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Contribution of $Na_V1.1$ to cellular synchronization and automaticity in spontaneous beating cultured neonatal rat ventricular cells

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Abstract. Two factors of cell coupling influence cellular synchronization and automaticity: gap junction coupling and ion channels activity. However, the role of Na^+ channel isoforms underlying cell-to-cell interaction and cellular automaticity is not well understood. To address these questions, we studied mRNA expression of Na^+ channel isoforms and the effects of TTX on spontaneously beating cultured ventricle cells. Using RT-PCR technique we demonstrated the presence of $Na_v1.1$ and $Na_v1.5$ channels. The reduction of $Na_v1.1$ channel activity disturbed cell-to-cell interaction and changed beating rates. Thus, $Na_v1.1$ channel is involved in cellular synchronization and automaticity.

Key words: Na^+ channel — Isoforms — Cultured ventricular cells — Synchronization — Rhythm generation

Introduction

Automatic phenomena of cells are observed under biological functions including the heart. The rhythm of the heart occurs from the sino-atrial node (SAN). It is due to intrinsic cellular property of pacemaker cell and cell-to-cell interaction (Jalife 1984; Boyett et al. 2000). For the intrinsic cellular property, the ion channels in pacemaker cell have been studied. The voltage-gated Na^+ channels, Ca^{2+} channels, K^+ channels and hyperpolarized-activated channels showed their activities on pacemaker cell (Irisawa et al. 1993). More than equal to intrinsic cellular property, cell-to-cell interaction is necessary for cellular automaticity (Jalife 1984; Masumiya et al. 2009). Two factors are accounted for cell-to-cell interaction: the membrane excitability of Na^+ channel and gap junction coupling (Dhein 2006).

The membrane excitability of Na^+ channel plays essential role for the action potential upstroke, generation and propagation of impulse in the heart (Bers 2001). During the last decade, ten α and four β subunits of the Na^+ channel isoforms have been cloned from various mammalian tissues (Goldin 2001). Several Na^+ channel isoforms, with $Na_v1.5$ channel being a main, were confirmed in the cardiac tissue (Haufe et al. 2005). However, $Na_v1.1$, $Na_v1.3$ and $Na_v1.6$ that preferentially expressed in neurons are also detected (Maier et al. 2003; Lei et al. 2004). Previous studies have demonstrated that $Na_v1.5$ is involved in action potential propagation, but both $Na_v1.1$ and $Na_v1.5$ contribute to the pacemaking in SAN (Haufe et al. 2005). However, knock-out of $Na_v1.5$ mice caused bradycardia, which elucidates the possibility of $Na_v1.5$ contribution to the pacemaking (Lei et al. 2005). Thus, the true nature of the spontaneous rhythm generation, synchronization and propagation of cells remains an open question with Na^+ channel isoforms participation.

To identify which Na^+ channel isoforms contribute to cellular synchronization and automaticity, we investigated mRNA expression of Na^+ channel isoforms. Moreover, we examined the effects of TTX on Na^+ channel activity by applying voltage imaging technique. We also studied the reduction of cell-to-cell coupling through gap junction to compare with the decrease of Na^+ channel activity. Those above-mentioned experiments were carried out by using spontaneously beating cultured neonatal rat ventricular cells. The isolated neonatal rat ventricular cells show individual beating rhythm in the early days of culture (Jongsma et al. 1987). Later, they start a synchronized spontaneous beating

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by establishing cell-to-cell interaction. Thus, the neonatal rat cultured ventricular cells are effective tool for study of cellular synchronization and automaticity.

Materials and Methods

Cell isolation and culture

All experiments were conducted in accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85–23, revised 1985). The experimental protocols were approved by the Animal Research Committee at Hyogo College of Medicine.

