Hirosaki Med. J. **70** : 163—171, 2020

ORIGINAL ARTICLE

Establishment of cardiac action potential recording using a membrane potential indicator in the mouse sinoatrial node

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Abstract The cardiac action potential in the sinoatrial node is important, as it propagates throughout the heart and determines cardiac rhythm. Because of the anatomical complexity, pacemaker cells in the sinoatrial node can be difficult to identify for electrophysiological analysis. Also, analysis of pacemaker cells requires a specialized technique. In this study, we tried to analyze pacemaking in the sinoatrial node. First, we evaluated the expression of various factors that participate in pacemaking or modify cardiac rhythm. Next, we evaluated recordings of the action potential in the sinoatrial node by a conventional electrophysiological technique and using oxonol V, a fluorescent voltage-sensitive dye.

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Key words: Channel; calcium; heart rate; mouse; fluorescence.

Introduction

The action potential is a brief change in the potential voltage across the cell membrane of cardiac myocytes[1, 2)]. Membrane potential is caused by movement of ions between the inside and outside of the cell through ion channels. The cardiac action potential differs from those in other types of electrically excitable cells, such as neurons. The action potential in skeletal muscle cells is initiated by activation of nicotinic acetylcholine receptors by nervous activity. Each contraction of the heart is initiated by the generation of action potential in the sinoatrial (SA) node, which propagates to the atria, cardiac conduction system, and ventricles. Action potentials in the heart vary due to the presence of various types of ion channels. The cardiac action potential originates from a group of specialized pacemaker cells, which exhibit automatic spontaneous action potential generation. Pacemaker cells are localized in the SA node in the right atrium. Interestingly, cardiac myocytes are electrically linked by gap junctions, which allow the action potential to pass from one cell to another. This connection leads to synchronized atrial or ventricular contraction. Although several studies concerning with pacemaker activity have been published, direct recording from mouse SA node has not been analyzed[3)]. As isolated cells usually show decreased responsiveness to pharmacological manipulations, and isolated cardiac myocytes (non-pacemaker cells) often show spontaneous beating, it seems to be important to record cardiac action potential directly in SA node. On the other hand, it is often difficult to record cardiac action potentials, as spontaneous contraction often

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Correspondence: Y. Toyama
Received for publication, November 27, 2019
Accepted for publication, December 23, 2019

stops by various conditions. Empirically, only one-third of preparations could be analyzed with conventional micro-electrode recording system.

Optical methods for monitoring the cardiac membrane potential with fast voltage-sensitive dyes, such as oxonol V, enable analysis of cardiac electrical function. Direct recording of electrical membrane potential should be more accurate than optical methods, while conventional microelectrode method could be adopted only once for one preparation. Therefore, it is apparent that optical method has advantage, as it could be re-analyzed. Optical methods have several advantages over conventional electrophysiological techniques[4, 5]. Optical recording can be performed on very small cells that are inaccessible to microelectrodes. Also, multiple regions of a preparation can be monitored simultaneously to provide spatial electrical activity mapping. Since the 1990s, several groups have reported recording action potentials in the SA node of rat or rabbit[6-8]. In this sense, we think that it is quite important to record cardiac action potentials directly in the heart, keeping intact tissue organization, such as SA node, atrioventricular (AV) node, and atrium with optical method. However, no study involving smaller rodents, such as mouse, has been published, concerning with the direct recording in the SA node. In the present study, we attempted action potential recording in the mouse SA node. In addition, we monitored the action potential in the SA node using the voltage-sensitive dye oxonol V.

Materials and Methods

1 Ethics approval

All experimental procedures were approved by the Institutional Animal Care and Research Advisory Committee of the Hirosaki University School of Medicine (M13040).

2 Model animals

The animals were housed under a constant 12/12-h light/dark cycle with *ad libitum* access to food and water. Twelve 20-week-old C57BL6 mice were used in the experiments.

