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Author(s): Vehniäinen, Eeva-Riikka; Haverinen, Jaakko; Vornanen, Matti

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Eeva-Riikka Vehniäinen ORCID iD: 0000-0001-6061-9014

Running head: PAHs affect cardiac ion currents in rainbow trout

Polycyclic aromatic hydrocarbons phenanthrene and retene modify the action potential via multiple ion currents in rainbow trout *Oncorhynchus mykiss* cardiac myocytes

Eeva-Riikka Vehniäinen[1*], Jaakko Haverinen[2], Matti Vornanen[2]

1 University of Jyväskylä, Department of Biological and Environmental Sciences, P.O. Box 35, FI-40014 University of Jyväskylä, Finland

2 University of Eastern Finland, Department of Environmental and Biological Sciences, P.O. Box 111, 80101, Joensuu, Finland

* Address correspondence to eeva-riikka.vehniainen@jyu.fi

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ABSTRACT

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous contaminants in aqueous environments. They affect the cardiovascular development and function in fishes. The three-ring PAH phenanthrene has recently been shown to impair cardiac excitation-contraction coupling by inhibiting Ca^{2+} and K^{+} currents in marine warm-water scombrid fishes. To see if similar events take place in a boreal freshwater fish, we studied if the PAHs phenathrene and retene (an alkylated phenanthrene) modify the action potential (AP) via effects on Na^{+} (I_{Na}), Ca^{2+} (I_{CaL}) or K^{+} (I_{Kr}, I_{K1}) currents in the ventricular myocytes of the rainbow trout *Oncorhynchus mykiss* heart. Electrophysiological characteristics of myocytes were measured using whole-cell patch-clamp. Micromolar concentrations of phenanthrene and retene modified the shape of the ventricular AP, and retene profoundly shortened the AP at low micromolar concentrations. Both PAHs increased I_{Na}, and reduced I_{CaL} and I_{Kr}, but retene was more potent. Neither of the PAHs had an effect on I_{K1}. Our results show that phenanthrene and retene affect the cardiac function in rainbow trout by a mechanism that involves multiple cardiac ion channels, and the final outcome of these changes (shortening of AP) is opposite to that observed in scombrid fishes (prolongation of AP). The results also show that retene, and aryl hydrocarbon receptor (AhR) agonist, has an additional mechanism of toxicity besides the previously known AhR-mediated, transcription-dependent one.

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Key words: aquatic toxicology, cardiotoxicity, mode of action, polycyclic aromatic hydrocarbons (PAHs)

1 INTRODUCTION

Polycylic aromatic hydrocarbons (PAHs) are ubiquitous contaminants that occur as complex mixtures in aquatic environments. They originate from petrogenic or pyrogenic sources, and they enter the waters via atmospheric deposition, oil accidents, municipal and industrial effluents, and urban runoff. Individual PAHs as well as PAH mixtures (such as oil) affect the development and function of the heart in several fish species (Billiard et al., 1999,Incardona et al., 2004,Incardona et al., 2006,Incardona et al., 2009,Incardona et al., 2011,Dubansky et al., 2013,Incardona et al., 2014,Brette et al., 2017,Raine et al., 2017).

Phenanthrene and retene (1-methyl-7-isopropyl phenanthrene) are three-ring PAHs. Phenanthrene is common in both petrogenic and pyrogenic mixtures of PAHs, and it causes reversible bradycardia and atrioventricular conduction block in zebrafish (*Danio rerio*) and slight increase in heart rate and reduction of circulation in marine medaka (*Oryzias melastigma*) (Incardona et al., 2004,Mu et al., 2014,Sun et al., 2015,Cypher et al., 2017). In Pacific bluefin tuna (*Thunnis orientalis*), phenanthrene affects the cardiac action potential (AP) and ion currents (Brette et al., 2017). Retene is an alkylated phenanthrene, and it has been found in sediments downstream from pulp and paper mills, in landfills, and in oil sands produced water (Leppanen and Oikari, 1999a,Leppanen and Oikari, 1999b,Legler et al., 2011,Cheng et al., 2018) . Retene is an aryl hydrocarbon receptor (AhR) agonist, and it activates the AhR and causes changes in the transcription

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of several genes, leading to developmental defects in the cardiovascular system (Billiard et al., 1999,Scott et al., 2011,Vehniainen et al., 2016).

Contraction of the vertebrate heart is triggered by cardiac AP, which originates from the primary pacemaker center at the border zone between the sinus venosus and the atrium (Yamauchi and Burnstock, 1968,Haverinen and Vornanen, 2007). From there AP spreads throughout the atrium, and via the atrioventricular canal further to the ventricular wall thereby triggering sequential contractions of atrium and ventricle (Sedmera et al., 2003). Cardiac AP is generated by the complex interaction between several voltage-gated ion currents in the sarcolemma of cardiac myocytes. In fish ventricular myocytes, there are two major inward currents, the fast Na^+ current (I_{Na}) and L-type Ca^{2+} current (I_{CaL}; long-lasting), and two major outward K^+ currents, the fast component of the delayed rectifier K^+ current (I_{Kr}) and the background inward rectifier K^+ current (I_{K1}) (Vornanen, 2016). Besides these major ion currents, fish ventricular myocytes may have T-type Ca^{2+} current (I_{CaT}; transient) and the slow component of the delayed rectifier K^+ current (I_{Ks}) (Nemtsas et al., 2010,Hassinen et al., 2011,Abramochkin et al., 2018,Haverinen, Hassinen, Dash et al., 2018). The shape of cardiac AP is regulated by time- and voltage-dependent opening and closing of the Na^+, Ca^{2+} and K^+ channels. Electrical excitability of cardiac myocytes, i.e. the ease with which the cardiac AP can be triggered, is dependent on the antagonistic effects of I_{Na} and I_{K1} on membrane potential and an important factor in uninterrupted propagation of cardiac AP (Varghese, 2016,Vornanen, 2016).

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The aim of this work was to study if phenanthrene and retene modulate the four major ion currents of the rainbow trout *Oncorhynchus mykiss* ventricle, which could reveal novel toxic effects of these PAHs on fish heart.

