Expression of calcium channel transcripts in the zebrafish heart: dominance of T-type channels

Haverinen, J

The Company of Biologists

https://erepo.uef.fi/handle/123456789/6750

Downloaded from University of Eastern Finland's eRepository
Expression of calcium channel transcripts in the zebrafish heart: dominance of T-type channels

Jaakko Haverinen¹, Minna Hassinen¹, Surjya Narayan Dash¹², and Matti Vornanen¹

¹Department of Environmental and Biological Sciences, University of Eastern Finland, P.O. Box 111, 80101 Joensuu, Finland

²Neuroscience Center and Institute of Biomedicine, Faculty of Medicine, University of Helsinki, P.O. Box 63, 00014 Helsinki, Finland

Address for correspondence:
Jaakko Haverinen, PhD
University of Eastern Finland
Department of Environmental and Biological Sciences
P.O. Box 111, 80101 Joensuu, Finland
Email: jaakko.haverinen@uef.fi
Phone: +358-45-78737316

http://jeb.biologists.org/lookup/doi/10.1242/jeb.179226
Abstract

Calcium (Ca) channels are necessary for cardiac excitation-contraction (e-c) coupling, but Ca channel composition of fish hearts is still largely unknown. To this end, we determined transcript expression of Ca channels in the heart of zebrafish (Danio rerio), a popular model species. Altogether 18 Ca channel α-subunit genes were expressed in both atrium and ventricle. Transcripts for 7 L-type (Cav1.1a, Cav1.1b, Cav1.2, Cav1.3a, Cav1.3b, Cav1.4a, Cav1.4b), 5 T-type (Cav3.1, Cav3.2a, Cav3.2b, Cav3.3a, Cav3.3b) and 6 P/Q-, N- and R-type Ca channels (Cav2.1a, Cav2.1b, Cav2.2a, Cav2.2b, Cav2.3a, Cav2.3b) were expressed. In the ventricle, T-type channels formed 54.9%, L-type channels 41.1% and P/Q-, N- and R-type Ca channels 4.0% of the Ca channel transcripts. In the atrium, the relative expression of T-type and L-type Ca channel transcripts was 64.1% and 33.8%, respectively (others accounted for 2.1%). Thus, at the transcript level T-type Ca channels are prevalent in zebrafish atrium and ventricle. At the functional level, peak densities of ventricular T-type (I_{CaT}) and L-type (I_{CaL}) Ca current were 6.3±0.8 and 7.7±0.8 pA pF⁻¹, respectively. I_{CaT} mediated a sizeable sarcolemmal Ca influx into ventricular myocyte: increment in total cellular Ca content via I_{CaT} was 41.2±7.3 µmol L⁻¹, which was 31.7% of the combined Ca influx (129 µmol L⁻¹) via I_{CaT} and I_{CaL}(88.5±20.5 µmol L⁻¹). The diversity of expressed Ca channel genes in zebrafish heart is high, but dominated by the members of the T-type subfamily. The large ventricular I_{CaT} is likely to play a significant role in e-c coupling.

Key words: adult zebrafish, calcium channels, cardiac myocytes, T-type Ca²⁺ current, zebrafish heart
**Introduction**

Voltage-gated Ca channels pass Ca influx into cells and mediate a wide variety of physiological responses including muscle contraction, hormone secretion, neuronal transmission, gene expression and cell division (McDonald et al., 1994; Hofmann et al., 2014). In the sarcolemma of cardiac myocyte two major types of Ca channels are usually present: L-type (long-lasting, high threshold) Ca channels of the subfamily Ca\(_v\)1 and T-type (transient, low threshold) Ca channels of the subfamily Ca\(_v\)3 (Ertel et al., 2000; Catterall et al., 2005).

In the mammalian heart, L-type Ca channels are abundantly expressed in atrial and ventricular myocytes, sinoatrial pacemaker cells and atrioventricular nodal cells (Hagiwara et al., 1988; Zamponi et al., 2015; Mesirca et al., 2015). L-type Ca current (I\(_{\text{CaL}}\)) is a critical component in excitation-contraction (e-c) coupling of atrial and ventricular myocytes by inducing Ca release (Ca-induced Ca release, CICR) from the sarcoplasmic reticulum (SR) (Fabiato, 1983; Bers, 2002). In sinoatrial myocytes, I\(_{\text{CaL}}\) generates the upstroke of pacemaker action potential (AP) (Irisawa et al., 1993). In cardiac myocytes of fetal and neonatal mammals and ectothermic vertebrates, I\(_{\text{CaL}}\) directly contributes to cytosolic Ca transient, since CICR is less powerful in these cells (Fabiato and Fabiato, 1978; Morad et al., 1981; Vornanen, 1996; Vornanen et al., 2002).

Distribution of T-type Ca current (I\(_{\text{CaT}}\)) and channels is more limited in the adult mammalian heart. T-type Ca channels are strongly expressed in sinoatrial and atrioventricular nodes, weakly expressed in atrial myocytes and are usually not present in healthy ventricular myocytes (Perez-Reyes, 2003; Ono and Iijima, 2010). It should be noted, however, that a small I\(_{\text{CaT}}\) is present in guinea-pig and dog ventricular myocytes (Nilius et al., 1985; Mitra and Morad, 1986; Balke et al., 1992; Wang and Cohen, 2003). I\(_{\text{CaT}}\) has a significant role in cardiac pacemaking by contributing to the diastolic depolarization of
pacemaker AP, while its significance in e-c coupling of atrial and ventricular myocytes is incompletely resolved (Zhou and January, 1998; Kitchens et al., 2003; Jaleel et al., 2008; Ono and Iijima, 2010; Mesirca et al., 2015). \( I_{CaT} \) is upregulated in diseased mammalian heart including genetic hypertension, cardiac hypertrophy and atherosclerosis (Takebayashi et al., 2006; Chiang et al., 2009).

