The mouse sino-atrial node expresses both the type 2 and type 3 Ca^{2+} release channels/ryanodine receptors

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Abstract Ca^{2+} released from intracellular Ca^{2+} stores is shown to be involved in pacemaker activity in the sino-atrial (SA)-node. However, little is known about the molecular identity of the Ca^{2+} release channel/ryanodine receptor (RYR) involved in pacemaker activity. We examined the mRNA distribution of three different RYR isoforms (RYR1, RYR2, and RYR3) in the mouse SA-node. RNase protection assay and in situ hybridization revealed that RYR2 mRNA expresses widely in the heart including the SA-node, while RYR3 mRNA expression is limited to the SA-node and to the right atrium. Thus, not only RYR2 but also RYR3 may participate in pacemaker activity.

Key words: Action potential; Heart; In situ hybridization; Mouse; Pacemaker; Protection assay

1. Introduction

Ryanodine receptors (RYRs) are a family of Ca^{2+} release channels located in the sarcoplasmic and endoplasmic reticulum (SR/ER) of various excitable or non-excitable tissues, and play a crucial role in muscle contraction and Ca^{2+} signaling [1–3]. Three RYR isoforms are identified in mammalian tissues. RYR1 is predominantly expressed in skeletal muscle, and RYR2 in the heart and brain, while RYR3 is expressed at a relatively low level in various tissues including the brain, diaphragm, and smooth muscle [4–6]. In cardiac muscle, RYR2 contributes to Ca^{2+}-induced Ca^{2+} release, and underlies the mechanism of cardiac excitation–contraction coupling [7].

Accumulating data using patch clamp experiments and confocal imaging of intracellular Ca^{2+} movement suggest that Ca^{2+} release through RYR on SR membrane is a critical factor in cardiac pacemaking, even though there is still controversy about the extent of its contribution to pacemaker activity. It is postulated that Ca^{2+} released from SR activates inward Na^{+}-Ca^{2+} exchanger current, which in turn helps to generate the pacemaker depolarization [8]. Stimulation of β-adrenergic receptor (β-AR) is an important modulator of cardiac beating, and regulation of RYR function by β-AR appears to be involved in the autonomic regulation of heart beating [9]. Despite these intriguing findings and exciting debates on the role of RYR in pacemaking, little is known about the molecules involved in the pacemaker activity in the sino-atrial (SA)-node. RYR2 is predominantly expressed in the heart including the SA-node, but it is not tested whether other RYR isoforms are expressed in the SA-node or not. To address these questions, we performed RNase protection assay and in situ hybridization to determine the mRNA expression of three RYR isoforms in the SA-node region.

2. Materials and methods

2.1. RNase protection assay

RNase protection assays were performed as described previously [10]. Total RNA from adult ICR mouse tissues was purified by acid-guanidine-thiocyanate-chloroform extraction methods. The probes were synthesized from pGEM1 vectors containing the indicated fragments of the following mouse RYR cDNAs: RYR1, GenBank accession number X83932, bp 1286–1503; RYR2, AF295105, bp 14519–14897; RYR3, D38218, bp 364–689. RYR1, RYR2, and RYR3 fragments were obtained by reverse transcription-polymerase chain reaction (RT-PCR) from skeletal muscle, left ventricle and brain, respectively. The plasmid vectors were linearized with *Nde*I or *Bgl*II. Antisense RNA probes were transcribed with T7 RNA polymerase in the presence of [α-^{32}P]uridine triphosphate (UTP) (Amersham Biosciences) using the Ambion RPA III Kit (Ambion). After purification, 8×10^{4} counts per minute (cpm) of cRNA probe were hybridized with the total RNA, and incubated with RNase A/RNase T1 mix and RNase digestion buffer. Subsequently, RNase inactivation buffer was added and incubated at −20°C for 15 min. Samples were obtained after centrifugation, and analyzed on 6% acrylamide gels containing 8 M urea in 0.5×TBE solutions. Autoradiographs were exposed for 1 day at room temperature for RYR1 and RYR2, and for 3 days at −80°C for RYR3.