2 MATERIAL AND METHODS

2.1 Animals

Hatchery-reared rainbow trout (*Oncorhynchus mykiss*) (73.43 ± 11.69 g, n=18) were obtained from the local fish farm (Kontiolahti, Finland). In the animal facilities of the University of Eastern Finland, the trout were maintained in 500 L metal aquaria for a minimum of 3 weeks before used in the experiments and fish were fed aquarium fish food (Ewos, Finland) at least five times a week. Water temperature was regulated at 14 ± 0.5°C (Computec Technologies, Joensuu, Finland) and oxygen saturation was maintained by aeration with compressed air. Ground water (average pH 8.0, conductivity 13 µS/cm) was constantly flowing through the aquaria at the rate of 150-200 L per day (permission ESAVI/2832/04.10.07/2015).

2.2 Myocyte isolation

All experiments were conducted *in vitro* on enzymatically isolated ventricular myocytes. The fish were killed by a cranial concussion and pithing, and the heart was rapidly excised. Ventricular myocytes were isolated using the retrograde perfusion of the heart and the standard concentrations of hydrolytic enzymes as reported for the method developed in our lab (Vornanen, 1997). Cell isolation was conducted at room temperature

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(20-22°C). Isolated myocytes were used in the experiments within 10 hours from isolation.

2.3 Whole-cell patch clamp

The whole-cell current-clamp recordings were made by using an Axopatch 1D amplifier (Axon Instruments, Saratoga, USA). Clampex 9.2 software was used for data acquisition, and off-line analysis of the recordings was done using the Clampfit 10.4 software package. During the experiments, myocytes were continuously superfused with the external saline solutions at the rate of 1.5-2 ml min^{-1}. The temperature of the external solution in the recording chamber was regulated at 14°C by using a Peltier device (CL-100, Warner Instruments, CT, USA or HCC-100A, Dagan, MN, USA), and continuously recorded on the same file with electrophysiological data. Patch pipettes were pulled (PP-83, Narishige, Tokyo, Japan) from borosilicate glass (King Precision, Claremont, CA) and had a resistance of 2.7 ± 0.06 MΩ when filled with the internal saline solutions. After gaining a giga ohm seal, the membrane under the pipette tip was ruptured by a short voltage pulse (zap) to get access to the cell, transients due to series resistance (7.3 ± 0.26 MΩ) and pipette capacitance were cancelled, and capacitive size of ventricular myocytes was determined.

For recording of APs and K^+ currents the external saline solution contained (mmol l^{-1}) 150 NaCl, 5.4 KCl, 1.2 $MgCl_2$, 1.8 $CaCl_2$, 10 HEPES, 10 glucose and pH was adjusted with NaOH to 7.6 at 20°C (giving a pH of 7.68 at the experimental temperature). The composition of the pipette (internal) solution was as follows (mmol l^{-1}): 140 KCl, 4

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MgATP, 1 $MgCl_2$, 0.03 Tris-GTP, 10 HEPES (pH adjusted with KOH to 7.2 at 20°C). To elicit APs, ventricular myocytes were stimulated with current pulses of constant duration (4 ms) and with increasing amplitude. The initial stimulus strength was 200 pA and it was raised with 20 pA increments until an all-or-none AP was elicited (Badr et al., 2018). The stimulation frequency was 1 Hz. The following AP parameters were analysed off-line: resting membrane potential (V_{rest}, mV), threshold potential of AP (V_{th}, mV), threshold current (I_{th}, pA), critical depolarization (CD = V_{th}-V_{rest}, mV), AP overshoot (OS, mV), AP amplitude (AMP, mV), AP duration at 50% repolarization level (APD_{50}, ms), maximum rate of AP upstroke (+dV/dt, mV ms^{-1}) and the maximum rate of AP repolarization (-dV/dt, mV ms^{-1}) (Fig. 1). V_{th}, I_{th} and CD are measures for electrical excitability of ventricular myocytes, i.e. the ease with which AP can be triggered by depolarizing current.

Voltage-dependency of the rapid component of the delayed rectifier K^+ current (I_{Kr}) and the inward rectifier K^+ current (I_{K1}) were measured using standard stimulation protocols (Vornanen, Ryökkynen et al., 2002) from the holding potential of -80 mV. When recording I_{K1}, the external saline included 2 µM E-4031 (1-[2-(6-methyl-2-pyridyl)ethyl]-4-(4-methylsulfonyl-aminobenzoyl)piperidine), 0.5 µM tetrodotoxin (TTX, Tocris Cookson, Bristol, UK) and 10 µM nifedipine to block I_{Kr}, I_{Na} and I_{CaL}, respectively. I_{Kr} was recorded in the presence of TTX (0.5 µM), nifedipine (10 µM) and 0.2 mM $BaCl_2$ (to block I_{K1}).

The fast Na^+ current (I_{Na}) was measured under a reduced Na^+ gradient (20 mM $[Na^+]_o$, 5 mM $[Na^+]_i$) across the sarcolemma to obtain good control of the membrane voltage. The

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composition of the external saline was (mmol l^{-1}): 20 NaCl, 120 CsCl, 1 $MgCl_2$, 0.5 $CaCl_2$, 10 glucose and 10 HEPES at pH 7.7 (adjusted with CsOH at 20°C)(Haverinen and Vornanen, 2004). Nifedipine (10 µmol l^{-1}) was included in the external solution to block I_{CaL}. The pipette solution consisted of (in mmol l^{-1}) 5 NaCl, 130 CsCl, 1 $MgCl_2$, 5 EGTA, 5 Mg_2ATP and 5 HEPES (pH adjusted to 7.2 with CsOH at 20°C). I_{Na} was elicited from the holding potential of -120 mV with established stimulus protocols (Haverinen and Vornanen, 2006,Haverinen, Hassinen, Korajoki et al., 2018).

The composition of the external saline solution for recording I_{CaL} was as follows (mmol l^{-1}): 150 NaCl, 5.4 CsCl, 1.8 $CaCl_2$, 1.2 $MgCl_2$, 10 HEPES and10 glucose (pH adjusted to 7.6 at 20°C with CsOH). TTX (0.5 µM) was included in this saline to block Na^+ current (I_{Na})(Vornanen, 1998). Since Cs^+ may flow through the Erg K^+ channels, 2 µM E-4031 was included in the external solution to prevent contamination by I_{Kr}. The pipette solution contained (mmol l^{-1}) 130 CsCl, 15 TEACl (tetraethylammonium chloride), 5 MgATP, 1 $MgCl_2$, 5 oxaloacetate, 10 HEPES and 5 EGTA (pH adjusted to 7.2 at 20°C with CsOH) (all chemicals from Sigma). I_{CaL} was elicited from the holding potential of -80 mV to +10 mV at the frequency of 0.2 Hz. Recording of I_{CaL} is complicated by time-dependent run-down (decline) of the current. To minimize the effect of run-down on results, time-dependent changes in I_{CaL} were monitored after getting access to the whole configuration. For the same reason the analysis of PAH effects was limited to two highest concentrations. Only those cells were accepted for analysis, where I_{CaL} stabilized within about 5 minutes from the start of recording.