\( I_{CaL} \) has been recorded in atrial and ventricular myocytes of several fish species including the zebrafish (\textit{Danio rerio}) (Maylie and Morad, 1995; Vornanen, 1997; Vornanen, 1998; Hove-Madsen and Tort, 1998; Vornanen et al., 2002; Shiels et al., 2006; Brette et al., 2008; Zhang et al., 2011; Galli et al., 2011; Haworth et al., 2014; Haverinen et al., 2014). \( I_{CaT} \) is shown to be present in atrial and ventricular myocytes of the dogfish (\textit{Squalus acanthias}) and zebrafish hearts, and in atrial myocytes of the Siberian sturgeon (\textit{Acipenser baerii}) heart (Maylie and Morad, 1995; Warren et al., 2001; Nemtsas et al., 2010; Haworth et al., 2014). In contrast to the rich data of the mammalian cardiac Ca channels, very little is known about Ca channels of the fish heart, even though sarcolemmal Ca channels play a central role in cardiac e-c coupling of fish (Vornanen et al., 2002; Zhang et al., 2011). Little is known about the molecular and genetic background of cardiac Ca channels in zebrafish (Rottbauer et al., 2001), which is somewhat surprising considering that this species is a popular model for cardiovascular drug screening and human cardiac diseases (Bakkers, 2011; MacRae and Peterson, 2015). To be a useful model for the human heart, it is crucial to know the molecular basis of Ca currents in the zebrafish heart, since Ca channels are important therapeutic targets for treatment of cardiovascular diseases (Belardetti and Zamponi, 2012). To this end, we measured Ca channel transcript expression in the zebrafish heart. In addition, Ca influx via \( I_{CaT} \) and \( I_{CaL} \) into zebrafish ventricular myocytes was determined using the whole-cell patch-clamp method. Because \( I_{CaL} \) is generally regarded as
the most important cardiac Ca current, our working hypothesis was that different L-type Ca channels form the dominant Ca channel subfamily in the zebrafish atrium and ventricle.

**Material and methods**

**Zebrafish**

The wild-type Turku zebrafish line (kindly donated by prof. Pertti Panula, University of Helsinki) was raised and maintained at the animal facilities of UEF (University of Eastern Finland, Joensuu) according to the established principles (Westerfield, 2007). The rearing temperature of the fish was 28°C. The experiments conform to the 'European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes' (Council of Europe No 123, Strasbourg 1985) and were authorized by the national animal experimental board in Finland (permission ESAVI/2832/04.10.07/2015).

**Total RNA isolation and quantification of gene expression**

Total RNA was isolated from three atrial and ventricular samples (each containing cardiac tissue of eight adult (1.5-year old; 0.37 ± 0.1g) zebrafish) using TriReagent (ThermoScientific). RNA was DNase treated with RNase free DNaseI (ThermoScientific) and reverse-transcribed with random hexamer and oligo (dT) primers and Maxima RNase H-reverse transcriptase (ThermoScientific) following manufacturer’s protocols. In order to design gene specific primers for qPCR, a and b isoforms of each *cacna* gene were aligned and primers were designed to non-homologous regions. Moreover, all 18 *cacna* genes were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) and the specificity of primers was checked. Finally, the PrimerBlast (https://www.ncbi.nlm.nih.gov/tools/primer-blast/) was used to validate the targets of each primer pair. qRT-PCR was performed using Maxima SYBR Green qPCR Master Mix.
(Thermo Scientific) in Aria MX Real-Time PCR machine (Agilent Technologies). Accession numbers of the genes and primers used in this study are listed in Table 1. The thermal conditions were as follows: enzyme activation at 95°C for 10 min followed by 40 cycles at 95°C for 10 s, 58°C for 20 s and 72°C for 30 s and final extension at 72°C for 3 min. After the qPCR reaction, the specificity of amplification was checked by melting curve analysis (from 65°C to 95°C) and by running the qPCR products on an agarose gel. Data were normalized to the geometric mean of dnapa2 (DnaJ homologue subfamily A member 2) and actb1 (β-actin) mRNA expression levels.

**Patch-clamp of I\text{CaT} and I\text{Cal}**

Electrophysiological experiments were conducted on enzymatically isolated ventricular myocytes of the adult (1.5-year old; 0.68 ± 0.03g) zebrafish. Firstly, fish were stunned with a quick blow to the head, the spine was cut and the heart was excised. The yield of atrial myocytes was low and therefore extensive characterization of atrial electrophysiology was not possible. Myocyte isolation procedure was essentially similar to our original isolation method for fish hearts (Vornanen, 1997), but scaled down to the size of small zebrafish hearts. For retrograde perfusion of the heart, a small cannula (34 gauge, TE734025, Adhesive Dispensing Ltd, UK) was inserted via bulbus arteriosus into the ventricle and the bulbus was secured with a fine thread around the cannula. The heart was perfused first with Ca-free solution for 5 min and then with the same solution but with added hydrolytic enzymes (Collagenase Sigma IA, Trypsin type VI from Serva together with fatty acid-free serum albumen) for 15-20 minutes. Myocytes were stored at 5°C and used within the same day they were isolated. The composition of the Ca-free solution contained (in mmol l\(^{-1}\)): 100 NaCl, 10 KCl, 1.2 KH\(_2\)PO\(_4\), 4 MgSO\(_4\), 50 taurine, 20 glucose and 10 HEPES with pH adjusted to 6.9 with KOH at 20°C.
Axopatch 1D amplifier (Axon Instruments, Saratoga, CA, USA) with a CV-4 1/100 head-stage was used for the whole-cell patch clamp. The external solution used for $I_{\text{CaT}}$ and $I_{\text{CaL}}$ recordings contained (in mmol l$^{-1}$): CsCl 20, tetraethylammonium chloride 120, MgCl$_2$ 1, CaCl$_2$ 2, glucose 10, Heps 10 (pH adjusted to 7.7 with CsOH). The solution was Na$^+$ ion-free and included 0.5 µM tetrodotoxin (Tocris Cookson, Bristol, UK) to prevent Na$^+$ current ($I_{\text{Na}}$). Since Cs$^+$ can flow through the Erg K$^+$ channels, 2 µmol l$^{-1}$ E-4031 (Tocris Cookson, UK) was included in the external saline solution to prevent the rapid component of the delayed rectifier K$^+$ current ($I_{\text{Kr}}$). Temperature was regulated at 28°C by using a Peltier device (HCC-100A, Dagan, MN, USA). The pipette solution contained (in mmol l$^{-1}$) 130 CsCl, 15 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) giving a mean resistance of 2.5 ± 0.03 (SEM) MΩ.

