ORIGINAL ARTICLE

Mechanical Characterization of Rabbit Pulmonary Vein Sleeves in *In Vitro* Intact Ring Preparation

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Background: Pulmonary vein (PV) sleeves, composed of cardiomyocytes, play certain roles in arrhythmogenesis. In the literature, it has been frequently reported that PV sleeves possess intrinsic spontaneous pacemaking activity and triggered activity in normal dogs and rabbits. In contrast, other research groups presented totally opposite findings which showed absence of such pacemakers in dogs, rabbits and rats. The present study was designed to clarify this puzzle and contradiction.

Methods: A novel methodology using *in vitro* experimentation was used to examine the electromechanical activity of whole segments of PV sleeves. The ring preparation was composed of a small piece of left atrial (LA) free wall, PV ostium and sleeve from rabbits. A circumferential contraction of the PV sleeve was measured when the preparation was electrically driven from the LA free wall. Mechanical force of the ring preparation was measured using a force transducer. The action potentials were recorded using conventional intracellular recording technique in strip preparation.

Results: In 15 rabbits, no spontaneous pacemaking activity or triggered activity was found in the *in vitro* ring preparation of PV sleeve. The circumferential contraction of PV sleeves was external calcium-dependent. Frequency-force relation displayed a negative staircase at 0.1–0.5 Hz and a positive staircase at 1–5 Hz. Post-rest potentiation was prominent between 15 s and 120 s. Intracellular action potential recording did not display any automaticity or triggered activity in PV sleeves.

Conclusion: In an intact ring preparation of rabbit PV sleeves, intrinsic spontaneous pacemaking activity or triggered activity was not found. [*J Chin Med Assoc* 2008;71(12):610–618]

Key Words: arrhythmias, automaticity, pulmonary veins, pulmonary vein sleeves, triggered activity

Introduction

Normal automaticity (also known as pacemaking or spontaneous activity) is typical for the sinoatrial node and atrioventricular node in the heart. Latent pacemakers, not regarded as abnormal, can be found in some atrial tissues and Purkinje fibers (also known as pseudotendon) and they will not manifest automaticity unless the normal driving impulses from the sinus node are suppressed. Abnormal automaticity, e.g. in ventricular tissues, can be seen only under pathophysiologic conditions such as ischemia, hypoxia, acidosis or stretch. The identification of pacemaker cells in the heart requires evidence from cellular electrophysiology, histology, immunocytochemistry, and molecular genetics.

Interestingly, pacemaker cells with spontaneous (or automatic) activity in cardiomyocytes in pulmonary vein (PV) sleeves have been enthusiastically found and documented by a particular research group since 2000.[1–12] Chen et al consistently observed pacemaking activity in normal healthy dog and rabbit PV sleeves. Amazingly, the incidence of automaticity being observed was always very high (Table 1[1–29]). Since 2000, after publishing numerous articles regarding the existence of automaticity

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Table 1. Discrepancies in the incidence of observing intrinsic pacemakers in pulmonary vein sleeves (reports from various independent research groups)

Lab	M & M	Pacemaker observed (%)	Lab	M & M	Pacemaker observed (%)
Chen et al[1]	Dogs: 48 tissues	71	Luk et al[13]	Dogs: 6 tissues	0
Chen et al[2]	Dogs: 60 cells	40	Luk et al[14]	Dogs: tissue	0
Chen et al[3]	Rabbits: 188 cells	51	Hocini et al[15]	Dogs: 35 tissues	0
Chen et al[4]	Rabbits: 156 cells	76	Wang et al[16]	Dogs: 50 tissues	0
Chen et al[5]	Rabbits: cells	60	Honjo et al[17]	Rabbits: 39 tissues	0
Chen et al[6]	Rabbits: 71 cells	65	Ehrlich et al[18]	Dogs: 83 cells	0
Chen et al[7]	Rabbits: 135 cells	100*	Miyauchi et al[19]	Rats: 14 tissues	0
Chen et al[8]	Rabbits: 21 tissues; 126 cells	55	Ehrlich et al[20]	Dogs: 40 cells	0
Wongcharoen et al[9]	Rabbits: 21 tissues; 98 cells	52	Wang et al[21]	Dogs: 80 tissues	0
Chang et al[10]	Rabbits: 35 tissues	22	Cha et al[22]	Dogs: 89 cells	0
Lee et al[11]	Rabbits: 177 cells	100*	Melnyk et al[23]	Dogs: 66 cells	0
Lo et al[12]	Rabbits: 34 tissues	49	Miyauchi et al[24]	Rats: 9 tissues	0
			Coutu et al[25]	Dogs: 56 cells	0
			Patterson et al[26]	Dogs: 43 tissues	0
			Patterson et al[27]	Dogs: 23 tissues	0
			Hirose et al[28]	Dogs: 9 tissues	0
			Maupoil et al[29]	Rats: 28 tissues	0
			Luk et al (this study)	Rabbits: 15 tissues	0

