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Ionic channels underlying the ventricular action potential in zebrafish embryo

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ABSTRACT

Over the last years zebrafish has become a popular model in the study of cardiac physiology, pathology and pharmacology. Recently, the application of the 3Rs regulation and the characteristics of the embryo have reduced the use of adult zebrafish use in many studies. However, the zebrafish embryo cardiac physiology is poorly characterized since most works have used indirect techniques and direct recordings of cardiac action potential and ionic currents are scarce. In order to optimize the zebrafish embryo model, we used electrophysiological, pharmacological and immunofluorescence tools to identify the characteristics and the ionic channels involved in the ventricular action potentials of zebrafish embryos. The application of Na^+ or T-type Ca^{+2} channel blockers eliminated the cardiac electrical activity, indicating that the action potential upstroke depends on Na^+ and T-type Ca^{+2} currents. The plateau phase depends on L-type Ca^{+2} channels since it is abolished by specific blockade. The direct channel blockade indicates that the action potential repolarization and diastolic potential depends on ERG K^+ channels. The presence in the embryonic heart of the Nav1.5, Cav1.2, Cav3.2 and ERG channels was also confirmed by immunofluorescence, while the absence of effect of specific blockers and immunostaining indicate that two K^+ repolarizing currents present in human heart, I_{to} and I_{Ks}, are absent in the embryonic zebrafish heart. Our results describe the ionic channels present and its role in the zebrafish embryo heart and support the use of zebrafish embryos to study human diseases and their use for drug testing.

1. Introduction

The zebrafish (*Danio rerio*) has been established as a biological model used in various scientific and technical fields such as biomedicine, environment, aquaculture and food. The high degree of genetic homology with mammals and humans [1–3], ease of handling and reproduction, as well as great prospects for the application of the new genomic and proteomic technologies, has fostered the use of zebrafish to spread rapidly in science and business.

Although the zebrafish heart is two-chambered, its electrical properties resemble those of humans [4], including embryonic and adult heart rates and the QT interval duration link to heart rate [5,6]. In addition, the zebrafish genome has orthologs of all major cardiac channels present in the human heart, such as the Na^+ channel Nav1.5, the Cav1.2 Ca^{2+} channel and the K^+ channels Kv4.3, Kv7.1 and ERG [7–14]. However, physiological studies indicate that some of these channels may solely be present in non cardiac tissues [15,16].

Action Potentials (AP) in myocytes isolated from adult zebrafish heart were first recorded in 2008 [17]. That work showed that the resting potential of cardiac myocytes from adult zebrafish is approximately −70 mV and the AP contains the plateau phase characteristic of the heart of large mammals including humans. Furthermore, the AP duration shortens by increasing the pacing rate, especially at the expense of a shortening of Phase 2 [17]. In 2010 a complete electrophysiological study of adult zebrafish cardiac myocytes was published [18]. Using electrophysiological and pharmacological tools authors demonstrated that: Na^+ channels are responsible for the Phase 0 of the cardiac AP; that both T-type and L-type Ca^{+2} currents contribute to the AP morphology; and

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that repolarisation relies exclusively on the potassium current I_{Kr}. In adult zebrafish the cardiac AP lacks the Phase 1 characteristic of human action potentials. However, the transient outward K^+ current, I_{to}, responsible for Phase 1 in human action potentials was not studied in any of the previous works.

Currently, a major zebrafish business application is the high-throughput testing of new compounds in preclinical studies. As a consequence of economic and ethical reasons, companies are no longer using adult fish to test compounds, but the embryo at early stages [19]. Moreover, the zebrafish embryo is transparent and allows direct observation of the heart rate and its variability. Thus, the capability of a compound for inducing bradycardia, arrhythmia, heart block or cardiac arrest is asserted mainly by stereomicroscopy, spectroscopy and other optical techniques [11,19–21].

