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Mini-Review

Electropharmacological properties of the pulmonary vein myocardium

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ABSTRACT

The pulmonary vein myocardium is receiving attention as the source of ectopic electrical activity underlying atrial fibrillation. Electrophysiological and pharmacological analysis in various experimental animal species have revealed the characteristics of the pulmonary vein myocardium such as lower repolarizing capacity and presence of intracellular Ca^{2+} oscillations. Pulmonary vein automaticity is affected by various neurohumoral substances and pharmacological agents. Studies on the mechanisms and regulation of pulmonary vein automaticity would lead to the development of novel therapeutic strategies for atrial fibrillation.

KEYWORDS: pulmonary vein myocardium, automaticity, intracellular Ca^{2+}

INTRODUCTION

The pulmonary vein is the blood vessel for the return of blood flow from the lung to the heart. Spontaneous pulsation of the pulmonary vein independent of the main body of the heart has been observed in the late 19th century [1]. The pulmonary vein contains a myocardial layer, which is a continuation from the left atrial myocardium and is capable of generating spontaneous or triggered action potentials [2, 3]. It was clinically reported that paroxysmal atrial fibrillation is initiated by trains of rapid discharges from the pulmonary veins [4, 5]. Since then, the electrical activity of the pulmonary vein myocardium is considered to play a central role in the generation and maintenance of atrial fibrillation, the most common type of arrhythmia in clinical practice. The pulmonary vein myocardium layer is composed of circumferential and parallel longitudinal fibers, which produce non-uniform anisotropy and discontinuities [6-9]. This provides a histological basis for micro reentry. The length and thickness of the myocardial sleeve in the pulmonary vein appeared to correlate with the patient's history of atrial fibrillation [10]. The pulmonary vein receives both sympathetic and parasympathetic innervation [9, 11], which are considered to play important roles in the generation of atrial fibrillation through its effects on the electrophysiological properties of the pulmonary vein cardiomyocytes. In this short review, we will summarize the characteristics of spontaneous and induced electrical activity in the pulmonary vein myocardium as revealed by electrophysiological and Ca^{2+} imaging analyses.

Basic action potential and membrane current properties

The action potential parameters and ionic current properties of the canine pulmonary vein was studied by standard microelectrode and voltage clamp experiments, and compared with the atrial myocardia [12]. The pulmonary vein myocardium had a lower resting membrane potential, which could be explained by a lower density of the inwardly rectifying K^+ current (I_{K1}). The upstroke velocity of the rapid depolarization phase of the

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pulmonary vein myocardium was less than half of that in the atria. As the density of the sodium current was not different between the two regions, the difference in upstroke velocity was probably the result of larger degree of voltage-dependent inactivation of the channel due to less negative resting membrane potential. The action potential duration was shorter in the pulmonary vein myocardium which could be explained by the lower density of L-type Ca^{2+} current and a larger density of the delayed rectifier K^+ currents (I_{Kr} and I_{Ks}). The difference in membrane current densities between the canine pulmonary vein and atrial myocardia was supported by western blot and immunohistochemical analyses [13].

Among the isolated cardiomyocytes from rabbit pulmonary vein, some show spontaneous pacemaker activity while others do not [14]. The cardiomyocytes showing pacemaker activity had a lower density of the inwardly rectifying K^+ current (I_{K1}), which appeared to allow spontaneous depolarization and pacemaking [14]. The densities of the hyperpolarization activated inward current (I_f) and the T-type Ca^{2+} current, which can probably contribute to spontaneous depolarization, were larger in pacemaking cells than in non-pacemaking cells [15, 16]. The density of the delayed rectifier K^+ current (I_K) was also larger, while that of the transient outward current (I_{to}) was smaller in pacemaking cells than in non-pacemaking cells [16]. No difference in the L-type Ca^{2+} current density was observed [14].

Microelectrode impalement of the guinea-pig pulmonary vein myocardium was conducted as early as in the 1960s [2]. The pulmonary vein myocardium had a less negative resting membrane potential, smaller maximum upstroke velocity and amplitude of action potential, and shorter action potential duration than the atrial myocardium; these differences were more prominent in the distal than in the proximal myocardium. The difference in action potential upstroke velocity of the three regions correlated with the action potential conduction velocity, as revealed by double microelectrode measurements. These characteristics of the action potential parameters in the guinea-pig pulmonary vein myocardium were confirmed by other researchers including ourselves [3, 17].

