Short Communication

Involvement of the Na⁺/Ca²⁺ Exchanger in the Automaticity of Guinea-Pig Pulmonary Vein Myocardium as Revealed by SEA0400

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Received June 21, 2008; Accepted March 19, 2009

Abstract. We examined the involvement of the Na⁺/Ca²⁺ exchanger in the automaticity of the pulmonary vein myocardium with a specific inhibitor, SEA0400. Action potentials were recorded from the myocardial layer of isolated guinea-pig pulmonary vein preparations, and Ca²⁺ transients were recorded from the cardiomyocytes. Spontaneous electrical activity was observed in 17.7% of the preparations, which was inhibited by either SEA0400 or ryanodine. In quiescent preparations, ouabain induced electrical activity and spontaneous Ca²⁺ transients, which were inhibited by SEA0400, as well as ryanodine. These results provide pharmacological evidence that the Na⁺/Ca²⁺ exchanger underlies the automaticity of the pulmonary vein myocardium.

Keywords: pulmonary vein myocardium, Na⁺/Ca²⁺ exchanger, automaticity

Pulmonary veins are considered to be involved in the initiation and maintenance of atrial fibrillation, one of the most frequent arrhythmias in clinical practice (1). Pulmonary veins contain a myocardial layer, whose electrical activity is considered to underlie their arrhythmogenic activity (2). The pulmonary vein myocardium has different electrophysiological properties from those of the working myocardium, including lower density of I_K1 and a less negative resting membrane potential (3). The precise mechanisms of the pulmonary vein electrical activity as well as its pharmacological properties are now receiving attention as the basis to develop an effective therapeutic strategy against atrial fibrillation.

The Na⁺/Ca²⁺ exchanger (NCX) is involved in the physiological and pathophysiological regulation of Ca²⁺ concentration in the myocardium. It functions both in the forward (Ca²⁺ extrusion) and reverse (Ca²⁺ influx) modes, and its functional role may vary with the region and the condition of the myocardium (4, 5). The forward mode NCX activity (inward current) is the major pathway for Ca²⁺ extrusion from the cytoplasm and is also considered to be involved in the normal pacemaking of the rabbit sinoatrial node (6). It was postulated that the Ca²⁺ released from the sarcoplasmic reticulum (SR) during the diastolic period is pumped out of the cell through the forward mode NCX, which generates an inward current that contributes to the diastolic depolarization of the pacemaker. Although it is possible that such a mechanism is involved in the automaticity of other myocardial regions including the pulmonary vein myocardium, pharmacological evidence is limited because of the lack of an NCX inhibitor with sufficient specificity.

SEA0400 {2-[4-[(2,5-difluorophenyl) methoxy] phenoxyl]-5-ethoxyaniline} is a potent and selective inhibitor of NCX in cultured neurons, astrocytes, microglia, dog sarcolemmal vesicles, and cultured rat myocytes with negligible affinities towards other transporters, ion channels, and receptors (7). We have previously shown that SEA0400 is a specific inhibitor of NCX in the myocardium (8, 9). SEA0400 (1 μM), which inhibited the NCX current by more than 80%, had no effect on the Na⁺ current, L-type Ca²⁺ current, delayed rectifier K⁺ current, inwardly rectifying K⁺ current (8), and the Ca²⁺ sensitivity of contractile proteins (5). This was in contrast with the effects of KB-R7943, a compound that has been widely used as an NCX inhibitor; KB-R7943 inhibited all of the above mentioned currents with equal potency (8). Thus, SEA0400 was established as the first specific pharmacological tool to study the role of NCX.
and was shown to be useful in studies on myocardial excitation–contraction mechanisms, regulation by autonomic transmitters, and ischemia–reperfusion injury (5, 10, 11). In the present study, we examined the effects of SEA0400 and ryanodine on the spontaneous and ouabain-induced electrical activity of the guinea-pig pulmonary vein myocardium to clarify the role of NCX in automaticity.