After gaining a giga ohm seal and access to the cell, current transients due to series resistance (6.17 ± 0.22 MΩ) and pipette capacitance (8.20 ± 0.10 pF) were cancelled out, and capacitive size of ventricular myocytes (27.77 ± 1.11 pF (n=62)) was determined. The total $I_{\text{Ca}}$, including both $I_{\text{CaT}}$ and $I_{\text{CaL}}$, was elicited by 300-ms square wave pulses from the holding potential (HP) of -90 mV with 10 mV increments to cover a voltage range from -80 to +50 mV. $I_{\text{CaL}}$ was elicited from the HP of -50 mV to voltages ranging from -40 to +50 mV. $I_{\text{CaT}}$ was obtained as a difference current from those two protocols.

Charge transfer through Ca channels was determined by integrating the inactivating portion of the Ca current for 300-ms voltage pulses from −90 mV to -30 mV for $I_{\text{CaT}}$ and from -50 mV to 0 mV for $I_{\text{CaL}}$. The change in total intracellular cellular Ca ([Ca$^\text{2+}$]$_i$) due to Ca influx via T-type and L-type Ca channels was calculated from the measured cell capacitance and the experimentally determined surface-to-volume ratio of the cells (1.15) as described.
earlier in detail (Vornanen, 1997). \([\text{Ca}]_i\) was expressed for the non-mitochondrial cell volume assuming a non-mitochondrial volume fraction of 0.55.

**Statistics**

Statistics were performed using SPSS version 21.0. Statistically significant differences between sarcolemmal Ca influxes by \(I_{\text{CaT}}\) comparison \(I_{\text{CaL}}\) were detected through Mann-Whitney U nonparametric-test for two independent samples, after checking normality of distribution and making necessary transformation of the data. Differences between mean transcript expression values of the five Ca channel types and concentration-dependent \(I_{\text{CaT}}\) inactivation by Ni\(^{2+}\) were compared using one-way ANOVA followed by Tukey’s *post hoc* test. Paired comparison between transcripts of Ca channel genes and T- and L-type Ca current were done by two-tailed Student’s *t*-test. The significance threshold was \(p < 0.05\).

**Results**

*Expression of Ca channel transcripts*

We performed quantitative RT-PCR to investigate the mRNA expression of T-type (\(Ca_{v3.1, -2a, -2b, -3a} \) and \(-3b\)), L-type (\(Ca_{v1.1a, -1b, -2, -3a, -3b, -4a} \) and \(-4b\)), P/Q-type (\(Ca_{v2.1a} \) and \(-b)\), N-type (\(Ca_{v2.2a} \) and \(-b)\) and R-type (\(Ca_{v2.3a} \) and \(-b)\) Ca channel \(\alpha\)-subunit genes in atrium and ventricle of 1.5-year-old zebrafish. Interestingly, in the atrium transcript expression of T-type Ca channels (64.1 ± 3.7% of all \(Ca_v\) transcripts) was significantly higher than that of L-type Ca channels (33.8 ± 3.4%) \((p < 0.05)\) (Figure 1). In the ventricle, T-type channel transcripts (54.9 ± 4.4%) seemed to be more numerous than L-type channel transcripts (41.1 ± 4.4%), but the difference was not statistically significant \((p > 0.05)\). Transcripts of P/Q-type channels constituted 1.7 ± 0.3% and 4.0 ± 0.5% of all Ca channel transcripts in atrium and ventricle, respectively (Figure 1). N- and R-type Ca channel
transcripts were also present, but the expression levels were low in both cardiac chambers (less than 0.5% of all Ca_v transcripts).

The main T-type Ca channel isoform in the zebrafish heart was α1G (Ca_v3.1), representing 99.7 ± 0.05% and 98.9 ± 0.3% of T-type transcripts in ventricle and atrium, respectively (Figure 2). The dominant L-type Ca channel isoform was α1C (Ca_v1.2) comprising 93.0 ± 1.2% and 95.9 ± 0.7% of all Ca_v1 transcripts in ventricle and atrium, respectively (Figure 2). Transcript expression of α1G was significantly ($p < 0.05$) higher - both in ventricle (54.8 ± 4.4% of all Ca_v transcripts) and atrium (63.4 ± 3.9%) - than that of the dominant L-type Ca channel, α1C (38.3 ± 4.6% in ventricle and 32.4 ± 3.4% in atrium) (Table 2). P/Q-type Ca channel isoform α1Ab (Ca_v2.1b) showed the third highest expression level representing 3.8 ± 0.5% and 1.6 ± 0.3% of all Ca_v transcripts in ventricle and atrium, respectively (Table 2). Collectively this data show that α1G mRNA expression is dominant in both cardiac chambers of the adult zebrafish heart.

**Density and voltage-dependence of I_{Cat} and I_{Cal}**

$I_{CaL}$ was determined as the current elicited from the HP of -50 mV. $I_{CaT}$ was obtained by subtracting $I_{CaL}$ from the current elicited from the HP of -90 mV, where both currents are available for opening. Ventricular myocytes of the zebrafish heart had large $I_{CaT}$ and $I_{CaL}$ (Figure 3). $I_{CaT}$ had the peak density of 6.3 ± 0.8 pA pF$^{-1}$ at -30 mV, whereas $I_{CaL}$ peaked at 0 mV with a density of 7.7 ± 0.8 pA pF$^{-1}$ ($p > 0.05$) (Figure 3B). Densities of $I_{CaT}$ and $I_{CaL}$ varied markedly between individual ventricular myocytes (Figure 3C). Typical densities for $I_{CaL}$ were 4-6 pA pF$^{-1}$. Frequency distribution of $I_{CaT}$ density was flatter than that of $I_{CaL}$ without any typical density value.
Nickel sensitivity of \( I_{\text{CaT}} \).