Intracellular Ca^{2+} oscillations and automaticity

There is increasing evidence that intracellular Ca^{2+} is involved in the generation of electrical activity in pulmonary vein cardiomyocytes. In case of the rabbit, pulmonary vein cardiomyocytes with pacemaker activity had higher diastolic Ca^{2+} concentrations [18] and higher Ca^{2+} spark incidence and amplitude [19] than those without pacemaker activity. The pacemaking pulmonary vein cardiomyocytes had a higher incidence of spindle/bifurcated morphology than those from non-pacemaking pulmonary vein cardiomyocytes and atrial cardiomyocytes [20]. Application of ryanodine to atrial preparations including the sinus node and the pulmonary vein ostium, induced a shift in the leading pacemaker from the sinus node to an ectopic focus near the right pulmonary vein-atrial junction [21]. In the pulmonary vein myocardium, 0.5 μM ryanodine, which probably caused an increase in cytoplasmic Ca^{2+} concentration, resulted in appearance of diastolic depolarization. Application of rapid pacing under such condition resulted in generation of automatic electrical activity, which was inhibited by cyclopiazonic acid, an inhibitor of sarcoplasmic reticulum Ca^{2+} pump [21]. The automatic activity of the rabbit pulmonary vein myocardium was inhibited by KB-R7943 [22] and K201 [23], compounds with inhibitory action on multiple ion channels including the Na^+-Ca^{2+} exchanger and sarcoplasmic reticulum Ca^{2+} release channel, respectively.

In the isolated guinea-pig pulmonary vein myocardium, microelectrode experiments revealed the presence of spontaneous electrical activity in about half of the tissue preparations [17, 24]. In quiescent preparations, interventions which increase intracellular Ca^{2+} load such as ouabain [17] or high-frequency pacing [25] induced automatic electrical activity. The spontaneous as well as pacing-induced electrical activity was completely inhibited by carbachol, which increases the repolarizing K^+ current density through activation of the acetylcholine- activated K^+ current. In ouabain-treated preparations, generation of action potentials was preceded by an oscillation of the resting membrane potential, which suggests the occurrence of intracellular Ca^{2+} oscillations. Fluorescence imaging of intracellular Ca^{2+} in

pulmonary vein cardiomyocytes revealed that this was indeed the case; ouabain induced an increase in intracellular diastolic Ca^{2+} concentration and Ca^{2+} waves and Ca^{2+} sparks preceding the generation of Ca^{2+} transients [17]. SEA0400, a highly selective inhibitor of the Na^{+}-Ca^{2+} exchanger [26], inhibited the automatic electrical activity without affecting the resting intracellular Ca^{2+} concentration or the Ca^{2+} oscillations. Inhibition of intracellular Ca^{2+} oscillations by ryanodine completely inhibited the automatic electrical activity.

These results obtained in the rabbit and guinea pig suggest the involvement of intracellular Ca^{2+} in the generation of automatic action potentials in the pulmonary vein myocardium [17]. Elevated intracellular Ca^{2+} concentration, either uniform elevation throughout the cytoplasm or localized elevation in the form of Ca^{2+} sparks and Ca^{2+} waves, activates the forward-mode Na^{+}-Ca^{2+} exchanger which extrudes Ca^{2+} from the cytoplasm and generates an inward current and slowly depolarizes the cell membrane. This diastolic depolarization drives the membrane potential to reach the threshold level and generates automatic action potentials. As the functional components of this mechanism are present not only in pulmonary vein cardiomyocytes but also in atrial and ventricular cardiomyoyctes, the question arises, why does such ectopic pacemaking occur only in the pulmonary vein myocardium? One possibility is that the Ca^{2+} handling properties of the pulmonary vein myocardium is different from those of the working myocardium. Observations in canine pulmonary vein cardiomyocyte suggest that this may not be the case [27]. Cardiomyocytes from the pulmonary vein and atrium from dogs subjected to 7-day rapid pacing were not different in their Ca^{2+} transients amplitude, half-decay time of, beat-to-beat regularity, propensity to alternans and β-adrenergic influence. Incidence of Ca^{2+} sparks by under Ca^{2+} loading and caffeine-induced Ca^{2+} transient amplitudes were also not different. These results do not support the hypothesis that intrinsic Ca^{2+} handling differences account for the occurrence of ectopic pacemaking only in the pulmonary vein myocardium. It rather appears that the difference in repolarizing capacity between regions is the underlying mechanism. The density of the inwardly rectifying K^{+} current (I_{K1}), the major current to maintain the resting membrane potential, was significantly smaller in pulmonary vein cardiomyocytes than in atrial cardiomyocytes [12], and in pacemaking pulmonary vein cardiomyocytes than in non-pacemaking [14]. That the electrical activity induced by rapid pacing (triggered activity) was completely inhibited by carbachol [25] is also consistent with this view. The rabbit pulmonary vein myocardial action potential could be well computer-simulated based on existing data [28]. In the model, the pulmonary vein cardiomyocyte had a minimal density of I_{K1}, and the major inward currents contributing to pacemaking (phase 4) depolarization were the L-type Ca^{2+} current, the Na^{+}-Ca^{2+} exchanger current and a background current.

