Pacing-Induced Spontaneous Activity in Myocardial Sleeves of Pulmonary Veins After Treatment With Ryanodine

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Background—Recent clinical electrophysiology studies and successful results of radiofrequency catheter ablation therapy suggest that high-frequency focal activity in the pulmonary veins (PVs) plays important roles in the initiation and perpetuation of atrial fibrillation, but the mechanisms underlying the focal arrhythmogenic activity are not understood.

Methods and Results—Extracellular potential mapping of rabbit right atrial preparations showed that ryanodine (2 μmol/L) caused a shift of the leading pacemaker from the sinoatrial node to an ectopic focus near the right PV-atrium junction. The transmembrane potential recorded from the isolated myocardial sleeve of the right PV showed typical atrial-type action potentials with a stable resting potential under control conditions. Treatment with ryanodine (0.5 to 2 μmol/L) resulted in a depolarization of the resting potential and a development of pacemaker depolarization. These changes were enhanced transiently after an increase in the pacing rate: a self-terminating burst of spontaneous action potentials (duration, 33.6 ± 5.0 s; n = 32) was induced by a train of rapid stimuli (3.3 Hz) applied after a brief rest period. The pacing-induced activity was attenuated by either depletion of the sarcoplasmic reticulum of Ca2+ or blockade of the sarcolemmal Na+-Ca2+ exchanger or Cl− channels and potentiated by β-adrenergic stimulation.

Conclusions—PV myocardial sleeves have the potential to generate spontaneous activity, and such arrhythmogenic activity is uncovered by modulation of intracellular Ca2+ dynamics. (Circulation. 2003;107:1937-1943.)

Key Words: electrophysiology • fibrillation • veins • action potentials

Attrial fibrillation (AF) is the most common of all sustained tachyarrhythmias and is one of the major causes of stroke. The most widely accepted mechanism of AF is multiple reentrant wavelets.1 However, recent clinical studies have shown that paroxysmal AF is initiated by bursts of premature excitations originating primarily in the pulmonary veins (PVs), and radiofrequency ablation or electrical isolation of these foci can eliminate AF.2,3 More recently, Haïssaguerre et al4 reported that in patients with drug-resistant paroxysmal AF, the pulmonary veins (PVs) entering the left atrium to form “myocardial sleeves” (MSs),6,7 and this is the origin of focal activity.8 Previous studies have suggested that PVMSs show a variety of spontaneous activities such as sinoatrial (SA) node-type automaticity, rapid spontaneous activities via early or delayed afterdepolarizations,8–12 and microreentry based on a marked heterogeneous tissue structure.12,13 However, the electrophysiological properties of PVMSs have not been fully characterized.

In the present study, we investigated the electrophysiological properties of rabbit PVMSs. The results show that addition of 0.5 to 2 μmol/L ryanodine to PVMSs uncovers pacing-induced spontaneous activity. Ryanodine at low concentrations locks the sarcoplasmic reticulum (SR) Ca2+ release channel, the ryanodine receptor (RyR), in a subconduction state, causing a Ca2+-independent Ca2+ release from the SR.14 Such Ca2+ leakage during diastole may alter intracellular Ca2+ and thus Ca2+-dependent ionic currents,15 and this may be the cause of the spontaneous activity. The results may be important clinically, because remodeling of Ca2+ handling proteins, including RyR, occurs in disease states.16

Methods

Rabbits weighing 1.5 to 2.0 kg (Chubu-Kagaku-Shizai, Nagoya, Japan) were anesthetized with pentobarbital (30 to 40 mg/kg IV), and the heart was quickly excised. The right atrium, including the SA node and the junction between the right superior PV and the atria, was isolated and opened to expose the endocardial surface (Figure 1, B and C). The preparation was fixed in a tissue bath equipped with an electrode array. Extracellular recordings were made from the epicardium (simultaneously from 120 sites) by use of the modified bipolar electrode array (interelectrode distance, 1 mm),17 and in some experiments, membrane potential was recorded by a glass
microelectrode from the endocardium near the PV-atrium junction (Figure 1, A and D). Alternatively, the MS was isolated from the right superior PV (Figure 2A, box), and membrane potential was recorded during regular pacing and a train of rapid stimuli after a brief rest.