All experiments were approved by the Ethics Committee of Toho University Faculty of Pharmaceutical Sciences and performed in accordance with the Guiding Principles for the Care and Use of Laboratory Animals approved by The Japanese Pharmacological Society. Hearts with lungs were quickly removed from male or female Hartley guinea pigs (weight, 350–450 g). The pulmonary veins were separated from the atrium at the left atrium–pulmonary vein junction, and separated from the lungs at the ending of the pulmonary vein myocardial sleeves. Tubular pulmonary veins were cut open and pinned down endocardial side up on the bottom of the 20-ml recording chamber. The extracellular solution contained 118.4 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 24.9 mM NaHCO₃, and 11.1 mM glucose (pH 7.4); it was gassed with 95% O₂–5% CO₂ and maintained at 36 ± 0.5°C. The low-sodium extracellular solution was prepared with the equimolar substitution of NaCl with LiCl so that the final Na⁺ concentration was 70 mM. Change to low-Na++ solution and return to normal solution were performed with superfused pulmonary-vein tissue preparations and an original flow-switching device; the time required for change of the solution was approximately 1 s.

Action potentials were recorded in pulmonary vein tissue preparations by standard microelectrode penetrations from the luminal side. The glass microelectrodes filled with 3 M KCl had resistances of 20 – 30 MΩ. The output of a microelectrode amplifier (MEZ8201; Nihon Kohden, Tokyo) with high input impedance and capacity neutralization was recorded and analyzed by an action potential analyzing system (Analog-Pro DMA and DSS type IV; Canopus, Tokyo). The action potential parameters: resting potential (RP); maximum diastolic potential (MDP); overshoot (OS); maximum rate of rise (Vmax); action potential duration at 20%, 50%, and 90% repolarization (APD₂₀, APD₅₀, APD₉₀, respectively); and the slope of the depolarization phase were measured under electrical stimulation at 1 Hz.

Confocal microscopic analyses of the Ca²⁺ dynamics in isolated pulmonary-vein cardiomyocytes were performed with LSM 510 (Carl Zeiss, Jena, Germany) and procedures basically the same as those used for ventricular myocytes as previously described (12). After Langendorff perfusion of the heart with the pulmonary veins attached and treatment with 1 mg/ml collagenase (YK-102; Yakult, Tokyo) for about 20 min, the pulmonary vein cardiomyocytes were isolated, treated with fluo-4/AM, and superfused with the extracellular solution of the following composition gassed with 100% O₂ at 36 ± 0.5°C: 143 mM NaCl, 5.4 mM KCl, 1.0 mM MgCl₂, 1.8 mM CaCl₂, 0.33 mM NaH₂PO₄, 5.5 mM glucose, and 5 mM HEPES. The cells were line scanned at a speed of line/960 µs. The excitation wavelength was 488 nm and fluorescence with wavelength above 505 nm was detected and analyzed.

SEA0400 was provided by Taisho Pharmaceutical Company, Ltd. (Saitama). Ryanodine and ouabain were purchased from Wako (Osaka) and Sigma (St. Louis, MO, USA), respectively. These compounds were dissolved in dimethyl sulfoxide (final concentration of 0.01%). All other chemicals used were of the highest commercially available quality. Data were expressed as the mean ± S.E.M. Statistical significance between means was evaluated by the paired t test or by the one-way repeated measures analysis of variance followed by Contrasts for mean values comparison or by Fisher’s exact test; a P value less than 0.05 was considered significant.