Single ventricular myocytes were isolated from 3 to 5 days old rats and cultured. Briefly, 10 to 20 ventricles were minced and dispersed with collagenase and protease solution for 40 min at 36°C. Isolated cardiomyocytes were subjected to percoll gradient centrifugation and cultured in Eagle's minimum essential medium (MEM) with 10% fetal bovine serum and plated onto collagen-coated coverslips in 35 mm culture dish (Sen et al. 1988). They were incubated for 3–6 days in a water-saturated CO_2 incubator at 36°C in 5% CO_2 and 95% air. The cells acquired automaticity and synchronicity within the first few days of culture.

RNA isolation and reverse transcriptase-polymerasec chain reactions (RT-PCR)

Total RNA from cultured cells were purified by acid-guanidine-thiocyanate -chloroform extraction method (Masumiya et al. 2003). The GenBank accession number for RT-PCR fragments were following; NM030875, bp 7355-8298; $Na_v1.1$, NM013119, bp 4935-5666; $Na_v1.3$, NM013125, bp 4920-5762; $Na_v1.5$, NM019266, bp 1137-1791; $Na_v1.6$, BC087743, bp 392-813; GAPDH. PCR reactions were performed in cDNA equivalent of 2 μg total RNA. The following temperature profile was used for amplification: denaturation for 1 cycle at 95°C for 30 s, and 30–50 cycles at 98°C for 45 s, 62°C for 1 min, and 72°C for 2 min. Reactions were terminated with 5 min extra DNA extension at 72°C and cooling down to 4°C.

Voltage imaging

Cells were incubated with a voltage-sensitive dye, di-4-ANEPPS (1 μM; Invitrogen, Carlsbad, California, USA) for 20 min in cultured medium, and rinsed with physiological solution with the following composition (in mM): NaCl 135, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 1.0, HEPES 5.0, and glucose 10 (pH 7.4). The voltage imaging measurements were performed at 36°C.

Cells were mounted on the stage of a fluorescence microscope (Nikon ECLIPSE TE2000-U) with viewing under an oil-immersion objective (Nikon DIC H/N2 1.30NA), and were illuminated at 480–550 nm with a tungsten-halogen lamp (150 W). The fluorescence emission (>590 nm) was recorded by a CMOS sensor array (MiCAM Ultima L-camera, Brainvision, Tokyo, Japan) as a relative measure of membrane potentials. The fluorescence images were captured every 20 ms. The change in fluorescence intensity (ΔF) relative to the initial intensity of the fluorescence (F_0) in each pixel was calculated. To normalize the difference in the amount of membrane-bound dye and illumination within the preparation, background fluorescence intensity at each pixel was divided by the maximal background fluorescence, and then the ratio of ΔF to the normalized background fluorescence intensity (F), i.e., the fractional change in fluorescence intensity ($\Delta F/F$), was calculated at each pixel in each frame.

The fractional change in fluorescence intensity (the amplitude of signal) indicated every cell beating. To determine the cell synchronization, we first calculated the every cell beat-to-beat interval that was measured by the intervals between upward or downward peaks of fluorescence intensity. The beat-to-beat intervals of multiple cells which we arbitrarily selected from the image were identical (Jongsma et al. 1987).

Statistical analysis

All values are presented as mean ± S.E.M. Statistical significance was determined by repeated-measures analysis of variance (ANOVA). $p < 0.05$ was considered significant.

Results

Expression of Na^+ channel isoforms in synchronized beating cells

We first investigated which Na^+ channel isoforms were expressed in spontaneous beating cultured ventricular cells. We examined the expression of neuronal ($Na_v1.1$, $Na_v1.3$, $Na_v1.6$) and cardiac ($Na_v1.5$) Na^+ channel isoforms with glyceraldehydes-e-phosphate dehydrogenase (GAPDH) as internal control. RT-PCR products of the sizes for the Na^+ channel isoforms are shown in Fig. 1A. $Na_v1.1$ and $Na_v1.5$ mRNA were detected, but no significant $Na_v1.3$ and $Na_v1.6$ mRNA were detected from the same samples. After normalization using the GAPDH mRNA, the $Na_v1.1$ and $Na_v1.5$ in spontaneous beating cultured cells were expressed at similar levels (Fig. 1B). The results shown are from 4–5 independent experiments.

