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 μM, 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 μM 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 potently 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.

Acknowledgments

This study was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (#21590602); the Pharmacological Research Foundation (Tokyo); the Science Research Promotion Fund from the Promotion and Mutual Aid Corporation for Private Schools of Japan; and "Research on the molecular mechanisms of appearance of age-related diseases by failure of cell function control system, and their prevention and treatment" by the "Research Center for Aging and Age-related Diseases" established in the Toho University Faculty of Pharmaceutical Sciences.

References

1 Kaarisalo MM, Immonen-Räihä P, Marttila RJ, Salomaa V, Kaarsalo E, Salmi K, et al. Atrial fibrillation and stroke. Mortality and causes of death after the first acute ischemic stroke. Stroke. 1997;28:311–315.

2 Haïssaguerre M, Jaïs P, Shah DC, Takahashi A, Hocini M, Quiniou G, et al. Spontaneous initiation of atrial fibrillation by ectopic beats originating in the pulmonary veins. N Engl J Med. 1998;339:659–666.

3 Cheung DW. Electrical activity of the pulmonary vein and its interaction with the right atrium in the guinea-pig. J Physiol. 1981; 314:445–456.

4 Chen YJ, Chen SA, Chang MS, Lin CI. Arrhythmogenic activity of cardiac muscle in pulmonary veins of the dog: implication for the genesis of atrial fibrillation. Cardiovasc Res. 2000;48: 265–273.

5 Chen YC, Chen SA, Chen YJ, Tai CT, Chan P, Lin CI. T-type calcium current in electrical activity of cardiomyocytes isolated from rabbit pulmonary vein. J Cardiovasc Electrophysiol. 2004; 15:567–571.

6 Wang TM, Luk HN, Sheu JR, Wu HP, Chiang CE. Inducibility of abnormal automaticity and triggered activity in myocardial sleeves of canine pulmonary veins. Int J Cardiol. 2005;104: 59–66.

7 Vermeulen JT, McGuire MA, Opthof T, Coronel R, de Bakker JM, Klopping C, et al. Triggered activity and automaticity in ventricular trabeculae of failing human and rabbit hearts. Cardiovasc Res. 1994;28:1547–1554.

8 De Bakker JM, Ho SY, Hocini M. Basic and clinical electrophysiology of pulmonary vein ectopy. Cardiovasc Res. 2002;54: 287–294.

9 Wit A, Cranefield P. Triggered and automatic activity in the canine coronary sinus. Circ Res. 1977;41:435–445.

10 Hiraoka M. Characteristics of triggered-activity and delayed afterdepolarization in responses to the electrical stimulation. Jpn Circ J. 1987;51:176–180.

11 Rosen MR, Danilo P Jr. Effects of tetrodotoxin, lidocaine, verapamil, and AHR-2666 on ouabain-induced delayed afterdepolarizations in canine Purkinje fibers. Circ Res. 1980;46:117–124.

12 Sugiyama A, Hashimoto K. Can the MAP technique be applied to detect delayed afterdepolarization? Electrophysiologic and pharmacologic evidence. J Cardiovasc Pharmacol. 1999;34: 46–52.

13 Honjo H, Boyett MR, Niwa R, Inada S, Yamamoto M, Mitsui K, et al. Pacing-induced spontaneous activity in myocardial sleeves of pulmonary veins after treatment with ryanodine. Circulation. 2003;107:1937–1943.

14 Chen YJ, Chen SA, Chen YC, Yeh HI, Chan P, Chang MS, et al. Effects of rapid atrial pacing on the arrhythmogenic activity of single cardiomyocytes from pulmonary veins: implication in initiation of atrial fibrillation. Circulation. 2001;104:2849–2854.

15 Inomata N, Ishihara T. Mechanism of inhibition by SUN 1165, a new Na channel blocking antiarrhythmic agent, of cardiac glycoside-induced triggered activity. Eur J Pharmacol. 1988;145: 313–322.

16 Noguchi K, Masumiya H, Takahashi K, Kaneko K, Higuchi S, Tanaka H, et al. Comparative effects of gallopamil and verapamil on the mechanical and electrophysiological parameters of isolated guinea-pig myocardium. Can J Physiol Pharmacol. 1997;75: 1316–1321.

17 Namekata I, Tsuneoka Y, Takahara A, Shimada H, Suginoto T, Takeda K, et al. Involvement of the Na^+/Ca^{2+} exchanger in the automaticity of guinea-pig pulmonary vein myocardium as revealed by SEA0400. J Pharmacol Sci. 2009;110:111–116.

18 Zucchi R, Ronca-Testoni S. The sarcoplasmic reticulum Ca^{2+} channel/ryanodine receptor: modulation by endogenous effectors, drugs and disease states. Pharmacol Rev. 1997;49:1–51.

19 Tan AY, Li H, Wachsmann-Hogiu S, Chen LS, Chen PS, Fishbein MC. Autonomic innervation and segmental muscular disconnections at the human pulmonary vein-atrial junction: implications for catheter ablation of atrial-pulmonary vein junction. J Am Coll Cardiol. 2006;48:132–143.

20 Arora R, Ng J, Ulphani J, Mylonas I, Subacius H, Shade G, et al. Unique autonomic profile of the pulmonary veins and posterior left atrium. J Am Coll Cardiol. 2007;49:1340–1348.

21 Lemola K, Chartier D, Yeh YH, Dubuc M, Cartier R, Armour A, et al. Pulmonary vein region ablation in experimental vagal atrial fibrillation: role of pulmonary veins versus autonomic ganglia. Circulation. 2008;117:470–477.

22 Ehrlich JR, Cha TJ, Zhang L, Chartier D, Melnyk P, Hohnloser SH, et al. Cellular electrophysiology of canine pulmonary vein cardiomyocytes: action potential and ionic current properties. J Physiol. 2003;551:801–813.

