Full Paper

Electrophysiological and Pharmacological Characteristics of Triggered Activity Elicited in Guinea-Pig Pulmonary Vein Myocardium

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Abstract. The pulmonary vein is known as an important source of ectopic beats, initiating frequent paroxysms of atrial fibrillation. We analyzed electrophysiological and pharmacological characteristics of triggered activity elicited in the isolated pulmonary vein from the guinea pig. Immediately after the termination of train stimulation (pacing cycle length of 100 ms), spontaneous activities accompanied with phase-4 depolarization were detected in 43 out of 45 pulmonary vein preparations. Such triggered activities were not observed in the isolated left atrium. The incidence of triggered activity was higher at a shorter pacing cycle length (100 – 200 ms), and the coupling interval was shorter at a shorter pacing cycle length. Verapamil (1 μ M), ryanodine (0.1 μ M), and pilsicainide (10 μ M) suppressed the occurrence of triggered activities. The resting membrane potential of the pulmonary vein myocardium was more positive than that of the left atrium. Carbachol (0.3 μ M) hyperpolarized the resting membrane potential and completely inhibited the occurrence of triggered activities. These results suggest that the pulmonary veins have more arrhythmogenic features than the left atrium, possibly through lower resting membrane potential. The electrophysiological and pharmacological characteristics of triggered activity elicited in the pulmonary vein myocardium were similar to those previously reported using ventricular tissues.

Keywords: triggered activity, pulmonary vein, left atrium, membrane potential, carbachol

Introduction

Atrial fibrillation is known as the most common cardiac arrhythmia in adults, which is a major cause of stroke (1). Whereas the arrhythmia has been recognized to be perpetuated by reentrant wavelets propagating in an abnormal atrial-tissue substrate, Haïssaguerre et al. found in 1998 that the origin of atrial ectopic beats was localized in the pulmonary vein myocardial sleeve of patients with drug-resistant atrial fibrillation (2). Cheung reported in 1980 that isolated pulmonary vein preparations from guinea pigs were capable of independent pace-making activity (3). Recently, electrophysiological characteristics of the pulmonary vein have been extensively analyzed in isolated rabbit or dog preparations, which show that the combination of reentrant and non-reentrant mechanisms is the underlying arrhythmogenic mechanisms of atrial

fibrillation from the pulmonary veins (4 – 6).

Triggered activity is one of the well-recognized mechanisms of ectopy aggravated by an increased rate of beating (7 – 9). Tactics to raise the intracellular Ca^{2+} concentration of the ventricular tissues, such as treatment with digitalis or a low K^+ / high Ca^{2+} extracellular environment, causes an oscillatory Ca^{2+} release from the sarcoplasmic reticulum and transient depolarization after completion of ventricular repolarization (10). The delayed afterdepolarization (DAD) and resulting triggered activity have been shown to be effectively suppressed by Na^+ -channel blockers, Ca^{2+} -channel blockers, and ryanodine in isolated ventricular preparations (11, 12). DAD-related triggered activity has been demonstrated in isolated pulmonary vein myocardium or its isolated cardiomyocytes (13, 14). However, fundamental electrophysiological characteristics of triggered activity have never been examined, such as the relationship between pacing rate to induce triggered activity and its incidence or coupling interval (10). In this study, to better understand the arrhythmogenic activity of the pulmonary vein

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itself, we analyzed electrophysiological and pharmacological characteristics of the triggered activity in isolated guinea-pig pulmonary vein. Triggered activity was induced by train stimulation, which is known as a useful methodology to reproducibly induce DAD-related triggered activity (10, 12, 15).

Materials and Methods

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. The heart and adjunct lungs were isolated from male or female Hartley guinea pigs weighing 350–450 g. The pulmonary veins were separated from the left atrium and lung at the end of the pulmonary vein myocardium sleeve in Krebs-Henseleit solution of the following composition: 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, gassed with 95% O₂ / 5% CO₂ (pH 7.4 at 37°C).

Histological examinations

Pulmonary veins were fixed with 10% formalin neutral buffer solution, and the segments were processed into paraffin blocks. The paraffinized tissue blocks were cut into 4- μ m-thick sections and mounted on charged slides. For each paraffin block, one slide each was stained with Masson trichrome to accentuate muscle and connective tissues. A serial section was incubated with antibodies against α -smooth muscle actin (α -SMA, 1:500; Dako, Glostrup, Denmark) followed by consecutive incubations with universal immuno-peroxydase polymer (Histofine[®], Simple Stain Rat MAX PO MULTI; Nichirei Bioscience, Tokyo). Antibody binding was demonstrated by staining with 3,3'-diaminobenzidine tetrahydrochloride.