Effects of TTX on the synchronized beating cells

Since $Na_v1.1$ mRNA was expressed in spontaneously beating cultured ventricular cells, we investigated whether $Na_v1.1$ has any contribution to the cellular synchronization and automaticity. We examined the effects of TTX on the optical membrane potentials of spontaneously beating cultured ventricular cells (Fig. 2). Cells were loaded with di-4-ANEPPS, and the optical membrane potentials were obtained from spontaneous beating cells.

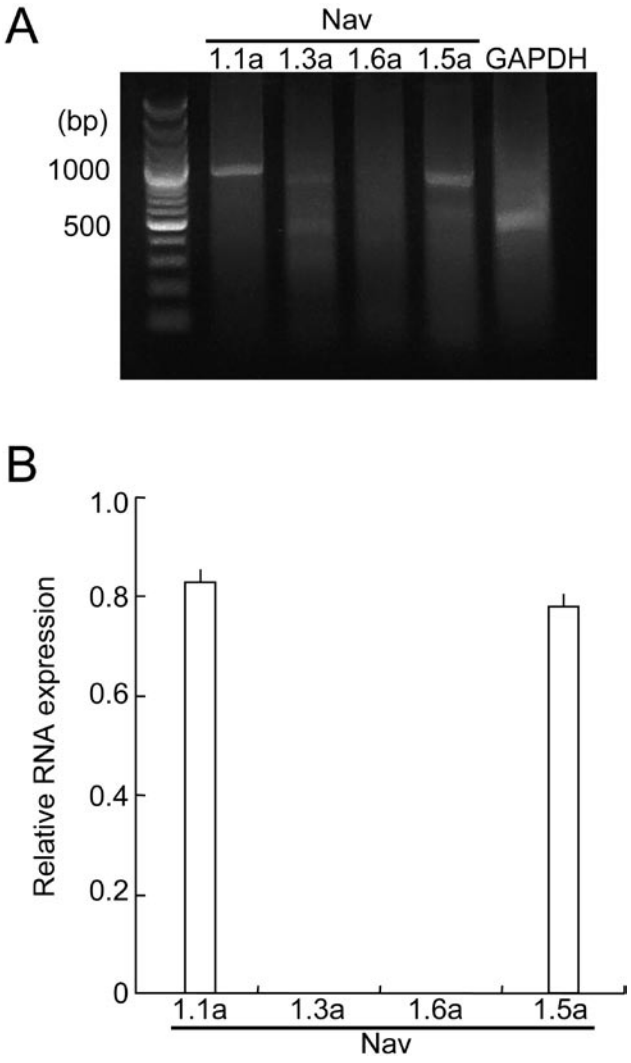

Figure 1. Expression of Na^+ channel isoforms in spontaneous beating cultured ventricular cells. **A.** RT-PCR products of neuronal ($Na_v1.1$, $Na_v1.3$, $Na_v1.6$) and cardiac ($Na_v1.5$) Na^+ channel isoforms. Total RNA from cells, 2 μg, was used for amplification. As an internal control, GAPDH was included in same sample. DNA markers were on the left lane. **B.** Quantification of $Na_v1.1$, $Na_v1.3$, $Na_v1.6$ and $Na_v1.5$ mRNA after normalization to GAPDH mRNA. The results shown are from 4–5 independent experiments.

TTX (10 nM) had little effect on optical membrane potentials of synchronized beating cells ($n = 3$, Fig. 2A). TTX (100 nM), which was classically used to inhibit $Na_v1.1$ as separate from $Na_v1.5$, disturbed the synchronization of cells and changed the spontaneous beating rate ($n = 4$, Fig. 2B). We obtained similar results at 300 nM TTX ($n = 3$, Fig. 2C). Subsequently, we examined the effect of 1 μM TTX on synchronized beating cells (Fig. 2D and E, Supplementary material; movie 1 and 2). TTX (1 μM) disturbed the synchronization and changed spontaneous beating rate of cells. TTX (1 μM) slowed down the spontaneous beating rate in 4 out of 5 experiments, but accelerated in 1 experiment. The traces in the absence and presence of 1 μM TTX are shown in Fig. 2D and 2E. These observations demonstrate that low concentration of TTX selectively inhibits $Na_v1.1$ and affects cellular synchronization and automaticity in spontaneous beating synchronized ventricular cells.

