

Medaka as a model for ECG analysis and the effect of verapamil

(心電図解析モデルとしてのメダカとベラパミルの効果)

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abstract

The heart of the medaka, a small fish native to East Asia, has electrophysiological aspects similar to mammalian hearts. We found that the heart rates of medaka were more similar to humans than mice or rats. Medaka exhibited similar electrocardiogram patterns to those of humans, suggesting a similarity in cardiac impulse formation and propagation. Their hearts also exhibited similar responsiveness to verapamil, a calcium channel antagonist; atropine, a parasympathetic nerve blocker; propranolol, a sympathetic β -adrenergic blocker; and isoproterenol, a sympathetic β -adrenergic agonist. We successfully analyzed action potentials and cardiac contractile forces in vivo. Verapamil affected action potential duration and reduced heart rate, suggesting the importance of voltage-dependent calcium channels in determining the heart rhythm of medaka. We also analyzed the expression of the voltage-dependent calcium channel $\beta 2$ subunit, which participates in channel formation in cardiac myocytes, and found that splice variant type-2 was the only major transcript in the heart. Our results indicate that medaka could be an appropriate animal model for studying cardiovascular pharmacology.

1. Introduction

The Japanese rice fish (*Oryzias latipes*), also known as the medaka or Japanese

killifish,(1) is a small (up to 3.2 cm in length) fish that has been popular for aquariums since the 17th century. Medaka has also been found in East and Southeast Asia (2) and has been used for biological experiments since 1921.(3) Similar to zebrafish (*Danio rerio*), which has been used widely since the 1980s,(4) medaka has become a major vertebrate model for genetic and embryological studies. Although medaka is a primitive vertebrate, it has several advantages as an animal model. One advantage is the high fecundity and low cost for animal care. These fish spawn 100 to 200 eggs every week and become adults only two to three months after hatching. In addition, they live in a wide range of temperatures (from 4 to 40°C in nature). Another advantage is trunk transparency, which is useful for the observation of internal organs in studies of morphology. In addition, medaka fish have 16 million single nucleotide polymorphisms (SNPs) in 700 Mbp of their genome, the mutagenesis of which is much higher than other vertebrates.(5) In the cardiovascular and physiology fields, the 120 beats per minute (bpm) heart rate in medaka and zebrafish is closer to humans (80 bpm) than mice (720 bpm) or rats (250 bpm).(6) Zebrafish have been adopted as a model for electrophysiology and pharmacology, and experiments have revealed that in terms of electrophysiological make-up, zebrafish are closer to humans than mice.(7) In contrast, medaka has received little attention in the cardiovascular field.

To fully realize the potential of the medaka as a cost-effective model for drug screens affecting heart function, basic analyses such as electrocardiography (ECG), we attempted to establish the first ECG recording in medaka. In addition, we analyzed the effect of verapamil, a calcium channel antagonist, on ECG, cardiac action potential formation, and cardiac contraction to characterize the involvement of voltage-dependent calcium channels in medaka cardiac rhythm formation. Furthermore, we characterized voltage-dependent calcium channels in the medaka heart. As a first step, we identified the major splice variant form of the voltage-dependent calcium channel $\beta 2$ subunit, which participates in cardiac voltage-dependent calcium channel formation.

2. Materials and methods

2.1. Animals

All experimental procedures were approved by the Institutional Animal Care and Research Advisory Committee of the Hirosaki University School of Medicine. We used 6-month-old adult HO5 medaka, which is a standard inbred line from Southern Japan.

2.2. ECG evaluation

ECG recording was performed as described by others, with modifications.(8) Two 30-gauge ECG electrodes were inserted into the left and right sides of the medaka (Fig. 1B). The preparation was allowed to stabilize for at least 5 min before starting the experiment. Electrical signals were amplified and translated with a PowerLab data acquisition unit (ML846 Power Lab system; AD Instruments, Dunedin, NewZealand) using LabChart 8 software (AD Instruments). The heart rate variability (HRV) analysis included both a time domain analysis (based on the deviation from the average R-R interval) and analysis of the HRV power spectrum.(9,10) An HRV power spectrum includes three components: very low frequency (VLF), low frequency (LF), and high frequency (HF). We defined the ranges of the spectral components as $VLF < 0.04 < LF < 0.15 < HF < 0.45$.(10)

2.3. Action potentials

The fish were mounted in an organ bath (10 mL) continuously perfused with oxygenated Tyrode's solution: 136.9 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 0.53 mM MgCl₂, 0.42 mM NaH₂PO₄, 5.0mMHEPES, and 5.6mMglucose. Action potentials were recorded with an amplifier (MEZ-8301, Nihon Kohden, Tokyo, Japan) The data acquisition and analysis was carried out using LabChart. Action potentials were recorded for analysis of the following parameters: resting membrane potential (RMP), action potential amplitude

(APA), maximum upstroke velocity (Max dV/dt), and action potential duration at 40% and 90% of repolarization (APD40 and APD90).