Preparations were superfused with Krebs-Ringer solution containing (in mmol/L): 120.3 NaCl, 4.0 KCl, 1.2 CaCl$_2$, 1.3 MgSO$_4$, 1.2 NaH$_2$PO$_4$, 25.2 NaHCO$_3$, and 11.0 glucose (pH 7.4, equilibrated with 95% O$_2$/5% CO$_2$) at 33°C. When Ni$^{2+}$/H$_{11001}$ was applied, HEPES-buffered solution gassed with 100% O$_2$ was used, with a composition of (in mmol/L): 145.0 NaCl, 4.0 KCl, 1.2 CaCl$_2$, 1.3 MgCl$_2$, 5.0 HEPES, and 11.0 glucose (pH 7.4). Ryanodine (Sigma) was dissolved in water to make a stock solution (5 mmol/L).

Data are presented as mean±SEM. Student’s t test and ANOVA were used to test differences, and a difference was considered significant at a value of $P<0.05$.

Results

Ectopic Atrial Pacemaker

Right atrial preparations showed regular spontaneous activity, and the center of the SA node was the leading pacemaker under control conditions ($n=9$). A representative activation map is shown in Figure 1B. For this and 2 other experiments, extracellular recordings were made from the crista terminalis (CT) and transmembrane recordings from the atrial muscle close to the PV-atrium junction (Figure 1A). Excitation propagated from the SA node to the CT, and the spread of excitation toward the interatrial septum was partially blocked (Figure 1B, thick line). The membrane potential recorded from the PV region showed typical atrial-type action potentials, with a stable resting potential (RP) ($-84.5±1.0$ mV, $n=3$) under control conditions. After ryanodine (2 μmol/L) was applied, there was a depolarization of the RP (by $11.8±0.6$ mV, $n=3$) and an elevation of the action potential plateau (Figure 1A). In addition, after application of ryanodine, the upstroke of the PV action potential preceded the activation of the CT, whereas before application of ryanodine, it followed (Figure 1A). The activation map obtained at this time shows that the pacemaker was shifted to an ectopic site close to the PV (Figure 1C). Further treatment with
Ryanodine caused complex changes in the membrane potential and the activation pattern (# in Figure 1A). Fast-time base recordings of the electrical signals during this period are shown in Figure 1D. The ectopic pacemaker close to the PV stopped beating, and this was associated with a sudden change in the extracellular potential at the CT. The activation map at this time (Figure 1E) shows that there was complete conduction block from the SA node toward the septum. During the quiescent period, the RP gradually hyperpolarized (by 13.2 ± 0.4 mV, n = 3) (Figure 1D). The ectopic pacemaker started beating again, and the activation map of the first beat (Figure 1F) shows that the conduction from the SA node to the septum recovered and the ectopic pacemaker was driven by the SA node. The ectopic pacemaker started to depolarize gradually after the resumption of beating (Figure 1D). The extracellular potential of the CT changed suddenly at the 10th beat, and this was associated with a takeover of the ectopic focus as the leading pacemaker (Figure 1G). Such dynamic equilibrium between the SA node and the ectopic pacemaker was repeated continuously (# in Figure 1A). Ryanodine (0.5 to 2 μmol/L) caused a similar intermittent shift of the leading pacemaker in 7 of 9 preparations. Histological examination (not shown) confirmed that the ectopic pacemaker corresponded to the wall of the right superior PV.