3 RNA isolation and RT-PCR

The SA node was dissected under a dissection microscope observing cardiac automaticity (MZ8, Leica GmbH, Wetzler, Germany). Total RNA was extracted from the mouse SA node using the RNeasy Kit (Qiagen, Valencia, CA). Reverse transcription was carried out using oligo-dT as a primer and the SuperScript IV reverse transcriptase (Invitrogen, San Diego, CA). Comparative reverse transcription polymerase chain reaction (RT-PCR) was performed using ExTaq™ polymerase (TaKaRa, Otsu, Japan) with 30–38 PCR cycles. The genes encoding β-actin, β1-adrenergic receptor, β2-adrenergic receptor, muscarinic M2 receptor, hyperpolarization-activated cyclic nucleotide (HCN)-gated channel 4, voltage-activated K^+ (BK) channel (KCa1.1), Cav1.2, Cav1.3, sodium–calcium exchanger (NCX) 1, sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) 1, SERCA2, SERCA3, inositol triphosphate receptor (IP3R) 2, ryanodine receptor (RyR) 1, RyR2, and RyR3 were amplified by RT-PCR using the primers listed in Table 1.

4 Cardiac action potential recordings

The spontaneously beating heart was mounted in an organ bath (10 mL) continuously perfused with an oxygenated Tyrode solution at 23°C. Tyrode solution contains (in mM) 126.7 mM NaCl, 5.5 mM KCl, 1.8 mM $CaCl_2$, 1.05 mM $MgCl_2$, 22 mM $NaHCO_3$, 0.42 mM $NaHPO_4$, and 5.6 mM glucose. Following equilibration with the air at room temperature, the pH of the solution was 7.4 (25°C). Action potentials in the right atrium or ventricle were recorded from all types of multicellular preparations with sharp glass microelectrodes filled with 3.0 M KCl (tip resistance, 20–30 MΩ). Action potentials were recorded with an amplifier (MEZ-8301, Nihon

Table 1. PCR primers in this study.

	For	Rev
β1	CATCGTTCTGCTCATCGTGG	ACACACAGCACATCTACCGA
β2	GAGCGACTACAAACCGTCAC	TGGTACTTGAAGGGCGATGT
M2	GGTAAGGACTGTGGAAGA	ACCAGGCATGTTGTTGTTG
HCN4	GGACCGCATAGGCAAGAAGA	GGCCACCGAAGTAGTAGCAG
KCa1.1	ACACCTCCTGGAATGGACAG	TGTGTCAGGGTCATCGTCAT
Cav1.2	CCCTTCTTGTGCTCTTCGTCATCA	GGCTTTGGCTTTCTCCCTCTCT
Cav1.3	ACTACAACTTTCCGCTCGGT	CTTGCATAGTTTGCCTCTGC
NCX1	CCTTGTGCATCTTAGCAATG	TCTCACTCATCTCCACCAGA
SERCA1	ACCAGATGTCAGTTTGCA	CAAGGTGGTGAGAGCAG
SERCA2	TGGCAGCATGTATATCTTTC	CTTTAATTCGTTGCACACTC
SERCA3	AATGTTGGCGAGGTTGTC	CAATAGCCAAATAGCGGAA
IP3R2	AATGTTGGCGAGGTTGTC	CAATAGCCAAATAGCGGAA
RyR1	AAGACGAAGCTGCCTAAGA	GGCTGTCCCTATTGCTG
RyR2	ATGTCGCTTGAAACCCTC	GATGCCGTAAGTCCAGC
RyR3	ACTGCCCTCCCTAAACAAAG	CACAGCCGAACATAAATACCTA
β-actin	CAACTGGGACGACATGGAGAA	CAGCCTGGATGGCTACGTACA

Names of the amplified genes and sequences of each forward and reverse primer are indicated.
PCR, polymerase chain reaction.