2.2. In situ hybridization

In situ hybridization analysis was performed as described previously [11]. The same linearized probes as were prepared for protection assay were used for in situ hybridization. Antisense RNA probes were transcribed with T7 or SP6 RNA polymerase (Invitrogen) in the presence of digoxigenin RNA labeling mix (Roche). Hybridization was carried

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Abbreviations: AMP, action potential amplitude; APD50, action potential duration at 50% amplitude; β-AR, β-adrenergic receptor; CL, cycle length; ES cell, embryonic stem cell; MDP, maximum diastolic potential; OS, overshoot; RYR, ryanodine receptor; SA-node, sino-atrial node; SR, sarcoplasmic reticulum; $+V_{max}$, maximum rate of rise

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out overnight at 55°C using digoxigenin-dUTP labeled probes. After washing and treatment with RNase, signals were detected by using an anti-digoxigenin-alkalinephosphatase-conjugated antibody (Roche). Nitroblue tetrazolium formulation (Invitrogen) and 5-bromo-4-chloro-3-indolyl-phosphate formulation (Invitrogen) were used for staining.

2.3. Action potential recording

The microelectrode study was performed as described previously [12]. Adult ddy mice were exsanguinated and the heart was quickly removed. Tissues, including the SA-node, were cut perpendicularly to the crista terminalis. Preparations were pinned down horizontally on a silicon block in an organ bath filled with oxygenated (95% O_2 and 5% CO_2) physiological salt solution of the following composition (in mM): NaCl, 118.4; KCl, 4.7; $CaCl_2$, 2.5; $MgSO_4$, 1.2; $NaHCO_3$, 24.9; and glucose, 11.1 (pH 7.4). The temperature of the organ bath was maintained at 32°C. Action potentials were recorded with glass microelectrodes filled with 3 M KCl having resistance of 50–80 MΩ. The output of the microelectrode amplifier (MEZ-8201, Nihon Kohden, Tokyo, Japan) was monitored through a dual beam cathode ray oscilloscope (VC-11, Nihon Kohden, Tokyo, Japan) and fed in to an AD converter (Analog Pro, Canopus, Kobe, Japan) attached to a computer (PC9801RA, NEC, Tokyo, Japan) for analysis. The action potential parameters measured were cycle length (CL), maximum diastolic potential (MDP), overshoot (OS), action potential amplitude (AMP), maximum rate of rise ($+V_{max}$), and action potential duration at 50% amplitude (APD50).

Significance of difference between means was evaluated by paired *t*-test. A *P*-value less than 0.05 was considered statistically significant. Ryanodine was purchased from Wako (Japan) and dissolved in water.

3. Results

3.1. Effects of ryanodine on the action potential of SA-nodal tissues

To investigate whether Ca^{2+} release through RYR is involved in pacemaker activity, we examined the effects of ryanodine on the action potential configurations of mouse SA-node tissue. Typical traces in the absence and presence of 30 nM ryanodine are shown in Fig. 1. The mean ± S.E.M. of control action potential parameters from three preparations were: CL, 235 ± 22 ms; MDP, −56.7 ± 2.6 mV; OS, 7.9 ± 1.0 mV; AMP, 64.4 ± 3.1 mV; APD50, 46.9 ± 4.8 ms; $+V_{max}$, 11.6 ± 1.5 V/s. In the presence of 30 nM ryanodine the values of each parameter were: CL, 278 ± 33 ms; MDP, −51.8 ± 2.3 mV; OS, 5.1 ± 1.1 mV; AMP, 58.5 ± 3.3 mV; APD50, 56.4 ± 6.4 ms; $+V_{max}$, 6.3 ± 0.5 V/s. Thus, CL prolonged by 24.3 ± 1.3%, APD50 by 17.2 ± 7.4%, while $+V_{max}$ decreased by 29.9 ± 1.7%. MDP, OS, and AMP were not significantly changed. Ryanodine also reduced the late phase of pacemaker potential (Fig. 1). These observations demonstrate that RYR affects pacemaker activity in the mouse SA-node, which is consistent with previous studies [13–15].

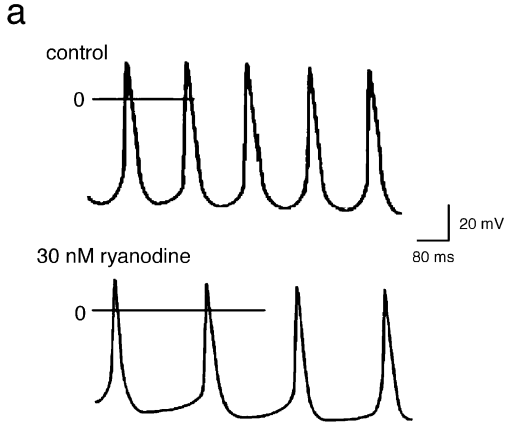

Fig. 1. Effects of ryanodine on action potential of mice SA-node. Typical action potential traces obtained in the absence and presence of 30 nM ryanodine.