Microelectrode recording of action potential configuration

The luminal side of the pulmonary vein at the middle region between the ostium and the distal end of myocardial sleeve or endocardial surface of the left atrium was impaled with glass microelectrodes filled with 3 M KCl to record transmembrane potential using a microelectrode amplifier (Intra 767; World Precision Instruments, Sarasota, FL, USA). The action potential signals were monitored by an oscilloscope (CS-5135; Kenwood, Tokyo) and fed into a waveform analysis system (DSS98-type IV, from Canopus, Tokyo or PowerLab, from ADInstruments, Castle Hill, Australia). All experiments were performed at $36.5 \pm 0.5^\circ\text{C}$.

Triggered activity was induced by a burst pacing of 100 train pulses at a pacing cycle length of 100, 150, or 200 ms using an electronic stimulator (SEN-2201; Nihon Kohden, Tokyo) with rectangular current pulses (3-ms duration, about 1.5 threshold) through bipolar platinum electrodes. Action potential parameters, including resting potential (RP), overshoot (OS), and action potential duration at 20% (APD₂₀), 50% (APD₅₀), and 90% (APD₉₀) repolarization, were measured under electrical stimulation at a constant frequency of 1 Hz. The action potentials of the pulmonary vein were of the fast-response type, and neither early afterdepolarizations (EADs) nor DADs were observed in the preparations electrically driven at 1 Hz.

Drugs

Verapamil hydrochloride and carbachol were purchased from Sigma-Aldrich (St. Louis, MO, USA), and ryanodine was obtained from Wako (Osaka). Pilsicainide hydrochloride was kindly provided by Daiichi-Sankyo Co., Ltd. (Tokyo). Ryanodine was initially dissolved in dimethylsulfoxide and diluted to 0.01% dimethylsulfoxide in the Krebs-Henseleit solution. Other drugs were dissolved in distilled water and small aliquots were added to the organ bath to obtain the desired final concentration. All other chemicals were commercial products of the highest available quality.

Statistical analyses

Statistical significance between means was evaluated by the one-way repeated measures analysis of variance followed by Contrasts for mean values comparison or by Dunnett's test. A *P*-value less than 0.05 was considered significant.

Results

Histology of the pulmonary vein

Typical photomicrographs of longitudinal sections of the left superior pulmonary vein obtained from the guinea pig are shown in Fig. 1. Vascular smooth muscle was detected on the luminal face of the pulmonary vein, whereas a myocardial sleeve was observed at mid-layer of the pulmonary vein.

Triggered activities in the pulmonary vein

Figure 2A shows a typical tracing of burst pacing-induced triggered activity in the pulmonary vein preparation. After the train stimulation at a pacing cycle length of 100 ms, spontaneous activities accompanied with phase-4 depolarization were detected in 43 out of 45 pulmonary vein preparations. On the other hand, phase-4 depolarization was not detected in the left atrium prepa-

rations examined (n = 8). As shown in the Table 1, the incidence of triggered activity and number of premature beats were greater after the faster train stimulation. The coupling interval decreased in a frequency-dependent manner.

Effects of pharmacological intervention on the triggered activity

At the pre-drug period, the number of triggered activity, which was induced after the 100-train stimulation at a pacing cycle length of 100 ms, was 2.4 ± 0.2 in the control group, 2.6 ± 0.9 in the pilsicainide group, 2.4 ± 0.7 in the ryanodine group, and 2.4 ± 0.4 in the verapamil group. There were no significant differences

Fig. 1. Photomicrographs of longitudinal sections of the left superior pulmonary vein of the guinea pig. Upper panel: masson trichrome staining. Lower panel: immunostaining for α -smooth muscle actin (α -SMA).

Fig. 2. Delayed afterdepolarization (DAD)-related triggered activity induced by train stimulation. **A:** A typical tracing of DAD-related triggered activity recorded from the pulmonary vein myocardium after the train stimulation at a pacing cycle length of 100 ms. Two premature beats were detected in this preparation. The DAD-related triggered activity was not observed in the left atrium. **B:** Summary of the effects of pilsicainide, verapamil, and ryanodine on the number of premature beats in the pulmonary vein preparation. Train stimulation (CL = 100 ms) was applied to the preparation before (C) and at 1, 3, 5, 7, 10, and 12 min after the drug administration. Data are means $\pm$ S.E.M. Pilsicainide: $10 \mu\text{M}$ (n = 6), verapamil: $1 \mu\text{M}$ (n = 5), ryanodine: $0.1 \mu\text{M}$ (n = 5), and control (n = 5). Closed symbols represent significant differences from the corresponding pre-drug values (C) at $P < 0.05$. CL: cycle length.