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2.4 PAHs

The stocks of phenanthrene (Sigma-Aldrich, Steinheim, Germany) and retene (MP Biomedicals LLC, Illkirch, France) were made in DMSO at 20 mM. Test solutions at the concentrations 0.3, 1.0, 10 and 30 µM for phenanthrene and 0.1, 1.0 and 10 µM for retene were made daily in external saline solutions. Effects of the highest DMSO concentration in the experimental solutions on AP parameters and ion currents were tested in separate experiments. No statistically significant effects were noticed.

2.5 Statistical analyses

After checking normality of distribution and equality of variances, one-way ANOVA (with Tukey's or Dunnett's T3 post hoc test) or nonparametric test (with Friedman's test) were used for evaluating the effect of different PAH concentrations on AP parameters and maximum ion currents. All the statistical tests were performed using SPSS (IBM; version 21.0) software. Data are presented as mean ± SEM and $P < 0.05$ was considered statistically different.

3 RESULTS

3.1 Phenathrene and retene differentially modify the action potential in rainbow trout ventricular myocytes

Phenanthrene had no effect on the duration of AP at the level of 50% repolarization (APD50) (Fig. 2A, E), but shortened it at the zero voltage level (APD_0) at 30 µM (Fig. 2A). Phenanthrene increased the maximum upstroke velocity (+dV/dt) at 1 and 10 µM,

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and accelerated the maximum rate of AP repolarization (-dV/dt) at 10 and 30 µM (Fig. 2C). Retene had more pronounced effects on APs than phenanthrene. The duration of the AP (APD50 and APD_0) was strongly shortened at 1 and 10 µM concentrations (Fig. 3A, E). Retene augmented the AP amplitude at 10 µM and increased the overshoot at 1 and 10 µM (Fig. 3B). The maximum rate of AP upstroke became faster at all concentrations of retene, but the effect on -dV/dt was significant only at 10 µM (Fig. 3C). Excitability of ventricular myocytes was decreased at the highest (10 µM) retene concentration, as CD needed to elicit AP was about 18% higher than in the control (Fig. 3B).

3.2 Phenanthrene and retene modulate cardiac I_{Na}, I_{CaL} and I_{Kr} currents but have no effect on I_{K1}

Phenanthrene and retene affected all studied ventricular ion currents except I_{K1}, but retene caused the effects at lower concentrations than phenanthrene. Under exposure of 10 µM phenanthrene or 1 µM retene, the peak density of I_{Na} was increased by 12 and 17 %, respectively (Fig. 4A, B). The effects of the highest concentrations (30 µM phenanthrene, 10 µM retene) were slightly less and statistically non-significant (Fig. 4B).

After getting electrical access to the cell, there was a clear increase in the amplitude of I_{CaL} due to the buffering of intracellular free Ca^{2+} by EGTA of the pipette solution (removal of Ca^{2+}-dependent inactivation of I_{CaL}). Then the current stabilized and enabled the recording of drug effects on I_{CaL} (Fig. 5A, B). Phenanthrene reduced I_{CaL}, but the effect was statistically significant only at the highest concentration tested, 30 µM (Fig. 5C). Retene diminished I_{CaL} at 1 and 10 µM (Fig. 5C).

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Whereas phenanthrene attenuated I_{Kr} at 10 and 30 µM, retene was effective even at the lowest test concentration (0.1 µM) (Fig. 6). Both phenanthrene and retene decreased the I_{Kr} tail currents at all voltages, where the tail current was activated (Fig. 6 C, D). The maximum inhibition of I_{Kr} tail at +40 mV was 79.3 and 59.2% for phenanthrene and retene, respectively. During the depolarizing prepulse, phenanthrene and retene inhibited I_{Kr} ($I_{Kractiv}$) in the voltage range between 0 and +20 mV, but did not have any effect at +40 and +60 mV (Fig. 6 E, F). This suggests that there is a phenanthrene- and retene-resistant current underlying I_{Kr}, probably the slow component of the delayed rectifier K^+ current, I_{Ks}. Neither of the PAHs had an effect on the background inward rectifier, I_{K1} (Fig. S1 A, B).

4 DISCUSSION

4.1 Effects on action potential

Both three-ring PAHs affected ventricular AP of the rainbow trout heart, but retene was a much stronger AP modifier than phenanthrene. Phenanthrene did not change V_{rest} or AP amplitude consistently with the findings from bluefin tuna cardiomyocytes (Brette et al., 2017). V_{rest} is maintained by the I_{K1}, which remained untouched by phenanthrene. Phenanthrene had only minor effects on APD; APD was slightly reduced at the zero-voltage level but remained unchanged at 50% repolarization level. In this respect, rainbow trout clearly differs from bluefin tuna, where phenanthrene lengthened ventricular APD (Brette et al., 2017). APD is regulated by a delicate balance between influx of Ca^{2+} via I_{CaL} and efflux of K^+ via I_{Kr}, I_{Ks} and I_{K1} (Grant, 2009). Since the resistance of the sarcolemma at AP plateau is high (Ca^{2+} and K^+ fluxes are small), small

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changes in the amplitude and activation/inactivation rate of Ca^{2+} and K^{+} currents will affect APD (Zaza, 2010). Shortening of AP at the zero-voltage level suggests that in the early plateau I_{CaL} is reduced more than I_{Kr} by phenanthrene. Our results show a small but clear increase in +dv/dt in rainbow trout with 10 and 30 µM phenanthrene. Phenanthrene also steepened the rate of repolarization (-dv/dt). These are novel actions of PAHs on AP of the fish heart. Collectively, the present findings show that phenanthrene has partly different effects on cardiac APs in rainbow trout and bluefin tuna.

In contrast to phenanthrene, retene strongly shortened APD and the effect occurred at the lower drug concentrations (1 and 10 µM). The strong shortening of APD suggest that the relative effect of retene on I_{CaL} is stronger than its effect on I_{Kr}, the main repolarizing current. The effects of retene differ from those of phenanthrene in that AP amplitude and overshoot were enhanced by retene but not by phenanthrene. This difference may be associated with slightly stronger stimulation of I_{Na} by retene. Notably, the highest retene concentration (10 µM) attenuated excitability of ventricular myocytes, as CD was increased. *In vivo*, the decrease in excitability could cause interruptions in AP propagation between ventricular myocytes (Vornanen, 2016).