2.4. Spontaneously beating heart

Hearts were exposed in an oxygenated tissue bath containing Tyrode's solution as in the action potential recording experiment. We measured isometric contractile force using a force transducer (CD200; Nihon Kohden).

2.5. RNA isolation and reverse transcription-polymerase chain reaction analysis

For reverse transcription-polymerase chain reaction (RT-PCR), total RNA was isolated from the heart using the RNeasy Kit (Qiagen, Valencia, CA, USA). Reverse transcription was performed using a solution of 10 pmol oligo-dT primer. We mapped the sequences of these transcripts using BLASTN (ver. 2.2.31), and the whole genome shotgun sequence of medaka from the National Center for Biotechnology Information (NCBI).

2.6. Western blot analysis

Cell lysates from medaka heart were prepared using TNE buffer in the presence of protease inhibitor cocktails (Roche Pharma, Basel, Switzerland). Lysates (100 mg) were

subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted with the following antibodies: anti-CaV1.2 (1:100; Alomone Labs, Jerusalem, Israel), anti-CaVb2 (1:200; SigmaAldrich, St. Louis, MO, USA), and anti-GAPDH (1:500; Abcam Inc., Cambridge, UK), and then incubated with alkaline phosphatase-conjugated secondary antibodies. The results were visualized with a colorimetric reaction using Western Blue stabilized substrate for alkaline phosphatase (Promega, Madison, WI, USA).

2.7. Statistics

Data are expressed as the means \pm SEM. Statistical differences were further determined with Student's t-test or analysis of variance with the Bonferroni post hoc test.

3. Results

3.1. Isolation of cardiomyocytes

We found that the ventricular myocytes of medaka were 119.2 ± 6.0 mm long and 9.6 ± 0.9 mm wide ($n = 7$), suggesting that medaka cardiomyocytes are larger than those of zebrafish (100.4 ± 3.7 mm long, 4.6 ± 0.3 mm wide).(11)

3.2. ECG recording

Typical ECG recordings showed P-, QRS-complex, and T-waves (Fig. 1C, upper panel). Propofol (5 mM) significantly prolonged the R-R interval (lower panel), suggesting that the ECG recording system was appropriate for physiological analysis.

Fig. 2 shows the results of statistical analyses of pharmacological manipulation using atropine (2.5, 5.0, and 7.5 mM), a parasympathetic nerve blocker, propranolol (2.5, 5.0, and 7.5 mM), a sympathetic nerve blocker, and isoproterenol, a sympathetic nerve agonist (25, 50, and 100 nM). Atropine increased the heart rate in a dose-dependent manner. Propranolol decreased the heart rate, to a lesser degree, while isoproterenol increased the heart rate in a dose-dependent manner. These data suggest that medaka could be useful as an animal model for pharmacological cardiovascular autonomic nerve research.

We evaluated HRV by analyzing changes in the R-R interval. In a Poincaré plot (also known as a return map, and named after Henri Poincaré), which is used to quantify self-similarity in periodic functions such as R-R intervals, a sequence for each interval is plotted against the subsequent interval. Fig. 3 shows typical results for beat-to-beat dynamics with Poincaré plots in a medaka (left upper panel) and a healthy human volunteer (left lower panel); the fish had a less stable R-R interval and some elongated intervals.

HRV is considered an indicator of autonomic nerve control.(9,10) Generally, the LF component reflects sympathetic/parasympathetic tone, whereas the HF component reflects parasympathetic tone. In the frequency domain analyses, LF and HF components were resolved in a power spectral density (Fig. 3). There was a smaller HF component for medaka than for humans. The smaller HF component might reflect the absence of pulmonary respiration.

To evaluate the possibility of medaka as an animal model for cardiac physiology, we decided to analyze the importance of voltage-dependent calcium channels. Currents through the voltage-dependent calcium channels are important for the long plateau phase in cardiac action potential formation.(12) Therefore, we next examined calcium channel antagonists. Of the several calcium channel antagonists, we chose verapamil, a derivative of phenylalkylamine calcium channel blockers. Verapamil is relatively selective for the myocardium and inhibits calcium channel currents through L-type voltage-dependent calcium channels, resulting in a decreased heart rate.