Among the 141 isolated guinea-pig pulmonary vein preparations examined, 116 preparations showed no spontaneous activity. The action potential parameters of such quiescent preparations when driven at 1 Hz were RP: −71.4 ± 1.9 mV, MDP: −75.4 ± 1.5 mV, OS: 30.9 ± 1.5 mV, Vmax: 135.6 ± 15.5 V/s, APD₂₀: 16.7 ± 2.0 ms, APD₅₀: 37.7 ± 2.8 ms, and APD₉₀: 92.9 ± 1.2 ms (n = 6). An obvious diastolic depolarization was observed, although the site of microelectrode penetration was not at the very center of the pacemaking region of the pulmonary vein myocardium. The slope of the diastolic depolarization was 9.5 ± 2.0 mV/s (n = 6). SEA0400 (1 µM) significantly shortened the action potential duration (APD₂₀ and APD₉₀) and decreased the slope of the diastolic depolarization without affecting other parameters. The action potential parameters after the application of SEA0400 were RP: −70.5 ± 1.6 mV, MDP: −73.7 ± 1.2 mV, OS: 30.7 ± 1.6 mV, Vmax: 128.3 ± 15.8 V/s, APD₂₀: 15.3 ± 1.8 ms, APD₅₀: 33.1 ± 2.2 ms, APD₉₀: 87.6 ± 2.1 ms, and the slope of the depolarization phase: 6.3 ± 1.1 mV/s (n = 6).

The other 25 preparations showed spontaneous electrical activity (17.7%, Fig. 1A). After confirming that the spontaneous activity persisted for 30 min, drugs were applied and the effects were observed for 30 min. SEA0400 (1 µM) prolonged the cycle length of the spontaneous action potential followed by complete suppression within 10 min (Fig. 1Aa, Table 1). Concerning the specificity of SEA0400, it was reported that
Fig. 1. Pharmacological properties of electrical activity and intracellular Ca\(^{2+}\) transient in pulmonary vein myocardia. A: Inhibition by SEA0400, ryanodine, and low-Na\(^+\) extracellular solution of the spontaneous activity in pulmonary vein myocardial tissue. Typical action potential traces showing the effects of 1 µM SEA0400 (a), 0.1 µM ryanodine (b), and rapid change of the extracellular solution to low-Na\(^+\) solution (c). B: Inhibition by SEA0400 and ryanodine of the ouabain-induced electrical activity in pulmonary vein myocardial tissue. Typical action potential traces showing the effect of 1 µM ouabain on a quiescent preparation in the absence (a) or presence of 1 µM SEA0400 (b) or 0.1 µM ryanodine (c). C: Confocal line-scan images of intracellular Ca\(^{2+}\) (a, c, e, g, i) and the time course of fluorescence intensity (b, d, f, h, j) in isolated pulmonary-vein myocardial cells. In a quiescent cell (a, b), ouabain induced an elevation of cytoplasmic Ca\(^{2+}\) (c, d) followed by generation of spontaneous Ca\(^{2+}\) transients (e, f). Further application of SEA0400 reduced the frequency of Ca\(^{2+}\) transients (g, h) and eventually completely inhibited spontaneous activity. In another cell, the Ca\(^{2+}\) sparks induced by ouabain, as well as Ca\(^{2+}\) transients, could be detected in the scanning line (i, j). The scanning line was placed along the longitudinal axis of the cardiomyocyte. Horizontal and vertical scale bars in a, c, e, g, and i indicate 500 ms and 10 µm, respectively; and the bars on the left side indicate the regions quantified.
TABLE 1. Inhibition by SEA0400 and ryanodine of pulmonary vein spontaneous activity

<table>
<thead>
<tr>
<th></th>
<th>Frequency (Hz)</th>
<th>Average of cessation</th>
<th>Cessation at 10 min (%)</th>
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<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>5 min</td>
<td>10 min</td>
</tr>
<tr>
<td>Control</td>
<td>1.1 ± 0.2</td>
<td>1.1 ± 0.2</td>
<td>1.2 ± 0.3</td>
</tr>
<tr>
<td>SEA0400 (1 μM)</td>
<td>1.1 ± 0.5</td>
<td>0.6 ± 0.6</td>
<td>0*</td>
</tr>
<tr>
<td>Ryanodine (0.1 μM)</td>
<td>0.9 ± 0.1</td>
<td>0.6 ± 0.2*</td>
<td>0.2*</td>
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</table>

Effects of SEA0400 or ryanodine on the spontaneous activity of pulmonary vein preparations are summarized in this table. SEA0400 or ryanodine was applied to spontaneously firing preparations. The frequency was sampled at 5 min because the drugs caused cessation in most of the preparations at 10 min. Asterisks indicate significant differences from the corresponding values at 0 min as evaluated by one-way repeated measures analysis of variance followed by Contrasts for mean values comparison (*P<0.05).