**Pacing-Induced Spontaneous Activity**

To characterize the electrical properties of the ectopic pacemaker without interference from the SA node, the right superior PVMS was isolated (Figure 2A) and membrane potential recorded. The majority of isolated PVMSs (39 of 41 preparations) were electrically quiescent without stimulation under control conditions, and the remaining 2 showed spontaneous activity. Figure 2B shows the membrane potential during regular pacing (2 Hz) before and after application of ryanodine (0.5 μmol/L) for 30 minutes resulted in an elevation of the action potential plateau, a depolarization of the RP, and a development of pacemaker depolarization (Figure 2B).
The pacemaker depolarization could be similar to that in the SA node, or it could be a pacing-induced delayed afterdepolarization such as that described in the coronary sinus. These changes were more marked during rapid pacing. Figure 2C shows an example in which a train of rapid stimuli (20 pulses at 3.3 Hz) was applied after a 1-minute rest to a preparation treated with ryanodine (0.5 μmol/L). Rest resulted in a large hyperpolarization of the RP (similar hyperpolarization was seen in preparations including the SA node when the ectopic pacemaker became quiescent after application of ryanodine; Figure 1D). The first action potential during the stimulation train after the rest was atrial-like with a stable RP, although the plateau of the action potential was low and short (Figure 2C). During rapid pacing, there was a broadening of the action potential plateau and a depolarization of the RP (Figure 2C). These changes were associated with a development of pacemaker depolarization, and by the end of pacing, the action potential had been converted from a typical atrial-type to an apparently SA node-type pacemaker action potential (Figure 2C). As a result, spontaneous action potentials developed from the pacemaker depolarization when pacing was stopped (Figure 2C). The slope of the pacemaker depolarization gradually declined during the train of spontaneous action potentials, and the spontaneous activity eventually ceased. Such behavior is analogous to that seen in preparations including the SA node (Figure 1).

Table 1 summarizes the pacing-induced spontaneous activity. A train of 3.3-Hz stimulation (after a 1-minute rest) caused self-terminating spontaneous activity in 82.1% of PVMS preparations (at 0.5 to 2 μmol/L; Table 1). A brief rest period before rapid pacing was not essential for the spontaneous activity, and an abrupt increase in the pacing rate (from 0.5 or 1.0 to 3.3 Hz) could also induce the activity in PVMSs treated with ryanodine (Table 1). Spontaneous action potentials were never induced by pacing in the normal atrial muscle of the right and left atrial appendages treated with ryanodine (Table 1).

### Steady-State Action Potentials

Development of pacemaker depolarization in PVMSs treated with ryanodine was accompanied by changes in the action potential configuration. We examined rate-dependent changes of the steady-state action potential in 6 PVMSs. Representative examples at 3 stimulation rates before and after application of ryanodine (2 μmol/L) are shown in Figure 3A, and changes in action potential parameters are summarized in Figure 3B. Under control conditions, there was a small but significant depolarization of the RP at pacing rates >3.3 Hz (Figure 3B). After treatment with ryanodine (2 μmol/L), there was a greater depolarization of the RP and a reduction of the action potential overshoot at high rates (Figure 3B). The ryanodine-induced depolarization of the RP at 2.0 Hz was significantly larger in PVMSs (9.5±1.1 mV, n=8) than in the normal atrial muscle (5.6±1.9 mV, n=6, P<0.01; Figure 3B). Ryanodine also caused biphasic changes in action potential duration: it was prolonged during pacing at 0.5 to 2 Hz, whereas it was shortened at faster rates (Figure 3B).

### Effects of Modulation of Intracellular Ca²⁺ Dynamics

The effect of depletion of SR Ca²⁺ on the pacing-induced spontaneous activity was examined. Figure 4A shows that an