3.3. Effect of verapamil on ECGs

A dose of 10 mM significantly prolonged the R-R interval and significantly reduced the heart rate (Fig. 4A), indicating the importance of voltage-dependent calcium channels in

cardiac pace-making in medaka. Although there was low amplitude and relatively high noise in the ECG recording, the QT interval was shortened by verapamil (0.284 ± 0.03 s, $n = 7$ and $0.18^* \pm 0.02$ s, $n = 6$, basal and verapamil, respectively, $*p < 0.05$ vs. basal). The calculated QTc interval (Bazett's formula) was also shortened (0.40 ± 0.06 s, $n = 7$ and $0.26^* \pm 0.02$ s, $n = 6$, basal and verapamil, respectively, $*p < 0.05$ vs. basal).

3.4. Effect of verapamil on action potentials

To further evaluate the electrophysiological properties of the hearts of adult medaka and the effect of verapamil, we recorded action potentials (Fig. 4B). We recorded ventricular action potentials from intact (in vivo) medaka hearts beating spontaneously at a physiological temperature of 25 °C by surgically opening the chest. In the basal state, a rapid upstroke of the action potential was followed by a long-lasting plateau phase, ending with a phase of rapid terminal repolarization. The overall shape of the action potential was similar to that of humans. Verapamil (10 mM) affected the formation of the action potential and significantly shortened its duration as previously reported (13) ; however, there were no significant effects on RMP, APA, or Max dV/dt (Supplemental Information Table 1, Fig. 4B inset, bottom panel).

3.5. Effect of verapamil on spontaneous cardiac contraction

Next, we examined cardiac contraction *in vivo*. Due to the small contractile force, the signal-to-noise ratio was relatively high. Cardiac muscle contractility was recorded for longer than 30 min (Fig. 4C). Verapamil significantly decreased the heart rate, whereas contractility was conserved (bottom panel), suggesting that verapamil mainly affected cardiac pace-making in the sinus node, rather than affecting cardiac contraction.

3.6. Molecular cloning of the voltage-dependent calcium channel $\beta 2$ subunit

To further characterize the mechanism of cardiac function and the calcium channel currents in cardiovascular physiology, it is essential to identify the corresponding gene. The voltage-dependent calcium channel is composed of five different subunits ($\alpha 1$, $\alpha 2/\delta$, β , and γ). Of these subunits, $\alpha 1$ is the main pore-forming subunit. Although auxiliary, the β subunit regulates channel population, peak amplitudes, and channel properties. Therefore, the β subunit is thought to be most important auxiliary subunit. Among the four different β subunits, we attempted to identify the $\beta 2$ subunit, which is involved in the formation of voltage-dependent calcium channels in cardiac myocytes in mammalian species. We also examined the expression of other β subunits, but no corresponding amplicons were detected (data not shown). We screened the medaka

genome databases (eEnsembl; http://asia.ensembl.org/Oryzias_latipes/Info/Index and NCBI; <https://www.ncbi.nlm.nih.gov/>) using the mouse $\beta 2$ subunit cDNA sequence.(14) We then identified ten splicing variants of the $\beta 2$ subunit (1-10, Supplemental Information Fig. 5 and Table 2). The mapping sequences of all the variants to the medaka genomic sequences revealed that this gene was located from 9,008400 to 9,066034 base pairs (bp), at a contig of chromosome 20 in its local position (NW_004088040.1), consisting of 18 exons. We constructed ten different oligonucleotide primers based on these exons. RT-PCR with these primers revealed that splice variant 2 is the major form in medaka hearts (Fig. 5).

3.7. Expression of voltage-dependent calcium channels (CaV1.2 and $\beta 2$ subunits)

Because of the pharmacological effect of verapamil, we expected the expression of voltage-dependent calcium channels. To confirm this, we performed immunoblotting of cardiac myocytes using calcium channel antibodies against CaV1.2 and the $\beta 2$ subunit. Anti-CaV1.2 antibody revealed a single band (160 kDa) with moderate expression in medaka heart (Fig. 5C upper panel) while anti-CaV $\beta 2$ antibody revealed a single band (60 kDa) with moderate expression (middle panel). The GAPDH band was used as an internal control (40 kDa, lower panel).

4. Discussion

We analyzed electrophysiological aspects of the medaka heart, which are similar to those of mammals. Interestingly, the medaka heart rate was more similar to that of a human heart (ca. 80 bpm) than mice (approximately 500-700 bpm) and rats (approximately 250-350 bpm), and was affected by atropine, a parasympathetic nerve blocker; propranolol, a sympathetic β -adrenergic blocker; isoproterenol, a sympathetic β -adrenergic agonist; and verapamil, a calcium channel antagonist.(6) The ECGs, cardiac action potentials, and expression of voltage-dependent calcium channels of medaka were also similar to those of mammals. Finally, we analyzed the expression of the voltage-dependent calcium channel $\beta 2$ subunit with RT-PCR and immunoblotting and found that the type 2 splice variant was the only major transcript in the hearts of medaka. To the best of our knowledge, these new electrophysiological parameters are presented here for the first time. These data will form an important basis for future screens and studies of heart function and regeneration.