Kohden, Tokyo, Japan) with low-pass filter (<10 Hz) and high-pass filter (>0.1 Hz). Data were acquired using the Power Lab system and analyzed with LabChart 8, as described previously[9]. For cardiac action potential recordings in the SA node, the SA node region was dissected from the right atrium along the superior vena cava and crista terminalis. For action potential recording in the SA node, the SA node was dissected under a dissection microscope observing cardiac automaticity. Then, Crista terminalis, sagittal bundle, and SA node were anatomically identified. Finally, the spontaneously beating SA node region was mounted in the organ bath. Then, action potential was recorded with glass microelectrode. Cardiac action potentials with slow depolarization phase, which is thought to be the fourth phase characteristic of pacemaker cells, were considered as action potentials in the SA node.

5 Membrane potential recording using oxonol V

As action potential recording in the SA node is difficult, we examined the utility of oxonol V, a fluorescent membrane potential indicator. After isolation of the SA node, tissue was incubated with oxonol V (200 nM) for 10 min at room temperature. The SA node was mounted in a glass chamber filled with Tyrode solution (~10 mL) with perfusion (3 mL/min). Fluorescence image (excitation wavelength 560 nm, emission wavelength 600 nm) was captured (sampling rate 30 frames/sec) using a high-sensitivity camera system (WAT-933, Watec Co., Ltd., Tsuruoka, Yamagata, Japan) mounted on a dissecting microscope (Stemi 508, Carl Zeiss AG, Oberkochen, Baden-Wurttemberg, Germany). Collected video data were captured with a capture system (GV-MPEG2/USB2, IO DATA, Kanazawa, Ishikawa, Japan) and saved in a personal computer. Changes in fluorescence were evaluated using a self-made photoresistor system with CdS (5-mm diameter, cadmium sulfide; Akizuki Co., Ltd., Tokyo, Japan). Data were acquired using the Power Lab system and

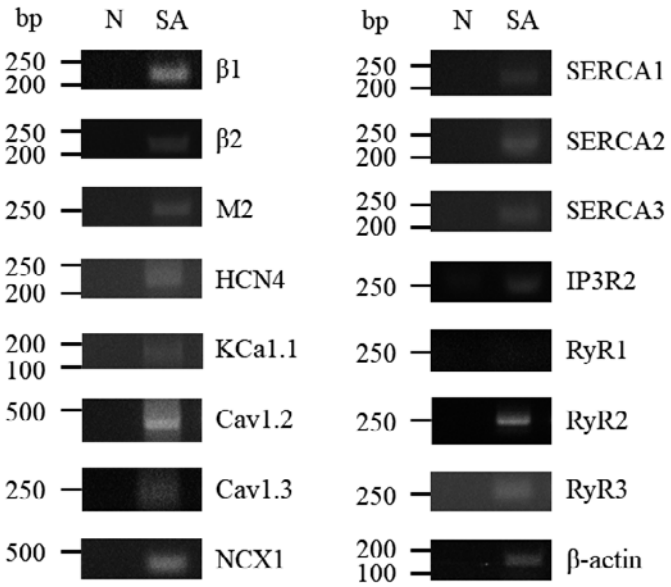

Figure 1. RT-PCR analysis of the mouse sinoatrial node.
N, negative control; SA, sinoatrial node; RT-PCR, reverse transcription polymerase chain reaction.

analyzed with LabChart 8. Membrane potential was estimated using the Goldman–Hodgkin–Katz flux equation:

$$E_{m,K_zNa_{1-z}Cl} = \frac{RT}{F} \ln \left(\frac{P_{Na}[Na^+]_{out} + P_K[K^+]_{out} + P_{Cl}[Cl^-]_{in}}{P_{Na}[Na^+]_{in} + P_K[K^+]_{in} + P_{Cl}[Cl^-]_{out}} \right)$$

Intracellular ion concentrations were calculated as follows (K^+; 150 mM, Na^+; 15 mM, Cl^-; 9 mM). Extracellular ion concentrations were calculated as follows (Na^+; 127 mM, Cl^-; 125 mM). At the basal status, extracellular potassium concentration was considered as 5.5 mM.