23 Pogwizd SM, Schlotthauer K, Li L, Yuan W, Bers DM. Arrhythmogenesis and contractile dysfunction in heart failure: roles of sodium-calcium exchange, inward rectifier potassium current, and residual β-adrenergic responsiveness. Circ Res. 2001;88: 1159–1167.

24 Tanaka H, Namekata I, Nouchi H, Shigenobu K, Kawanishi T, Takahara A. New aspects for the treatment of cardiac diseases based on the diversity of functional controls on cardiac muscles: diversity in the excitation-contraction mechanisms of the heart. J Pharmacol Sci. 2009;109:327–333.

J Pharmacol Sci 115, 235 – 238 (2011)

Journal of Pharmacological Sciences

Short Communication

Developmental Changes in Action Potential Prolongation by K^+-Channel Blockers in Chick Myocardium

Hideaki Nouchi[1], Naoaki Kiryu[1], Mikio Kimata[1], Yayoi Tsuneoka[1], Shogo Hamaguchi[1], Iyuki Namekata[1,*], Akira Takahara[1], Koki Shigenobu[1], and Hikaru Tanaka[1]

[1]*Department of Pharmacology, Toho University Faculty of Pharmaceutical Sciences, Miyama 2-2-1, Funabashi, Chiba 274-8510, Japan*

Received August 7, 2010; Accepted December 12, 2010

Abstract. The effects of K^+-channel blockers on the action potential duration of the myocardium were examined in isolated right ventricles from the 7 – 10-day-old, 11 – 13-day-old, and 14 – 20-day-old embryo and 1 – 7-day-old hatched chicks. E-4031 significantly prolonged action potential duration at all developmental stages examined; the prolongation was largest in the 11 – 13-day-old embryo and was accompanied by early after-depolarizations. Chromanol 293B showed smaller prolongation at all stages examined. Terfenadine prolonged action potential duration in the 11 – 13-day-old embryo, but not in other stages. Thus, the chick ventricular myocardium changes its repolarization properties during development.

Keywords: chick, K^+-channel blocker, action potential duration

The chick embryo heart has been used to study various aspects of myocardial excitation and contraction. Although the chick heart shares common basic mechanisms with the mammalian heart, characteristic features in the electrophysiological properties, Ca^{2+} handling, contraction, and their physiological and pharmacological regulation has been reported (1 – 4). Concerning its electrophysiological properties, the resting membrane potential and action potential amplitude was reported to increase progressively during embryonic development (1), and many reports have been published concerning the depolarizing membrane currents involved (2). On the other hand, relatively little is known about its repolarization process, which is the determinant of the action potential duration. In the case of mammalian myocardia, information on the species difference and developmental changes in repolarization properties have accumulated, which enables appropriate selection of preparations for different experimental purposes (4). In the present study, we intended to obtain information about the repolarization mechanisms of the chick heart. We applied standard microelectrode techniques to the chick embryonic ventricular myocardia from four different developmental stages and examined the effect of inhibitors of the delayed rectifier potassium current, the major repolarizing current in the myocardium.

Standard microelectrode experiments were performed as previously described (5 – 7). Briefly, fertilized chicken eggs were incubated at 37°C, and the right ventricular free wall was isolated from 7 – 10-day-old embryo, 11 – 13-day-old embryo, 14 – 20-day-old embryo, and 1 – 7-day-old hatched chicks. They were driven at 1 Hz in an organ bath containing physiological salt solution of the following composition: 118.4 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1.2 mM KH_2PO_4, 24.9 mM $NaHCO_3$, and 11.1 mM glucose. The solution was gassed with 95% O_2 – 5% CO_2 and maintained at 37°C (pH 7.4). All experimental data are expressed as the mean ± S.E.M., and statistical significance of differences between means were evaluated by the paired *t*-test. A P value less than 0.05 were considered significant. The drugs used were E-4031 (Wako, Osaka), chromanol 293B (Sigma, St. Louis, MO, USA), and terfenadine (Wako). All other drugs and chemicals were commercial products of the highest available quality.

The resting potential, overshoot and the maximum rate of rise increased progressively during the embryonic period (Table 1, Fig. 1A). Action potential duration increased progressively during the embryonic period. The action potential duration of 1 – 7-day-old hatched chicks

*Corresponding author. iyuki@phar.toho-u.ac.jp
Published online in J-STAGE on January 20, 2011 (in advance)
doi: 10.1254/jphs.10205SC

Table 1. Developmental changes in action potential parameters

Parameters (units)	7 – 10-day-old embryo	11 – 13-day-old embryo	14 – 20-day-old embryo	1 – 7-day-old hatched chicks
RP (mV)	−78.8 ± 0.3	−80.8 ± 0.4	−82.4 ± 0.3	−82.8 ± 0.3
OS (mV)	34.4 ± 0.5	36.7 ± 0.4	37.7 ± 0.5	37.3 ± 0.3
APD_{20} (ms)	103.1 ± 2.7	103.4 ± 2.8	106.9 ± 2.9	102.3 ± 2.7
APD_{50} (ms)	152.8 ± 3.0	154.3 ± 2.7	163.9 ± 2.6	145.5 ± 2.8
APD_{90} (ms)	180.4 ± 2.6	185.6 ± 2.6	197.8 ± 1.7	168.5 ± 2.8
$\dot{V}_{max}$ (V/s)	118.2 ± 6.3	149.3 ± 8.4	180.1 ± 4.1	177.8 ± 3.7

Action potential parameters of ventricular myocardia from 7 – 10-day-old embryos, 11 – 13-day-old embryos, 14 – 20-day-old embryos, and 1 – 7-day-old hatched chicks were obtained in the absence of agents. RP, OS, and $\dot{V}_{max}$ indicate resting potential, overshoot, and maximum rate of phase-0 depolarization, respectively. APD_{20}, APD_{50}, and APD_{90} indicate action potential duration at 20%, 50%, and 90% repolarization, respectively. Each value is the mean ± S.E.M. from 36 experiments.

was shorter than that of the 14 – 20-day-old embryos.