In the ECG recording, the ECG wave consisted of PQ and QRS sections immediately followed by an ST section, which is similar in both zebrafish and humans.(8) The calculated PR interval in medaka was 82.6 ± 5.6 ms ($n = 7$), which is longer than mouse

(~37 ms) or rat (~56 ms) intervals, but shorter than human intervals (~164 ms). The medaka PR (~63 ms) and QT intervals (284 ± 33.8 ms ($n = 7$); 287 in zebrafish) were similar to that of zebrafish.(7,8) Human QT intervals are longer (401 ms). In contrast, QT intervals in mice and rats are shorter than that of medaka (45 and 80 ms in mice and rats, respectively). (8,15) Since ECG reflects cardiac action potentials, which are composed of various types of ionic currents, the similarity in heart rate and PR and QT intervals in medaka seems to be preferable in an animal model.

In addition to the electrophysiological experiments, we analyzed the expression of the voltage-dependent calcium channel $\beta 2$ subunit and found that the type 2 splice variant was the only major transcript in the hearts of medaka. Western blot analysis with anti- $\beta 2$ antibody revealed a single band, suggesting that there is one splice variant as the major transcript.

In this study, the cardiac action potential of medaka was examined for the first time. The shape of the action potential varies widely between species, indicating prominent differences in ion channel expression and the composition of subunits. The overall action potential shape in medaka was similar to that of mammals. Due to the prominent plateau phase, the action potential in zebrafish is also closer in shape and duration to that of humans than mice.(16) Verapamil, a calcium channel antagonist, significantly shortened

APD90, suggesting that L-type calcium channel currents were affected. Compared with other zebrafish studies, such as Nemtsas et al.,⁽⁷⁾ APA was apparently low, while other parameters (RMP, Max dV/dt, APD40, and APD90 repolarization) were comparable (Supplemental Information Table 1).

We also conducted a contractile analysis of medaka ventricles. Although Scheid et al., reported contractile force measurement with zebrafish,⁽¹⁷⁾ to the best of our knowledge, this is the first study to report the cardiac contractile force of medaka. Verapamil (10 mM) significantly affected the contractile rate of its heart, whereas the contractile force remained unchanged (Fig. 4), suggesting that verapamil mainly affects pace making in our in vivo experimental conditions.

Through these electrophysiological experiments, we found that the characteristics of the medaka heart are more similar to humans than mice, whereas detailed analysis with various types of channels will be needed. Compared with zebrafish, we showed that medaka has an equal or potentially greater homology to mammals in terms of genetic sequence since zebrafish have two different $\beta 2$ subunits ($\beta 2.1$ and $\beta 2.2$). This is an important point since as our biological analysis aimed to establish an animal model for human cardiac rhythm.

Zebrafish have a number of advantages as a tool for molecular biology, including

CRISPR/Cas9, an established transgenic overexpression system. However, ethylnitrosourea-based mutagenesis has been applied to medaka,(18) and comparison of two related species, such as zebrafish and medaka, is one of the most successful approaches to identifying general genetic principles in eukaryotes. The CRISPR/Cas9 system has also been applied to medaka.(19) Taken together, this study indicates that medaka has sufficient potential as an animal model for cardiovascular research.

5. Conclusions

We report several similarities between the electrical activity of medaka and mammalian hearts. Our data support the possibility of using medaka as a model for cardiac research or drug screening. We successfully recorded action potentials and cardiac contractile force in vivo, indicating that future studies on gene-manipulated transgenic fish could be performed on this species, making it a strong animal model for cardiovascular pharmacology.

Conflict of interest

The authors declare no conflicts of interest associated with this manuscript.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <https://doi.org/10.1016/j.jpshs.2018.04.003>

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Figure legends

Fig. 1. (A) Adult medaka. (B) Real-time acquisition of electrocardiogram (ECGs) in medaka. Electrodes were inserted through a Teflon O-ring (M-ring), which was fixed on a damp sponge. (C) Representative ECG traces in the basal state (upper panel) and during anesthesia (propofol 5 mM, lower panel). Propofol significantly elongated the R-R interval.

Fig. 2. Pharmacological manipulation via atropine and propranolol. (A) Atropine, a

parasympathetic nerve blocker, significantly increased heart rate in a dose-dependent manner. (B) Propranolol, an adrenergic β -blocker, significantly decreased heart rate in a dose-dependent manner. (C) Isoproterenol, an adrenergic β -agonist, significantly increased heart rate in a dose-dependent manner. A significant difference from the basal condition is determined at $*P < 0.05$.