**Only automatic cardiomyocytes were used in the experiment. Tissues: conventional intracellular recording technique in multicellular preparations; cells: patch-clamp technique in isolated single cardiomyocytes. It is noted that spontaneous pacemaking activities in both tissues and cells were reported only by Chen et al.[1–12] Lab = laboratory; M & M = materials and methods.*

in dog and rabbit PV veins sleeves, Chen et al reached a definitive conclusion.[30–32] Namely, no matter what kind of experimental methodology (multicellular tissue preparations or isolated single cardiomyocytes) was used, they could always identify the automatic rhythm from PV sleeves under normal experimental conditions. Without knowing any possible implication of such normal automaticity in PV sleeves, their results have been regarded as a convenient explanation for the role of PV sleeves in arrhythmogenesis (e.g. atrial fibrillation).

PV sleeves were found as anatomic sites of arrhythmogenesis in patients with focal atrial fibrillation, according to the pioneering work of Haïssaguerre et al in 1998.[33] In addition to pharmacologic therapeutic modalities, trans-septal radiofrequency ablation and surgical ablation proved to be effective in rhythm conversion of atrial fibrillation. Although it has been found that PV sleeves are composed of complex muscle bundle geometry, the genuine physiologic or pathophysiologic roles of such sleeve regions in arrhythmogenesis, however, remain to be identified.

The major cellular mechanisms responsible for cardiac arrhythmias include triggered activity, abnormal automaticity and reentry.[34] The exact mechanisms for arrhythmogenesis in PV sleeves, however, remain to be explored. Our first preliminary studies found that, in normal healthy dogs and under normal experimental conditions, there was no spontaneous pacemaking activity in PV sleeves.[13,14] Whether the cardiomyocytes in PV sleeves that possess intrinsic spontaneous pacemaking activity is a fact or an artifact has become a prime controversy to be resolved. The present study, to our knowledge, is the first to use isolated intact ring preparation of rabbit PV sleeves. In such an *in vitro* experiment, we attempted to characterize the mechanical property and possible source of arrhythmogenesis in PV sleeves.

Methods

Ring preparations of PV sleeves

New Zealand rabbits (either sex, 4.1 ± 0.7 kg, $n = 15$) were used. Experiments were performed following national guidelines on animal care. Study approval was obtained from the Taichung Veterans General Hospital animal institutional review board before the experiments began.

The rabbits were anesthetized by intramuscular injection of pentobarbital (50 mg/kg). Ketamine (5 mg/kg,

intramuscular) was supplemented if the anesthesia depth was not adequate. Sternotomy and thoracotomy were performed while positive mechanical ventilation was applied. The pericardium was opened, and heparin (1,000 U/kg) was injected into the right atrial appendage. The heart and lungs were removed together from the thorax and immediately placed in a dissecting Petri dish filled with oxygenated normal Tyrode solution. The composition of normal Tyrode solution was as follows (in mM): NaCl, 130; $CaCl_2$, 2.7; KCl, 4; $MgCl_2$, 0.5; $NaHCO_3$, 20; glucose, 5. The pH of normal Tyrode solution was kept at 7.4 by bubbling with 95% O_2 and 5% CO_2. The temperature of the Tyrode solution was kept at 37°C.

The heart-lungs preparation was placed in the dish with the posterior side of the heart upwards. An incision line was made along the atrioventricular groove and the left auricle. The left superior PV ostium was clearly identified from the open left atrium. A strip of left atrial free wall (around 4 mm) attached to the intact PV ostium and sleeve (< 4 mm) was carefully dissected (Figure 1). The preparation was quickly mounted into a 2-mL tissue bath. The intrapulmonary part of the PV was completely dissected away. The tissue was oxygenated with superfusate of normal Tyrode solution with a flow rate of 5 mL/min.

Electrical stimuli to the tissue were applied via a bipolar platinum electrode and an isolation unit (S88; Grass Technologies, West Warwick, RI, USA). Point stimulation was composed of pulses as follows: 2 ms of duration, suprathreshold intensity, frequency at 1 Hz (except for study of frequency response). The intact PV sleeve was vertically hooked with an S-shaped wire pin,