In spite of the resolution of the image techniques, the electrical behavior of the embryo zebrafish is poorly understood. In this sense, it is known that the zebrafish embryo cardiac myocytes depolarize after few hours of development; Connexin 43 is clearly detectable and the heart starts beating in a synchronous manner at 24 hpf [22]. By 48 hpf the impulse propagation is fast in both the atrium and the ventricle and slow in the sinoatrial and the atrioventricular nodes. The transmural and apex to base electrical gradient, characteristic of the mammalian heart, is also established at this stage [22–27].

Thus, the electrical characterization of the embryonic heart is a crucial point for determining its physiological similarity to adult human heart and to support the use of zebrafish embryos in a regulatory environment. The aim of this work is to describe the different ionic channels responsible for the cardiac action potential in the early zebrafish embryo.

2. Methods

The investigation fulfills the Spanish (RD 1201/2005) and European (D2003/65/CE and R2007/526/CE) rules for care of animals used for experimental and other research purposes, has been approved by the Ethics Committee for Animal Care (CEBA-131-2010) and by the Ethics Committee for the use of genetically modified organisms (CEIAB-2-2010) of the Universidad del País Vasco, and conforms to the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health.

2.1. Isolation of embryonic hearts

Zebrafish were raised under standard laboratory conditions at 28 °C. In this work, we used a transgenic zebrafish line expressing the fluorescent copGFP protein under the control of a cardiomyocyte promoter (Cmlc2). The transgenic embryos were obtained from Biobide S.L. (San Sebastian, Spain) (Fig. 1A).

Decorionated 48 hours post-fertilization (hpf) embryos were anaesthetized with tricaine 0.02%. The medium was removed and the embryos were suspended in an enzymatic solution containing 4 mg/ml of collagenase type I and 1 mg/ml of protease type XIV in Leibovitz L15 medium. Embryos were homogeneized by 10 gentle upstrokes with a glass homogenizer and incubated for 30 min at 31 °C in agitation. They were homogenized again and centrifuged 10 min at 1000 rpm. The pellet was resuspended in L15 medium and maintained at 4 °C. Only the heart and some small fragments of skeletal muscle are resistant to the process of isolation. Cardiac tissue was easily identifiable because of its specific green fluorescence and atrium and ventricle are differentiated by their different morphology (Fig. 1B).

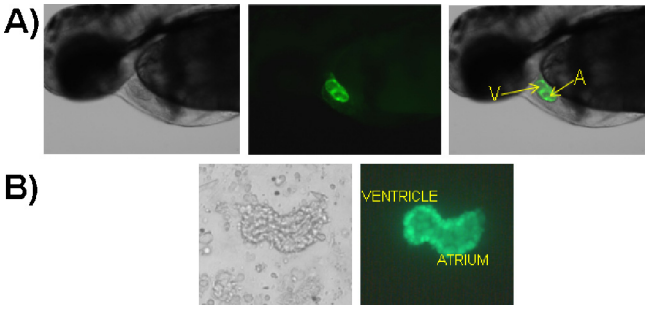

Fig. 1. Characteristics of the transgenic zebrafish line. (A) Left panel: The bright field micrography shows the transparency of the 48 hpf zebrafish embryo. Middle panel: Micrography showing the green fluorescent heart thanks to the green fluorescent protein copGFP expressed under the control of the cardiomyocyte specific promoter Cmlc2. Right panel: Merged image showing the position of the atrium and the ventricle. The blood flows through the sinus venosus to the atrium and upwards to the ventricle; then is pumped through the bulbus arteriosus to the aorta. Magnification 100×. (B) Micrography of the embryonic heart isolated from the surrounding tissues. Magnification 400×. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Action potential recording

Only spontaneously beating hearts were used. All experiments were performed at room temperature (22–24 °C). The hearts were perfused with an external solution containing (in mM): NaCl 150, KCl 5.4, $CaCl_2$ 2, $MgSO_4$ 1.5, HEPES-Na 10, NaH_2PO_4 0.4 and glucose 10 (pH 7.7). Suction pipettes were made from borosilicate capillary tubes (Sutter Instruments Inc.) and had a tip resistances of 4–6 MΩ when filled with the internal solution (mM): KCl 139, NaCl 10, $MgCl_2$ 0.5, EGTA-K 0.5, HEPES-K 10 and ATP-Na 5 (pH 7.2). Transmembrane potential was measured by using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) with the disrupted patch technique as published [28]. The pipette was positioned adjacent to the ventricle and a seal was formed by application of minimal suction. Spontaneous ventricular APs were recorded for 10 min and then perfused with the different drugs.