Effects of gap junction blocker on the synchronized beating cells

Gap junction is another factor of cell coupling that influences cellular synchronization and automaticity. We examined the effects of gap junction blocker, carbenoxolone (1 μM), on optical membrane potential of spontaneous beating cultured cells. This experiment allowed comparing the effect of carbenoxolone to 100 nM TTX on spontaneous beating cells. Carbenoxolone prolonged the beat-to-beat interval of cells that means decreasing in the rate of beating. However, carbenoxolone did not disturb cellular synchronization (Fig. 3, Supplementary movie 3 and 4). It was consistent with three different experiments.

Discussion

In this study, we detected mRNA expression of $Na_v1.1$ and $Na_v1.5$ in spontaneously beating cultured ventricular cells (Fig. 1). The specific expression of these Na^+ channel isoforms suggests that they may have specific functional role on cellular synchronization and automaticity.

To investigate the role of $Na_v1.1$ on spontaneously beating cultured ventricular cells, we took advantage of the different sensitivity of TTX concentration and voltage-dependence of inactivation and activation between $Na_v1.1$ and $Na_v1.5$. Low concentration of TTX, 100–200 nM, inhibited $Na_v1.1$, while full inhibition of $Na_v1.5$ required more than 20–30 μM TTX (Antoni et al. 1988; Maier et al. 2003; Lei et al. 2004). These pharmacological treatments could easily separate $Na_v1.1$ from $Na_v1.5$. Meanwhile, the different voltage-dependence of inactivation and activation between $Na_v1.1$ and $Na_v1.5$ is counted to circumvent latent problems of TTX sensitivity. $Na_v1.5$ is half inactivated at –82 mV and completely inactivated at –60 mV in ventricular myocytes. In another study,