E-4031 (1 μM), a blocker of I_{Kr}, significantly prolonged action potential duration at all developmental stages (Fig. 1: A, Ca). The magnitude of prolongation by E-4031 was larger in 7 – 10-day-old embryo and 11 – 13-day-old embryo than in the 14 – 20-day-old embryo and 1 – 7-day-old hatched chick. In the 11 – 13-day-old embryo, the prolongation by E-4031 was prominent and was accompanied by early after-depolarizations (EADs); phase 3 repolarization was interrupted followed by a single or a series of partial depolarization toward 0-mV level (Fig. 1Ac). Early after-depolarizations were observed in all of the preparations from the 11 – 13-day-old embryo (n = 6), but was not observed in those from other stages.

Chromanol 293B (30 μM), a blocker of the slowly activating component of the delayed rectifier K^+ current (I_{Ks}), prolonged action potential duration at all developmental stages (Fig. 1Cb); the prolongation by chromanol 293B was statistically significant, but was much smaller than that by E-4031 at all developmental stages.

Terfenadine (30 μM) produced a prolongation of the action potential duration in the 11 – 13-day-old embryo. Such prolongation was not observed at other stages (Fig. 1B, Cc).

The action potential duration of the chick ventricular myocardium increased during the embryonic period and was shortened in the hatched chick. This was in agreement with previous reports (1, 8) and also appeared to be similar to the case in the guinea pig where the action potential duration increased during the embryonic period followed by a decrease during the perinatal period (9). The developmental change in action potential duration is likely to be accompanied by changes in the balance of underlying membrane currents, as was shown to be the case in guinea pig (10). Such changes would greatly affect the responsiveness of the myocardium to pharmacological agents acting on myocardial ion channels. E-4031 markedly prolonged the action potential duration at all ages examined, while the prolongation by chromanol 293B was small (Fig. 1C). This suggests that in the chick ventricular myocardium, the rapid component (I_{Kr}), but not the slow component (I_{Ks}), of the delayed rectifier potassium channel is largely responsible for the repolarization, which is the same as in many mammalian species including the guinea pig, rabbit, and human. The prolongation by E-4031 was most prominent in the 11 – 13-day-old embryo (Fig. 1). The most likely explanation is that the prolongation of the repolarization phase of the embryonic myocardia reflects less density of repolarizing currents. In other words, the embryonic myocardia have less "repolarization reserve" (11), which makes the action potential highly sensitive to I_{Kr} blockade. Early afterdepolarizations, which are considered to be one of the causes of cardiac arrhythmia, were induced by E-4031 in all of the preparations from the 11 – 13-day-old embryo (Fig. 1Ac). In mammalian myocardia, I_{Kr} blockade is known to induce action potential prolongation, but does not always induce early after-depolarization. For example, in guinea-pig ventricular myocardium, E-4031 (1 μM) prolonged the action potential duration by about 70 ms, but did not induce early after-depolarizations (12).

Clinical treatment with certain cardiovascular and non-cardiovascular drugs has been reported to induce QT prolongation and serious ventricular arrhythmia including *torsades de pointes*. Drugs such as terfenadine and cisapride were withdrawn from clinical practice because of their arrhythmogenic risk. Since then, the assessment of the risks incurred with noncardiovascular therapeutic agents for cardiac function has received great attention (13). Terfenadine inhibits the I_{Kr} current in freshly isolated myocardial cells (14) and the hERG channel current expressed in HEK293 cells (15), but does not prolong the