Results

1 RT-PCR analysis

Membrane potential is caused by movement of ions through ion channels. We therefore examined the mRNA levels of various channels expressed in the SA node by RT-PCR (Figure 1). To confirm that the excised tissue with spontaneous beats was the SA node, the expression of HCN4 was evaluated. RT-PCR using HCN4-specific DNA primers confirmed the expression of HCN4. Moreover, the expression of voltage-dependent calcium channels Cav1.2 and 1.3 was confirmed. Also, the expression of RyR2 and 3 was verified, whereas the RyR1 primer did not yield a PCR product. It was interesting that we confirmed the expression of CaV1.3 and RyR3 in the SAnode, as these molecules were thought to be not expressed in the cardiac myocytes. CaV1.3 and RyR3 might be related to action potential formation in the SA node. Expression of SERCA 1–3 was detected. Primers for KCa1.1 and NCX1 also yielded PCR products. Another calcium carrier, IP3R2, also yielded a PCR product. The expression of autonomic nerve-related receptors, such as β1- or β2-adrenergic receptors and muscarinic M2 receptor, was also confirmed.

2 Action potential recordings

To evaluate cardiac pacemaking and electrophysiological properties, we recorded the action potential of the SA node, atrium, and ventricle (Figure 2). The action potential at the SA node showed slow depolarization (Figure 2 left panel, arrow) followed by a sharp depolarization wave.

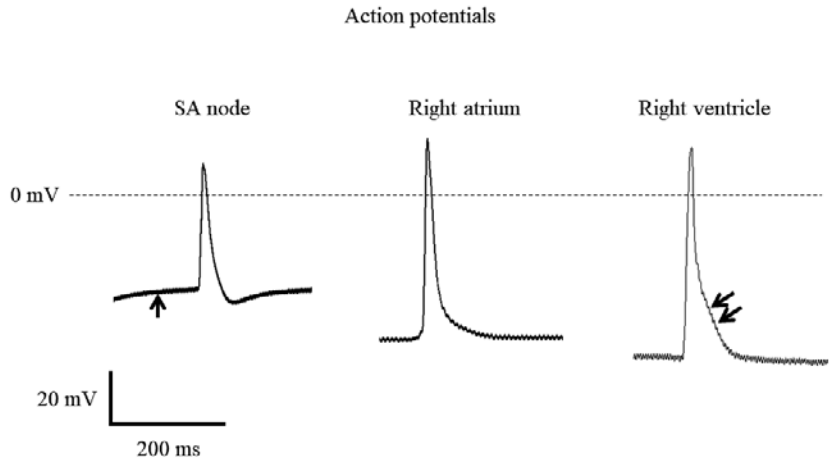

Figure 2. Action potentials in the SA node, right atrium, and right ventricle.
Representative action potentials derived from the SA node (left panel), right atrium (middle panel), and right ventricle (right panel) of mice. Action potentials of the SA node exhibited spontaneous slow depolarization (phase 4; indicated), a feature of SA nodal action potentials. Atrial action potentials were similar to ventricular action potentials, except that the former had a narrower plateau phase due to the smaller calcium influx.
SA, sinoatrial.

In the atrium and ventricle, rapid upstrokes of the cardiac action potential were evident (Figure 2 middle and right panels). The repolarization phase of the action potential is thought to result both from rapid inactivation of most of the Na^+ current and from activation of a transient outward current (Ito), carried mainly by K^+ ions, in the atrium and ventricle. Relatively slow action potential repolarization was detected in the ventricle (Figure 2 double arrows).

Action potentials in an atrium or ventricle can be recorded easily, whereas only one-third of the SA node preparations showed action potentials. During the preparation of SA node dissection, SA node often stops spontaneous activity, resulting low success rate of action potential recording. Furthermore, the SA node is an anatomically small area near the right atrium where accurate recording of the action potential is difficult. Therefore, identifying the SA node morphologically can be problematic. Additionally, the small size of the mouse heart, which necessitated dissection microscopy, prevented identification of the SA node. Using the conventional action potential recording system, only one recording could be performed at a time, whereas video images captured and saved as digital data could be re-analyzed. Therefore, we examined the utility of oxonol V for monitoring changes in membrane potential.