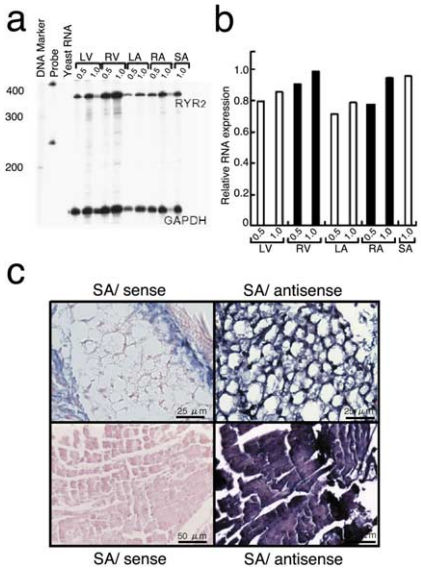

Fig. 2. RYR2 mRNA expression in mouse cardiac tissue. a: RNase protection analysis of RNAs from various regions of mouse heart. RYR2 probes were hybridized with 5 μg of total RNA from yeast tRNA, 0.5 or 1 μg of total RNA from left and right ventricle, left and right atrium, and 1 μg of total RNA from SA-node. As an internal control, GAPDH probe was also included in each sample. b: Quantification of RYR2 mRNA after normalization to GAPDH RNA. c: In situ hybridization of mice SA-node regions and right atrial myocardium with sense and antisense RYR2 cRNA probes. Antisense RYR2 probe hybridized with SA-node (right upper panel), and right atrium (right lower panel) samples, while no hybridization signals were detectable in the samples with sense probes.

3.2. RYR2 is expressed in the SA-node and other regions of the heart

To demonstrate the expression of RYRs in the SA-node, we carried out RNase protection assays and in situ hybridization analyses. Fig. 2a shows that RYR2 expression was observed in the SA-node as well as in other regions of the heart. After normalization using the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) signal, the RYR2 expression in different regions of the heart is slightly higher in right atrium and ventricle than in the left atrium and ventricle (Fig. 2b). After in situ hybridization, intense staining with the antisense RYR2 probe was detected both in the SA-node and in atrial tissues, which is consistent with those obtained by RNase protection assay (Fig. 2c). No significant signal was detected with the sense RYR2 probe that was used as a negative control.

3.3. RYR3 is expressed in the SA-node and right atrium

We employed the same approaches to examine the expression of RYR3 in the SA-node. Fig. 3a shows that RYR3 signals were clearly detected in the SA-node, right atrium, and brain. A faint signal was also detected in the right ventricle. After in situ hybridization analysis, the RYR3 antisense probe exhibited signals in the SA-node regions and right atrium, whereas the sense probe that was used as a negative

control did not reveal any staining in these tissues (Fig. 3c). These results confirm that RYR3 is expressed both in the SA-node and in the right atrium. RYR3 expression was not detected in the left ventricle or atrium under the present experimental conditions (Fig. 3a).

3.4. *RYR1 is not expressed in the SA-node or other regions of the heart*

We also examined the expression of RYR1 in the SA-node and other regions of the heart. Using RNase protection assays, we detected RYR1 expression in skeletal muscle, but not in the SA-node, atrium, or ventricle (Fig. 4). In the same experiment, probes for RYR2 and RYR3 showed a similar expression pattern to that described above (Figs. 2 and 3). The corresponding protected fragments of the RYR1, RYR2, and RYR3 probes were not detected in the yeast tRNA, further confirming the specificity of the RNase protection assays.

4. Discussion

The presence of RYR2 in the SA-node has been demonstrated by immunohistochemical labeling and functional analysis [16,17]. In the present study, we provide the first evidence that RYR3 in addition to RYR2 is expressed in the mouse SA-node (Figs. 2 and 3).