4.2 Effects on cardiac ion currents

The Na^{+} current (I_{Na}) is active during the upstroke of the AP causing the depolarization of the sarcolemma by fast and large influx of Na^{+}. Both phenanthrene and retene increased the peak I_{Na} density in the ventricular myocytes of rainbow trout. Retene was more potent than phenanthrene in enhancing I_{Na}, which is in line with its larger effect on +dv/dt and overshoot of the AP. At the level of intact tissue the larger I_{Na} means a faster propagation

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of AP in the ventricular wall. To our knowledge, there is no earlier data on the effects of PAHs on fish cardiac I_{Na}. However, in the bluefin tuna ventricular myocytes, phenanthrene did not affect the upstroke velocity of the AP, thus suggesting species-specific differences in PAH modulation of I_{Na}.

I_{CaL} and I_{Kr} are the main determinants of the long AP plateau. They are counteracting currents, as I_{CaL} is depolarizing and I_{Kr} repolarizing. The net outcome of the inhibition of these currents can be seen as changes in APD. Notably, both PAHs caused shortening of APD, but retene was much more powerful than phenanthrene. Strong shortening of APD by retene indicates that the net charge influx via I_{CaL} is inhibited more than the K^+ efflux via I_{Kr}. The final phase 3 repolarization is accelerated by the background inward rectifier I_{K1}. The increase in the rate of -dV/dt by PAHs is probably attributable to the resistance of I_{K1} to retene and phenanthrene whereby the uninhibited I_{K1} overwhelms the reduced I_{CaL}.

The lowering of Ca^{2+} influx in phenanthrene-treated rainbow trout cardiac myocytes is in line with previous research showing that phenanthrene decreased Ca^{2+} transients in ventricular myocytes of scombrid fishes, even though scombrids may be more dependent on intracellular Ca^{2+} stores for contractile activation (Brette et al., 2017). The magnitude of I_{CaL} inhibition by phenanthrene seems to differ between tuna and rainbow trout: Whereas 5 µM phenanthrene decreased I_{CaL} by ~30 % and 25 µM phenanthrene by ~75 % in bluefin tuna, in rainbow trout 10 and 30 µM phenanthrene attenuated I_{CaL} only by ~20 and ~40 %, respectively (Brette et al., 2017). On the other hand, in tuna the reduction of I_{CaL} should be partly compensated by the prolonged AP plateau. Brette et al. (2017) did

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not report whether the run-down of I_{CaL} was taken into account, which might have also affected their results. In rainbow trout, retene was more potent than phenanthrene in attenuating I_{CaL}, as the same reduction of I_{CaL} by ~20 and ~40 % was achieved with lower concentrations of retene, 1 and 10 µM, respectively.

The strong shortening of APD and inhibition of I_{CaL} by retene is expected to have a strong reducing effect on the intracellular free Ca^{2+} concentration. In trout ventricular myocytes, the activation of contraction is largely dependent on the sarcolemmal Ca^{2+} influx during the AP plateau, as about 2/3 of the activator Ca^{2+} is estimated to come from the extracellular space (Vornanen, Shiels et al., 2002). Inhibition of I_{CaL} and shortening of the plateau means that Ca^{2+} influx is smaller and there is less time for Ca^{2+} entry. In the intact ventricle, this should appear as reduced force of contraction. Indeed, exposure to PAHs or oil reduces atrial and ventricular contraction and diminishes cardiac stroke volume in larval fish (Incardona et al., 2013,Jung et al., 2013,Edmunds et al., 2015,Esbaugh et al., 2016,Sørhus et al., 2016,Khursigara et al., 2017,Perrichon et al., 2018). In rainbow trout yolk sac larvae, retene causes pericardial and yolk sac edemas (Billiard et al., 1999,Scott et al., 2011,Vehniainen et al., 2016), phenomena often seen with PAH and oil exposures, and proposed to be caused by reduced cardiac output (Incardona and Scholz, 2016). Taken together, inhibition of I_{CaL} and shortening of AP plateau would compromise contractility and cardiac output of the heart with the outcome of reduced physical performance level and fitness of the fish. These effects would be particularly strong under the intoxication by retene.

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I_{Kr} channels are notorious about their susceptibility to inhibition by low concentrations of various small molecule compounds (Sanguinetti and Tristani-Firouzi, 2006). The wide pore cavity of the channel allows access of small molecules to the pore (Vandenberg et al., 2001). Therefore, it is no surprise that also PAHs can block these channels in fish cardiac myocytes. In rainbow trout ventricular myocytes, 10 and 30 µM phenanthrene reduced I_{Kr} by 43 and 75 %, respectively. This is slightly less than the inhibition in bluefin tuna, where 5 and 25 µM phenanthrene decreased I_{Kr} by 60 and over 85 %, respectively (Brette et al., 2017). In rainbow trout, the effect of retene on I_{Kr} was more pronounced, as 10 µM retene caused a 60 % reduction in I_{Kr} (Fig. S2).

In mammalian heart, blockade of I_{Kr} by many drugs is shown to be proarrhythmic and able to induce chaotic ventricular tachycardia, *torsades de pointes* (Vandenberg et al., 2001). However, if both I_{Kr} and I_{CaL} are inhibited simultaneously and at similar drug concentrations, the effect is antiarrhythmic, even when drugs prolong, shorten or triangulate ventricular APs (Kramer et al., 2013,Obejero-Paz et al., 2015). A typical example is verapamil, a useful human cardiovascular medicine, which inhibits human I_{Kr} and I_{CaL} at similar concentrations (Shetuan et al., 1999,Kang et al., 2012). As PAHs inhibit both I_{Kr} and I_{CaL} at similar micromolar concentrations, they should not be proarrhythmic in fish ventricle. However, I_{Kr} and I_{CaL} are essential components of the cardiac pacemaker, which determines the rate and rhythm of the heartbeat (Schram et al., 2002). Half-maximal inhibition of I_{Kr} by E-4031 is known to reduce the beating rate of rainbow trout sinoatrial preparations, and therefore inhibition of I_{Kr} might explain the PAH-induced bradycardia of larval fish (Haverinen and Vornanen, 2007). Inhibition of I_{CaL} is likely to affect impulse generation and conduction of the nodal tissues (sinoatrial

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pacemaker and atrioventricular canal), since I_{CaL} is the main determinant for the rate of AP upstroke and impulse conduction (I_{Na} is absent or small in nodal cells)(Schram et al., 2002). Inhibition of I_{CaL} might therefore appear as atrioventricular block and ventricular bradycardia, phenomena seen in larval fish exposed to PAHs or oil (Incardona et al., 2004,Zeltser et al., 2004,Incardona et al., 2005,Incardona et al., 2009,Incardona et al., 2011,Perrichon et al., 2016,Perrichon et al., 2018). However, care must be taken when applying results from mature fish to embryos or larval fish.