2.3. Data analysis

Action potential parameters were measured with the Clampfit 10.2 software. Values are presented as mean ± SEM. Student's *t*-test for paired data was used to compare the difference between two means. $p < 0.05$ was considered significant.

2.4. Immunofluorescence

Zebrafish embryos were anaesthetized with tricaine and fixed in paraformaldehyde 4%. With the help of a stereomicroscope the chest was opened and the yolk sac was removed to expose the heart to the bathing solutions. The embryos were washed with phosphate buffer and dehydrated in 50% methanol. They were sequentially rehydrated with 50% metOH, 30% metOH and PBS-Tween and incubated 15 min at room temperature with proteinase K 10 μg/ml. After washing with PBS-Triton X-100 0.1%, the embryos were immersed in blocking solution (PBS with 5% Normal Donkey Serum, 4 mg/ml BSA and 5% Triton X-100) for 1 h at RT. Primary anti-Nav1.5, Cav1.2, Cav3.2, ERG, Kv4.3 and Kv7.1 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in blocking buffer were applied overnight at 4 °C. Secondary donkey anti-goat Cy5-conjugated antibodies (Invitrogen, Grand Island, NY, USA) diluted in blocking buffer were applied for 2 h at RT. Coverslips were mounted in Mowiol reagent (Calbiochem) and analyzed in a Zeiss Axio Observer Z1 fluorescence microscope (Zeiss, Jena, Germany).

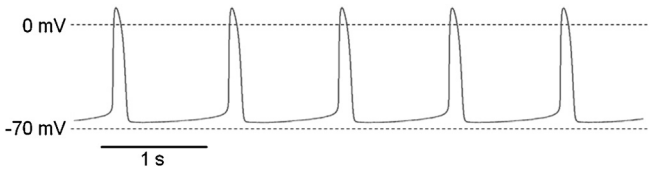

Fig. 2. *Ventricular action potentials from spontaneously beating hearts.* The 48 hpf heart has a resting potential of −66.3 ± 0.5 mV, an action potential amplitude of 91.9 ± 0.9 mV and an APD90 of 230.9 ± 5.2 ms (n = 55).

3. Results

3.1. Ventricular action potential (AP) characteristics

Ventricular action potentials were recorded from spontaneously beating hearts isolated from 48 hpf zebrafish embryos (Fig. 2). Ventricular APs were generated by spontaneous electrical activity initiated in the atrium at room temperature. Under these experimental conditions the ventricular action potential morphology showed minor differences to those reported in adult ventricular myocytes (see Section 4). The cardiac cycle length is 1073 ± 40 ms, the resting membrane potential was −66.3 ± 0.5 mV, the action potential amplitude was 91.9 ± 0.9 mV and the action potential duration at 90% of repolarization (APD90) was 230.9 ± 5.2 ms. These action potentials had distinguishable phases 0, 2, 3 and 4, but lacked the phase 1 characteristic of large mammals (Fig. 2; Figs. 3 and 4, Control).

3.2. Pharmacological dissection of ionic channels

The functional presence or absence of the different ionic channels is commonly explored by using specific blockers. In this work we employed several widely used blockers of the different ionic channels at concentrations slightly over the reported IC50.