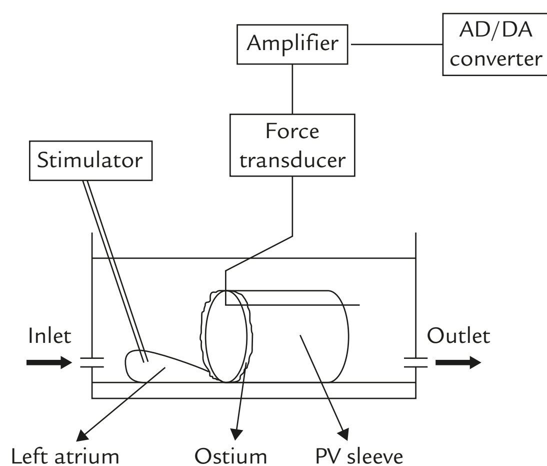

Figure 1. Experimental set-up of *in vitro* intact ring preparation of pulmonary vein (PV) sleeves. The preparation was composed of left atrium (LA) free wall–ostium-PV sleeves, superfused with Tyrode solution, and driven by electrical stimuli. The contractile force was measured by an S-hook, force transducer and amplifier.

and its contractile force was measured using a force transducer and an amplifier. In order to minimize the effect of horizontal contraction on the PV sleeve, the atrial tissue was carefully and extensively secured by insect pins. The analog signal was digitized and analyzed with Chart software version 5.0 (PowerLab Data Acquisition System, AD Instruments Inc., Colorado Springs, CO, USA). The force transducer was calibrated before each experiment. The baseline tension of the PV sleeve was adjusted to reach 90% of its maximal contractile force elicited by basal electrical stimuli. Experiments started after a stabilization period of 30 minutes and were performed at 37°C.

Action potential measurement was performed in two rabbit PV sleeves. The PV sleeve was opened and cut into a square piece, which was attached to a small piece of left atrial tissue and ostium. The stimulation protocol and measurement method were the same as previously reported.[13,14,16,21]

Data analyses and chemicals

The amplitude of contractile force of PV sleeves was measured and analyzed. Data are presented as mean ± standard deviation. Values were obtained from different rabbits. Statistically significant level of difference (α) was set at 0.05, using Wilcoxon signed-rank test. All the chemicals were purchased from Sigma-Aldrich Corporation (St Louis, MO, USA).

Results

In 15 rabbits, no spontaneous activity (pacemaker) was found. Every intact PV sleeve ring preparation was electrically driven by the impulses from the atrial free wall or inside the PV sleeve.

Extracellular calcium response

In 6 rabbits, elevation of extracellular calcium (from 2.7 mM to 5.4 mM and then 8.1 mM) increased the contractile force of the PV sleeve. Up to 8.1 mM, there was no sign of calcium overload or spontaneous contraction. The dose-response relationship of calcium to contractile force of PV sleeve is demonstrated in Figure 2. In comparison to 2.7 mM, external calcium concentrations of 5.4 mM and 8.1 mM increased contractile force to 209 ± 96% and 424 ± 321%, respectively ($n = 6$, $p = 0.028$).

Frequency-force relation

In 8 rabbits, frequency response (ranging from 0.1 Hz to 5 Hz) of electrical stimuli to contractile force of PV sleeves was examined. A typical response

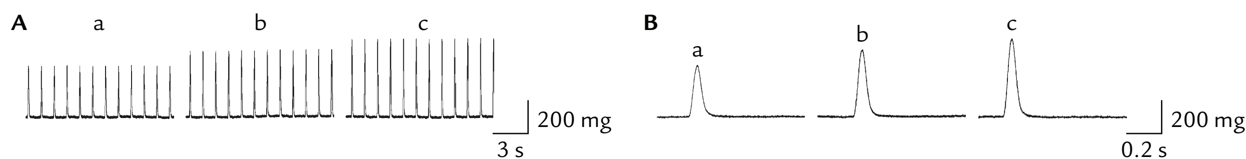

Figure 2. Effect of high external calcium on pulmonary vein (PV) sleeve contraction. The PV ring preparation was driven by 1 Hz of electrical stimuli from the LA free wall and superfused with normal Tyrode solution. (A) Slow sweep speed; (B) fast sweep speed. a = Ca 2.7 mM; b = Ca 5.4 mM; c = Ca 8.1 mM.

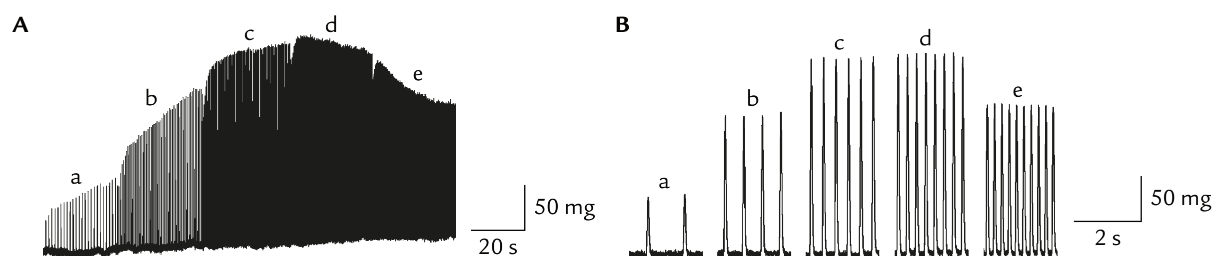

Figure 3. Effect of different pacing frequencies on pulmonary vein (PV) sleeve contraction. The PV ring preparation was driven by electrical stimuli from the LA free wall and superfused with normal Tyrode solution. (A) Slow sweep speed; (B) fast sweep speed. a = 1 Hz; b = 2 Hz; c = 3 Hz; d = 4 Hz; e = 5 Hz.