Fig. 3. Representative Poincaré plots of ECGs of medaka (upper left panel) and a healthy human volunteer (lower left panel). The fish has relatively unstable R-R intervals (thick arrows) and some supraventricular bradycardia (double arrows). The human ECG exhibits a stable R-R interval. The frequency domain measurements of basal ECG in medaka (upper right panel) and a healthy human volunteer (lower right panel). The fish displays low amounts of the HF component.

Fig. 4. Effects of verapamil, a calcium channel blocker. Verapamil (10 mM) significantly elongated the R-R interval (upper panels), resulting in a decreased heart rate (lower panel). (B) Representative cardiac action potential recordings (upper panel). Verapamil significantly affected action potentials. Statistical analyses revealed significant reduction in action potential duration at 90% (APD90) (lower panel). (C) Verapamil significantly

decreased the heart rate, but contractile force was conserved.

Fig. 5. (A) Reverse-transcription polymerase chain reaction (RT-PCR) analysis to determine the major splice variants of the calcium channel $\beta 2$ subunit in the hearts of medaka. Primers a and b resulted in a 333 base pair (bp) single band, whereas a primer set including primer b resulted in no amplification (g/b, h/b, i/b, and j/b). Primers c and d resulted in a 297-bp single band, and primers e and f resulted in a 278-bp band. (B) Using primers a and k, we observed a 2.2 kb PCR product. (C) Western-blot analysis of medaka cardiac myocytes. Representative immunoblots analyzed for CaV1.2, CaV $\beta 2$, and GAPDH are shown as indicated.

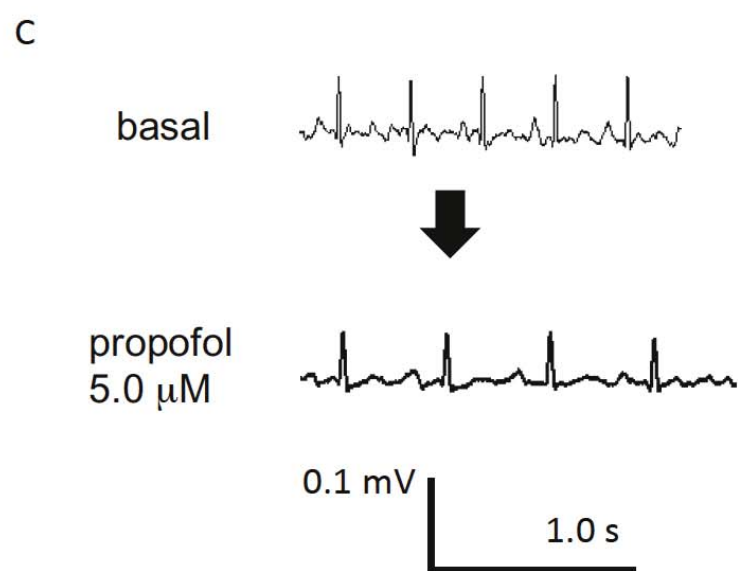
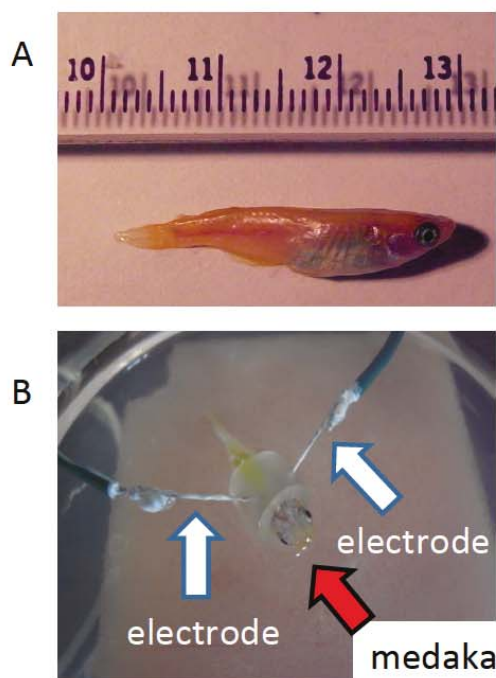