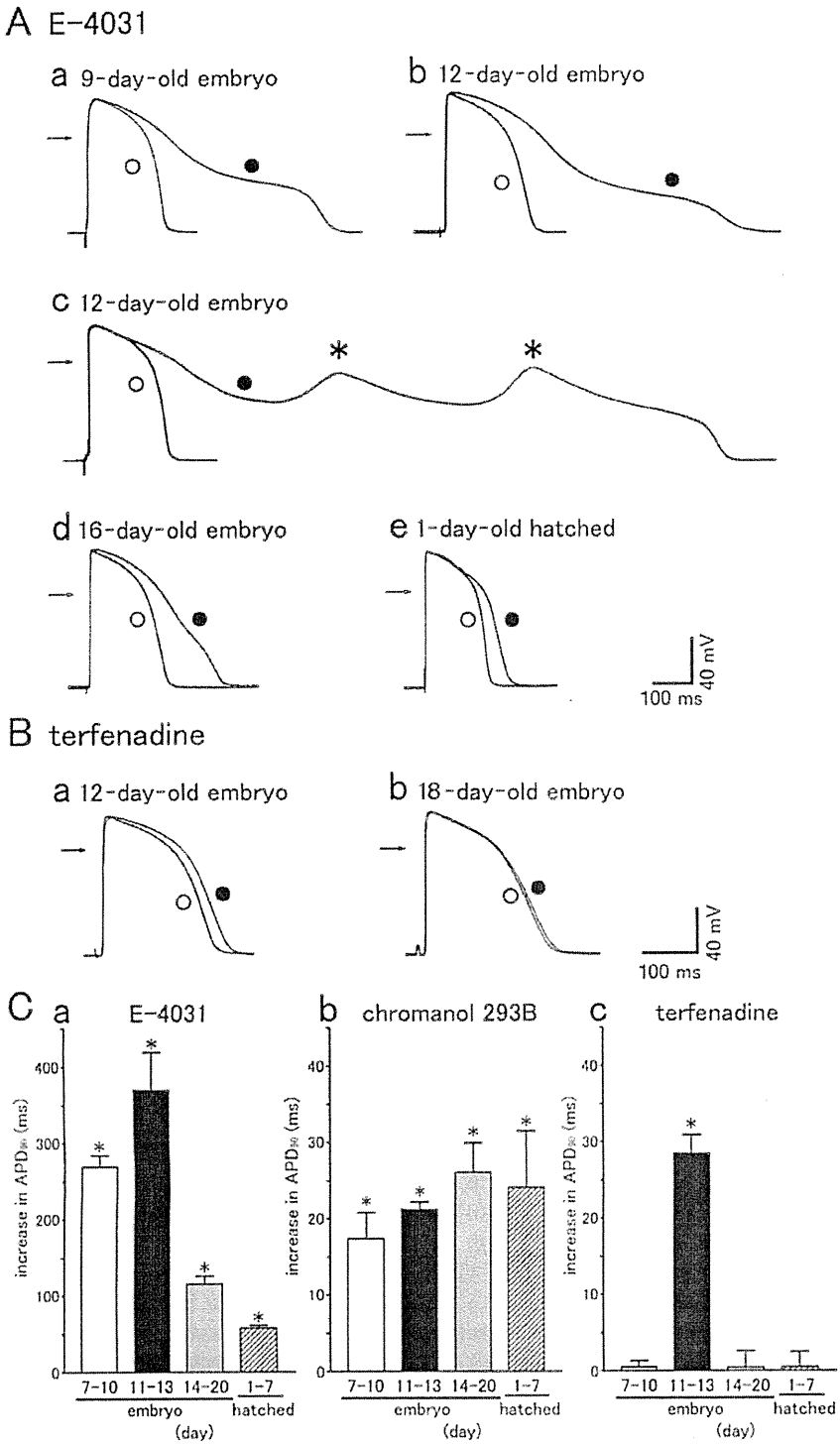

Fig. 1. Effect of K^+-channel blockers on the action potential of isolated chick ventricular myocardium. A: Typical action potential recordings before (open circles) and after (closed circles) the addition of E-4031 (1 μM) in the 9-day-old embryo (a), 12-day-old embryo (b, c), 16-day-old embryo (d), and 1-day-old hatched chick (e). Note that E-4031 induced early after-depolarizations in the 12-day-old embryo (c). B: Typical action potential recordings before (open circles) and after (closed circles) the addition of terfenadine (30 μM) in the 12-day-old embryo (a) and in the 18-day-old embryo (b). Horizontal arrows indicate zero-mV level. Voltage and time calibrations apply to all panels. C: Summarized results for the prolongation of the action potential duration at 90% repolarization (APD_{90}) by 1 μM E-4031 (a), 30 μM chromanol 293B (b), and 30 μM terfenadine (c) in the 7 – 10-day-old embryo (open columns), 11 – 13-day-old embryo (closed columns), 14 – 20-day-old embryo (gray columns), and 1 – 7-day-old hatched chick (hatched columns). Columns and vertical bars are the mean ± S.E.M. from 5 to 6 experiments. Asterisks indicate that the prolongation was statistically significant. Note the difference in the scale of the ordinate.

action potential duration in isolated ventricular tissue from mammals even at 20 μM (15,16). Such presence of "false positive" drugs complicate drug evaluation through action potential duration in tissue preparations (17).

Terfenadine did not prolong action potential duration in the 14 – 20-day-old embryo, which was the same as in isolated mammalian myocardial tissue. However, terfenadine prolonged the action potential duration in the 11 – 13-day-old embryo (Fig. 1: B, Cc). Thus, the 11 – 13-day-old chick embryonic myocardium may be a sensitive model to detect the proarrhythmic activity of drugs. Details such as the density of depolarizing and repolarizing membrane currents and the molecular structure of the delayed rectifier potassium channel in the chick ventricle await further investigation.

In conclusion, we found that the chick ventricle changes its repolarization reserve during development, which provides an interesting model for further studies on myocardial repolarization mechanisms and drug evaluation.

Acknowledgments

This study was supported in part by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science, and Technology of Japan (#21590293, #21590602, and #22790262) and the Science Research Promotion Fund from the Promotion and Mutual Aid Corporation for Private Schools of Japan. This study was performed in part as "Research on the molecular mechanisms of appearance of age-related diseases by failure of cell function control system, and their prevention and treatment" by the "Research Center for Aging and Age-related Diseases" established in the Toho University Faculty of Pharmaceutical Sciences.