In most species, the main depolarizing currents are the Na^+ and the Ca^{+2} currents and their role in zebrafish embryo action potentials were similar (Fig. 3). After the application for 5 min of either lidocaine 30 μM to block the Na^+ channel, or NNC55-0396 20 μM to block the T-type Ca^{2+} channel, the action potential disappeared, indicating that the phase 0 is dependent on Na^+ and T-type Ca^{+2} channels. The L-type Ca^{+2} channels blockade with nitrendipine 10 μM lead to the elimination of the plateau phase of the action potential.

In the human heart there are three main repolarizing currents: I_{to}, responsible for the action potential phase 1, I_{Kr}, responsible for the phase 3 and I_{Ks}, considered the repolarization reserve. Fig. 4 shows the effect of blocking each one of these currents on the ventricular action potential of 48 hpf zebrafish embryos. The I_{Kr} blocker terfenadine 100 nM depolarized the hearts (−56.1 ± 4.6, $p < 0.01$) and prolonged the APD90 to 244.5 ± 6.5 ms ($p < 0.05$) indicating its role in the action potential phase 3 and in the maintenance of the resting potential. Higher terfenadine concentrations 1 (μM) further depolarized the membrane and abolished action potential generation (not shown). However, the blockade of either I_{to} with heteropodatoxin (HPTX) 50 nM or I_{Ks} with Chromanol 293B 10 μM had no effect on action potentials, indicating the absence of functional I_{to} or I_{Ks} currents in the membrane of the myocytes (Fig. 4). Higher concentrations of HPTX or Chromanol 293B also had no effect on action potentials (100 nM and 100 μM respectively; not shown).

3.3. Ion channel forming proteins present in the zebrafish embryonic heart

The pharmacological dissection of the cardiac action potential suggests that the membrane of the myocytes contains functional Na^+, T- and L-type Ca^{+2} and ERG K^+ channels, whereas it lacks functional I_{to} and I_{Ks} currents. We performed immunofluorescence experiments to determine whether the channel proteins supposed to drive each of these currents are present or absent in the cardiomyocytes of the zebrafish embryo.

Immunofluorescence experiments corroborated the functional experiments. The hearts of 48 hpf zebrafish embryos showed clear staining of the of Nav1.5 Na^+ channels. The Cav3.2 and Cav1.2, responsible for T- and L-type Ca^{+2} currents respectively were also present in zebrafish embryos. The immunohistological experiments also confirmed the presence of the ERG channel in the heart of 48 hpf zebrafish embryos (Fig. 5).

Also as expected from the pharmacological and functional experiments, immunohistochemistry failed to detect the Kv4.3 and Kv7.1 channels, responsible for the I_{to} and slow delayed rectifier I_{Ks} currents respectively. One possible explanation could be that these channels appear in later stages of heart development. To