As retene is quite hydrophobic ($\log K_{ow}$ ~6), the actual concentrations in the test chamber have most probably been lower than nominal. It must also be borne in mind that retene is quickly metabolized by CYP1A in fish, and this may lower the concentration of parent retene that reaches cardiac myocytes *in vivo* (Hawkins et al., 2002). In nature, however, fish are exposed to PAH mixtures that frequently contain CYP1A inhibitors, which in turn decrease the metabolism of PAHs and thus increase the concentration of parent compounds (Hawkins et al., 2002).

Retene is an AhR agonist, and it disturbs the cardiovascular development in fish via activating AhR and altering transcription (Scott et al., 2011,Vehniainen et al., 2016). Our study shows that in addition to this transcriptional route, retene has a direct effect on cardiac function via modulating voltage-gated ion channel activity. As normal cardiac function is important for cardiovascular development (Glickman and Yelon, 2002,Incardona et al., 2015), as well as the development of other tissues and organs (Incardona et al., 2004), retene may cause developmental defects also independent of

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AhR. The ability to modulate the activity of cardiac ion channels also means that in addition to early-life stages, retene may be cardiotoxic to juveniles and adults.

5 CONCLUSION

Three-ring PAHs phenanthrene and retene differentially modified ventricular APs in rainbow trout cardiomyocytes. Retene was more potent and strongly reduced the duration of ventricular AP. Although phenanthrene and retene had qualitatively similar effects on ion currents, phenanthrene only slightly affected AP duration, probably due to its weaker inhibition of I_{Kr} and I_{Ca} in comparison to retene. Furthermore, the effects of phenanthrene on ventricular AP differed from those reported earlier for the marine warm-water scombrid fish bluefin tuna. These results suggest that different PAHs may have different direct effects on cardiac function, and that these effects may be partly species-specific. This further complicates the environmental risk assessment of PAHs.

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Figures

Figure 1. AP parameters measured in the current clamp experiments. (A) The first 150 ms of a rainbow trout ventricular action potential (AP) showing the parameters that were determined from the recordings. The continuous line shows the all-or-none AP and the dotted line the local passive response of the membrane. V_{rest}, resting membrane potential; CD, critical depolarization; V_{th}, threshold voltage; overshoot (OS) and amplitude (AMP) of AP. The maximum rate of depolarization (upstroke) (+dV/dt) and repolarization (-dV/dt) of AP were measured from the first derivative of the AP tracing. (B) The stimulus protocol of increasing current strengths (duration 4 ms) used to search the trigger level for APs. The strength of current just able to trigger an AP is the threshold current (I_{th}). (C) A typical response of a ventricular cell to increasing stimulus strength.

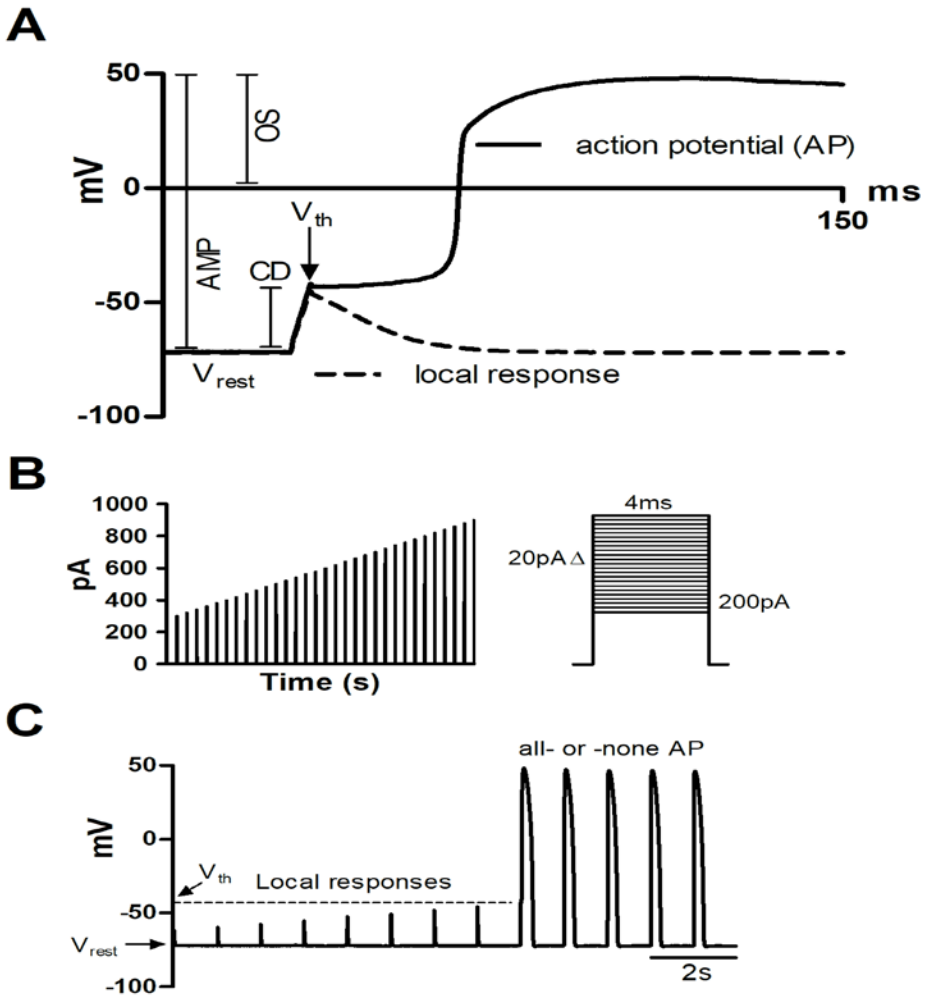

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Figure 2. Phenanthrene modifies the shape of the action potential (AP) but has no effect on the AP duration in rainbow trout cardiac ventricle cells. (A) A representative experiment showing the effect of cumulatively increasing concentrations of phenanthrene on AP. (B,D,E) Phenanthrene has no effect on the resting membrane potential (V_{rest}), threshold voltage (V_{th}), AP amplitude (AMP), overshoot (OS), critical depolarization (CD), threshold current (I_{th}) and AP duration at 50% repolarization level (APD50). (C) Phenanthrene steepens both the maximum rate of AP depolarization (+dV/dt) and repolarization (-dV/dt). An asterisk indicates statistically significant difference from control.