Figure 1

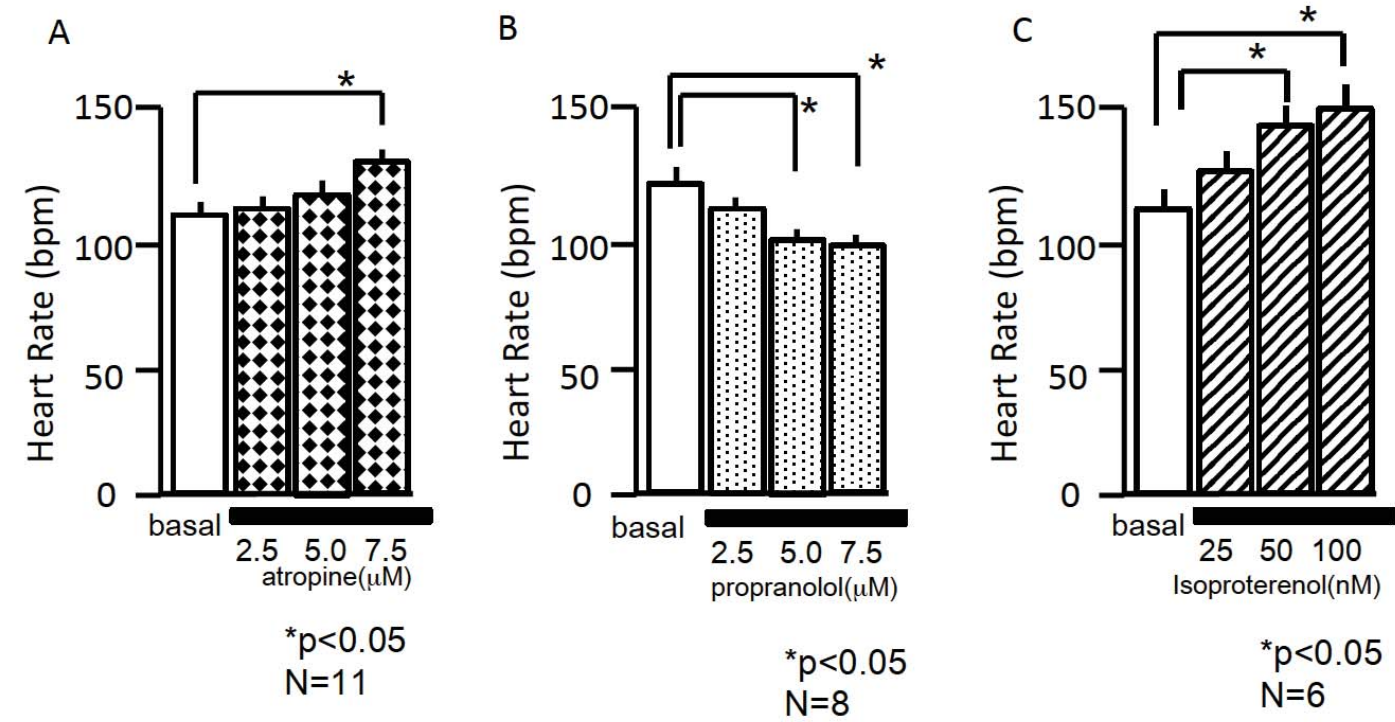
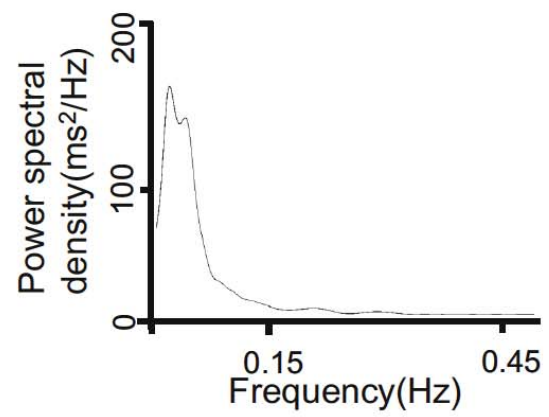
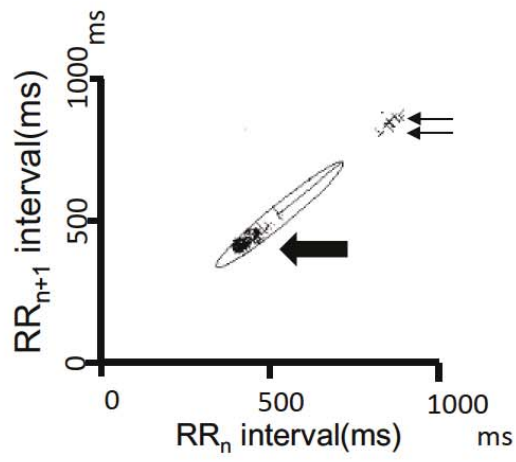