Table 1. Frequency-dependent induction of the triggered activity in pulmonary vein

Pacing cycle length	Pulmonary vein			Left atrium
	200 ms	150 ms	100 ms	100 ms
Incidence	18/45 (40.0%)	33/45 (73.3%)	43/45 (95.6%)	0/8 (0%)
Number of premature beats	0.7 ± 0.1	$1.4 \pm 0.2^*$	$2.1 \pm 0.2^*$	0 ± 0
Coupling interval (ms)	613 ± 24	564 ± 18	$490 \pm 16^*$	—

Data are means $\pm$ S.E.M. $^*P < 0.05$, compared with the corresponding values at a pacing cycle length of 200 ms.

among the groups. The number of triggered activity was unchanged during the observation period in the absence of drugs (control), whereas it was significantly decreased by pilsicainide at 10 μ M, verapamil at 1 μ M, and ryanodine at 0.1 μ M, as shown in the Fig. 2B.

Effects of carbachol on the triggered activity

At the pre-drug period, the number of triggered activity was 2.2 ± 0.5 in the carbachol group. Carbachol at 0.3 μ M decreased the number of triggered activity, as shown in the Fig. 3. The same concentration of carbachol shortened the action potential duration of the left atrium electrically driven at 1 Hz without affecting the resting membrane potential, as shown in Table 2. On the other hand, carbachol shortened the action potential duration of the pulmonary vein myocardium together with significant hyperpolarizing effects on the resting membrane potential.

Discussion

In this study, we investigated the inducibility of triggered activity in the guinea-pig pulmonary vein myocardium, which is distributed at the mid-layer of the pulmonary vein tissue, as shown in Fig. 1. The shorter pacing cycle length (100 ms) of train stimulation provoked triggered activity in the pulmonary vein preparation, whereas such phenomenon was not observed in the left atrial preparations (Fig. 2A). In previous studies using isolated ventricular tissues, triggered activity is often induced by train stimulation in the presence of intracellular Ca^{2+} overload by using cardiac glycoside or low K^+ / high Ca^{2+} extracellular solution (10, 12, 15). In this study, however, the triggered activity could be induced in the pulmonary vein preparation under the normal experimental condition consisting of a standard physiological solution without cardiac glycoside, as shown in Fig. 2A. These results suggest that the pulmonary vein prepara-

Fig. 3. Effects of carbachol on the DAD-related triggered activity elicited in the pulmonary vein preparation. A: Typical tracings of effects of carbachol on the DAD potentials after the train stimulation at a pacing cycle length of 100 ms. B: Summary of the effects of carbachol (0.3 μ M, $n = 5$) on the number of premature beats. Train stimulation (CL = 100 ms) was applied to the preparation before (C) and at 1, 3, 5, 7, 10, and 12 min after the drug administration. Data are means $\pm$ S.E.M. Closed symbols represent the significant differences from corresponding pre-drug values (C) by $P < 0.05$.

Table 2. Effects of carbachol (0.3 μ M) on the action potential parameters of the pulmonary vein and left atrium

	Pulmonary vein		Left atrium	
	Before	Carbachol	Before	Carbachol
RP (mV)	-70.3 ± 1.0	$-75.4 \pm 0.9^*$	-80.6 ± 1.1	-80.0 ± 0.6
OS (mV)	27.9 ± 2.3	26.4 ± 1.4	27.1 ± 0.8	$18.2 \pm 1.4^*$
APD ₂₀ (ms)	13.2 ± 0.6	$9.8 \pm 0.5^*$	23.3 ± 1.2	$12.7 \pm 0.8^*$
APD ₅₀ (ms)	28.0 ± 1.9	$18.9 \pm 1.1^*$	40.7 ± 1.6	$21.3 \pm 1.1^*$
APD ₉₀ (ms)	75.5 ± 2.5	$57.6 \pm 3.5^*$	76.1 ± 2.7	$45.5 \pm 1.9^*$

Data are means $\pm$ S.E.M. of 5 experiments. The preparations were electrically driven at 1 Hz. Resting potential (RP); overshoot (OS); action potential duration at 20% (APD₂₀), 50% (APD₅₀), and 90% (APD₉₀) repolarization.