Autonomic influence

Atrial fibrillation is known to be greatly influenced by the sympathetic and parasympathetic nerve activity. The automaticity of the pulmonary vein myocardium is also reported to be influenced by adrenergic and cholinergic stimuli. In the canine pulmonary vein myocardium, isoproterenol, a β-adrenergic agonist, induced diastolic depolarization but it was not enough to trigger automatic action potentials [29]. When applied after automatic action potentials were induced by Ba^{2+}, the frequency was increased by isoproterenol, and decreased by acetylcholine [29]. An interesting observation with acetylcholine is that its washout in the presence of isoproterenol induced automatic electrical activity.

In the rabbit pulmonary vein, either α- or β-adrenergic stimulation induced automatic electrical activity; both effects were inhibited by KN-93, which suggests the involvement of calmodulin kinase II [30]. In the guinea pig pulmonary vein, application of noradrenaline to quiescent preparations induced a gradual depolarization of the resting membrane potential followed by generation of automatic electrical activity [24].

In the rat pulmonary vein, application of noradrenaline to quiescent preparations induced a transient hyperpolarization followed by a gradual depolarization of the resting membrane potential, which lead to generation of automatic electrical activity [24, 31]. The hyperpolarization and depolarization were mediated by β- and α- adrenergic

receptors, respectively. Pharmacological analyses revealed that activation of either α- or β-adrenergic receptors alone is not enough and simultaneous activation of both receptor types are necessary for the generation of automatic electrical activity [31, 32]. Noradrenaline-induced activity appeared in the form of repetitive bursts. Ryanodine either completely inhibited or decreased the frequency and duration of bursts, and the residual automatic electrical activity was inhibited by further application of nifedipine [24]. This indicates that both intracellular Ca^{2+}-dependent and Ca^{2+}-independent components of automatic activity exists in the rat pulmonary vein myocardium. Inhibition by nifedipine indicates the involvement of L-type Ca^{2+} channels. This is consistent with the observation that the take-off potential during the burst is in the range of -55 to -35 mV, which overlaps the activation voltage range of L-type Ca^{2+} channels. It is interesting that the maximum diastolic potential gradually shifts towards negative direction during the burst. It is probable that during the bursts, accumulation of intracellular Ca^{2+} gradually activates some Ca^{2+}-dependent hyperpolarizing currents, which eventually inhibits the generation of action potentials.

Results obtained from pulmonary vein myocardia from various animal species indicate that sympathetic neuronal influence on the pulmonary vein myocardium leads to the generation of automatic electrical activity through activation of both α- and β-adrenergic receptors. Acetylcholine, which appear to have inhibitory influence on automatic electrical activity when simply applied, may have stimulatory effects when its concentration is altered in the presence of adrenergic influence. These mechanisms may be involved in the generation and maintenance of atrial fibrillation of pulmonary vein origin under autonomic nerve influence.

Effect of pathophysiological status, humoral factors and drugs

The pulmonary vein automaticity, as well as atrial fibrillation, is known to be greatly influenced by the pathophysiological status of the myocardium. It was shown that acute mechanical stretch to the atrium makes the pulmonary vein excitable, leading to generation of atrial fibrillation [33]. Membrane currents activated by hypotonicity [34] or stretch [35] have been reported to be present on the cell membrane in the pulmonary vein cardiomyocyte, and to play important roles in their automaticity. Atrial dilatation induced by chronic volume overload may produce a substrate of atrial fibrillation in chronic atrioventricular block goat and dog models [36, 37]. The action potential of the pulmonary vein myocardium became significantly shorter after chronic atrioventricular block while that of the left atria did not [38]. This indicated that the pulmonary vein is more sensitive to volume overload than the atrium. Furthermore, the difference in action potential duration between the pulmonary vein and left atria was larger in atrioventricular block dogs, which may underlie the generation of atrial fibrillation. Interestingly, charybdotoxin, but not iberiotoxin, prolonged the action potential duration in the pulmonary vein after chronic atrioventricular block. This suggested that the volume overload-induced electrical remodeling of the heart involved expression of the intermediate Ca^{2+}-activated K^+ channels in the pulmonary vein cardiomyocyte. This channel is generally considered to be abundantly expressed in immune cells or epithelia tissue especially when they are in the proliferating mode. Thus, the pulmonary vein myocardium of the atrioventricular block dog may be under remodeling and is shifted towards a dedifferentiated state [38].

