myocytes to investigate the effects of DHA and EPA on action potentials and I_{to}.

There have been many reports about the electrophysiological effects of n-3 PUFA on potassium channels of cardiomyocytes and vascular smooth muscle cells in recent years [29–33]. Interestingly, it seems that n-3 PUFA inhibit or block potassium channels of cardiomyocytes [29–31], whereas they activate potassium channels of vascular smooth muscle cells [32, 33]. So far, n-3 PUFA protect against arrhythmia and sudden cardiac death using largely unknown mechanisms [34–36]. In order to investigate the mechanisms of n-3 PUFA on anti-arrhythmias and prevention of sudden death, we performed this experiment to study the effects of DHA and EPA on the action potential and I_{to} of rat ventricular myocytes. The reasons that we chose the rat as the experimental animal are not only because the rats have many advantages, e.g. cheap, strong vitality, and easily bred, but also because there are many similar electrophysiological characteristics between rat and human cardiomyocytes [17, 37].

After DHA and EPA at various concentrations were applied, APDs were gradually prolonged and I_{to} current densities were decreased by degrees with the increased concentrations of DHA and EPA. DHA and EPA could inhibit I_{to} currents, prolong APDs, and extend effective refractory period of cardiomyocytes [38]. The effects of DHA and EPA on action potentials and I_{to} may be one of their anti-arrhythmia mechanisms.

Figure 4 clearly showed that DHA and EPA could inhibit I_{to} currents. However, from the morphologic changes of action potentials in Fig. 3, we found that the morphologic changes of action potentials mainly appeared in phase 2 and phase 3 with the increased concentrations of

DHA and EPA. In contrast, there were no significant changes in phase 1 of action potential formed mainly by I_{to} current efflux. This means DHA and EPA may have effects on other ion currents in addition to the I_{to} current.

The present study has some limitations, e.g., we only investigated the effects of DHA and EPA on action potentials and I_{to} of rat ventricular myocytes. The effects of DHA and EPA on other ion currents such as I_{Na}, $I_{Ca\text{-}L}$, I_K, and I_{K1} still need to be studied further. Only if we explore the anti-arrhythmia mechanisms of n-3 PUFA completely, can we apply them correctly in clinical practice to prevent and treat cardiovascular diseases [39–43].

In summary, the present findings obtained by the patch-clamp technique have clearly shown that APDs are prolonged, and I_{to} current densities are gradually reduced with the increased concentrations of DHA and EPA. DHA and EPA have the similar effects on action potentials and I_{to} of ventricular myocytes. The effects of n-3 PUFA on action potentials and I_{to} may be one of the important mechanisms of anti-arrhythmias.

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