* $P < 0.05$, compared with the corresponding control values (Before).

tion has more arrhythmogenic characteristics than the ventricular tissues and the left atria of this study.

Next, we analyzed electrophysiological and pharmacological characteristics of triggered activity elicited in the guinea-pig pulmonary vein myocardium. The higher incidence of triggered activity and the shortening of coupling interval were detected in a frequency-dependent manner, as summarized in Table 1, which is in accordance with earlier literature demonstrating typical electrophysiological features of the DAD-related triggered activity in the ventricular tissues (10). As shown in the Fig. 2B, optimum concentrations of pilsicainide and verapamil (15, 16) effectively inhibited the occurrence of triggered activity in the pulmonary vein preparation, suggesting that inhibition of Na^+ and Ca^{2+} influx through voltage-dependent channels might relieve Ca^{2+} accumulation in the pulmonary vein cardiomyocytes induced by train stimulation. In this study, ryanodine at $0.1 \mu\text{M}$, which suppressed ouabain-induced excitability in the guinea-pig pulmonary vein (17), effectively inhibited occurrence of train stimulation-induced triggered activity. On the other hand, Honjo et al. have demonstrated that $0.5 - 2 \mu\text{M}$ of ryanodine promoted occurrence of triggered activity in rabbit pulmonary vein preparations (13). Ryanodine has been known to have a unique pharmacological profile that can either stimulate or inhibit Ca^{2+} release, depending on the experimental conditions including its concentration or incubation time (18). Taken together, these observations at least suggest that generation of the train stimulation-induced triggered activity is closely associated with oscillatory Ca^{2+} release from the sarcoplasmic reticulum, leading to transient depolarization after completion of ventricular repolarization (10). Each of the drugs used in this study has been demonstrated to suppress train stimulation-induced triggered activity in the canine ventricular tissues intoxicated with cardiac glycoside (12). Therefore, these results suggest that electrophysiological and pharmacological characteristics of triggered activity elicited in the pulmonary vein myocardium were fundamentally similar to those previously reported in the ventricular tissues (10, 12), which may be important information on the therapeutic strategy for the triggered activity elicited in the pulmonary vein myocardium.

It is known that the pulmonary vein as well as the atrium is densely innervated by the autonomic nerves (19, 20), and parasympathetic nerve activation usually encourages reentry via shortening of the effective refractory period, acting as a maintenance factor of atrial fibrillation. Indeed, in the canine model of vagally induced atrial fibrillation, ablation of the autonomic ganglia near the ostia of pulmonary vein prevented atrial fibrillation, showing the importance of cholinergic activation in the

pulmonary veins for this type of atrial fibrillation (21). On the other hand, information is still limited regarding the influence of muscarinic receptor activation on the occurrence of triggered activity, as an initiation factor of atrial fibrillation. As shown in Fig. 3, application of carbachol to the pulmonary vein preparation hyperpolarized the resting membrane potential and potentially suppressed the train stimulation-induced triggered activity. These results imply that parasympathetic nerve activation may act differently on atrial fibrillation, depending on the type or underlying mechanisms of atrial fibrillation.

In this study, triggered activity could be induced by train stimulation in the pulmonary vein preparation under the normal experimental condition, whereas ventricular cells usually exhibit triggered activity under pathological conditions (10, 12, 15). Previous studies using the isolated cardiomyocytes from the pulmonary vein myocardium have demonstrated that the density of inward rectifier current (I_{K1}) was about half of that in the left atrial cells, which is potentially accounting for the less negative resting membrane potentials in the pulmonary vein myocardium (22, 23). As shown in Table 2, application of carbachol to the pulmonary vein myocardium hyperpolarized the membrane potential through increment of G protein-activated inward-rectifier currents (24), which might contribute a decrease of cell excitability, leading to counteraction of the train stimulation-induced triggered activity (Fig. 3). Thus, it is speculated that arrhythmogenicity of the pulmonary vein myocardium is closely associated with its lower resting membrane potential, which may explain the induction of triggered activity under the normal experimental condition.

In conclusion, these results suggest that the pulmonary vein has more arrhythmogenic features than the left atrium possibly through lower resting membrane potential. The electrophysiological and pharmacological characteristics of triggered activity elicited in the pulmonary vein myocardium were fundamentally similar to those previously reported using the ventricular tissues.

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