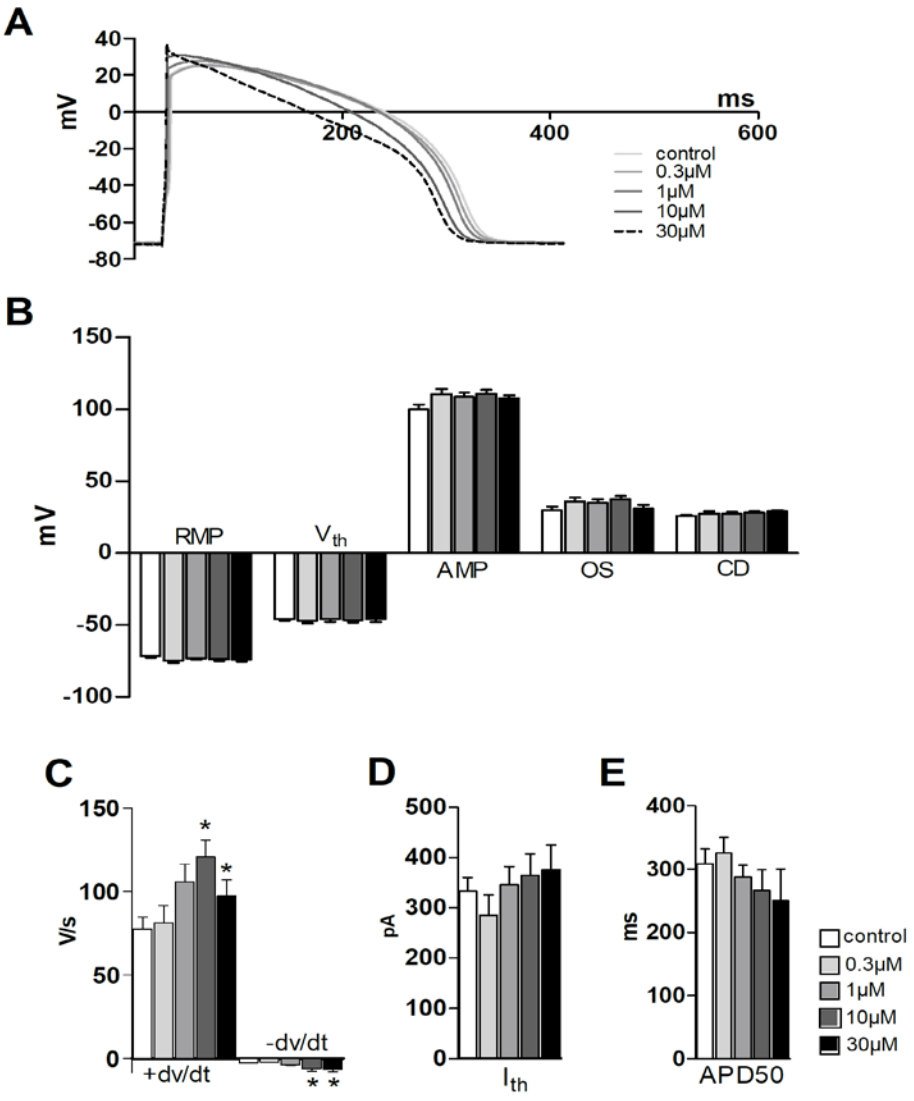

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Figure 3. Retene shortens the action potential duration (APD) and modulates the shape of the AP in ventricular cardiomyocytes of the rainbow trout. (A) A representative experiment showing the effect of cumulatively increasing concentrations of retene on AP. (B,D) Retene has no effect on the resting membrane potential (V_{rest}), threshold voltage (V_{th}) and threshold current (I_{th}). (B) AP amplitude (AMP), overshoot (OS) and critical depolarization (CD) are increased by retene. (C,E) Retene shortens the AP duration (APD50) and steepens both the maximum rate of depolarization (+dV/dt) and the maximum rate of repolarization (-dV/dt) of the AP. An asterisk indicates statistically significant difference from control.