The pulmonary vein automaticity is reported to be affected by various humoral factors. Thyroid hormone was reported to change the electrophysiological properties of the pulmonary vein cardiomyocyte to increase the arrhythmogenetic activity of the pulmonary vein myocardium [39]. Hyperthyroid pulmonary vein cardiomyocytes had a shorter action potential duration and higher incidences of early and late afterdepolarizations. Tumor necrosis factor α, a proinflammatory cytokine which is known to induce cardiac arrhythmias, was reported to increase the arrhythmogenecity of the pulmonary vein myocardium through enhancement of its abnormal intracellular Ca^{2+} homeostasis [40]. The pulmonary vein cardiomyocytes treated with tissue necrosis factor α had a larger amplitude of delayed afterdepolarizatons, larger Na^+-Ca^{2+} exchanger current density, and a decreased sarcoplasmic reticulum ATPase expression. Hypoxia reduced the pulmonary vein beating rate in the rabbit; this effect was

mimicked by the ATP-sensitive K^+ channel opener, pinacidil, and was attenuated by the ATP-sensitive K^+ channel blocker, glibenclamide [41]. Increased repolarizing capacity could suppress the pulmonary vein automaticity. On the contrary, adenosine, which hyperpolarized the canine pulmonary vein myocardium, restored the dormant conduction, which could be explained by restoration of excitability through removal of voltage-dependent inactivation of the Na^+ channel [42]. Thus, increased repolarizing capacity may either suppress or enhance pulmonary vein automaticity depending on the situation.

Several strategies for the pharmacological therapy of atrial fibrillation can be postulated based on the present understanding of the electrical activity in the pulmonary vein myocardium. Inhibition of the intracellular Ca^{2+} based mechanisms of depolarization can markedly reduce the electrical automaticity of the pulmonary vein cardiomyocytes. Compounds such as the Na^+-Ca^{2+} exchange inhibitor SEA0400 [17] and the ryanodine receptor channel stabilizer K201 [23] appears to be promising for this purpose. However, as the functional proteins involved in automaticity are present not only in pulmonary vein myocardium but also in the working myocardium, the selectivity of these agents must be clarified. The pathways to provide Ca^{2+} to the pulmonary vein cardiomyocytes, such as the stretch activated channels, may serve as a specific target to inhibit the pulmonary vein electrical activity. Increasing the repolarizing capacity of pulmonary vein cardiomyocytes might be effective in suppressing their automaticity [25, 29], but this has to be achieved without shortening the refractory period of the working myocardium. Modifying the effect of various neurohumoral factors may also be effective. Targetted G_i protein inhibition [43], as well as inhibitors of the acetylcholine-activated K^+ channel [44, 45], was reported to be effective for the treatment of experimental atrial fibrillation; effects on the pulmonary vein myocardium may possibly be involved. Further studies on the precise mechanisms of pulmonary vein automaticity would lead to the discovery of novel drugs for the treatment of atrial fibrillation.

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Cardioprotective effects of Na^+-Ca^{2+} exchanger inhibition

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Abstract

The Na^+-Ca^{2+} exchanger acts both in the Ca^{2+} extrusion and Ca^{2+} influx modes, and is involved in the pathophysiolgy of arrhythmia and ischemia-reperfusion injury. Pharmacological inhibition of the Na^+-Ca^{2+} exchanger showed anti-arrhythmic effects in ventricular and pulmonary vein myocardium. It enhanced the recovery of contractile force after ischemia-reperfusion through preservation of mitochondrial function. Thus, inhibition of the Na^+-Ca^{2+} exchanger appears to be a promising therapeutic strategy for ischemia-reperfusion injury and arrhythmia.