is demonstrated in Figure 3. A positive staircase phenomenon was shown from 1 Hz to 5 Hz. Calcium overload, hypoxia, and energy depletion was established at higher frequencies (4 Hz and 5Hz) and therefore reduced the contractile force (e.g. Figure 3E). It should be noted that 1-to-1 stimulus-contraction relation was sustained even at 5 Hz. In contrast, at very low frequency (from 0.1 Hz to 0.5 Hz), a negative staircase phenomenon was observed. It should be noted that no spontaneous pacemaking activity was observed from 0.01 Hz to 10 Hz. Statistical analysis of the results of frequency-force relation is depicted in Figure 4. In comparison to 1 Hz, the contractile forces at 2, 3, 4 and 5 Hz increased to 224 ± 63%, 336 ± 146%, 373 ± 188%, and 336 ± 189%, respectively ($n = 8$, $p = 0.012$).

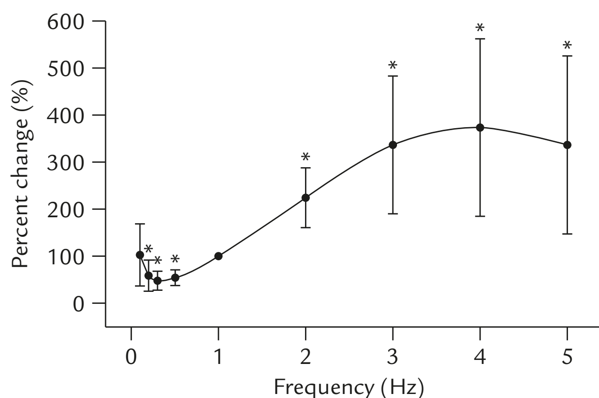

Figure 4. Plot of frequency-response of pulmonary vein (PV) sleeve contraction. The PV ring preparation was driven by electrical stimuli from the LA free wall and superfused with normal Tyrode solution. Frequency of electrical stimuli ranged from 0.1 Hz to 5 Hz. Data are expressed as mean ± standard deviation. $*p < 0.05$ *vs.* value at 1 Hz (as a control, 100%).

Post-rest potentiation

As shown in Figure 5, after a period of rest, the magnitude of the first electrical stimuli-elicited contraction was potentiated. The post-rest potentiation (PRS) reflected the extent of intracellular calcium in sarcoplasmic reticulum. The longer the rest period was, the larger the PRS was. Figure 6 demonstrates such an effect. After the steady state of driven contractions (SS) was obtained, the electrical stimuli were ceased for different periods of time. The ratio of PRS to SS was plotted against rest period (from 1 second to 2 minutes). PRS was significant when the rest period of time was longer than 15 seconds. The electrical quiescence decreased the steady-state contractile force. In addition, the longer the rest period was, the slower the recovery to steady-state contraction of PV sleeves was (Figure 7). In comparison to a rest of 1 second, the ratio of PRS to SS after rest of 30 seconds, 60 seconds, and 120 seconds increased to 219 ± 92%, 247 ± 109%, and 267 ± 126%, respectively ($n = 8$, $p = 0.012$, 0.012, and 0.017).

Electrical activity

When conventional intracellular recording technique was applied in 2 rabbit PV sleeves (in strip preparation), the recorded action potentials were fast-response action