T-type Ca channels are blocked by \( \text{Ni}^{2+} \). Since \( \alpha1\text{G}, \alpha1\text{H} \) and \( \alpha1\text{I} \) isoforms are differently sensitive to \( \text{Ni}^{2+} \) (Lee et al., 1999), inhibition of \( I_{\text{CaT}} \) by \( \text{Ni}^{2+} \) can be used to trace isoform composition of cardiac myocytes. To this end, \( \text{Ni}^{2+} \) sensitivity of \( I_{\text{CaT}} \) was examined by cumulatively additions of \( \text{NiCl}_2 \) (0.3-1000 µM) to the external saline solution. \( I_{\text{CaT}} \) was inhibited by \( \text{Ni}^{2+} \) in concentration-dependent manner (Figure 4A). Half-maximal inhibition (IC\(_{50}\)) occurred at 92.1 ± 0.1 µM \( \text{Ni}^{2+} \) (Figure 4B). \( \text{Ni}^{2+} \) also slowed inactivation of \( I_{\text{CaT}} \) (inset Figure 4B).

Sarcolemma Ca entry via \( I_{\text{CaT}} \) and \( I_{\text{CaL}} \).

Sarcolemmal Ca influx was obtained from the integrals of \( I_{\text{CaT}} \) and \( I_{\text{CaL}} \) at -50 and 0 mV, respectively (Figure 5A). In ventricular myocytes, cytosolic Ca increments via \( I_{\text{CaT}} \) and \( I_{\text{CaL}} \) were 41.2 ± 7.3 and 88.5 ± 20.5 µmol L\(^{-1}\) per non-mitochondrial cell volume, respectively (Figure 5B). This means that \( I_{\text{CaT}} \) was responsible for 31.7% of the total Ca influx via sarcolemmal Ca channels.

Discussion

Ten Ca channel \( \alpha \)-subunit genes, divided in three subfamilies (\( \text{Ca}v1-3 \)), exists in vertebrate genomes (Catterall et al., 2005). Due to the whole genome duplication in the early teleosts, fish genomes include a number of gene paralogs. Therefore, a higher diversity of Ca channel genes is expected to exist in fish genomes (Jegla et al., 2009). Indeed, 21 \( \alpha \)-subunit genes were found in the genome of the fugu (\( \text{Fugu rubripes} \)) (Wong et al., 2006). In the current study, we provide a complete survey of Ca channel \( \alpha \)-subunit expression in the zebrafish heart. Transcript abundance of 18 Ca channel \( \alpha \)-subunit genes were quantified in atrium and
ventricle of the zebrafish heart. Several studies have shown that expression of ion channel proteins in heart is predominantly determined at the level of transcription (Rosati and McKinnon, 2004; Marionneau et al., 2005; Gaborit et al., 2007; Chandler et al., 2009; Abd Allah et al., 2012). Therefore, we believe that relative abundancies of gene transcripts are good surrogates for Ca channel composition and expression of atrial and ventricular muscle of the zebrafish heart. Overall, the present findings show that the diversity of Ca channels expressed in the zebrafish heart is higher than in mammalian hearts and T-type channels are more abundant than L-type Ca channels.

**T-type Ca channel transcript expression**

To our knowledge, this is the first quantitative analysis of Ca channel composition and expression of the fish heart. The main finding of the present study is that at the transcript level, T-type Ca channels with the relative abundance of 55-64% form the biggest Ca channel subfamily in the zebrafish heart. This is in striking contrast to the human heart, where L-type Ca channels form 96-98% of the Ca channel transcripts in both atria and ventricles, while T-type Ca channels represent only 2-4% of the transcripts (Gaborit et al., 2007). The predominance of T-type Ca channels is probably not common among fish hearts in the light of the Ca current recordings. \( I_{CaL} \) is the main Ca current in atrial and ventricular myocytes of many fish species including crucian carp (Carassius carassius), rainbow trout (Oncorhynchus mykiss), burbot (Lota lota) and bluefin tuna (Thunnus orientalis) (Vornanen, 1997; Vornanen, 1998; Hove-Madsen and Tort, 1998; Shiels et al., 2006; Shiels et al., 2015). It remains to be shown, whether the strong expression of T-type Ca channels in ventricle is typical for Danio species in general (a phylogenetic trait), or is it an adaptation of tropical fishes to high environmental temperatures.
In zebrafish heart, five isoforms of T-type Ca channels are expressed, while in the human heart only two gene products (α1H, α1G) are present (Gaborit et al., 2007). Differently from mammalian genomes, in the zebrafish genome two paralogues exist for α1H and α1I, and both gene products are expressed to some extent in the heart. Interestingly, the main T-type Ca channel isoform of the zebrafish ventricle is α1G. In the right ventricle and Purkinje fibers of the human heart T-type Ca channels are weakly expressed, and α1H is the predominant isoform in both tissues (Gaborit et al., 2007). In the human right atrium α1G is the main T-type Ca channel isoform comprising 73.6% of T-type Ca channel transcripts and 3.0% of all Ca channel transcripts (Gaborit et al., 2007).

The density of $I_{CaT}$ is significantly higher in the ventricles of fetal and neonatal mammals than in the ventricles of adult mammals (Perez-Reyes, 2003; Ono and Iijima, 2010). The developmental decrease in $I_{CaT}$ density of the rat ventricle is associated with a significant shift in isoform composition from predominantly α1G channels to α1H channels (Ferron et al., 2002). Messenger RNA expression of T-type Ca channel α1-subunits (α1G, α1H) in the diseased (cardiac hypertrophy and failure) mammalian heart is significantly higher than in healthy controls (Perez-Reyes, 2003; Ono and Iijima, 2010) (see Pathophysiology of T-type Ca channels).