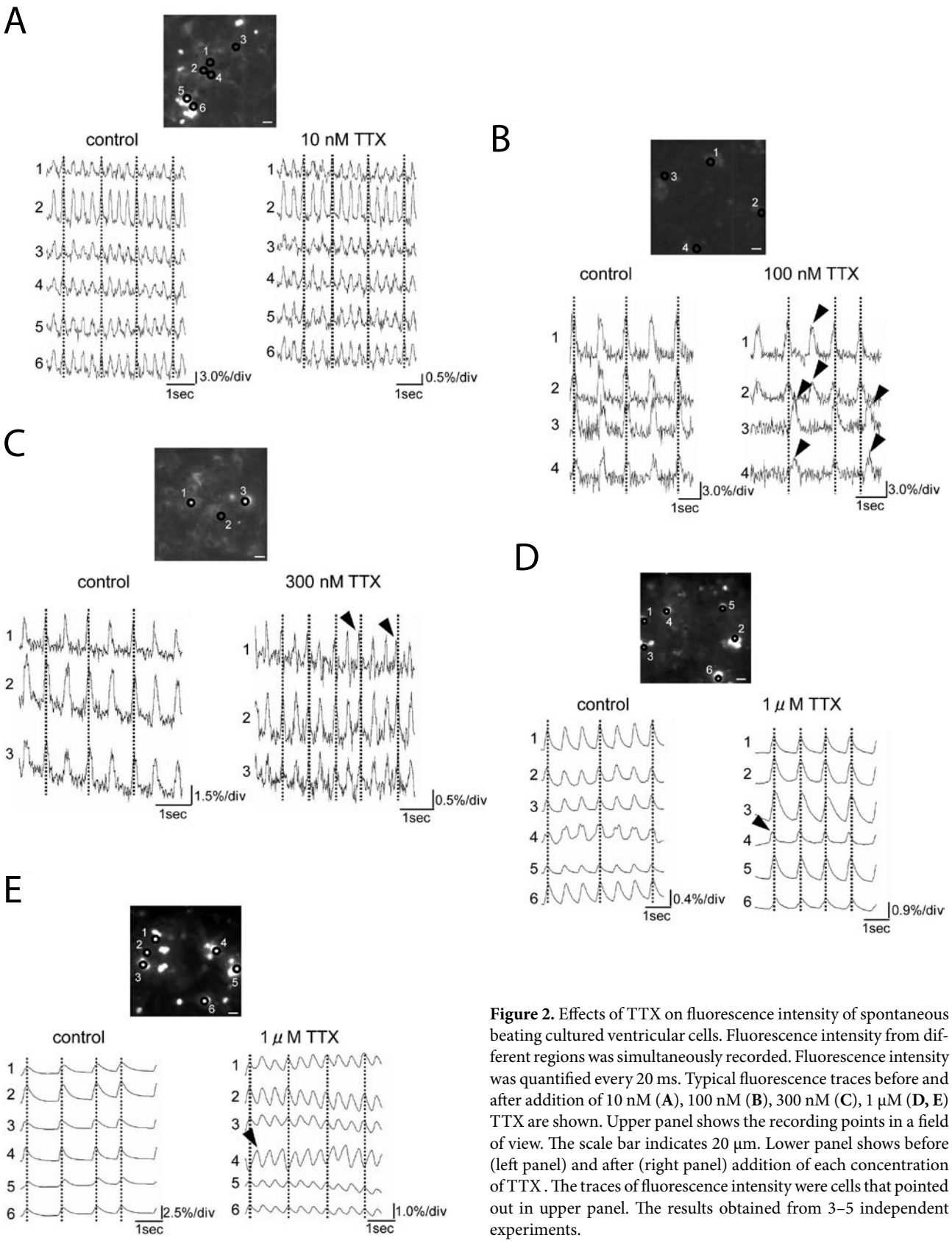

Figure 2. Effects of TTX on fluorescence intensity of spontaneous beating cultured ventricular cells. Fluorescence intensity from different regions was simultaneously recorded. Fluorescence intensity was quantified every 20 ms. Typical fluorescence traces before and after addition of 10 nM (**A**), 100 nM (**B**), 300 nM (**C**), 1 μM (**D, E**) TTX are shown. Upper panel shows the recording points in a field of view. The scale bar indicates 20 μm. Lower panel shows before (left panel) and after (right panel) addition of each concentration of TTX . The traces of fluorescence intensity were cells that pointed out in upper panel. The results obtained from 3–5 independent experiments.

$Na_v1.1$ inactivates at a potential 25 mV more negative than $Na_v1.5$ (Cohen et al. 1981; Maier et al. 2003). Furthermore, $Na_v1.5$ activated at a potential 20 mV more negative voltages than Nav1.1 (Lei et al. 2004). These data provide the enablement to separate $Na_v1.1$ from $Na_v1.5$ at resting potential of cultured ventricular cells. Because, the average values of resting potentials were between –60 mV and –40 mV in cultured ventricular cells (Matsuki and Hermsmeyer 1983; Mitcheson et al. 1996). The effects of 0.1–1 μM TTX on spontaneous beating cultured cells could consider to reflect the functional portion of $Na_v1.1$. In the present study, inhibition of $Na_v1.1$ by TTX disturbed synchronization and changed beating rates of spontaneous beating cultured ventricular cells (Fig. 2). This result indicates that $Na_v1.1$ is involved in cellular synchronization and automaticity.