Introduction

The Na^+-Ca^{2+} exchanger (NCX) is involved in myocardial Ca^{2+} regulation; it functions both in the forward (Ca^{2+} extrusion) and reverse (Ca^{2+} influx) modes. The major role of NCX is Ca^{2+} extrusion from the cytoplasm during diastole through its forward mode [1]. It is also considered to function in the reverse mode to provide a pathway for the influx of Ca^{2+} on reperfusion after myocardial ischemia [2, 3]. Some researchers postulate that, during early systole, Ca^{2+} influx through the reverse mode NCX might contribute to triggering of Ca^{2+} release from the sarcoplasmic reticulum [4, 5]. As the NCX is electrogenic, Ca^{2+} extrusion by its forward mode causes depolarization, which may be the trigger for arrhythmic activity. Specific pharmacological agents to modify NCX activity would be a powerful tool for studies on its role in myocardial Ca^{2+} handling.

SEA0400, a Selective NCX Inhibitor

SEA0400 (2-[4-[(2,5-difluorophenyl) methoxy] phenoxy]-5-ethoxyaniline) is an aniline derivative which was shown to be a potent and selective inhibitor of NCX in cultured neurons, astrocytes and microglia [6]; SEA0400 inhibits NCX in cultured neurons, astrocytes and microglia with EC_{50} values of 5 to 33 nM [6]. To establish SEA0400 as a selective NCX inhibitor in cardiac muscle, we examined its effects on various myocardial preparations. In voltage-clamped ventricular myocytes, SEA0400 inhibited the NCX current with an EC_{50} value of 30 to 40 nM [7]. The potency of SEA0400 was about 10 fold higher than that of KB-R7943 [8], a widely used NCX inhibitor with less selectivity. SEA0400 had no effect on the Na^+ current, L-type Ca^{2+} current, inwardly rectifying K^+ current and delayed rectifyer K^+ current at 1 μM, a concentration at which NCX was inhibited by more than 80% [7]. SEA0400 was also shown to inhibit the cardiac type NCX (NCX1) expressed in HEK293 cells [9].

In isolated myocardial tissue from the guinea-pig ventricle, SEA0400 had no significant effect on the action potential configuration, which is consistent with its lack of effect on sodium, calcium and potassium currents [10] . SEA0400 shortens the late plateau phase of the action potential in the rat and mouse ventriaular myocardium, which reflects Ca^{2+} extrusion by the NCX [11] . In the guinea-pig ventricle, SEA0400 significantly inhibited the contracture induced by low Na^{+} solution, which reflects Ca^{2+} influx through the reverse mode NCX [12].

These studies showed that SEA0400 is a potent and highly selective inhibitor of NCX in the myocardium, and would be a powerful tool for further studies on the role of NCX in the heart and the therapeutic potential of its inhibition. In this review, we will summarize the results obtained by ourselves and by other researchers on the cardioprotective and antiarrhythmic effects of SEA0400.

Effect of NCX Inhibition on Ischemia Reperfusion

Concerning the development of myocardial ischemia-reperfusion damage, studies with ionic manipulations and transgenic mice have implicated the involvement of reverse mode NCX [13]. During ischemia, cytoplasmic acidification stimulates the Na^{+}-H^{+} exchanger, which in turn favors the reverse mode NCX activity and thus leads to intracellular Ca^{2+} overload. This process is accelerated upon reperfusion, when the transsarcolemmal H^{+} gradient is increased by washout of extracellular H^{+}. Thus, the NCX is considered to be the key transporter in the development of myocardial ischemia-reperfusion injury and its inhibition may protect the myocardium through attenuation of Ca^{2+} overload. To obtain pharmacological evidence for the involvement of NCX in myocardial ischemia-reperfusion injury, we constructed an ischemia-reperfusion model based on coronary-perfused ventricular tissue preparation, and analyzed the effects of SEA0400.

The contractile force of the coronary-perfused ventricular preparations decreased rapidly during early ischemia and was almost abolished at 10 min after the onset of ischemia [10]. This could be explained by factors such as reduction of Ca^{2+} influx due to shortening of the action potential duration, decreased release of Ca^{2+} from the SR [14], decrease in tissue ATP content [15], and intracellular acidosis [16, 17]. During the last 10 min of the 30 min ischemic period, gradual increases in resting tension was observed in control preparations. Such phenomenon has been attributed to decrease in intracellular ATP leading to rigor shortening [18], increased diastolic Ca^{2+} concentration

due to abnormal Ca^{2+} release from the sarcoplasmic reticulum [19], and also to Ca^{2+} influx through reverse mode NCX [20, 21]. SEA0400, applied from 30 min before the onset of experimental ischemia, greatly reduced the increase in basal tension. This indicates that Ca^{2+} influx through the reverse mode NCX does occur during myocardial ischemia. It is likely that inhibition of this abnormal Ca^{2+} influx by SEA0400 would contribute to the overall cardioprotection by the drug. This is supported by the fact that SEA0400 enhanced the recovery of contractile force after reperfusion even when applied during the pre-ischemic and ischemic periods and washed out on reperfusion.