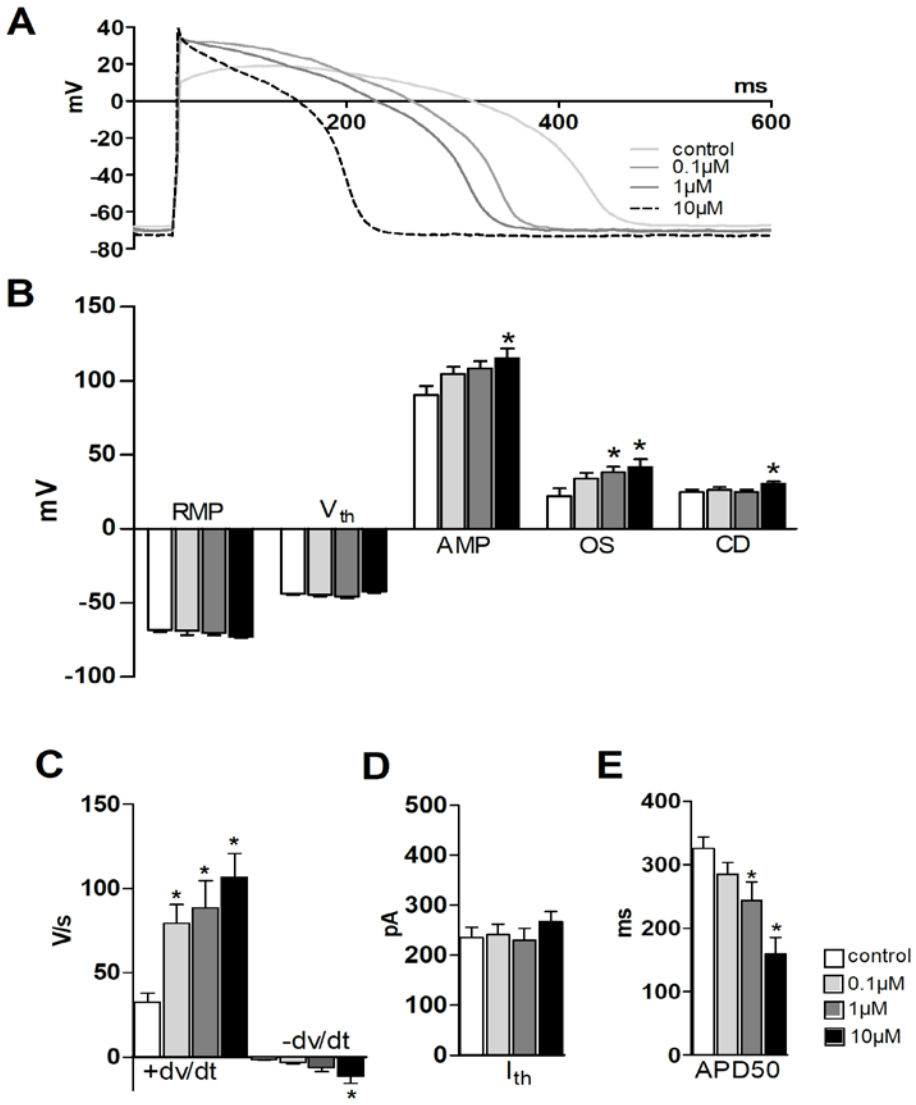

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Figure 4. Phenanthrene and retene increase the fast Na+ current (I_{Na}) in rainbow trout ventricular cardiomyocytes. (A) Current-voltage relationship of I_{Na} in the absence and presence of phenanthrene (left) and retene (right). The stimulus protocol is shown between the graphs. (B) Effects of phenanthrene (10, 30 μM) and retene (1, 10 μM) on the peak density of I_{Na}. The results are means ± SEM of 12-14 myocytes from at least 3 animals. Groups denoted by the same letter do not differ significantly from each other.

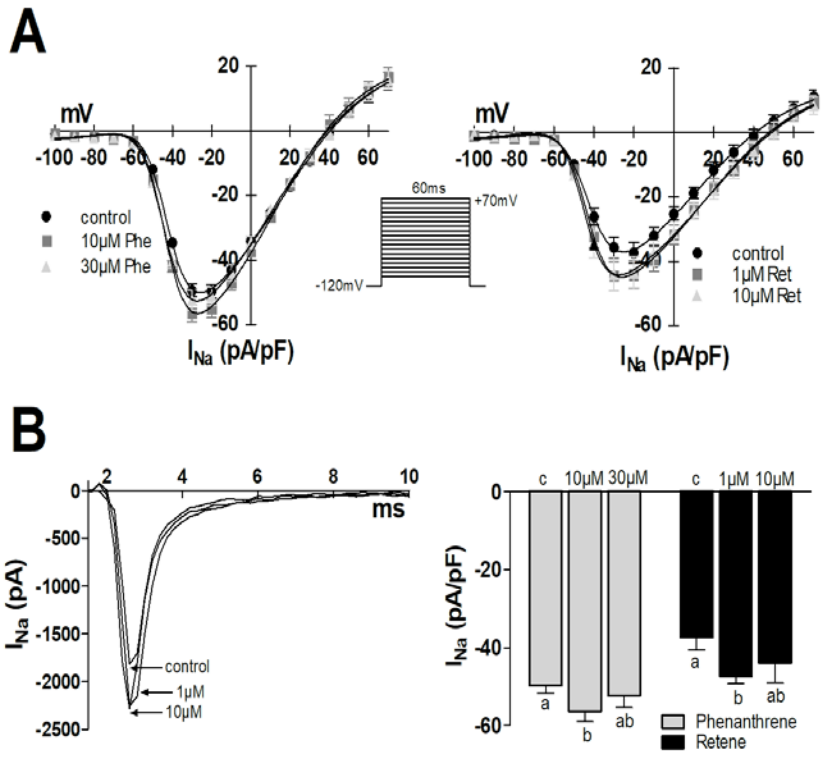

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Figure 5. Phenanthrene and retene attenuate the L-type Ca2+ current (I_{CaL}) in rainbow trout ventricular cardiomyocytes. (A) A representative experiment on L-type Ca^{2+} current. Immediately after having access into the cell, I_{CaL} starts to increase due to the buffering of intracellular Ca^{2+} by EGTA (pipette solution is perfusing the cell from the inside). Then I_{CaL} stabilizes (b) and the cell is then cumulatively exposed to 1 (c) and 10 μM retene (d). (B) Fast time-based tracings of I_{CaL} under control conditions and in the presence of 1 and 10 μM retene at the positions shown by letters b, c and of the panel A. The stimulus pulse is shown below the tracings. (C) Effects of phenanthrene (10, 30 μM) and retene (1, 10 μM) on the peak density of I_{CaL}. The results are means ± SEM of 12-16 myocytes from at least 3 animals. Groups denoted by the same letter do not differ significantly from each other.