<table>
<thead>
<tr>
<th>Changes in Pacing Rate, Hz</th>
<th>Control: Incidence of Spontaneous Activity</th>
<th>Depolarization of Resting Potential, mV</th>
<th>Incidence of Spontaneous Activity (Duration, s)</th>
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<tbody>
<tr>
<td>PV</td>
<td></td>
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<tr>
<td>(2.0)→0→3.3</td>
<td>0/39</td>
<td>13.1±0.6</td>
<td>32/39 (33.6±5.0)</td>
</tr>
<tr>
<td>0.5→3.3</td>
<td>0/7</td>
<td>13.0±1.2</td>
<td>6/7 (35.9±5.6)</td>
</tr>
<tr>
<td>1.0→3.3</td>
<td>0/6</td>
<td>10.2±1.7</td>
<td>5/6 (13.0±3.5)</td>
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<tr>
<td>RAA, LAA</td>
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<tr>
<td>(2.0)→0→3.3</td>
<td>0/7</td>
<td>8.9±0.8</td>
<td>0/7</td>
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RAA and LAA indicate right and left atrial appendages.
There are various Ca$^{2+}$-regulated ionic currents, and the possible contribution of Na$^+$-Ca$^{2+}$ exchange current and Ca$^{2+}$-activated Cl$^-$ current to the spontaneous activity was investigated. Figure 4B shows the effects of Ni$^{2+}$ to inhibit the Na$^+$-Ca$^{2+}$ exchanger. Application of Ni$^{2+}$ (5 mmol/L) abolished the development of the pacemaker depolarization during rapid pacing and spontaneous activity after pacing. It is unlikely that these effects of Ni$^{2+}$ are the result of its blocking action on Ca$^{2+}$ current (and a decrease of SR Ca$^{2+}$ content), because Ni$^{2+}$ abolished the pacing-induced spontaneous activity (duration, from 35.7±11.1 s to 0 s; n=5), whereas a partial block of Ca$^{2+}$ current by nifedipine (3 μmol/L), which caused a similar shortening of the action potential to that induced by Ni$^{2+}$, had no appreciable effects on the duration of the activity (from 35.2±9.8 s to 29.2±6.3 s, n=4).

Figure 4C shows that niflumate, a Cl$^-$ channel blocker, attenuated the development of the pacemaker depolarization induced by rapid pacing, and there were no spontaneous action potentials after pacing. The duration of the pacing-induced spontaneous activity was decreased significantly by the Cl$^-$ channel blockers. 50 μmol/L niflumate or 100 μmol/L DIDS from 26.2±5.2 s to 2.9±1.7 s (n=7, P<0.01). These results suggest that the contribution of the Na$^+$-Ca$^{2+}$ exchanger is major but that of the Ca$^{2+}$-activated Cl$^-$ channel is also significant in the pacing-induced spontaneous activity.

Effects of β-Adrenergic Stimulation
Figure 5 shows that isoproterenol (0.1 μmol/L) markedly increased the development of pacemaker depolarization during pacing and prolonged the spontaneous activity after rapid pacing in the PVMSs treated with ryanodine. The duration of the spontaneous activity was significantly increased after application of isoproterenol from 32.1±3.1 s to 70.9±38.9 s (n=6, P<0.01). These findings support the hypothesis that the pacing-induced spontaneous activity is the result of changes in intracellular Ca$^{2+}$ dynamics. Spontaneous action potentials were never induced by rapid pacing in the presence of isoproterenol (0.1 μmol/L) in PVMSs without treatment with ryanodine (n=3).

Effects of Strophanthidin
The effects of strophanthidin were investigated on right atrial preparations including the SA node and isolated PVMS. In all the right atrial preparations tested (n=5), strophanthidin (0.1 μmol/L) induced spontaneous activity in an ectopic site close to the PV-atrium junction (Figure 6A), as in the case of ryanodine. Figure 6B shows the effects of strophanthidin (0.1 μmol/L) on the isolated PVMS. In the presence of strophanthidin, a train of rapid pacing at 3.3 Hz induced a short burst of spontaneous action potentials (duration, 3.5±0.8 s; n=4), but the activity induced by strophanthidin was not associated with the development of pacemaker depolarization during diastole.