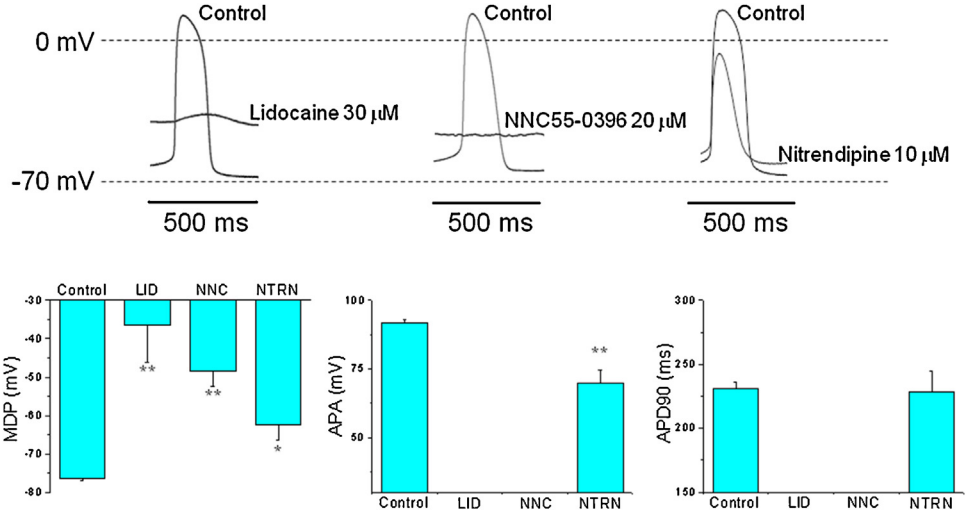

Fig. 3. *Pharmacological dissection of depolarizing currents.* Lidocaine (Na^+ current inhibitor) and NNC55-0396 (T-type Ca^{+2} current blocker) abolish the electric activity of the heart and reduce the maximum diastolic potential (n = 18 paired hearts). The L-type Ca^{+2} channel blocker Nitrendipine eliminates the plateau and reduces both the AP amplitude and the maximum diastolic potential (*$p < 0.05$, **$p < 0.01$. n = 12 paired hearts). MDP: Maximun Disatolic Potential; APA: Action Potential Ampitude; APD90; Action Potential Duration at 90% of repolarization; LID: Lidocaine 30 μM; NNC: NNC55-0396 20 μM; NTRN: Nitrendipine 10 μM.

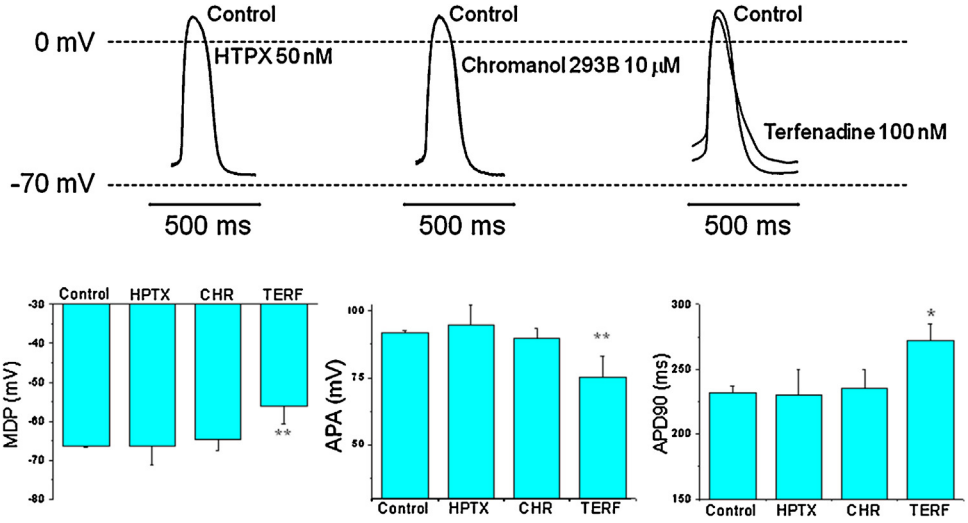

Fig. 4. *Pharmacological dissection of repolarizing currents.* Neither I_{to} blockade with heteropodatoxin (HPTX) nor I_{Ks} inhibition with Chromanol 293B induce any effect on the action potential morphology ($n = 14$ paired hearts). However, the I_{Kr} current inhibitor Terfenadine induces a cell depolarization that leads to an AP amplitude reduction and slightly prolongs the APD90 ($^{*}p < 0.05$, $^{**}p < 0.01$. n = 11 paired hearts). MDP: Maximun Disatolic Potential; APA: Action Potential Ampitude; APD90; Action Potential Duration at 90% of repolarization; HPTX: Heteropodatoxin 50 nM; CHR: Chromanol 293B 10 μM; TERF: Terfenadine 100 nM.

test this possibility we performed the immunofluorescence experiments using 72 and 96 hpf embryos and obtained similar negative results. We found no signal for Kv4.3 and Kv7.1 K^+ channels in any of the tested stages (Fig. 6). On the contrary, Nav1.5, Cav3.2, Cav1.2 and ERG channels expression was maintained at 72 and 96 hpf (not shown).