Figure 2

Medaka



Human

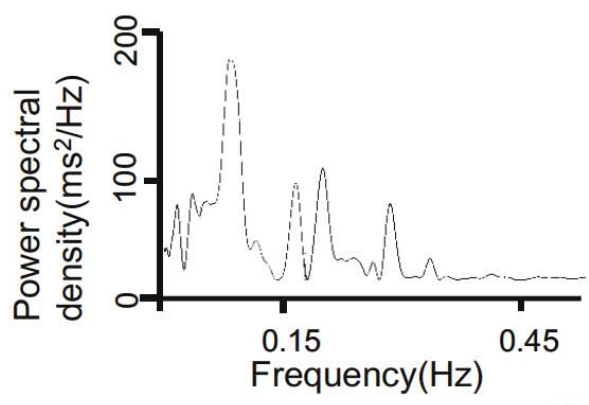
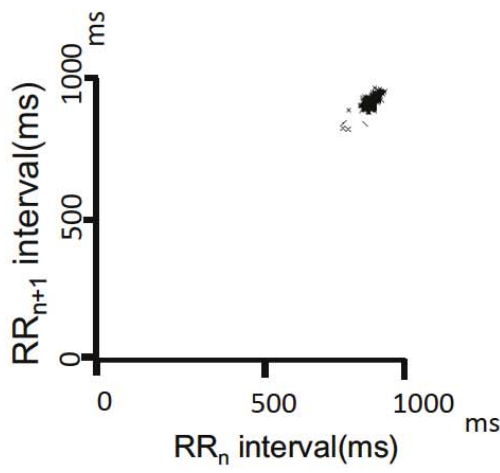