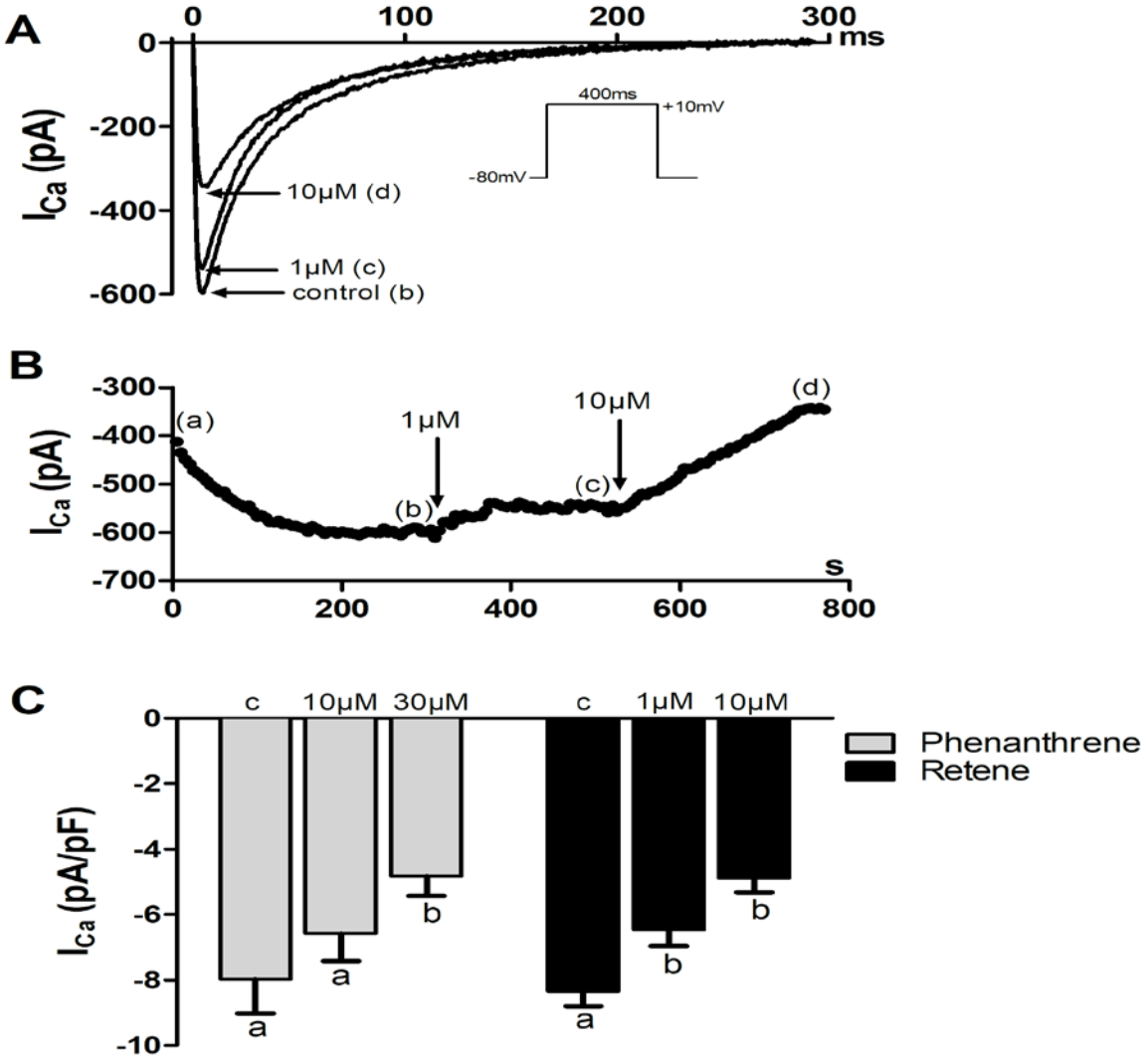

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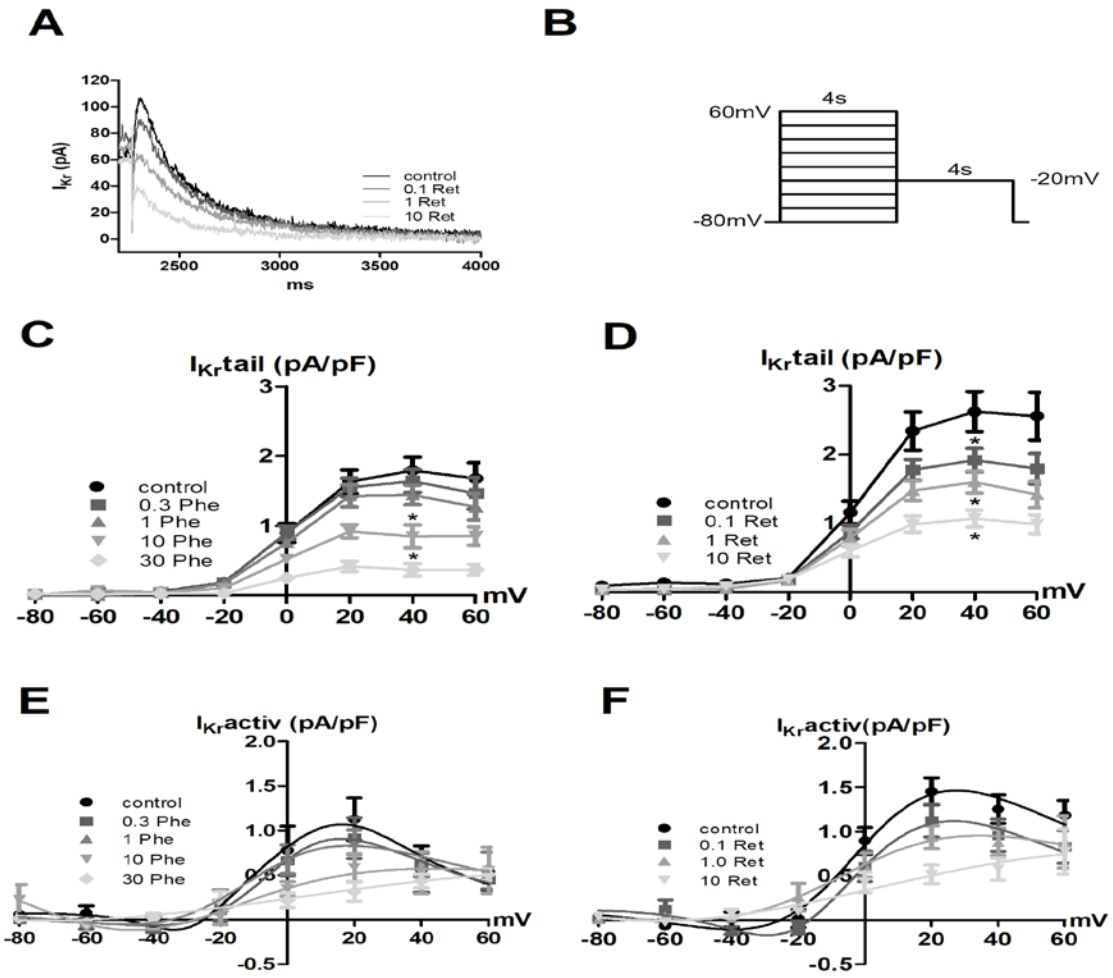
Figure 6. Phenanthrene and retene reduce the rapid component of the delayed rectifier K+ current (I_{Kr}) in rainbow trout ventricular cardiomyocytes. (A) A representative experiment showing the inhibitory effect of retene on I_{Kr}. The two-step stimulus protocol is shown on right. (C and D) Current-voltage relationship of the I_{Kr} tail current ($I_{Kr,tail}$ during the repolarizing pulse at -20 mV) in the absence and presence of phenanthrene (C) and retene (D). (E, F). Current-voltage relationship of the I_{Kr} during the depolarizing pulses from +80 to -60 mV in the absence and presence of phenanthrene (E) and retene (F). The results are means ± SEM of 11-12 cells from at least 3 animals. An asterisk indicates statistically significant difference from control.



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