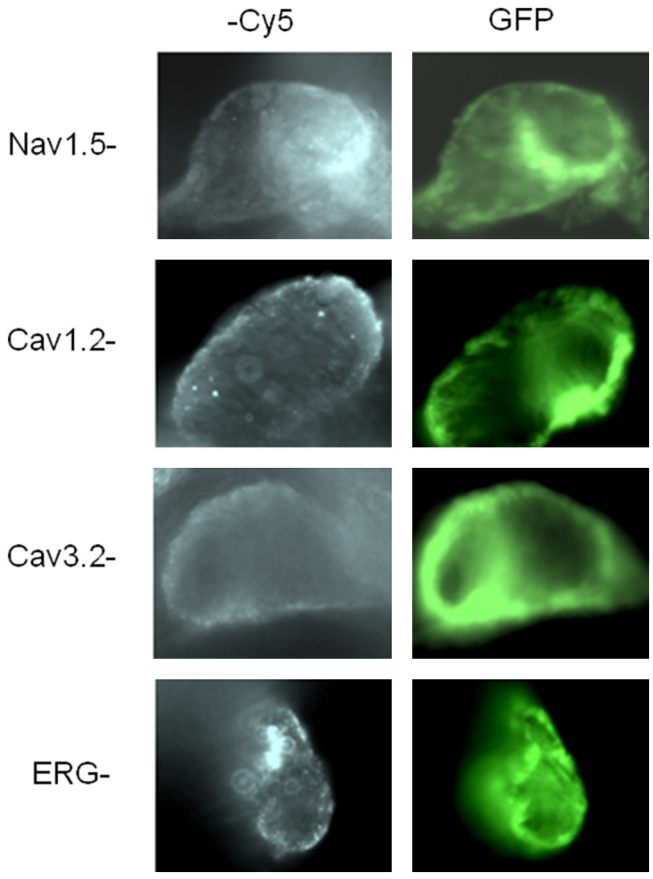

Fig. 5. *Immunolocalization of ionic channels responsible for the action potential in cardiac myocytes.* The Na^+, L-type and T-type Ca^{+2} and rapid delayed rectifier K^+ currents are carried through Nav1.5, Cav1.2, Cav3.2 and ERG channels respectively. Fluorescence microscopy imaging showing Cy5 labeled antibodies shows the presence of the four channel types (left column) in the Green Fluorescent (right) 48 hpf zebrafish heart. $n = 10–15$ embryos. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

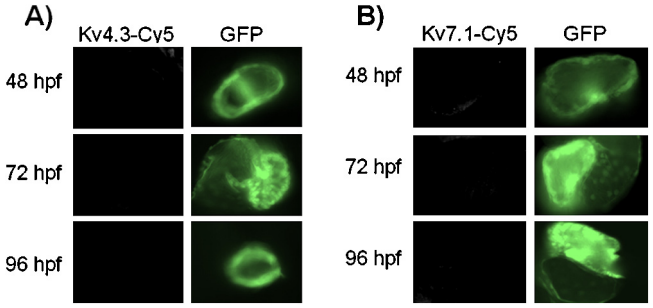

Fig. 6. *The membrane of zebrafish cardiomyocytes lacks Kv4.3 and Kv7.1 channels.* Fluorescence microscopy imaging of Cy5 labeled antibodies (left column) in the Green Fluorescent (right) 48 hpf zebrafish heart detects no signal when Kv4.3 (A) or Kv7.1 (B) specific antibodies are used. The fluorescence does not appear after 72 or 96 h of development. $n = 10–15$ embryos. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

4. Discussion

Mouse is probably the most commonly used model for the study of human heart diseases, but some electrophysiological aspects, such as heart rate and action potential duration, differ significantly to those observed in the human heart. These characteristics are a consequence of different ion channels present in the membrane of cardiomyocytes of both species. Thus, new animal models capable to translate cardiovascular properties into human are being explored for the study of human electrophysiology as well as to be applied to evaluate the cardiotoxic properties of new drugs for human use. In this sense, zebrafish is as a vertebrate model in which genetic, optical and physiological techniques can be easily employed to study heart function. In recent years, zebrafish embryos have been proposed as a suitable model for drug testing because they are easy to use, inexpensive to raise, they develop in hours and are optically transparent [26,29]. To better support this option, specific electrophysiological, pharmacological and molecular comparative studies should be made.