Figure 3

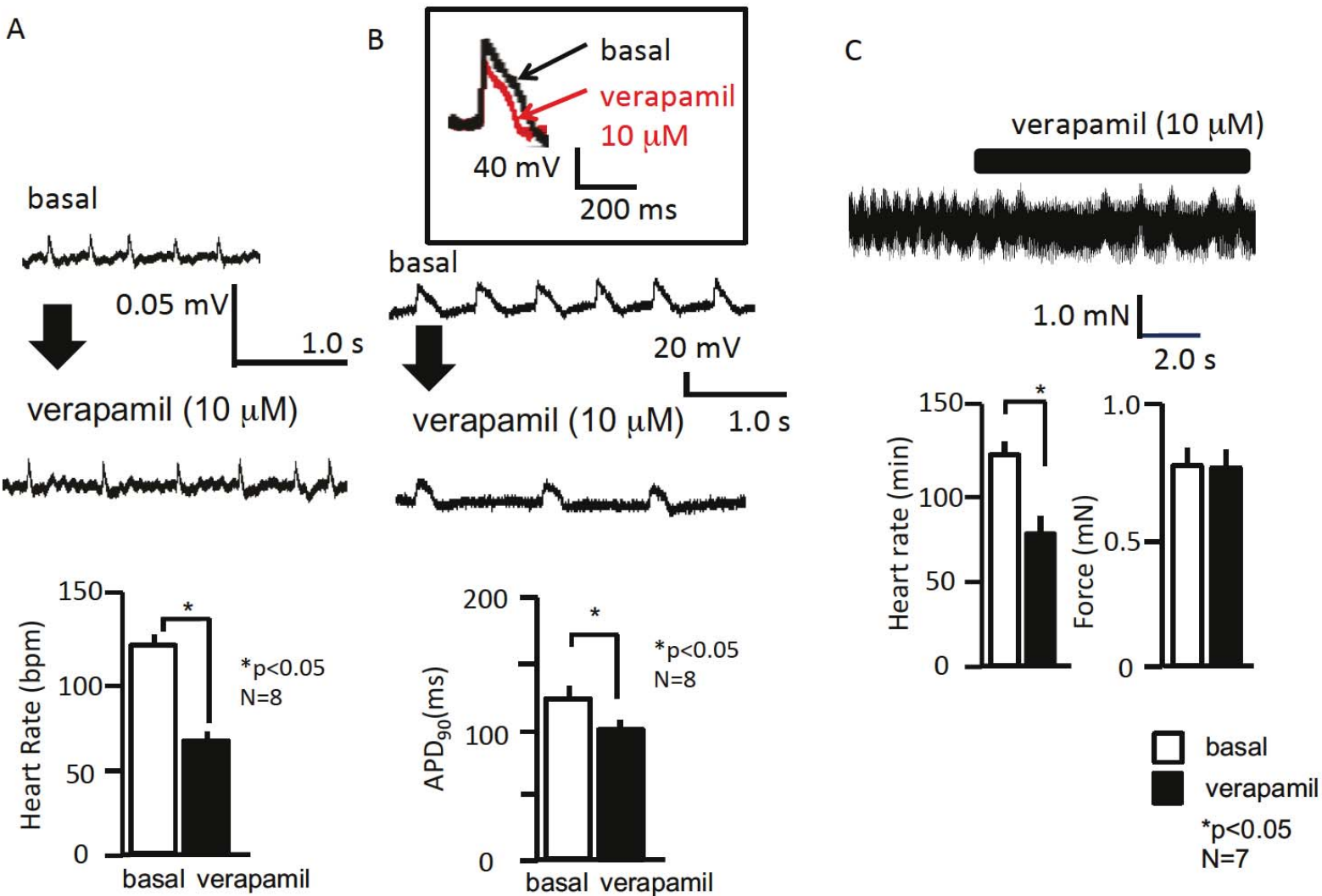


Figure 4

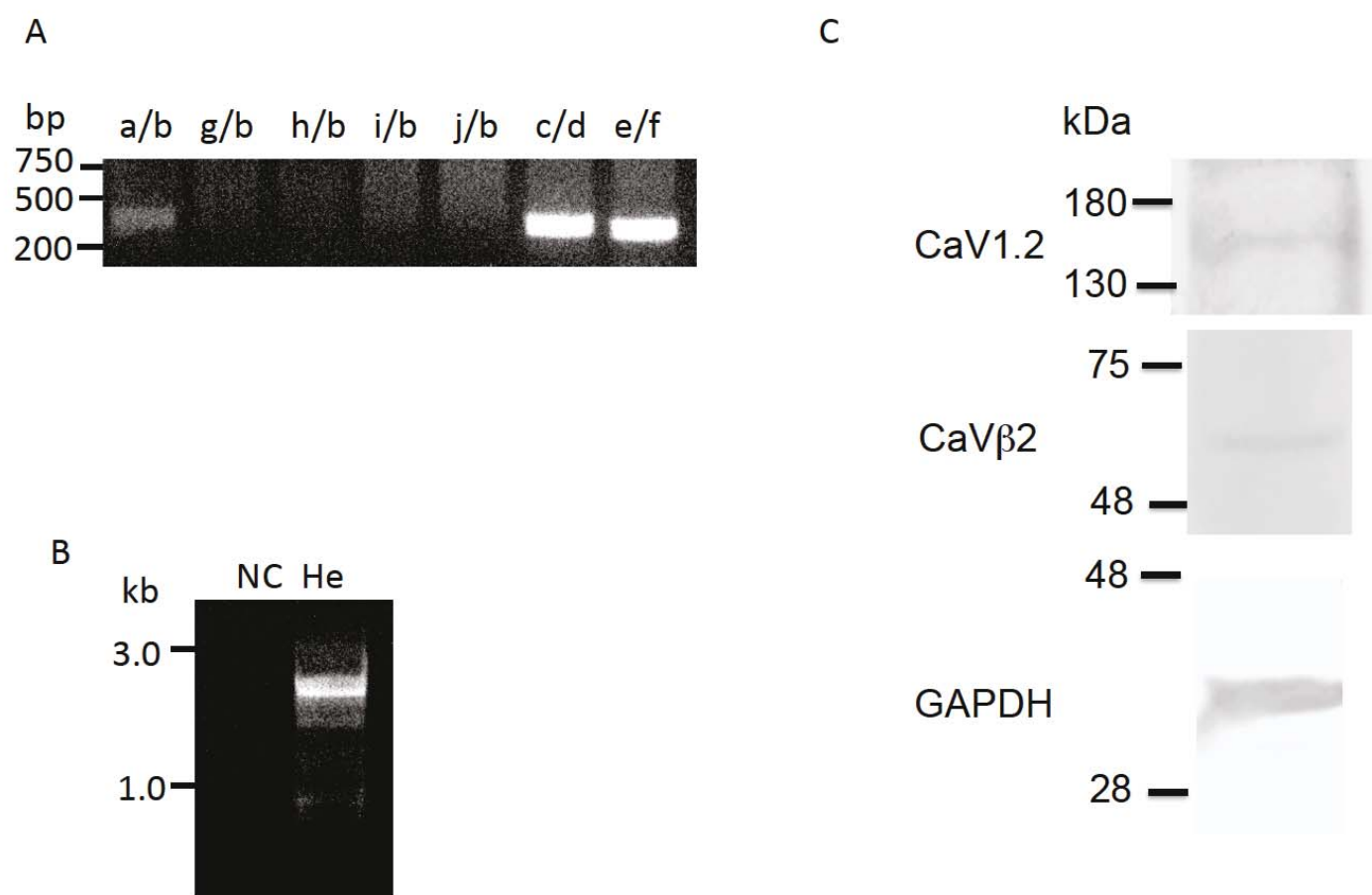


Figure 5