Discussion
In the present study, we have demonstrated that ryanodine, presumably by altering intracellular Ca$^{2+}$ dynamics, causes pacing-induced spontaneous activity in the rabbit PVMS.
Spontaneous action potentials induced by rapid pacing were associated with pacemaker depolarization during diastole. Various types of spontaneous activities, including SA node–like spontaneous action potentials, have been recorded from guinea pig and dog PVs in previous studies. However, spontaneous activity was observed under control conditions in the previous studies, whereas in the rabbit, spontaneous activity under control conditions was rare, and interventions affecting intracellular Ca$^{2+}$ (rapid pacing and ryanodine) were necessary to generate the activity. Electron microscopy of rat PVMSs demonstrated the presence of "clear cells" possessing the characteristic feature of SA node cells, although such cells have not been found in other species and may be artifacts. In addition, immunohistochemical studies of embryonic hearts using specific antibodies for the cardiac conduction system suggest that the PVMSs share a common embryonic origin with the SA node. It is therefore possible that the electrophysiological characteristics of PVMSs are similar to those of the SA node.

The fact that the spontaneous activity could be induced only in PVMSs and not in the normal atrial muscle (Table 1) suggests that PVMS cells have distinct electrophysiological characteristics. Chen et al reported that canine PVMS cells with spontaneous activity have a significantly lower density of inward rectifier K$^+$ current, $I_{K_1}$. It is well known that SA node cells lack $I_{K_1}$, and this is essential for their normal pacemaking. The low density of $I_{K_1}$ may also be an essential feature for the ectopic pacemaker activity of the PVMSs. The present study suggests that Na$^+$-Ca$^{2+}$ exchange current along with Ca$^{2+}$-dependent Cl$^-$ current may be involved in the spontaneous activity. It is possible that during diastole, these inward currents are able to cause depolarization of the

Figure 5. Effect of β-adrenergic stimulation on pacing-induced spontaneous activity in ryanodine-treated PVMS. Stimulation protocol of Figure 2C was repeated after treatment with 2 μmol/L ryanodine (Ry) and in presence of 0.1 μmol/L isoproterenol (ISP) after washoff of ryanodine. Top, stimuli; middle, membrane potential before and after ISP application; bottom, superimposed recordings of selected action potentials during period of rapid pacing (number of stimuli shown) and first spontaneous action potential (*) before and after ISP application.

Figure 6. Triggered activity induced by strophanthidin in PVMS. Top, Activation maps before (control) and after treatment with strophanthidin (0.1 μmol/L). Format of activation maps and abbreviations are same as those in Figure 1. Leading pacemaker site. Bottom, Spontaneous activity induced by rapid pacing in presence of strophanthidin (0.1 μmol/L). Preparation stimulated at 0.5 Hz and then at 3.3 Hz (20 pulses). Fast time base recording of spontaneous action potentials shown at right. Top, stimuli; middle, membrane potential.
membrane (and thus spontaneous activity) because of the low density of $I_{K1}$.

The results obtained from rabbit PVMSs may help to explain why AF begets AF.24 When there is already AF (by any mechanism), the high-frequency activity may provoke the arrhythmogenic activity of the PVMSs and thereby initiate another episode of AF. Clinical electrophysiology studies in patients with AF have demonstrated that rapid focal activity originating in one PV can trigger focal activity in another PV to maintain AF.25

Atrial tachyarrhythmias, including AF, are more common under pathological conditions, such as hypertrophy, hyperthyroidism, and heart failure, and these conditions result in a remodeling of Ca$^{2+}$-handling proteins and thus a modification of intracellular Ca$^{2+}$ dynamics.16 For example, heart failure is associated with upregulation of the Na$^+$–Ca$^{2+}$ exchanger and downregulation of SR Ca$^{2+}$–ATPase, causing an increase in the propensity for intracellular Ca$^{2+}$ overload.26 In addition, it has recently been reported that in failing hearts, RyR molecules are hyperphosphorylated.27 Hyperphosphorylation of RyR dissociates its stabilizing subunit, FKBP12.6, resulting in altered channel function (increased open probability and subconductance states).27 This is comparable to the action of low concentrations of ryanodine. It is reported that mutations in RyR result in catecholamine-sensitive tachyarrhythmias.28 However, there is no evidence at present for remodeling of intracellular Ca$^{2+}$ handling in PVMSs of AF patients, and therefore, it is uncertain whether the pacing-induced spontaneous activity observed in this study has any relationship to rapid the PV activity seen in AF patients.

References
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