3 Fluorescent images recording system under a dissection microscope

Figure 3A shows an overview of the fluorescence imaging system. A sample was mounted on the stage of a dissecting microscope (sample arrow). Fluorescence image (excitation wavelength 525 nm, emission wavelength 600 nm) was captured using a high-sensitivity camera system (WAT-933) mounted on a dissecting microscope (Stemi 508, Carl Zeiss AG). Collected video data were captured with a capture system (GV-MPEG2/USB2, IO DATA) and saved in a personal computer. Changes in fluorescence were evaluated using a self-made photoresistor system with CdS (5-mm diameter, cadmium sulfide; Akizuki Co., Ltd., Tokyo, Japan). Data were acquired using the Power Lab system and analyzed with LabChart 8.

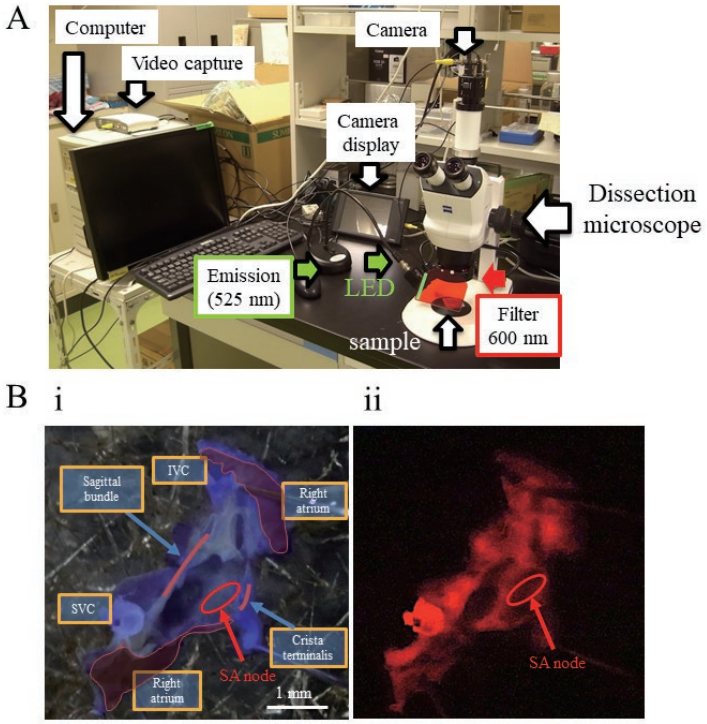

Figure 3. A. Overview of the video-imaging system.

A sample is mounted on the stage of a dissecting microscope (sample arrow) equipped with a digital camera. Visual data were monitored on a camera display and transmitted to a video-capture system. Converted data were saved in a personal computer. The green LED (excitation wavelength 525 nm) and red-fluorescent filter (emission wavelength 600 nm) are indicated.

B. Location of the SA node.

The image of a right atrium was shown (i). The magnification was 30. The sagittal bundle, inferior *vena cava* (IVC), superior *vena cava* (SVC), and crista terminalis are indicated. The SA node area is shown (red circle). Fluorescence image (wavelength 600 nm) of an oxonol V-loaded heart (ii).
SA, sinoatrial.

4 Location of the SA node

A representative isolated heart is shown in Figure 3Bi. The right atrium was opened and pinned to a board. The SA node area was indicated (red circle). Fluorescence image (wavelength 600 nm) of an oxonol V-loaded heart was also shown (ii).

5 Analysis of fluorescent images due to oxonol V

First, fluorescence due to changes in membrane potential was examined. To calibrate the fluorescence as a function of the membrane potential, a mouse atrium was perfused with Tyrode solution containing various concentrations of K^+ as KCl. The other constituents were identical to those in the Tyrode solution. Figure 4 shows high-potassium-induced oxonol V-related fluorescence and membrane potential changes in the mouse atrium. First, cardiac contraction was suppressed by 45 mM KCl. Next, the membrane potential was changed by adjusting the KCl concentration (5.5, 11, 45, and 60 mM) in Tyrode solution. Figure 4A shows the time-dependent changes in the response to high-potassium Tyrode solution. Figure 4B shows the relationship between the calculated membrane potential and oxonol V-related fluorescence. A correlation between fluorescence and the calculated membrane potential was identified.