References

1 Sperelakis N, Shigenobu K. Changes in membrane properties of chick embryonic hearts during development. J Gen Physiol. 1972;60:430–453.
2 Satoh H, Sada H, Tohse N, Shigenobu K. [Developmental aspects of electrophysiology in cardiac muscle]. Folia Pharmacol Jpn (Nippon Yakurigaku Zasshi). 1996;107:213–223. (text in Japanese with English abstract)
3 Nouchi H, Tanaka H, Shigenobu K. Pharmacological properties of the developing chick myocardium. Trends Comparative Biochem Physiol. 2005;11:37–45.
4 Tanaka H, Namekata I, Nouchi H, Shigenobu K, Kawanishi T, Takahara A. New aspects for the treatment of cardiac diseases based on the diversity of functional controls on cardiac muscles: diversity in the excitation-contraction mechanisms of the heart. J Pharmacol Sci. 2009;109:327–333.
5 Nouchi H, Kaeriyama S, Muramatsu A, Sato M, Hirose K, Shimizu N, et al. Muscarinic receptor subtypes mediating positive and negative inotropy in the developing chick ventricle. J Pharmacol Sci. 2007;103:75–82.
6 Tanaka H, Komikado C, Namekata I, Nakamura H, Suzuki M, Tsuneoka Y, et al. Species difference in the contribution of T-type calcium current to cardiac pacemaking as revealed by *R*(−)-efonidipine. J Pharmacol Sci. 2008;107:99–102.
7 Namekata I, Tsuneoka Y, Takahara A, Shimada H, Sugimoto T, Takeda K, et al. Involvement of the Na^+/Ca^{2+} exchanger in the automaticity of guinea-pig pulmonary vein myocardium as revealed by SEA0400. J Pharmacol Sci. 2009;110:111–116.
8 Kasuya Y, Matsuki N, Shigenobu K. Changes in sensitivity to anoxia of the cardiac action potential plateau during chick embryonic development. Dev Biol. 1977;58:124–133.
9 Agata N, Tanaka H, Shigenobu K. Developmental changes in action potential properties of the guinea-pig myocardium. Acta Physiol Scand. 1993;149:331–337.
10 Kato Y, Masumiya H, Agata N, Tanaka H, Shigenobu K. Developmental changes in action potential and membrane currents in fetal, neonatal and adult guinea-pig ventricular myocytes. J Mol Cell Cardiol. 1996;28:1515–1522.
11 Sugiyama A. Sensitive and reliable proarrhythmia *in vivo* animal models for predicting drug-induced torsades de pointes in patients with remodeled hearts. Br J Pharmacol. 2008;154:1528–1537.
12 Matsuda T, Takeda K, Ito M, Yamagishi R, Tamura M, Nakamura H, et al. Atria selective prolongation by NIP-142, an antiarrhythmic agent, of refractory period and action potential duration in guinea pig myocardium. J Pharmacol Sci. 2005;98:33–40.
13 Nakaya H, Hashimoto K. QT PRODACT: database construction for the evaluation of the risk of QT interval prolongation by drugs in Japan. J Pharmacol Sci. 2005;99:421.
14 Tanaka H, Nishimaru K, Sekine T, Shijuku T, Shigenobu K. Effects of terfenadine, betotastine and ketotifene on the action potential and membrane currents in isolated guinea-pig myocardium. Res Commun Pharmacol Toxicol. 1997;2:163–174.
15 Masumiya H, Saito M, Ito M, Matsuda T, Noguchi K, Iida-Tanaka N, et al. Lack of action potential-prolonging effect of terfenadine on rabbit myocardial tissue preparations. Biol Pharm Bull. 2004;27:131–135.
16 Tanaka H, Masumiya H, Kato Y, Shigenobu K. Inhibitory effects of terfenadine on the rising phase of action potentials in isolated guinea-pig myocardium. Gen Pharmacol. 1996;27:337–340.
17 Kii Y, Hayashi S, Tabo M, Shimosato T, Fukuda H, Itoh T, et al. QT PRODACT: evaluation of the potential of compounds to cause QT interval prolongation by action potential assays using guinea-pig papillary muscles. J Pharmacol Sci. 2005;99:449–457.

Blocking Effect of NIP-142 on the KCNQ1/KCNE1 Channel Current Expressed in HEK293 Cells

Iyuki NAMEKATA,[a] Noriko TSURUOKA,[a] Yayoi TSUNEOKA,[a] Tomoyuki MATSUDA,[a,b] Akira TAKAHARA,[a] Yoshio TANAKA,[a,c] Takeshi SUZUKI,[a,d] Tetsuo TAKAHASHI,[e] Naoko IIDA-TANAKA,[a,f] and Hikaru TANAKA*,[a]

[a] *Department of Pharmacology, Faculty of Pharmaceutical Sciences, Toho University;* [c] *Department of Chemical Pharmacology, Faculty of Pharmaceutical Sciences, Toho University;* [e] *Department of Biophysical Chemistry, Faculty of Pharmaceutical Sciences, Toho University; Funabashi, Chiba 274–8510, Japan:* [b] *Biological Research Laboratories, Nissan Chemical Industries, Ltd.; Shiraoka, Saitama 349–0294, Japan:* [d] *School of Material Science, Japan Advanced Institute of Science and Technology; Tatsunokuchi, Ishikawa 923–1292, Japan: and* [f] *Department of Food Science, Otsuma Women's University; Chiyoda-ku, Tokyo 102–8357, Japan.*

Received September 14, 2010; accepted October 29, 2010; published online November 5, 2010

We examined the effect of NIP-142, a benzopyran compound with terminating effect on experimental atrial arrhythmia, on the KCNQ1/KCNE1 channel, which underlies the slow component of the cardiac delayed rectifier potassium channel (I_{Ks}). NIP-142, as well as chromanol 293B, showed concentration-dependent blockade of the current expressed in HEK293 cells; the EC_{50} value of NIP-142 and chromanol 293B for the inhibition of tail current was 13.2 μM and 4.9 μM, respectively. These results indicate that NIP-142 has blocking effect on the KCNQ1/KCNE1 channel current.

Key words NIP-142; KCNQ1; KCNE1; atrial fibrillation

Atrial fibrillation is one of the most frequent types of arrhythmia in clinical practice. It is reported to double the risk of deaths due to cardiovascular diseases and to be the major risk factor for thromboembolism, especially cerebral embolism.[1] At present, atrial fibrillation is mainly treated with class I antiarrhythmic agents such as pilsicainide and flecainide, or class III antiarrhythmic agents such as dofetilide and amiodarone,[2—4] but the major problem with these agents is that they also affect ventricular excitation and repolarization. Thus, drugs with atrial selectivity are desired for the treatment of atrial fibrillation.