Although SR Ca^{2+} release is involved in pacemaking activity, it is still controversial how much extent it contributes to the pacemaking activity. Lakatta et al. [18] suggested that SR

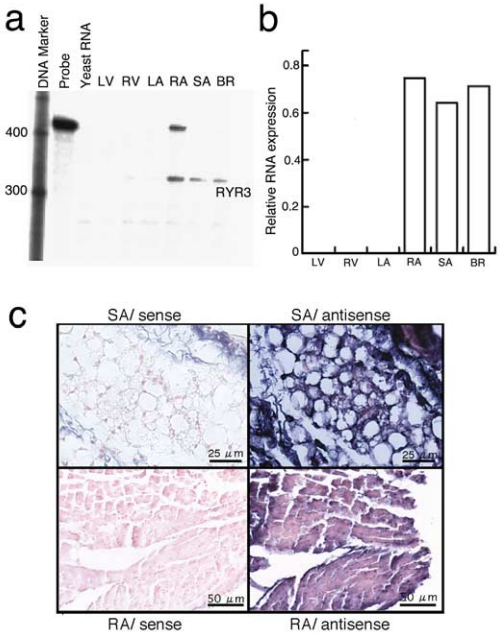

Fig. 3. RYR3 mRNA expression in mouse cardiac tissue. a: RNase protection analysis of RNAs from various regions of mouse heart. RYR3 probe was hybridized with 5 μg of total RNA from yeast tRNA, 1 μg of total RNA from left and right ventricle, left and right atrium, or SA-node, and 5 μg of total RNA from whole brain. b: Quantification of RYR3 mRNA after normalization to GAPDH RNA. c: In situ hybridization of mice SA-node regions and right atrial myocardium with sense and antisense RYR3 cRNA probes. Antisense RYR3 probe hybridized with SA-node (right upper panel), and right atrium (right lower panel) samples, while no hybridization signals were detectable in the samples with sense probes.

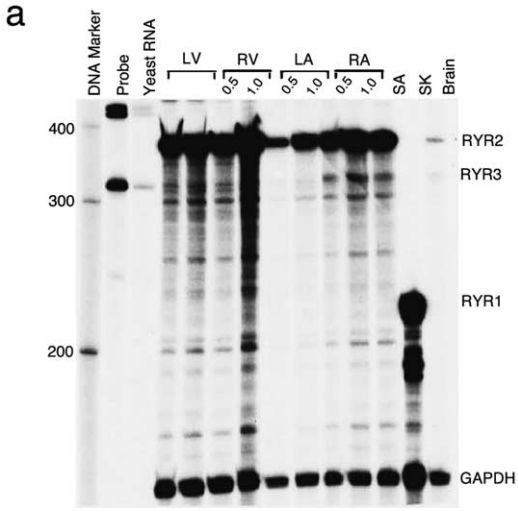

Fig. 4. RYR1 mRNA expression in mouse cardiac tissues. Total RNA from mouse cardiac tissues (left and right ventricle, left and right atrium, SA-node), skeletal muscle and brain were analyzed by RNase protection. RYR1, RYR2, RYR3, and GAPDH probes were hybridized with 5 μg of total RNA from yeast tRNA 0.5 or 1 μg of total RNA from left and right ventricle, left and right atrium, 1 μg of total RNA from SA-node, 5 μg of total RNA from skeletal muscle, and 5 μg of total RNA from brain.

Ca^{2+} release might be obligatory for pacemaking, because 30 mmol/l ryanodine abolishes the spontaneous activity of isolated SA-node cells from rabbit. On the other hand, Honjo et al. [19] showed that 30 mmol/l ryanodine slowed spontaneous rate only by about 20% in the same species, and question the dominant role of SR Ca^{2+} release in pacemaking. Embryonic stem (ES) cells exhibited spontaneous beating after they differentiate into cardiac cells. RYR2 knockout ES cells displayed spontaneous beating with a slower rate than that of the wild-type ES cells did [20], indicating that RYR2 partially contributes to the pacemaker activity. However, these data do not resolve the controversy, because it is not known whether RYR2 is the only RYR expressed in the SA-node. In fact, in the present study we found that not only RYR2 but also RYR3 expressed in the SA-node. It is of worth, therefore, to test the effects of RYR2 and RYR3 double knockout on the pacemaking.

What is the rationale for the coexistence of RYR2 and RYR3 in the SA-node? RYR2 and RYR3 might function in a complementary manner to each other, or they might have their distinct functions. In rat heart, expression levels of RYR2 and RYR3 mRNA differ regionally and vary developmentally [21]. Thus, it is possible that RYR2 and RYR3 express in different regions of the heart or even of the SA-node, or in the different developmental stages of the SA-node. Thus, they have distinct functions in a region- or an age-specific manner. Interestingly, RYR2 is expressed in various regions of the heart, while RYR3 expression is mainly detected in the SA-node and right atrium (Fig. 3). Consistent with these findings, RYR3 has been shown to localize primarily in the conduction system of the ventricle [22]. Thus, RYR3 may have a unique role in rhythm generation and conduction.

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