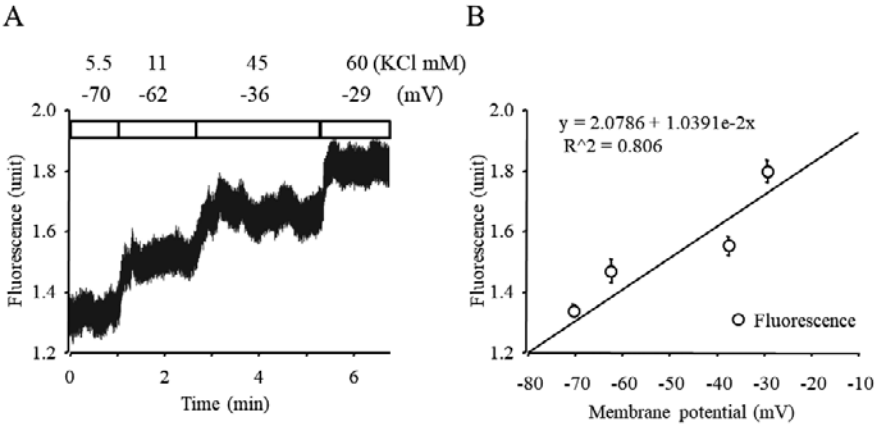

Figure 4. Calibration of the fluorescence of oxonol-V against calculated membrane potentials.

A. Changes in fluorescence intensity in Tyrode solution containing potassium (5.5, 11, 45, and 60 mM) in mouse atrium stained with oxonol V (200 nM).

B. Correlation of the fluorescence of oxonol V with the calculated membrane potential; N = 4. The relationship between oxonol V fluorescence and the calculated membrane potential was fitted to a linear regression.

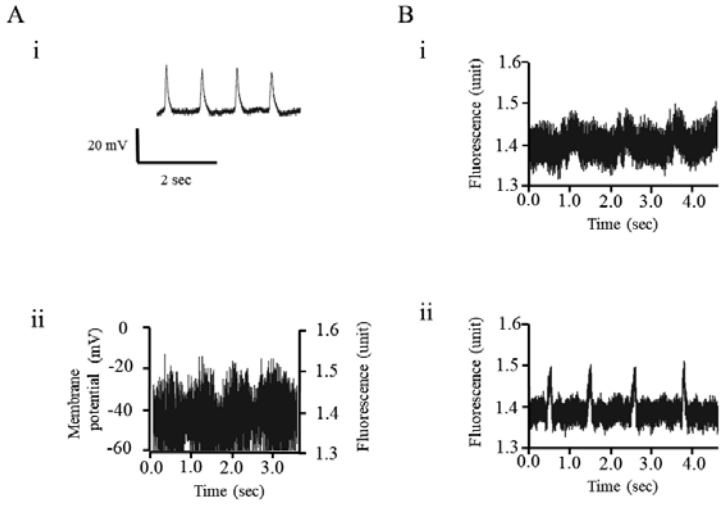

Figure 5. A. Action potential recording.

Representative trace of action potential recording using a conventional microelectrode (i). Representative changes in oxonol V-related fluorescence compared to baseline (ii).

B. Pharmacological responses to isoproterenol.

Changes in fluorescence: before (i) and after application of isoproterenol (30 nM) (ii).

6 Action potential recording at the SA node using oxonol V

As KCl-induced membrane changes could be monitored using the oxonol V-loaded mouse atrium, the action potential was recorded using a conventional microelectrode and an oxonol V-loaded SA node (Figure 5A). The conventional method showed a typical SA node action potential (i). In contrast, the oxonol V method was little affected by strong noise (ii), whereas contraction in the SA node was readily detectable.

Adrenergic stimulation with isoproterenol (30 nM) significantly increased the action potential formation rate in the SA node (ii). The oxonol

V-induced fluorescence changes were easily monitored using isoproterenol (Figure 5B). Thus, action potential can be monitored in the oxonol V-loaded SA node, but accurate recording is difficult.