NIP-142, (3*R**,4*S**)-4-cyclopropylamino-3,4-dihydro-2,2-dimethyl-6-(4-methoxyphenylacethylamino)-7-nitro-2*H*-1-benzopyran-3-ol, is a benzopyran derivative with terminating effects on canine vagal stimulation-induced atrial fibrillation model and on canine Y-shaped incision-induced atrial flutter model.[5—7] These effects have been attributed to prolongation of atrial refractory period. The prolongation of the refractory period and action potential duration (APD) by NIP-142 was observed in the atrium,[5,8] but not in the ventricle.[8] This may indicate that NIP-142 is less likely to disturb ventricular repolarization when applied for the treatment of atrial arrhythmia.

Concerning the molecular mechanisms for the action potential prolongation by NIP-142, blocking effects on potassium currents such as the acetylcholine-activated potassium current (I_{KACh})[8,9] and the ultrarapid component of the delayed rectifier potassium current (I_{Kur})[10] has been reported. The blocking effect on the rapid component of the delayed rectifier potassium current (I_{Kr}) was observed only at higher concentrations. The fast component (I_{Kr}) and the slow component (I_{Ks}) of the delayed rectifier potassium current are the two major currents responsible for atrial repolarization in species including the guinea-pig and human.[11] However, the effect of NIP-142 on I_{Ks} has not yet been reported. In the present study, we examined the effect of NIP-142 on currents through expressed potassium channel subunits KCNQ1/KCNE1, which underlie I_{Ks}.

MATERIALS AND METHODS

Preparation of HEK293 Cells Expressing Human KCNQ1/KCNE1 Channel Currents cDNA fragments for human KCNQ1 and KCNE1, which encode the two subunits of the I_{Ks} channel, were amplified by polymerase chain reaction from a human heart cDNA library (Takara Shuzo Co., Ltd., Kyoto, Japan) with oligonucleotide primers designed based on the published mouse cDNA sequence of KCNQ1 (GenBank accession number U89364) and KCNE1 (GenBank accession number NM000219), and assembled to obtain the full length cDNAs. They were inserted into the vector pIRES (Clontech, Palo Alto, CA, U.S.A.) to yield a tricistronic expression vector from which a single mRNA coding KCNQ1, KCNE1, and the neomycin resistance protein is transcribed. This vector, together with the vector pIRES-hrGFP-1α (Stratagene, Garden Grove, CA, U.S.A.), which encodes the green fluorescence protein (GFP), was introduced into HEK293 cells with lipofectamine (Invitrogen, Tokyo) and stable transformants were obtained by clone culture in the presence of G418 (500 μg/ml), a neomycin analog. Stable transformants of HEK293 cells expressing KCNQ1/KCNE1 channels were obtained as described in our previous report.[9] The cells expressing either channel were plated on glass coverslips 48 to 72 h before electrophysiological experiments.

Electrophysiological Recording of Expressed KCNQ1/KCNE1 Channel Current Whole-cell voltage clamp experiments were performed with HEK293 cells expressing KCNQ1/KCNE1 channels in a chamber mounted on the stage of an inverted microscope. The chamber was perfused continuously at a flow rate of 1.0 to 2.0 ml/min and the temperature was maintained at 22—25 °C. The external solution

* To whom correspondence should be addressed. e-mail: htanaka@phar.toho-u.ac.jp

was of the following composition: 128.0 mM NaCl, 20.0 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.33 mM NaH_2PO_4, 5.0 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid (HEPES), 10.0 mM glucose (pH 7.4 with NaOH). The patch pipette solution was of the following composition: 100 mM KOH, 40 mM KCl, 70 mM aspartic acid, 1 mM $MgCl_2$, 5 mM ATP-K_2, 5 mM creatine phosphate-K_2, 5 mM HEPES, 10 mM ethylene glycol bis(2-aminoethylether)-*N,N,N',N'*-tetraacetic acid (EGTA) (pH 7.2 with KOH). Pipette tip resistances were 2 to 4 MΩ when filled with the patch pipette solution. The KCNQ1/KCNE1 channel current was measured as the amplitude of the peak tail current on return to a holding potential of −40 mV from 3 s voltage clamp pulses. Data acquisition and analyses were performed with the system described previously[9] including a patch-clamp amplifier (Axopatch 1D; Axon Instruments, Foster City, CA, U.S.A.), a personal computer (Prolinea 486; Compaq, Houston, TX, U.S.A.) and pCLAMP software (Axon).

Drugs and Chemicals NIP-142 was synthesized and provided by Nissan Chemical Industries (Tokyo, Japan). NIP-142 was added to the bath solution from a stock solution (100 mM) in 0.1 M HCl. The final concentration of HCl in the measuring bath (<0.1 mM) did not affect any of the experimental parameters measured. All other chemicals were commercial products of the highest available grade of quality.

RESULTS

In whole cell voltage clamped HEK293 cells expressing the KCNQ1/KCNE1 channel, depolarization to membrane potentials more positive than −10 mV induced voltage- and time-dependent outward currents. On repolarization to −40 mV, outward tail currents were observed (Figs. 1A, C). The peak amplitude of the tail current after a depolarizing pulse to 20 mV was 8.6±0.8 pA/pF (n=10).

Chromanol 293B, at 1 to 30 μM, concentration-dependently reduced the outward currents on depolarization and the tail currents on repolarization (Figs. 1A, B). The current density of the tail current after a depolarizing pulse to 20 mV in the absence and presence of 1, 3, 10 and 30 μM chromanol 293B was 7.5±1.4 pA/pF (n=10), 5.4±0.4 pA/pF (n=4), 4.5±0.9 pA/pF (n=5), 2.2±0.2 pA/pF (n=6) and 0.1±0.2 pA/pF (n=4), respectively. The EC_{50} value for the inhibition of tail current on repolarization from 20 mV was 4.9 μM (Fig. 1E).