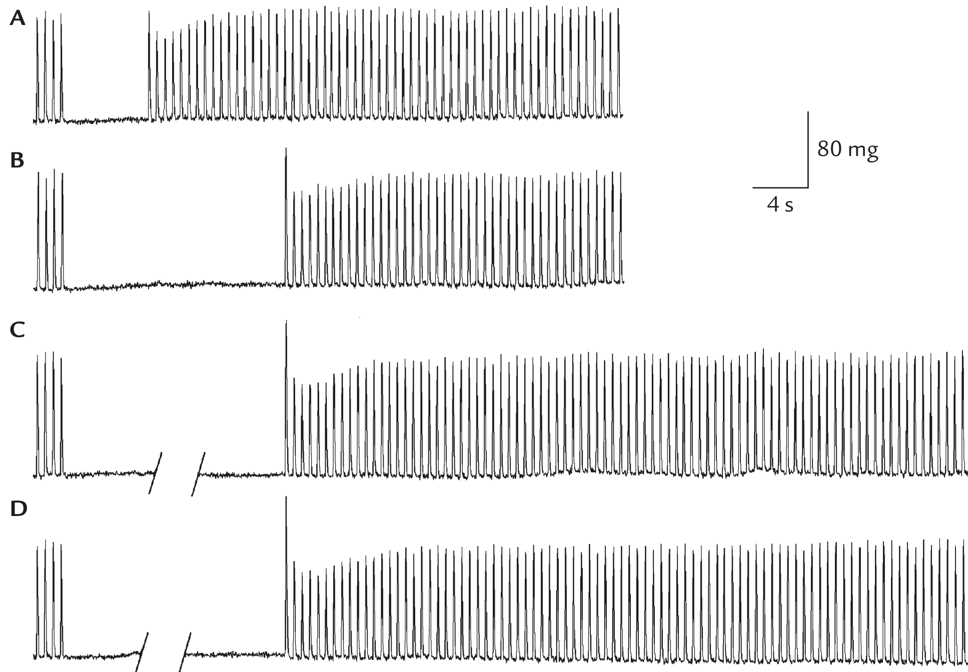

Figure 5. Post-rest potentiation of pulmonary vein (PV) sleeve contraction. The PV ring preparation was driven by electrical stimuli from the LA free wall and superfused with normal Tyrode solution. Basal frequency of electrical stimuli was 1 Hz. Stimuli were ceased for: (A) 5 seconds; (B) 15 seconds; (C) 30 seconds; (D) 60 seconds, and then reapplied. The double slash denotes omission of part of the records. It is noted that no spontaneous contraction was recorded during the rest period in all cases. The last four steady-state contractions before rest are presented. The first contraction after a period of rest was potentiated.

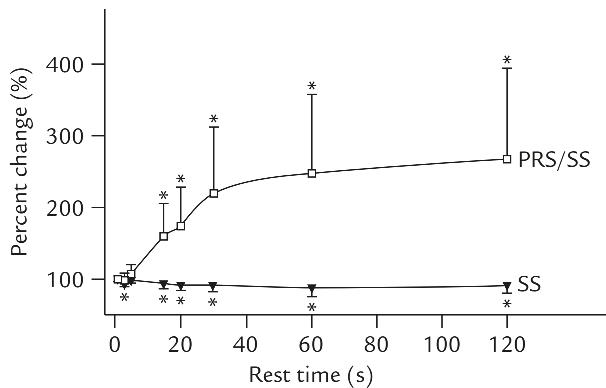

Figure 6. Relation between rest duration and post-rest potentiation of pulmonary vein (PV) sleeve contraction. The PV ring preparation was driven by electrical stimuli from the LA free wall and superfused with normal Tyrode solution. Basal frequency of electrical stimuli was 1 Hz. Stimuli were ceased for 1, 2, 3, 5, 10, 15, 20, 30, 60, and 120 seconds, respectively. Data are expressed as mean ± standard deviation. $*p < 0.05$ *vs.* value at rest period of 1 second (as a control, 100%). PRS = post-rest first contraction; SS = pre-rest steady-state contraction.

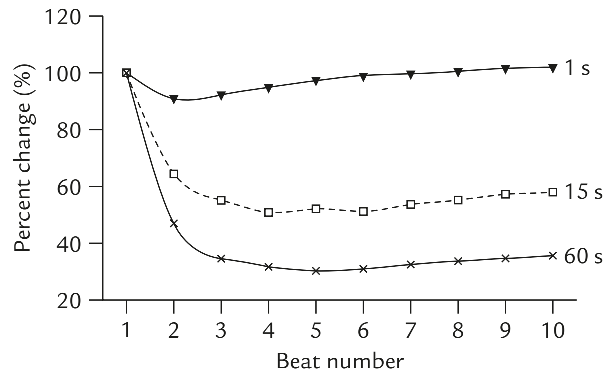

Figure 7. Relation between rest duration and post-rest force development of pulmonary vein (PV) sleeve contraction. The PV ring preparation was driven by electrical stimuli from the LA free wall and superfused with normal Tyrode solution. Basal frequency of electrical stimuli was 1 Hz. Stimuli were ceased for 1, 15, and 60 seconds, respectively. The contractile force of the first 10 beats after reapplied electrical stimuli is presented. The amplitude of the first post-rest contraction was used as control (100%). The longer the tissue rested, the longer was the recovery needed.

potentials with stable resting membrane potential. No spontaneous diastolic depolarizations were observed (Figure 8). All the action potentials were electrically driven with 1-to-1 relation from 1 Hz to 5 Hz. The action potential duration increased at 2 Hz and then progressively decreased from 3 Hz to 5 Hz. The plateau region of the action potentials was more prominent at 2 Hz and 3 Hz. When the driven stimuli were stopped,