Taken together, the diversity of isoform composition of T-type Ca channels is wider in zebrafish ventricle than in human (mammalian) ventricles and the major isoform is α1G instead of the human α1H. With respect to expression of T-type Ca channels, the zebrafish ventricle resembles more perinatal and diseased mammalian ventricles than the healthy adult mammalian ventricles. Overall, isoform composition of T-type Ca channels in zebrafish ventricle is more diverse and relative transcript abundance markedly higher than in adult human ventricles.
**L-type Ca channel transcript expression**

Instead of the four mammalian L-type Ca channels, α1C, α1D, α1F and α1S, seven isoforms are known for the zebrafish, because α1D, α1F and α1S genes are duplicated. All seven L-type Ca channel genes are expressed to some extent in the zebrafish heart. Similar to the human heart (Gaborit et al., 2007) α1C is the main L-type Ca channel isoform in zebrafish atrium and ventricle, and it is almost equally expressed in both cardiac chambers. Another L-type Ca channel expressed in the human heart is α1D, but the expression level is quite low representing 0.04% and 1.13% of all Ca\textsubscript{v1} transcripts in ventricular and atrial myocardia, respectively (Gaborit et al., 2007) (Table 2). In the zebrafish heart, both α1D isoforms are very weakly expressed, whereas α1Sa shows the second highest expression level among the L-type channels in both atrium and ventricle (Figure 2). Collectively, the relative expression of L-type Ca channel transcripts is much lower (41.1% vs. 98.4%), and L-type Ca channel composition is more diverse in zebrafish than human heart.

**Density of I_{CaT} in comparison to I_{CaL}**

Ventricular myocytes of the zebrafish heart have a large I_{CaT}. The peak current density of I_{CaT} is 83.1% of the respective value of I_{CaL}. The slightly smaller density of I_{CaT} in comparison to I_{CaL} is probably due to the lower single channel conductance of the T-type Ca channels (Nilius et al., 1985). It should be also noted that the variability of current densities between cells is large for both I_{CaT} and I_{CaL}. Our findings are similar to those of Nemtsas et al., although our values for I_{CaT} are slightly higher and those of I_{CaL} slightly smaller than theirs (Nemtsas et al., 2010). Notably, I_{CaT} has not been found in human atrial and ventricular myocytes, which accords with the minimal expression of the T-type Ca channels in the human heart (Beuckelmann et al., 1991; Ouadid et al., 1991; Leuranguer et al., 2001). In contrast, a large cardiac I_{CaT} has been previously documented for a few endo-
and ectothermic vertebrates. A relatively large $I_{CaT}$ exists in ventricular myocytes of finch (5.9 pA/pF; 56% of $I_{CaL}$) (Bogdanov et al., 1995) and shark ($Squalus acanthias$) (-9.8 pA/pF; 92.4% of $I_{CaL}$) (Maylie and Morad, 1995), and atrial myocytes of the Siberian sturgeon ($Acipenser baerii$) (3.52 pA/pF; 242% of $I_{CaL}$) (Haworth et al., 2014). The peak density of $I_{CaT}$ in zebrafish ventricular myocytes is 2-6 times higher than the reported $I_{CaT}$ densities in atrial and nodal tissues of other vertebrate species (for references see (Maylie and Morad, 1995)). The presence of a large $I_{CaT}$ in zebrafish ventricular myocytes is one of the most prominent differences in ion current compositions between zebrafish and human hearts. $I_{CaT}$ density is significantly higher in the neonatal than adult mammalian heart. For example in the rat ventricle, the density of $I_{CaT}$ decreases from about 3 pA/pF at the fetal stage F16 to practical absence of the current in the adult ventricle (Ferron et al., 2002).

$Ni^{2+}$ block of T-type Ca channels

$Ni^{2+}$ blocks different T-type Ca channels with different affinities, and $Ni^{2+}$ sensitivity of the channels is dependent on the cellular environment (Lee et al., 1999). When expressed in $Xenopus$ oocytes $IC_{50}$ were 167, 5.7 and 87 µM $Ni^{2+}$ for $\alpha_1G$ (from rat), $\alpha_1H$ (human) and $\alpha_1I$ (rat), respectively. In HEK-293 cells, the respective $IC_{50}$-values were markedly higher (250, 12 and 216 µM) even though the relative sensitivity order remained the same. Furthermore, the $Ni^{2+}$ block of $\alpha_1G$ and $\alpha_1I$ channels (but not of $\alpha_1H$) is voltage-dependent, e.g. $IC_{50}$-value of $\alpha_1G$ is 200 µM at 0 mV and only 70 µM at -40 mV (Lee et al., 1999). In the present study $Ni^{2+}$ inhibition of $I_{CaT}$ was determined at -30 mV. The $IC_{50}$-value of the zebrafish ventricular $I_{CaT}$ was 92.1 µM, which is consistent with the T-type Ca channel composition dominated by $\alpha_1G$ (70 µM at -40 mV) (Lee et al., 1999). Others have reported a slightly higher $IC_{50}$ value (124 µM) for the zebrafish ventricular $I_{CaT}$ at -40 mV (Nemtsas et al., 2010). The high $Ni^{2+}$ sensitivity of $\alpha_1H$ channels is related to a unique histidine
residue at position 191 in the S3-S4 loop of the domain I (Kang et al., 2007). Sequence analysis of zebrafish T-type Ca channel genes indicates α1H and α1G, but not α1I, share the histidine-191 with the mammalian α1H.

Ca influx via I_{CaT} and its physiological significance

In canine Purkinje myocytes, guinea-pig ventricular myocytes and mouse ventricular myocytes overexpressing α1G T-type Ca channels Ca admitted through T-type Ca channels is capable of activating contraction via CICR from the SR (Sipido et al., 1998; Zhou and January, 1998; Jaleel et al., 2008). However, contractions initiated by Ca entry through T-type Ca channels are characterized by a long delay to the onset of shortening, slow rates of shortening and relaxation, low peak shortening, and long time-to-peak shortening (Zhou and January, 1998; Sipido et al., 1998; Jaleel et al., 2008). These findings show that Ca entry through I_{CaT} is less effective than I_{CaL} in triggering CICR. T-type Ca channels are primarily located in the peripheral sarcolemma and therefore more distant from the Ca release channels of SR than L-type Ca channels of the T-tubule membrane (Jaleel et al., 2008).