NIP-142, at 10 and 30 μM, concentration-dependently reduced the outward currents on depolarization and the tail current on repolarization (Fig. 1C). The current density of the tail current after a depolarizing pulse to 20 mV in the absence and presence of 1, 10 and 30 μM NIP-142 was 9.7±0.7 pA/pF (n=10), 9.5±1.4 pA/pF (n=4), 5.4±1.0 pA/pF (n=5) and 1.8±0.5 pA/pF (n=5), respectively. The EC_{50} value for the inhibition of tail current on repolarization from 20 mV was 13.2 μM (Fig. 1E).

DISCUSSION

NIP-142 concentration-dependently blocked the KCNQ1/KCNE1 channel current with an EC_{50} value of 13.2 μM (Fig. 1). The reported EC_{50} value of NIP-142 for the GIRK1/4 channel current is 0.64 μM,[9] that for the Kv1.5

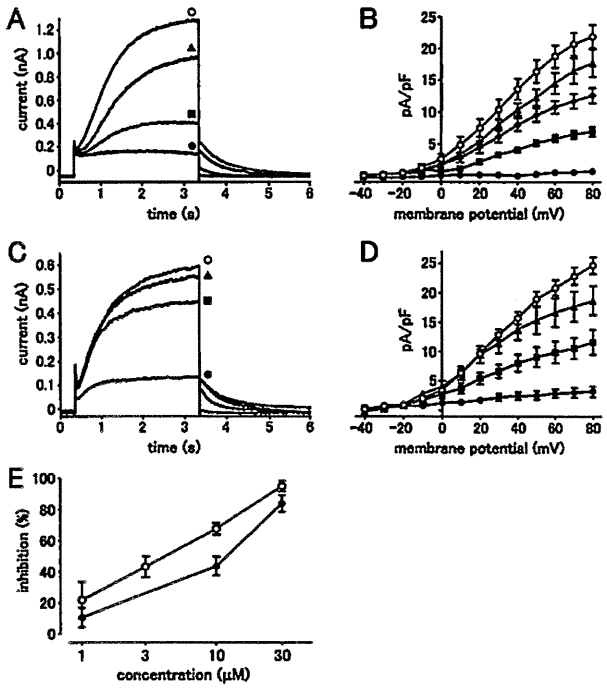

Fig. 1. Effect of Chromanol 293B and NIP-142 on KCNQ1/KCNE1 Channel Currents Expressed in HEK293 Cells

(A) Typical recordings in the absence (open circles) and presence of 1 μM (closed triangles), 10 μM (closed squares) and 30 μM (closed circles) chromanol 293B. (B) Summarized current–voltage relationships for the peak outward tail current on repolarization to −40 mV from a 3 s depolarization to various test potentials in the absence (open circles) and presence of 1 μM (closed triangles), 3 μM (closed diamonds), 10 μM (closed squares) and 30 μM (closed circles) chromanol 293B. (C) Typical recordings of the effects of NIP-142. (D) Summarized current–voltage relationships for the peak outward tail current on repolarization to −40 mV from a 3 s depolarization to various test potentials in the absence (open circles) and presence of 1 μM (closed triangles), 10 μM (closed squares) and 30 μM (closed circles) NIP-142. (E) Concentration–response relationship for blockade by chromanol 293B (open circles) and NIP-142 (closed circles) of the peak tail current on repolarization from 20 mV. Symbols and bars represent the mean±S.E.M from 4 to 8 experiments.

channel current is 4.8 μM,[10] and that for the HERG channel current is 44 μM.[9] The blocking potency of NIP-142 on the KCNQ1/KCNE1 channel current was relatively low, suggesting that the prolongation of atrial action potential by the compound is mainly produced by blockade of potassium currents other than I_{Ks}, especially the acetylcholine-activated potassium current. However, as the concentration range of NIP-142 (10 to 100 μM) to prolong the atrial action potential[8] overlaps that to inhibit the KCNQ1/KCNE1 channel current (Fig. 1E), the possibility that I_{Ks} blockade contributes to the effect of NIP-142 can not be totally excluded. The I_{Ks} current is known to be increased by factors such as β-adrenergic stimulation[12] or angiotensin II.[13] A gain-of-function mutation of KCNQ1 was reported to cause atrial fibrillation.[14] Development of novel I_{Ks} blockers with antiarrhythmic activity are in progress.[15] Thus, the I_{Ks}-blocking effect of NIP-142 may contribute to its antifibrillatory activity under certain pathological conditions.

NIP-142 was reported not to prolong the action potential duration in isolated guinea-pig ventricular tissue preparations,[8] despite its inhibitory effect on the KCNQ1/KCNE1 channel current. One explanation for this apparent discrepancy is the variation in sensitivity among experimental sys-

tems. There are cases in which potency of ion channel inhibitors are higher in voltage-clamped single cells than in myocardial tissue preparations (*S*(+)-Efonidipine[16]). The extent of I_{Ks}-blockade by NIP-142 in tissue preparations might be smaller than expected from the present results. Another possible explanation for the lack of action potential prolongation by NIP-142 in the ventricle is its blocking effect on calcium channels. We have observed that NIP-142 has blocking effect on the L-type calcium channel,[17] which may counteract its action potential-prolonging effect through potassium channel blockade. In any case, the details for the mechanisms of action of NIP-142 remain to be investigated.

Acknowledgments This study was supported in part by the Science Research Promotion Fund from the Promotion and Mutual Aid Corporation for Private Schools of Japan (for Y. Tanaka and H. Tanaka). This study was partly performed as a part of the project "Research on the molecular mechanisms of appearance of age-related diseases by failure of cell function control system, and their prevention and treatment" by the "Research Center for Aging and Age-Related Diseases" established in the Toho University Faculty of Pharmaceutical Sciences.