Ouabain (1 μM) was applied to quiescent pulmonary vein preparations in the presence of vehicle (control), SEA0400, or ryanodine; and the incidence of electrical activity at 30 min is summarized. For the preparations in which ouabain induced electrical activity, the latency before the first firing and the frequency at 15 min are summarized; it took an average of 7–13 min before activity was induced. SEA0400 or ryanodine was present from 30 min before the application of ouabain. Asterisks indicate significant differences from the control values as evaluated by Fisher’s exact test (*P<0.05).

SEA0400 depresses Ca²⁺ transients in NCX1-knockout mouse (13). Although such non-specific effects of SEA0400 has not been reported in normal animals, the possibility that the compound is acting through mechanisms other than NCX inhibition can not be totally excluded. Thus, we intended to inhibit forward-mode NCX activity with low Na⁺ extracellular solution. Changing the extracellular solution to low Na⁺ solution caused slowing of the spontaneous electrical activity within a few seconds and this recovered after return to normal solution (Fig. 1Ac); the frequency of the spontaneous activity under normal and low-Na⁺ condition was 1.2 ± 0.1 and 0.6 ± 0.1 (P<0.05, n = 6), respectively. Ryanodine (0.1 μM) suppressed spontaneous activity in most of the preparations (Fig. 1Ab, Table 1).

In the pulmonary vein preparations without spontaneous activity, 1 μM ouabain induced membrane potential oscillations, leading to the repetitive generation of action potentials (Fig. 1Ba). Pretreatment with SEA0400 suppressed the ouabain-induced electrical activity in a concentration dependent manner (Fig. 1Bb, Table 2). Concerning the preparations in which ouabain-induced electrical activity persisted in the presence of SEA0400, the latency was longer and the frequency was lower than those in the preparations treated with ouabain alone (Table 2). In the presence of ryanodine (0.1 μM), ouabain (1 μM) did not induce electrical activity (Fig. 1Bc, Table 2).

To clarify the effect of ouabain and SEA0400 on intracellular Ca²⁺, the drugs were applied to isolated pulmonary-vein myocardial cells loaded with the fluorescent Ca²⁺ indicator, fluo-4, under line-scan mode confocal microscopy (Fig. 1C). In quiescent pulmonary-vein cardiomyocytes (Fig. 1Ca, b), ouabain (1 μM) induced an increase in cytoplasmic Ca²⁺ concentration (Fig. 1Cc, d) and generation of spontaneous Ca²⁺ transients (Fig. 1Ce, f); the basal fluorescence intensity of the cells after the induction of spontaneous activity was 281.8 ± 60% (n = 6) of intensity under quiescence. Further application of SEA0400 decreased the frequency of the Ca²⁺ transients and eventually completely inhibited the Ca²⁺ transients without decreasing the diastolic Ca²⁺ level (Fig. 1Cg, h); the fluorescence was 100.8 ± 2.1% (n = 6) of the value before application of SEA0400. In some cells, the induction by ouabain (1 μM) of Ca²⁺ sparks, as well as spontaneous Ca²⁺ transients, could be detected within the scanning line (Fig. 1Ci, j). Ryanodine (0.1 μM) completely inhibited Ca²⁺ transients (n = 6).