The contraction of zebrafish ventricle is strongly dependent on sarcolemmal Ca influx, while CICR from the SR makes only a small contribution (about 15%) to Ca transient (Zhang et al., 2011; Bovo et al., 2013). In keeping with this, the combined sarcolemmal Ca influx via I_{CaL} and I_{CaT} in zebrafish ventricular myocyte (129 μmol L^{-1} at 28°C) is markedly large, bigger than in other fish species like rainbow trout (32.1-45.8 μmol L^{-1} at 21°C) and crucian carp (14.7-42.9 μmol L^{-1} at 19-23°C) (Vornanen, 1997; Vornanen, 1998; Hove-Madsen and Tort, 1998). Moreover, in zebrafish ventricular myocytes, a significant portion of this Ca influx is mediated by I_{CaT}. Indeed, T-type Ca channels could be important for e-e coupling of cardiac myocytes in zebrafish and other tropical ectotherms, which rely strongly on sarcolemmal Ca influx for cardiac contraction. Due to its low voltage-threshold
(about -70 mV), $I_{\text{CaT}}$ starts to contribute to intracellular Ca transient earlier in AP than $I_{\text{CaL}}$, which has a more depolarized threshold (about -40 mV). This is expected to reduce the delay between membrane depolarization and onset of intracellular Ca transient, and make the Ca transient fast rising. Fast Ca transients might be adaptive for the tropical *Danio* species, which have high heart rates at temperatures close to their upper thermal tolerance limit (Sidhu et al., 2014). For example, in *Danio rerio* at 36°C heart beats about 300 times per min and ventricular AP lasts (AP duration at 50% repolarization) only 66 ms (Vornanen and Hassinen, 2016). At high temperatures, $I_{\text{CaT}}$ and fast Ca transients may be needed to cope the requirements of short cycle lengths and AP durations. The role of $I_{\text{CaT}}$ in cardiac e-c coupling of zebrafish and other tropical fish species will be an interesting future topic.

*Pathophysiology of T-type Ca channels*

T-type Ca channels and $I_{\text{CaT}}$ are present in ventricular myocytes of perinatal mammalian (e.g. rat and mouse) heart, but during the early postnatal development they gradually disappear (Ferron et al., 2002; Yasui et al., 2005). T-type Ca channels and $I_{\text{CaT}}$ reappear in ventricular myocytes of hypertrophied mammalian heart under different pathological stresses (Nuss and Houser, 1993; Martinez et al., 1999; Takebayashi et al., 2006) suggesting that they are somehow involved in the pathogenesis of hypertrophy. Indeed, studies on transgenic mouse models suggest that $\alpha1H$ channels might be pro-hypertrophic and $\alpha1G$ channels anti-hypertrophic (Chiang et al., 2009; Nakayama et al., 2009). It should be noted, however, that in ventricular myocytes of perinatal mammals and adult zebrafish $I_{\text{CaT}}$ is a normal physiological component of the sarcolemmal ion current complex. In the ventricle of zebrafish, $I_{\text{CaT}}$ is largely generated by $\alpha1G$ channels, while in the ventricles of embryonic and neonatal mouse $\alpha1H$ channels predominate (Ferron et al., 2002; Yasui et al., 2005).
Hypertrophic heart is prone to cardiac arrhythmias, but the role of the re-expressed T-type Ca channels in arrhythmogenesis of mammalian is not yet resolved (Kinoshita et al., 2009). SS activation and inactivation curves of mammalian $I_{CaT}$ overlap, which shows that a small portion of the channels do not inactivate (Vassort et al., 2006). The non-inactivating T-type Ca channels could be involved in arrhythmogenesis by inducing early and delayed afterdepolarizations and associated arrhythmias (Kinoshita et al., 2009). The role of $I_{CaT}$ is, however, difficult discern since many other ion currents are changed in parallel with $I_{CaT}$ (Kinoshita et al., 2009). It should be noted, however, that delayed afterdepolarization are induced by spontaneous Ca release from the SR and SR leak may be also involved in triggering early afterdepolarizations (Choi et al., 2002). In fish ventricular myocytes, Ca release channels of the SR have low affinity to Ca and therefore spontaneous Ca releases are rare (Shiels and White, 2005; Vornanen, 2006; Bovo et al., 2013). The minor role of calcium-induced calcium release (CICR) in fish cardiac e-c coupling is suggested to make fish heart less susceptible to early and delayed afterdepolarizations and therefore for triggered arrhythmias (Vornanen, 2017).

Conclusions and future perspectives
Zebrafish ventricular myocytes have a large $I_{CaT}$, which contributes a distinct depolarizing current and relatively large sarcolemmal Ca influx. This is a prominent difference to human ventricles, where $I_{CaL}$ is apparently the sole Ca current type (Beuckelmann et al., 1991; Leuranguer et al., 2001). These differences are due to the dominance of T-type Ca channels in the zebrafish heart (present study) and L-type Ca channels in the human heart (Gaborit et al., 2007). Since zebrafish is a popular model for drug screening and human cardiac toxicology (Barros et al., 2008; Chakraborty et al., 2009; Peterson and MacRae, 2012), these species-specific differences in electrical excitation and e-c coupling warrant some care.
when adapting results from zebrafish studies to human heart. Due to significant differences in Ca and K currents/channels between zebrafish and human ventricles (Hassinen et al., 2015; Vornanen and Hassinen, 2016), zebrafish is probably not an optimal model for screening of cardiovascular drugs. Interspecies differences in cardiac ion currents are so marked (even among mammals) that non-human cells and tissues are considered unsatisfying for preclinical drug screening and safety pharmacology. According to the novel CiPA (Comprehensive In vitro Proarrhythmia Assay) initiative of preclinical drug screening, only human cardiac ion channels, human stem cell-induced cardiomyocytes and in silico models of human cardiac AP are considered acceptable for drug screening and safety pharmacology (Gintant et al., 2016). CiPA is based on analysis of drug effects on multiple (7) human cardiac ion channels (Colatsky et al., 2016; Vicente et al., 2016), and I_{CaT} is not among those channels. If zebrafish are used for preclinical drug screening and safety pharmacology, the potential impact of the large I_{CaT} on drug responses and arrhythmia propensity should be carefully examined.