The first electrophysiological recordings in the zebrafish heart were done more than 15 years ago [30] and the ionic channels responsible for action potential in adult cardiac myocytes have been recently described in some detail [17,18]. However, the ionic channel composition of the membrane of embryonic cardiac myocytes has never been described. Our immunofluorescence experiments prove the presence of the voltage-dependent Nav1.5, Cav3.2, Cav1.2 and ERG channels, responsible for I_{Na}, I_{Ca}-T, I_{Ca}-L and I_{Kr} respectively already in the 48 hpf embryonic hearts. However, 48 hpf, 72 hpf embryos and 96 hpf larvae lack the Kv4.3 and Kv7.1 channels, responsible for I_{to} and I_{Ks} indicating a first important difference to be taken into account when positioning the zebrafish embryo as a human cardiotoxic surrogate model.

Previous works recorded the spontaneous action potentials in hearts dissected one by one with fine forceps from 48 hpf zebrafish embryos [28,31], this method ensures the integrity of the macroscopic structure and of the membrane proteins. Here, taking advantage of the fact that the heart is fluorescent we perform an enzymatic digestion quicker and easier and isolate hearts form many fishes at once. However, the isolation procedure could alter the cardiac electrophysiological behavior. In our work, the cardiac cycle length and the action potential characteristics such as maximum diastolic potential, action potential amplitude or action potential duration, are similar to those reported by other groups [28,31]. The slight differences observed are attributable to small variations in the composition of the internal and external recording solutions. Due to the loss of neuroendocrine control, the cardiac cycle length is slightly shorter in the isolated organ than in the intact organism as described in mammalian hearts [19].

The action potential characteristics of 48 hpf zebrafish hearts described in ours work are similar to those observed in adult hearts, supporting the use of embryos to substitute adult animal use but with some specificities. The resting membrane potential reported in adult ventricular myocytes is stable and slightly under −70 mV. In contrast, we have showed that the membrane potential between action potentials spontaneously depolarizes in embryonic ventricular myocytes, with a maximum diastolic potential around −66 mV. Besides, the APD90 is about 100 ms longer in embryonic *vs* adult ventricular myocytes [17,18,28].

In embryonic ventricular myocytes, the resting membrane potential was proposed to be dependent on ERG channels because channel block causes the cell membrane depolarization [28]. However, the resting membrane potential is not affected by ERG channel blockade in adult myocytes [18]. In our work, we have observed that the ERG channel blockade with low terfenadine concentration (100 nM) in embryonic myocytes depolarizes the cell and reduces the action potential amplitude in line with what had previously observed. Moreover, higher terfenadine concentration (1 μM), which causes maximum ERG channel blockade, further depolarizes the myocyte and causes a loss of electrical activity. These results indicate that, unlike what had been described in adult myocytes, the ERG channels in zebrafish embryonic myocytes are the main responsible for the maximum diastolic potential. This important role could be due to the later development of the inward rectifier channels responsible for I_{K1}, which could explain the more stable resting potential and the shorter APD in adult myocytes.

Once the ventricle is reached by a stimulus generated in the atrium, it generates its own action potential. Our experiments using the Na^+ channel blocker Lidocaine demonstrate that, as reported in adult zebrafish and mammalian cardiac myocytes [17,18], the action potential upstroke is strongly dependent on Na^+ channels, since Na^+ channel blockade fully eliminates the action potential. In addition, action potential generation is also abolished by the T-type Ca^{+2} channel blocker NNC55-0396. These results together indicate that the action potential upstroke is strongly dependent both on both Na^+ and T-Type Ca^{+2} channels. T-type Ca^{+2} channels are also expressed in the mammalian fetal heart, and disappear during heart development [32]. In zebrafish ventricular myocytes T-type Ca^{+2} channels have also been observed in later stages of development and in the adult fish [18,30].