The mechanical and electrical parameters that were altered during ischemia tended to recover towards pre-ischemic values after reperfusion. Resting potential, over shoot and Vmax after 10 min of reperfusion was almost the same as pre-ischemic values, which could be explained by rapid washout of extracellular potassium ions accumulated during the ischemic period. Reperfusion is also considered to result in washout of the accumulated extracellular protons, which generates a huge pH-gradient between the cytoplasm and extracellular space. Consequently, the Na^{+}-H^{+} exchange is activated which will cause a large Na^{+} influx. Increased intracellular Na^{+} will lead to additional Ca^{2+} overload through activation of reverse mode NCX [2, 3]. Inhibition of NCX would attenuate this rapid influx of Ca^{2+} and can possibly result in cardioprotection. In fact, in the present study, SEA0400, applied either throughout ischemia-reperfusion or only during reperfusion, significantly enhanced the recovery of contractile force after reperfusion. Similarly, in coronary perfused hearts of the rabbit [22] and rat [23], SEA0400 was reported to enhance the recovery of left ventricular developed pressure and +dP/dt, respectively, even when applied just before reperfusion.

Intracellular mechanisms for the protective effect of NCX inhibition

Cytoplasmic Ca^{2+} overload may lead to cellular dysfunction through multiple pathways. There is increasing evidence suggesting that Ca^{2+} accumulation in the mitochondria during ischemia is one of the major triggers for irreversible cell injury [24]. To clarify the intracellular mechanisms for the cardioprotective effects of NCX inhibition, we applied fluorescence microscopy on isolated cardiomyocytes loaded with fluorescent probes for intracellular Ca^{2+} and mitochondria function, and observed the effects of changing the extracellular solution to that mimicking ischemia [25]. During experimental ischemia, an increase in mitochondrial Ca^{2+} was observed in Rhod 2-loaded myocytes during experimental ischemia, which paralleled the increase in

cytoplasmic Ca^{2+} concentration. The increase in cytoplasmic and mitochondrial Ca^{2+} was significantly reduced by SEA0400. This indicates that inhibition of the sarcolemmal NCX can reduce Ca^{2+} overload both in the cytoplasm and in the mitochondria. The NCX, is present not only on the sarcolemma but also on the mitochondrial inner membrane [26]. This mitochondrial NCX is considered to serve as a Ca^{2+} efflux pathway rather than a Ca^{2+} influx pathway, and its inhibition can not explain the reduction of mitochondrial Ca^{2+} overload [27]. Elevated mitochondrial Ca^{2+} is considered to result in mitochondrial dysfunction through pathways that include the opening of the permeability transition pore [28, 29]. This leads to a loss of key cofactors of mitochondrial metabolism and substrate oxidization, and to subsequent irreversible loss of the capacity to maintain the electrochemical gradient of protons ($\Delta\varphi m$) across the mitochondrial inner membrane. According to the chemiosmotic theory [30], $\Delta\varphi m$ is the sole energy-transduction intermediate between the respiratory chain and proton-translocating ATP synthetase. Thus, loss of $\Delta\varphi m$ indicates the loss of mitochondrial ATP synthesis. The time course of mitochondrial depolarization during ischemia was reported to be related to ATP exhaustion in cardiomyocyte-derived HL-1 cells [31]. Experimental ischemia induced a loss of $\Delta\varphi m$ in TMRE-loaded cardiomyocytes. The time course of the $\Delta\varphi m$ loss, which was monitored by decrease in TMRE fluorescence, was significantly delayed by SEA0400, suggesting that SEA0400 maintains mitochondrial integrity and function [25].

Preservation of the cellular ATP level through maintenance of mitochondrial function appears to be the main mechanism by which SEA0400 exerts its cardioprotective effect. In fact, the decrease in tissue ATP content during ischemia was significantly smaller in SEA0400-treated myocardial preparations [25]. These results suggest that attenuation of mitochondrial Ca^{2+} overload during ischemia results in the preservation of mitochondrial ability to produce ATP. This view is also supported by the observation in the rat heart that SEA0400-induced recovery of myocardial phosphocreatine and ATP levels after reperfusion closely correlates with the recovery of left ventricular developed pressure [32]. Ischemia reperfusion injury is a complex process involving apoptosis, production of physiologically active substances, and changes in cytoplasmic pH [33] etc., which may all be related to NCX activity. Further investigation with SEA0400 should clarify the effect of specific NCX blockade on these cellular processes.