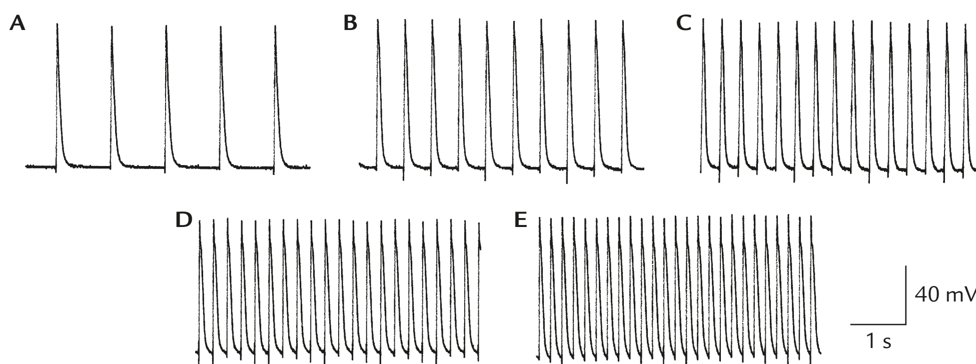

Figure 8. Action potentials of rabbit pulmonary vein (PV) sleeves at different frequencies of stimulation. The PV preparation was in rectangular strip and driven by electrical stimuli for 30 seconds at: (A) 1 Hz; (B) 2 Hz; (C) 3 Hz; (D) 4 Hz; (E) 5 Hz. Basal frequency of electrical stimuli was 1 Hz. There was no diastolic spontaneous depolarization. Every fast-response action potential was captured in 1-to-1 form.

the PV sleeves became electrically quiescent. No spontaneous pacemaking activity was observed.

Discussion

Absence of intrinsic pacemakers in PV sleeves

In the present study, we did not find any normal spontaneous pacemaking activity in PV sleeves from rabbits. Although Chen et al consistently reported a high incidence (from 22% to 76%) of intrinsic pacemaking activity in normal canine and rabbit PV sleeves,[1–12] such existence of normal automaticity in PV cardiomyocytes has never been reproducibly identified by other independent research groups.[13–29] As shown in Table 1,[1–29] we first reported in 2001 that, in multicellular tissue preparation, there were no sinoatrial nodal cell-like cells in canine PV sleeves.[13] Up to now, in more than 300 dogs, we have not found normal automaticity in PV sleeve tissues, either in proximal or distal PV sleeves. Such evidence from cellular cardiac electrophysiology was later supported by histologic evidence which showed no sinoatrial node-like cardiomyocytes in normal human PV sleeves. Although nodal cell-like cardiomyocytes in PV sleeves have been observed in very few human autopsy studies, it should be noted that those PV sleeves were from patients with atrial fibrillation. It then becomes a puzzle as to why Chen et al have consistently found such a high incidence of pacemaking cardiomyocytes in normal healthy dogs and rabbits,[1–12] while others have not.[13–29] Some researchers have proposed possible explanations for this contradiction, such as species difference (rats, rabbits, guinea pigs, dogs), different methodologies (tissues versus single isolated cells), different recording techniques (patch clamp techniques versus conventional intracellular recording system), and different temperature and composition of pipette and bath solutions. Unfortunately, none of them can adequately explain the contradictory findings.

Comparison of findings in literature

As clearly shown in Table 1,[1–29] Chen et al reported a high incidence of finding automatic cells in PV sleeves from dogs and rabbits.[1–12] Their experimental results were obtained from both multicellular tissue preparations and isolated single cells. This fact just contradicts the hypothesis, which suggested that automatic cells might be suppressed by surrounding normal cardiomyocytes with fast-response action potentials in multicellular (i.e. tissue preparation) PV sleeves. Once the automatic cells were enzymatically isolated from tissues, they manifested their intrinsic automaticity. Unfortunately, this hypothesis broke down because Chen et al consistently found normal automatic activity not only in isolated single cells[2–9,11] but also in multicellular preparations.[1,10,12] Although Chen et al consistently found automatic cells in canine PV sleeves,[1,2] others found fast-response action potentials with rather stable resting membrane potentials in dogs.[13–16,18,20–23,25–28] The species difference hypothesis has been proposed to explain the discrepancy. (Chen et al later shifted the study materials from dog to rabbit PV sleeves.) But even in rabbit tissue preparations, no automaticity has ever been observed in PV sleeves by other research groups.[17] The species difference hypothesis does not stand up either. Before discussing the possible artifacts made in a series of publications from Chen et al, several arguments should be noted. First, different "bizarre configurations" of the action potentials in PV sleeves were reported by Chen et al (Figure 1C;[1] Figures 2A and 7B;[2] Figures 2 and 3;[3] Figure 1A;[7] Figures 1A and 2B;[8] Figures 1C and 9B;[9] Figure 1[12]), but did not reappear