Although zebrafish may not be an optimal general model for human cardiac safety pharmacology and screening of cardiac drugs, they may be useful in solving more specific problems of ion channel function in cardiac excitation and e-c coupling. Owing to the large ventricular I_{CaT}, zebrafish could be useful model species, when the role of I_{CaT} in cardiac e-c coupling and as a putative target for cardiovascular drugs is examined. In having large I_{CaT}, being less dependent on CICR from the SR and not having t-tubules, zebrafish ventricular myocytes resemble more perinatal ventricular myocytes than adult ventricular myocytes of the human heart. Considering that there is a paucity of information regarding the cardiac safety pharmacology of human neonates and premature infants (Pesco-Koplowitz et al., 2018), zebrafish could a useful model for this population.
Acknowledgements

This work was supported by Jane and Aatos Erkko Foundation (project No 64579 to MV). Prof. Pertti Panula (University of Helsinki) is acknowledged for donating us zebrafish for the establishment of our own zebrafish population in Joensuu. Anita Kervinen kindly prepared the solutions for electrophysiological experiments.

Author contributions

MV, MH, and JH conceived the project and designed the experiments, MH and SD performed the molecular studies, JH conducted the electrophysiological experiments, MV drafted the manuscript, MH and JH modified the manuscript, and SD agreed on the final version of the manuscript.

Disclosures

The authors declare no conflict of interests.
References


Figure 1. Relative transcript expression of the five Ca channel types in zebrafish atrium and ventricle as percentage of all Ca channel transcripts. The results are means ± SEM of three samples, each representing pooled tissue from 8 fishes. An asterisk (*) indicates a statistically significant difference ($p < 0.001$) between T-type channels and other Ca channel types (pairwise comparisons between T-type channels and L-, P/Q-, N- and R-type channels using one-way ANOVA followed by Tukey’s post-hoc test).
Figure 2. Transcript expression of Ca channel α-subunit genes in zebrafish atrium and ventricle. (A) The pie charts indicating relative portions (%) of each Ca\textsubscript{v} transcripts from the total Ca channel expression of each calcium channel type. (B) Transcript expression of Ca\textsubscript{v} genes in zebrafish atrium and ventricle normalized to the geometric mean expression of the reference genes, DnaJA2 and β-actin. The results are means ± SEM of three samples, each representing pooled tissue from 8 fishes. An asterisk (*) indicates statistically significant difference between atrium and ventricle (p < 0.05, two-tailed Student’s t-test).
**Figure 3.** T-type Ca current of the zebrafish ventricular myocytes in comparison to L-type Ca current. (A) Representative recordings of voltage-dependence of the total cardiac Ca current (left) elicited from the holding potential of -90 mV, $I_{CaL}$ elicited from the holding potential of -50 mV (middle) and the $I_{CaT}$ (right) obtained as the difference between the former two. (B) Current-voltage dependence of $I_{CaT}$ and $I_{CaL}$ in zebrafish ventricular myocytes. The results are means ± SEM of 34 and 44 myocytes for $I_{CaT}$ and $I_{CaL}$, respectively. (C) Current density histograms of ventricular $I_{CaT}$ and $I_{CaL}$. 
Figure 4. Concentration-dependent inhibition of zebrafish ventricular \( I_{CaT} \) by \( Ni^{2+} \). (A) Representative patch-clamp recordings showing inhibition of \( I_{CaT} \) by 10, 300 and 1000 \( \mu \)M \( Ni^{2+} \). (B) The mean concentration-response curve of \( Ni^{2+} \) inhibition of \( I_{CaT} \). The inset bar graph indicate concentration-dependent slowing of \( I_{CaT} \) inactivation by \( Ni^{2+} \). The results in B are mean values (± SEM) from 6-8 myocytes. An asterisk (*) indicates statistically significant difference between means in the absence and presence of \( Ni^{2+} \) (\( p < 0.05 \), one-way ANOVA followed by Tukey’s post hoc test).
Figure 5. Sarcolemmal Ca influx by T-type Ca current: comparison with L-type Ca current.