The cardioprotective effect of SEA0400 during ischemia appears to persist until

after reperfusion. In fact, SEA0400 enhanced the recovery of contractile force after reperfusion, even when it was applied only during the pre-ischemic and ischemic periods and washed out on reperfusion [10]. Also, in coronary perfused rabbit [22] and rat [23] hearts, SEA0400 has been reported to enhance the recovery of left ventricular developed pressure and +dP/dt, respectively, even when applied only before ischemia. This could be attributed to the reduction of irreversible mitochondrial damage by SEA0400. It has been reported that, in cardiomyocytes, an elevated mitochondrial Ca^{2+} concentration during experimental ischemia correlated with the occurrence of cell hypercontracture after reperfusion [34]. On the other hand, enhanced recovery of contractile force was observed when SEA0400 was applied only during reperfusion [10]. On reperfusion, the washout of extracellular protons accelerates extrusion by the Na^+-H^+ exchanger of intracellular protons accumulated during ischemia. This causes an increase in intracellular Na^+ concentration, which in turn causes rapid influx of Ca^{2+} through the reverse mode NCX and leads to intracellular Ca^{2+} overload. SEA0400 applied on reperfusion could inhibit this rapid Ca^{2+} influx. Thus, the inhibition of the reverse mode NCX could be cardioprotective both during ischemia and during reperfusion.

Pharmacological agents, such as Na^+ and Ca^{2+} channel blockers [3], β-blockers [35], and K^+ channel openers [36] have cardioprotective effects against ischemia-reperfusion damage. A common feature of these agents appears to be cardiosuppression; a reduced heart rate and a reduced contractile force result in reduced oxygen requirements and the preservation of high-energy phosphonucleotides. We examined the effects of these types of drugs on guinea pig coronary-perfused right ventricular tissue preparations during experimental ischemia and reperfusion, and our results were consistent with this view [16, 37]. However, the suppression of cardiac function may be a disadvantage of these drugs. These agents, either directly or indirectly, inhibit Ca^{2+} influx through the L-type Ca^{2+} channel, which is the major trigger for Ca^{2+} release from the sarcoplasmic reticulum. In ventricular cardiomyocytes, L-type Ca^{2+} channels exist on the T-tubular membrane in close proximity to the ryanodine receptor/Ca^{2+} release channel located on the junctional sarcoplasmic reticulum [38]. Functional experiments suggest that a Ca^{2+} microdomain exists and includes the L-type Ca^{2+} channel and the ryanodine receptor/Ca^{2+} release channel [39]. On the other hand, the NCX protein exists on the T-tubular membrane but appears to be excluded from the Ca^{2+} microdomain; the efficiency of Ca^{2+} influx through the NCX to trigger Ca^{2+} release is through the

ryanodine receptor/Ca^{2+} release channel is too low to contribute to myocardial contraction. Thus, it is reasonable that inhibition of Ca^{2+} influx through the reverse mode Na^{+}-Ca^{2+} exchanger has no cardiosuppressive effects. Therefore, inhibition of the reverse mode NCX appears to be an ideal mechanism by which to attenuate Ca^{2+} overload under ischemia.

Ischemia-Reperfusion-Induced Arrhythmia

The NCX also appears to be involved in arrhythmia induced by ischemia-reperfusion. In the guinea-pig coronary perfused ischemia-reperfusion model, intermittent arrhythmia was observed after reperfusion in all of the preparations either untreated or treated with SEA0400 [10]. SEA0400 could not prevent the occurrence of arrhythmia on reperfusion, but reduced the incidence of arrhythmia during the period between 10 and 60 min after reperfusion. In the ischemia-reperfusion arrhythmia model of anesthetized rats, SEA0400 significantly reduced the incidence of ventricular fibrillation and mortality rate [23]. Direct or indirect effects of SEA0400 on myocardial refractory period might be involved in its anti-arrhythmic action. In this connection, an interesting coincidence is that enhanced recovery of APD and reduction of arrhythmic contraction both became prominent at 10 min after reperfusion [10]. Ischemia produces a decrease in tissue ATP content, which is only partially recovered after reperfusion [15]. A decrease in tissue ATP results in opening of the ATP sensitive potassium channel, shortening of the APD, and decrease in contractile force. As SEA0400 enhanced the recovery of tissue ATP after reperfusion, the most probable explanation for the antiarrhythmic effect of SEA0400 is enhanced recovery of APD and refractory period. However, as NCX is present not only on the sarcolemma but also on the mitochondrial inner membrane and that its inhibition results in augmentation of ATP production [40], the direct and indirect effects of SEA0400 on mitochondrial NCX and ATP production remain to be investigated.