in their later articles. Second, "high-frequency rhythm" (e.g. Figure 3 in Reference 1) reported in canine PV sleeves was not reproduced in their later publications. Third, Chen et al did not explain why they could observe so many early and delayed after-depolarizations (and triggered activity) in PV sleeve cardiomyocytes from normal healthy dogs and rabbits. Fourth, if the rapid-pacing atrial fibrillation dog model was the reason for the high incidence of automatic cells (93% *vs.* 71% in normal control group) in tissue preparations from dog PV sleeves,[1] it would be very difficult to explain why they only observed fewer automatic cells (25% *vs.* 40% in normal control group) in isolated cardiomyocytes from dog PV sleeves.[2] Apparently, the effects of rapid pacing and cell isolation procedure produced ambiguity and inconsistency in the results. Fifth, it is difficult to explain why mapping or recording technique did not regularly detect any ectopic firing in normal healthy dogs and rabbits in *in vivo* condition if there were so many automatic cells in PV sleeves. Namely, there should be many normal healthy dogs and rabbits exhibiting atrial fibrillation if there are so many intrinsic automatic cells in their PV sleeves. Finally, if high incidence of intrinsic automatic cells in normal dog and rabbit PV sleeves (reported by Chen et al[1–12]) were not associated with ectopic firing and atrial fibrillation, it would be difficult to interpret the original pioneering work of Haïssaguerre et al,[33] who found ectopic foci in PV sleeves from patients with atrial fibrillation. Radiofrequency ablation of such ectopic foci could convert atrial fibrillation into sinus rhythm.

Possible artifact sources of intrinsic automaticity

The claim of high incidence of intrinsic automatic cells in normal dog and rabbit PV sleeves has lasted for more than 8 years since Chen et al first reported them in 2000.[1] Although the hypothesis of existence of automaticity in PV sleeves is a promising and welcome explanation for the role of PV sleeves in atrial fibrillation, it is difficult to interpret the role of intrinsic automatic cells in PV sleeves under normal physiologic conditions. It should be emphasized that, if the high incidence of automatic cells in PV sleeves reported by Chen et al were true,[1–12] other independent laboratories should have the same chance of obtaining the same results. Up to now, they have not.[13–29] Therefore, one should be careful in reexamining the electrophysiologic data reported by Chen et al in order to exclude any possibility of experimental artifacts.[1–12] One of the possible artifacts proposed by Chen et al is "unstable electrode impalement" while intracellularly recording the action potentials in PV sleeves. For example, the action potentials in Figures 3–7 in Reference 1 all showed fluctuations in membrane potentials due to unstable impalement. Such examples of poor quality of recording appeared everywhere in their publications.[1–4,7,11] It should be noted that "high-frequency irregular rhythm" (Figures 3–7 in Reference 1), a sign of unstable recording, was not reported again in their later publications.[2–12]

The second possible artifact is unsatisfactory experimental conditions, namely, stretch, ischemia and hypoxia. Cardiac myocytes are susceptible to hypoxic insults which cause depolarization, shortening of the action potentials and abnormal automaticity. In the articles from Chen et al, many such examples could be identified, e.g. Figure 2C,[1] Figure 2B,[2] Figure 2A,[4] and Figure 2.[10] Either a delay in harvesting the tissue preparations in the oxygenated superfusate or damaging the single myocytes during the isolation procedure could cause changes in the action potentials in PV sleeves. In addition, rescue of injured tissue preparation with Tyrode solution containing catecholamines and high extracellular potassium could cause similar hypoxic changes in the action potentials. In fact, we have demonstrated the effects of hypoxia, catecholamines, and high external potassium on PV sleeve cardiomyocytes. Namely, we could manipulate and reproduce the slow-response action potentials under pathological experimental conditions.[21]

The third artifact arose from inadequate voltage-clamping and current-clamping in the articles from Chen et al.[1–12] The quality of clamping was poor due to technical problems. Loss of clamping and oscillations appeared on many occasions, such as shown in Figures 2E and 3,[2] Figures 7 and 8,[7] Figure 8,[8] and Figure 5.[11] Again, due to inadequate clamping, what Chen et al described as triggered activity (early and delayed after-depolarizations) in PV sleeves is not convincing. Fluctuations and oscillations in membrane potentials and currents are noted everywhere, e.g. Figure 5,[3] Figure 8,[7] Figure 8,[8] and Figure 5.[11] The time course and the amplitude of DAD is not usual, for example, in Figure 2,[2] Figures 2 and 3,[3] Figure 3,[6] and Figure 2.[8] Up to now, only very few researchers have ever indicated discrepancies between the findings of Chen et al and others (Table 1[1–29]). Schram et al wrote, "a number of discrepancies make that study difficult to interpret…".[35] Ehrlich et al wrote, "the properties of PV currents reported by Chen et al showed some discrepancies from corresponding currents previously characterized in other systems".[18] Wit and Boyden wrote, "In our opinion, studies on automaticity and triggered activity in isolated myocytes should be repeated by other laboratories."[34] To explain the discrepancy by

stating "electrotonic inhibition of pacemaking cells by non-pacemaking cells may occur in tissues and not in isolated myocytes" might not be helpful,[34] because Chen et al also found tremendous numbers of automatic cells in tissue preparations of PV sleeves in both dogs and rabbits.[1,10,12]