In the present study, about 18% of the guinea-pig pulmonary vein preparations showed spontaneous activity (Fig. 1A); the incidence was increased by ouabain. Ouabain has been used as a pharmacological
tool to induce intracellular Ca\(^{2+}\) overload through inhibition of the Na\(^+/K^+\) ATPase (14), although other mechanisms of action have also been postulated (15). Increased cellular Ca\(^{2+}\) load can cause elevation of the diastolic Ca\(^{2+}\) level and/or induce spontaneous Ca\(^{2+}\) release from the SR. These would accelerate the diastolic depolarization through enhancement of the forward-mode NCX and thereby elicit electrical activity. Our present results that ouabain induced elevation of diastolic Ca\(^{2+}\) level and induced Ca\(^{2+}\) sparks and that ouabain-induced electrical activity was inhibited by ryanodine and SEA0400 suggest that this is indeed the case. The spontaneous electrical activity was inhibited by ryanodine and SEA0400 and also by low Na\(^+\) extra-cellular solution. These results indicated that for both the ouabain-induced and spontaneous electrical activities, the basic mechanism is activation of the forward-mode NCX by Ca\(^{2+}\) released from the SR. Acceleration of late repolarization (reduction of APD\(_{90}\) and APD\(_{50}\)) and reduction of the slope of diastolic depolarization by SEA0400 supports the view that Ca\(^{2+}\) extrusion through forward mode NCX occurs during the repolarization phase and the diastolic depolarization phase.

Ouabain, at a concentration of 1 \(\mu\)M, induced electrical activity in the guinea-pig pulmonary vein myocardium (Fig. 1B, Table 2). This concentration of ouabain did not induce electrical activity in the ventricular myocardium (11). The pulmonary vein myocardium is reported to have different electrophysiological properties from those of the working myocardium such as lower density of I\(_{K_1}\) and a less negative resting membrane potential (3). The resting membrane potential of the guinea-pig pulmonary vein myocardial cells in the present study was \(-71.4\) mV, which was less negative than that of the guinea-pig atria and ventricle (\(-78\) to \(-85\) mV, ref. 16). The smaller contribution of I\(_{K_1}\) around the resting membrane potential would allow underlying depolarizing mechanisms to contribute to diastolic depolarization, leading to the generation of spontaneous action potentials in the pulmonary vein myocardium. In the guinea-pig ventricular myocardium, SEA0400 (1 \(\mu\)M) decreased the incidence of ouabain-induced arrhythmic contraction from 73% to 46% (11). This potency of SEA0400 to inhibit automaticity is similar to that in the pulmonary vein preparations (87% to 47%, Table 2). This suggests that the depolarizing mechanisms involving the forward-mode NCX is intrinsically present in both the ventricular and pulmonary vein myocardium, but manifests itself as automaticity preferentially in the pulmonary vein because of the limited ability to maintain the resting membrane potential.

The pulmonary vein is considered to be a source of ectopic electrical activity that leads to paroxysms of atrial fibrillation (2). Abnormalities in intracellular Ca\(^{2+}\) homeostasis induced by factors such as increased mechanical stretch has been suggested to underlie the generation of ectopic activity in the pulmonary vein (17). The Ca\(^{2+}\) influx through stretch-activated cation channels may load the SR above its capacity and cause spontaneous focal Ca\(^{2+}\) release from the SR. This would lead to the generation of arrhythmias through forward-mode NCX activity. The present results with SEA0400 indicate that Ca\(^{2+}\) overload–induced electrical activity in the pulmonary vein myocardium can be reduced by NCX inhibition. Thus, NCX inhibition may be an effective therapeutic strategy for the treatment of atrial fibrillation of pulmonary vein origin. Extension of the present study with experiments, such as direct recording from the center of the pacemaking region of the pulmonary vein myocardium, simultaneous recording of Ca\(^{2+}\) concentration and membrane potential, and evaluation of the effect of drugs in clinical use, would lead to a deeper understanding of the pulmonary vein automaticity and its therapeutic significance.

In conclusion, the present results provide pharmacological evidence that the forward-mode NCX activated by Ca\(^{2+}\) released from the SR is involved in the automaticity of the pulmonary vein myocardium.

Acknowledgments

This study was in part performed as a part of the project “Research on the molecular mechanisms of appearance of age-related diseases by failure of cell function control system, and their prevention and treatment” by the “Research Center for Aging and Age related Diseases” established in the Toho University Faculty of Pharmaceutical Sciences. This study was supported in part by Grants-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan to I.N. (#20890233), A.T. (#19590532), and H.T. (#18590243). It was also supported in part by a Grant-in-Aid for Research on Publicly Essential Drugs and Medical Devices to K.S. and T.K. from the Japan Health Science Foundation.

References

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