Effect of NCX Inhibition on Ouabain-induced Arrhythmia

Ouabain has been considered to increase intracellular Na^{+} concentration, shift the balance of the two modes of NCX to favor the reverse mode and increase cellular Ca^{2+} load. When this Ca^{2+} load exceeds the capacity of the SR, abnormal Ca^{2+} release from the SR occurs, which in turn triggers abnormal electrical activity and arrhythmic contractions. In isolated guinea-pig ventricular preparations, the ouabain-induced

increase in basal tension and arrhythmic contractions were significantly reduced by SEA0400 [12]. Arrhythmic contractions were preceded by small oscillations in resting tension suggesting that intracellular Ca^{2+} oscillations were acting as their trigger. This provides pharmacological evidence that NCX plays a crucial role in ouabain-induced arrhythmogenesis. It was also reported that SEA0400 attenuated ouabain-induced arrhythmia in a canine *in vivo* model [41] and *in* isolated Purkinje fibers [42]. Further, long term administration of SEA0400 to Dahl salt-sensitive rats attenuated the ouabain-induced rise in intracellular Ca^{2+}, and fibrosis, which suggests that Ca^{2+} entry via reverse mode NCX induced by the endogenous digitalis-like factor is involved in the development of fibrosis and heart failure [43].

Automaticity of the Pulmonary Vein Myocardium

Pulmonary veins are considered to be involved in the initiation and maintenance of atrial fibrillation, one of the most frequent arrhythmia in clinical practice [44]. Pulmonary veins contain a myocardial layer, whose electrical activity is considered to underlie their arrhythmogenic activity [45]. 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 [46]. 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. We applied microelectrode techniques to the myocardial layer of isolated guinea-pig pulmonary veins, and examined the effects of SEA0400 and ryanodine on the spontaneous and ouabain-induced electrical activity. About 15% of the guinea-pig pulmonary vein preparations showed spontaneous activity. The incidence was increased to 95% by ouabain, which induces intracellular Ca^{2+} overload through inhibition of the Na^+/K^+ ATPase [12]. Increased cellular Ca^{2+} load can cause spontaneous Ca^{2+} release from the SR. If this occurs during the diastolic period, this would accelerate the diastolic depolarization through activation of the forward-mode NCX, and elicit spontaneous electrical activity. Our present results that both spontaneous and ouabain-induced electrical activity were inhibited by ryanodine and SEA0400 suggest that this is indeed the case in the pulmonary vein myocardium. Acceleration of late repolarization and reduction of the slope of diastolic depolarization by SEA0400 [47] supports the view that Ca^{2+} extrusion through forward mode NCX occurs during the repolarization phase to the diastolic

depolarization phase. However, functional components of the intracellular Ca^{2+}-induced depolarization such as the sarcoplasmic reticulum and NCX are present not only in the pulmonary vein, but also in the working myocardium. In fact, depolarization induced by the forward mode NCX was reported to be involved in the generation of fibrillation and torsades de pointes in the ventricular myocardium [48-50]. Thus, the pulmonary vein myocardium possibly has some characteristic feature which underlies its tendency to generate spontaneous activity.

The pulmonary vein myocardium appears to have different electrophysiological properties from those of the working myocardium. Ouabain, at a concentration of 1 μM, induced spontaneous activity in the guinea-pig pulmonary vein myocardium but not in the ventricular myocardium [12]. The resting membrane potential of the guinea-pig pulmonary vein myocardial cells was about -75.4 mV, which was less negative than that of the guinea-pig atria and ventricle (-78 to -85 mV; [51]). The current density of I_{K1}, the major membrane current responsible for the maintenance of the resting membrane potential, was reported to be smaller in the pulmonary vein cardiomyocyte [46]. The smaller contribution of I_{K1} around the resting membrane potential would allow underlying depolarizing mechanisms to cause diastolic depolarization leading to the generation of spontaneous action potentials in the pulmonary vein myocardium. On the other hand, 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 [52]. 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 spontaneous 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.

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