The contribution of $Na_v1.1$ to cellular synchronization and automaticity resembled the pacemaker activity of the SAN. In the previous study, $Na_v1.1$ was identified together with $Na_v1.5$ in SAN (Maier et al. 2003; Lei et al. 2004; Haufe et al. 2005). $Na_v1.1$ only expressed in the center of SAN, while Nav1.5 was dominantly recognized in the periphery of the SAN (Maier et al. 2003; Lei et al. 2004). Another interpretation, both $Na_v1.1$ and $Na_v1.5$ contribute to the pacemaker activity, but only $Na_v1.5$ contribute to the impulse propagation (Lei et al. 2004). Those works support the gradient model that indicates the gradual transition of cell properties from the center to the periphery (Bleeker et al. 1980; Zhang et al. 2001). However, the SAN is functionally and anatomically more complex than those studies (Bleeker et al. 1980; Roberts et al. 1989). The cell-to-cell interaction underlies the cellular synchronization and automaticity in SAN (Jalife 1984; Masumiya et al. 2009). Our present finding of $Na_v1.1$ on cell-to-cell interaction would underlie synchronization and automaticity as SAN. The existence of $Na_v1.1$ would be immutable cellular synchronization and automaticity.

Reduction of intercellular coupling through gap junctions did not disturb cellular synchronization but slowed beating rate of cells (Fig. 3). The alteration of conduction velocity has been studied through Cx43 that is the major gap junction channel protein in the ventricle (Desplantez et al. 2007). The knockout of Cx43 mice, specific and dissected downregulation of intercellular coupling, preserved the effective conduction though it slowed conduction velocity (van Rijen et al. 2004; Stein et al. 2009). Because our study used cultured cells, it was not able to detect conduction velocity of longitudinal and transverse cells in the same way of ventricular tissue. We could not sufficiently measure the conduction velocity of excitation propagation under attenuation of intercellular coupling through gap junction in cultured cells. However, our result shows that reduction of intercellular coupling through gap junction affected the beating rate of cells, i.e., cellular automaticity (Fig. 3). Slowing conduction velocity due to reduce intracellular coupling through gap junction are required to cellular automaticity but not cellular synchronization.

It has been known that reduction of the membrane excitability and the intercellular coupling causes arrhythmia (Dhein 2006). Disturbing cellular synchronization and changing beating rate of cells by blocking Na^+ channel activity could cause arrhythmia. The decrease of Na^+ channel activity lead to the ectopic impulse and abnormal electrical propagation (Frame and Simson 1988). The reduction of $Na_v1.1$ activity could be responsible arrhythmia that was thought to be due to reduction of $Na_v1.5$ activity.

To summarize, reduction of membrane excitability of $Na_v1.1$ activity disturbed cellular synchronization and automaticity. By further extension, reduction of $Na_v1.1$ activity could be lead the arrhythmia as well as $Na_v1.5$.

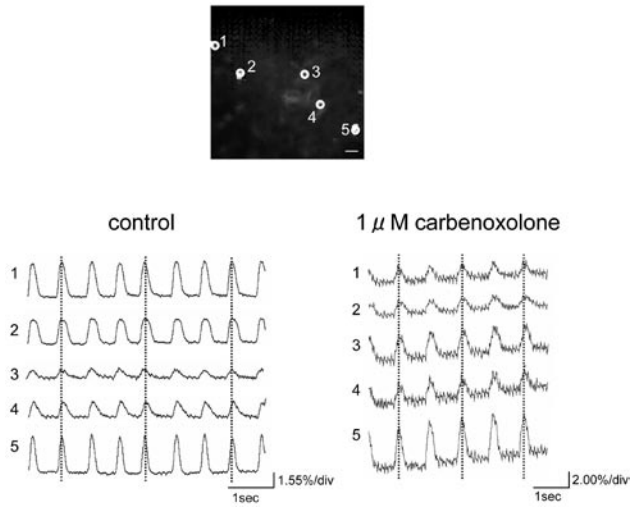

Figure 3. Effects of 1 μM carbenoxolone on fluorescence intensity in spontaneous beating cultured ventricular cells. Upper panel shows the location of the recording points of fluorescence intensity. Fluorescence intensity of 5 different cells was simultaneously recorded. The scale bar indicates 20 μm. Lower panels show fluorescence intensity of cells before (left) and after (right) addition of 1 μM carbenoxolone. The traces of fluorescence intensity were from 5 different cells that pointed out in upper panel. Fluorescence intensity was quantified every 20 ms.

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