Discussion

We analyzed action potential changes in the mouse SA node. By RT-PCR, we confirmed the expression of various types of ion channels and β–adrenergic receptors in the SA node. We also examined the utility of the fluorescent membrane indicator, oxonol V, for action potential recording in the SA node.

The RT-PCR analysis revealed the expression of HCN4 channels, which are responsible for the SA funny Na^+ current. The SA node contains the most excitable pacemaker cells in the heart, which have an unstable resting membrane potential that spontaneously depolarizes due to funny current. Subsequently, the L-type Ca^{2+} channels (CaV1.2 and 1.3) start to open gradually. Calcium influx through L-type calcium channels is followed by calcium-induced calcium release from the sarcoplasmic reticulum through ryanodine receptors (RyR-2 and -3). The expression patterns in other mammalian species show that the SA node preparation was successful.

Specific primers for Cav1.2 and Cav1.3 yielded PCR products, suggesting the expression of two types of L-type calcium channels. Christel *et al.* reported colocalization of Cav1.3 and ryanodine receptors, as well as Cav1.3 involvement in voltage-dependent facilitation in the SA node[10]. Specific primers for RyR-2 and -3, but not RyR-1, yielded specific PCR products, suggesting that RyR-2 and -3 are expressed in the SA node. Our data are in agreement with the RNase protection analysis by Masumiya *et al.*[11].

KCa1.1-specific primers yielded a single band in the SA node, indicating the expression of big potassium (BK) channels. Lai *et al.* reported that decreased K^+ currents through BK channels at the SA node reduced HR[12]. Our data suggest that BK channels, encoded by the KCa1.1 gene, participate in membrane potential formation in the SA node.

In the present study, we examined possible usage of oxonol V, which enabled to monitor membrane potential changes by recording fluorescent changes. Despite of its high background, we could measure beat rate by optical fluorescence recording, while strong noise prevented to analyze detailed parameters, such as 90 % action potential duration (APD_{90}), or other factors, which could be analyzed in the conventional microelectrode method.

Reasons of the high background in the oxonol V method are not completely understood, but we speculate several possibilities. One possibility is background from tissue preparation (surrounding regions of SA node). As we observed marginal fluorescence with SA node preparation without oxonol-V loading, it is evident that the recorded fluorescence was originated from oxonol V. On the other hand, fluorescence from surrounding regions of SA node should affect the recording, whereas we tried to isolate SA node as small as possible. Second possibility is movement of tissue preparation. As tissue preparation contains oxonol V, it is inevitable to record fluorescence changes due to muscle contraction with oxonol V loaded tissue. Despite of these problems, fluorescent changes in this study was between 1.3 to 1.5, which might suggest possible usage of optical recording method for further analysis.

Oxonol V resulted in greater noise then the conventional microelectrode method. However, oxonol V showed several advantages. Because fluorescent images were saved in a computer, the data could be stably analyzed, whereas data obtained using the conventional method can be analyzed only once. In addition, fluorescence changes could be analyzed in different areas, such as the SA or atrium from one recording.

Despite the disadvantages (low time resolution or low signal/noise ratio), fluorescent membrane potential indicators could be used for analysis of medicines that may affect action potential formation in the SA node. In addition, optical method could be adopted for other fluorescent indicator, such as calcium indicator fluo-8. Nevertheless, further analysis and improvement of the detection systems are needed.

Conclusion

We evaluated various types of ion channels and heart rate modulators, such as β-adrenergic receptors, in the mouse SA node. Our data suggest the utility of oxonol V for action potential recording in the mouse SA node.

Conflict of interest

All authors have no conflicts of interest directly relevant to the content of this article.

Acknowledgments

We thank Mr. Maximilian Murakami for critically reading the manuscript.

Role of the funding source

This research was sponsored in part by Grants-in-Aid for Scientific Research from the JSPS, KAKENHI (17K08527, 17H04319, and 16K09489).

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