Characteristics of PV sleeves in ring preparation

To our knowledge, we are the first to use *in vitro* intact ring preparation of rabbit PV sleeve to study its role in arrhythmogenesis. The main advantage of such methodology is to keep the whole segment of PV sleeve intact and to record the electromechanical activity of the PV sleeve more physiologically. It should be mentioned here that PV sleeves are composed of complex musculature which then provides substrates for arrhythmogenesis. In conventional rectangular strip preparation, any existing intrinsic pacemakers in the PV sleeve might not be preserved during dissection of the tissue. On the other hand, if we cannot record any ectopic or spontaneous mechanical activity in *in vitro* intact ring preparation, it would be appropriate to conclude that there is a lack of intrinsic automatic cells in normal rabbit PV sleeves. In the present study, as expected, we did not find any spontaneous pacemaking activity in PV sleeves from 15 rabbits. The contraction of *in vitro* ring preparation of PV sleeves is characterized by calcium-response relation (Figure 2), frequency-force relation (Figures 3 and 4), and PRS (Figures 5, 6 and 7). The contractile force of *in vitro* ring preparation of PV sleeves is dependent on external calcium concentration (i.e. L-type calcium current) and intracellular calcium dynamic handling (i.e. SR calcium reuptake and Na-Ca exchanger). It should be noted that frequency-force relation displayed a positive staircase phenomenon from 1 Hz to 5 Hz. At high frequencies, the contractile force might decrease due to hypoxia and energy depletion. From 0.1 Hz to 0.5 Hz, however, a negative staircase phenomenon was shown. It should be stressed that no spontaneous activity was found even at very low stimulation frequency of 0.01 Hz. In the PRS protocol, the rest time (without electrical stimuli) varied from 1 second to 2 minutes. Even during electrical silence up to 4 minutes, spontaneous activity was not found. Absence of intrinsic pacemaking activity in PV sleeves is confirmed by intracellular recording of the action potentials (Figure 8). Fast-response action potentials were elicited by electrical stimuli at 1–5 Hz. No spontaneous diastolic depolarizations were observed. This is consistent with previous findings in tissue preparations of PV sleeves in rabbits[17] and dogs.[15,16,21,26–28]

Study limitations

Although *in vitro* intact ring preparation of rabbit PV sleeves is a novel methodology with advantages, it has some limitations. First, such a preparation was composed of a small area of LA free wall proximal to the PV ostium. This piece of LA free wall was used to receive the electrical stimuli from a bipolar electrode. Despite the direction of impulse conduction from the LA free wall to the distal end of the PV sleeve being of physiological significance, ectopic impulse (if there is any) in the LA free wall could be conducted into PV sleeves and cause premature or spontaneous contraction. Therefore, it would be difficult to differentiate the origin of premature contraction from LA free wall or PV sleeves. Fortunately, under normal experimental conditions, no spontaneous activity was ever observed. Second, the contractile force of ring preparation of PV sleeves was measured using S-hook and a force transducer in a vertical way (Figure 1). Although the ring preparation was secured with many insect pins, its contraction could not be completely excluded from the effect of horizontal contraction of the LA free wall. To solve this problem, we dissected the LA free wall from the PV sleeve and identified the contractile force of isolated PV sleeves to be 40–80 mg. It means that if the contraction of ring preparation of PV sleeves is not contaminated by that of the LA free wall, the contractile force should not be larger than 100 mg. Meticulously securing the tissue preparation with pins could lessen the problems from horizontal contraction. Third, our recording system of contractile force reflected only the circumferential contraction (more physiological), not that from longitudinal contraction. Since the goal of our study was to observe the rhythm of contraction, the force amplitude itself was not under consideration. Last, the sensitivity to detect any ectopic or spontaneous activity was limited by the amplitude of contractile force. Namely, any miniature contraction elicited by ectopic impulse might be underestimated. Fortunately, intracellular recording of the action potentials showed no such spontaneous action potentials (Figure 8).

In conclusion, using a novel methodology of measuring the contractile force of *in vitro* intact ring preparation of PV sleeves, we could not find any intrinsic spontaneous pacemaking activity in normal rabbits under normal experimental conditions.

Acknowledgments

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