We observed that inhibition of the L-Type calcium current with nitrendipine suppresses the phase 2 of the action potential. Thus, this current is the main responsible for the plateau phase of the cardiac action potential in mammals and adult zebrafish as well as in the 48 hpf zebrafish embryo [17,18,33].

Finally, the main repolarizing K^+ currents in human heart are the transient outward I_{to}, carried through Kv4.3 channels and responsible for the AP phase 1; the rapid delayed rectifier I_{Kr}, carried through ERG channels and responsible for phase 3; and the slow delayed rectifier I_{Ks}, carried by Kv7.1 channels which also participate in phase 3. Only the I_{Kr} current has been described both in adult and embryonic zebrafish cardiac myocytes [18,28,30] and the zERG channel is the only one biophysically characterized [34]. In adult zebrafish cardiomyocytes the presence of the I_{Ks} current was discarded and the absence of I_{to} was suggested [18,19] although the presence of a functional I_{to} has been described only in zebrafish skeletal muscle as early as 48 hpf [15,16]. In our work, the absence of phase 1 in the action potential of embryonic ventricular myocytes indicates the absence of a relevant I_{to}. The absence of a functional I_{to} was confirmed with the use of the specific blocker Heteropodatoxin. However, the absence of a functional I_{to} could be due to different physiological factors. The absence of Kv4.3 channel proteins in the membrane of the cardiomyocytes was then confirmed with immunofluerescence staining. Moreover, these channels do not develop in later stages of embryonic development. Similar results also confirmed that the absence of Kv7.1 channel proteins in the membrane of the cardiomyocytes is the responsible for the absence of a functional I_{Ks} current in the heart of embryonic and adult zebrafish. It can be argued against the specificity of the antibodies. However, the absence of immunostaining only corroborates the electrophysiological findings.

In summary, in spite of some differences, these results indicate that the 48 hpf zebrafish embryonic heart has similar electrophysiological characteristics to the adult zebrafish and mammalian heart. As in the human heart, in the 48 hpf zebrafish embryo the cardiac action potential upstroke is dependent on Na^+ channels but, both in adults and 48 hpf embryos a T-type Ca^{+2} current is also present. The T-type Ca^{+2} current is present in the heart of mammalian embryos, but in contrast to zebrafish it disappears on development. Thus, since the action potential upstroke depends both on Na^+ and T-Type Ca^{+2} channels, for drug toxicity testing it must take into account that false positives due to drug binding to the T-type Ca^{2+} and not the Na^+ channel can be at the time of assessing potential drugs that alter the Action Potential Generator.

In the 48 hpf embryo L-Type Ca^{2+} channels are responsible for the action potential phase 2, supporting the concept that drug safety assessment in zebrafish embryos correlates with drug cardiotoxicity in mammals. Finally, I_{Kr} has been the main focus of cardiotoxicity studies and regulatory interest as a consequence of its significant involvement in heart ventricular repolarization and Torsade des Pointes incidence related to I_{Kr} disfunction. In humans and in zebrafish embryos the action potential repolarization and diastolic potential is dependent on ERG K^+ channels.

Taken together, these results describe for the first time the complete set of ion channels regulating the heart beating in zebrafish embryonic hearts and their role in the AP. They also indicate that, being non-expensive and easier to use, the zebrafish embryo is a model to be considered for the study of human diseases and could offer significant advantages for high-throughput drug testing.

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Potential conflicts of interest

Carles Callol and Ainhoa Letamendia were employees of Biobide S.L. when the work was carried out. Currently, the company Biobide S.L. does not longer exist.

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