(A) Representative recordings of $I_{CaT}$ at -50 mV and $I_{CaL}$ at 0 mV (top) and the respective charge integrals (bottom). The voltage protocol is shown on the top. (B) Sarcolemmal Ca influx by $I_{CaT}$ and $I_{CaL}$ in zebrafish ventricular myocytes. The results are means ± SEM from 34 and 44 myocytes for $I_{CaT}$ and $I_{CaL}$, respectively. An asterisk (*) indicates a statistically significant difference between mean values of $I_{CaL}$ and $I_{CaT}$ ($p < 0.05$, Mann-Whitney U nonparametric test).
Table 1. Primers used for qPCR. Primers are in 5'-3' orientation.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Gene</th>
<th>Accession number</th>
<th>Primer sequences</th>
<th>Amplicon length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca, 1.1a</td>
<td>α1Sa</td>
<td>NM_001146150</td>
<td>TCTATAGGGCGTGCTGGAGGT GCTATCTGCCAGCTGTAGGG</td>
<td>113</td>
</tr>
<tr>
<td>Ca, 1.1b</td>
<td>α1Sb</td>
<td>NM_214726</td>
<td>GTTGGCTGAAGACCCACAGT GCCCATTGAATACCTCCCA</td>
<td>110</td>
</tr>
<tr>
<td>Ca, 1.2</td>
<td>α1C</td>
<td>XM_009300335</td>
<td>ACGGAGTCACCTCCGACAC AGAGAGGCACAGGTGGATT</td>
<td>105</td>
</tr>
<tr>
<td>Ca, 1.3a</td>
<td>α1Da</td>
<td>NM_203484</td>
<td>ACTGGGCCACAGAGACTACG TTCATCTGAAGACCCACAGT</td>
<td>106</td>
</tr>
<tr>
<td>Ca, 1.3b</td>
<td>α1Db</td>
<td>ENSDARG000000101589</td>
<td>CGAAGACCCACAGAGACTACG TTCATCTGAAGACCCACAGT</td>
<td>105</td>
</tr>
<tr>
<td>Ca, 1.4a</td>
<td>α1Fa</td>
<td>XM_021478483</td>
<td>AGCAAGCCTCGAGCATACACT CCAAGCCTCGAGCATACACT</td>
<td>115</td>
</tr>
<tr>
<td>Ca, 1.4b</td>
<td>α1Fb</td>
<td>NM_001324496</td>
<td>ATTCACCAACTGCACAGAGT GCCATTGAAGAGGGAGGTAAC</td>
<td>115</td>
</tr>
<tr>
<td>Ca, 2.1a</td>
<td>α1Aa</td>
<td>ENSDARG00000037905</td>
<td>TGCTACCCAGCCACATGATA TGCTACCCAGCCACATGATA</td>
<td>113</td>
</tr>
<tr>
<td>Ca, 2.1b</td>
<td>α1Ab</td>
<td>ENSDARG0000006923</td>
<td>CTTCGGTGACAGATTGAATG GCCATTGAAGAGGGAGGTAAC</td>
<td>105</td>
</tr>
<tr>
<td>Ca, 2.2a</td>
<td>α1Ba</td>
<td>ENSDARG00000021735</td>
<td>CAAATGCTGAGATGCAGAGGA CTGTGGTGCAAAAGTGGATG</td>
<td>106</td>
</tr>
<tr>
<td>Ca, 2.2b</td>
<td>α1Bb</td>
<td>ENSDARG00000079295</td>
<td>TGCTTCCTCAGACCTCGACACATG AAAAGGGTGATGGTGATGGT</td>
<td>106</td>
</tr>
<tr>
<td>Ca, 2.3a</td>
<td>α1Ba</td>
<td>ENSDARG00000062346</td>
<td>GCAGCTCGAGGAACAGAAAC CCTGCACTTGAAC CTGTGACATG</td>
<td>114</td>
</tr>
<tr>
<td>Ca, 2.3b</td>
<td>α1Eb</td>
<td>ENSDARG00000095614</td>
<td>ACTACGACGCCTCAGACCTGTT TGGAACCTGAGGACCATG</td>
<td>115</td>
</tr>
<tr>
<td>Ca, 3.1</td>
<td>α1G</td>
<td>ENSDARG00000089913</td>
<td>CTTGGCAAGCGTGGTGGTTTT CATACTGCCTTGCCCTTG</td>
<td>100</td>
</tr>
<tr>
<td>Ca, 3.2a</td>
<td>α1Ha</td>
<td>ENSDARG00000060496</td>
<td>TCCATCGAGCATTTATCA GCCAGAGTTAATGAGCAC</td>
<td>107</td>
</tr>
<tr>
<td>Ca, 3.2b</td>
<td>α1Hb</td>
<td>ENSDARG00000099708</td>
<td>TGTTTGGGTCCTGTGTCTG ACTCTGGTGATTTCGGGAGATT</td>
<td>120</td>
</tr>
<tr>
<td>Ca, 3.3a</td>
<td>α1Ia</td>
<td>ENSDARG00000070522</td>
<td>TGTTTCAGGAAAGAGCCACAG TGTTTGGGTCCTGTGTCTG</td>
<td>103</td>
</tr>
<tr>
<td>Ca, 3.3b</td>
<td>α1Ib</td>
<td>ENSDARG00000096307</td>
<td>TTCCCCATCGAAAACCTCTAC TGAGATCGTGGGCAATT</td>
<td>107</td>
</tr>
<tr>
<td>β-actin</td>
<td>actb1</td>
<td>NM_131031</td>
<td>CTTCAGCAGATGTGGATCA GCCATTTAAGGTGCAACA</td>
<td>102</td>
</tr>
<tr>
<td>DnaJA2</td>
<td>dnaja2</td>
<td>NM_213493</td>
<td>CTATGGGGAACAGGGTGCTGC ATATGGGGAACAGGGTGCTGC</td>
<td>104</td>
</tr>
</tbody>
</table>
Table 2. Comparison of different calcium ion currents and genes (% of all transcripts) between zebrafish (present study) and human heart (Gaborit et al., 2007).

<table>
<thead>
<tr>
<th>Ca-current</th>
<th>Channel</th>
<th>Zebra fish (atrium)</th>
<th>Zebra fish (ventricle)</th>
<th>Human (atrium)</th>
<th>Human (ventricle)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$I_{Ca,T}$</td>
<td>Ca,3.1</td>
<td>63.44</td>
<td>54.76</td>
<td>3.02</td>
<td>0.14</td>
</tr>
<tr>
<td></td>
<td>Ca,3.2a</td>
<td>0.58</td>
<td>0.06</td>
<td>1.08</td>
<td>1.46</td>
</tr>
<tr>
<td></td>
<td>Ca,3.2b</td>
<td>0.02</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ca,3.3a</td>
<td>0.04</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ca,3.3b</td>
<td>0.04</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I_{Ca,L}$</td>
<td>Ca,1.2</td>
<td>32.43</td>
<td>38.33</td>
<td>94.77</td>
<td>98.36</td>
</tr>
<tr>
<td></td>
<td>Ca,1.3a</td>
<td>0.03</td>
<td>0.07</td>
<td>1.13</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Ca,1.3b</td>
<td>0.05</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ca,1.4a</td>
<td>0.03</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ca,1.4b</td>
<td>0.01</td>
<td>0.001</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ca,1.1a</td>
<td>1.20</td>
<td>2.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ca,1.1b</td>
<td>0.02</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I_{Ca,P/Q}$</td>
<td>Ca,2.1a</td>
<td>0.06</td>
<td>0.02</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ca,2.1b</td>
<td>1.64</td>
<td>3.84</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I_{Ca,N}$</td>
<td>Ca,2.2a</td>
<td>0.08</td>
<td>0.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ca,2.2b</td>
<td>0.19</td>
<td>0.05</td>
<td></td>
<td></td>
</tr>
<tr>
<td>$I_{Ca,R}$</td>
<td>Ca,2.3a</td>
<td>0.02</td>
<td>0.004</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Ca,2.3b</td>
<td>0.12</td>
<td>0.04</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>