REFERENCES

1) Sugimoto T., Hayakawa K., Kasanuki H., "Atrial Fibrillation, Flutter, and Tachycardia," ed. by Hayakawa K., Kasanuki H., Igaku-Shoin, Tokyo, 1999, pp. 14—18.
2) Aliot E., Denjoy I., *Am. J. Cardiol.*, **77**, 66A—71A (1996).
3) Iwasaki H., Takahara A., Nakamura Y., Satoh Y., Nagai T., Shinkai N., Sugiyama A., *J. Pharmacol. Sci.*, **110**, 410—414 (2009).
4) Lenz T., Hilleman D., *Drugs Today* (Barc), **36**, 759—771 (2000).
5) Nagasawa H., Fujiki A., Fujikura N., Matsuda T., Yamashita T., Inoue H., *Circ. J.*, **66**, 185—191 (2002).
6) Tanaka H., Hashimoto N., *Cardiovas. Drug Rev.*, **25**, 342—356 (2007).
7) Hashimoto N., Tanaka H., "Progress in Cardiac Arrhythmia Research," ed. by Tarkowicz I. R., Nova Science Publishers, Hauppague, 2009, pp. 125—139.
8) Matsuda T., Takeda K., Ito M., Yamagishi R., Tamura M., Nakamura H., Tsuruoka N., Saito T., Masumiya H., Suzuki T., Iida-Tanaka N., Itokawa M., Yamashita T., Tsuruzoe N., Tanaka H., Shigenobu K., *J. Pharmacol. Sci.*, **98**, 33—40 (2005).
9) Matsuda T., Ito M., Ishimaru S., Tsuruoka N., Saito T., Iida-Tanaka N., Hashimoto N., Yamashita T., Tsuruzoe N., Tanaka H., Shigenobu K., *J. Pharmacol. Sci.*, **101**, 303—310 (2006).
10) Matsuda T., Masumiya H., Tanaka N., Yamashita T., Tsuruzoe N., Tanaka Y., Tanaka H., Shigenobu K., *Life Sci.*, **68**, 2017—2024 (2001).
11) Bosch R. F., Gaspo R., Busch A. E., Lang H. J., Li G. R., Nattel S., *Cardiovas. Res.*, **38**, 441—450 (1998).
12) Sanguinetti M. C., Jurkiewicz N. K., Scott A., Siegl K. S., *Circ. Res.*, **68**, 77—84 (1991).
13) Zankov D. P., Omatsu-Kanbe M., Isono T., Toyoda F., Ding W. G., Matsuura H., Horie M., *Circulation*, **113**, 1278—1286 (2006).
14) Chen Y. H., Xu S. J., Bendahhou S., Wang Y., Xu W. Y., Jin H. W., Sun H., Su X. Y., Zhuang Q. N., Yang Y. Q., Li Y. B., Liu Y., Xu H. J., Li X. F., Ma N., Mou C. P., Chen Z., Barhanin J., Huang W., *Science*, **299**, 251—254 (2003).
15) Salataa J. J., Scinickb H. G., Lynch J. J. Jr., *Curr. Med. Chem.*, **11**, 29—44 (2004).
16) Tanaka H., Namekata I., Ogawa T., Tsuneoka Y., Komikado C., Takahara A., Iida-Tanaka N., Izumi-Nakaseko H., Tsuru H., Adachi-Akahane S., *Eur. J. Pharmacol.*, **649**, 263—267 (2010).
17) Matsuda T., Masumiya H., Saito T., Tanaka H., Shigenobu K., *Jpn. J. Pharmacol.*, **79** (Suppl. I), 178P (1999).

Current Topics in Pharmacology

Mini-Review

Electropharmacological properties of the pulmonary vein myocardium

Hikaru Tanaka*, Yayoi Tsuneoka, and Iyuki Namekata

Department of Pharmacology, Toho University Faculty of Pharmaceutical Sciences, Miyama 2-2-1, Funabashi, Chiba 274-8510, Japan

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

*Corresponding author
htanaka@phar.toho-u.ac.jp

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.

RERERENCES

1. Brunton, T. L. Fayrer, J. 1876, Proc. Royal Soc. London., 25, 174.
2. Tasaki, H. 1969, Jpn. Circ. J., 33, 1087.
3. Cheung, D. W. 1981, J. Physiol., 314, 445.
4. Haissaguerre, M., Jais, P., Shah, D. C., Takahashi, A., Hocini, M., Quiniou, G., Garrigue, S., Le Mouroux, A., Le Metayer, P., and Clementy, J. 1998, N. Engl. J. Med., 339, 659.
5. Chen, S. A., Hsieh, M. H., Tai, C. T., Tsai, C. F., Prakash, V. S., Yu, W. C., Hsu, T. L., Ding, Y. A., and Chang, M. S. 1999, Circulation, 100, 1879.
6. Saito, T., Waki, K., and Becker, A. E. 2000, J. Cardiovasc. Electrophysiol., 11, 888.
7. Hocini, M., Ho, S. Y., Kawara, T., Linnenbank, A. C., Potse, M., Shah, D., Jais, P., Janse, M. J., Haissaguerre, M., and De Bakker, J. M. 2002, Circulation, 105, 2442.
8. Hamabe, A., Okuyama, Y., Miyauchi, Y., Zhou, S., Pak, H. N., Karagueuzian, H. S., Fishbein, M. C., and Chen, P. S. 2003, Circulation, 107, 1550.
9. Tan, A. Y., Li, H., Wachsmann-Hogiu, S., Chen, L. S., Chen, P. S., and Fishbein, M. C. 2006, J. Am. Coll. Cardiol., 48, 132.
10. Kholova, I. and Kautzner, J. 2003, Pacing Clin. Electrophysiol., 26, 1348.
11. Gao, C. H., Wang, F., Jiang, R., Zhang, J., Mou, H., and Yin, Y. H. 2011, Auton. Neurosci., 162, 42.
12. Ehrlich, J. R., Cha, T. J., Zhang, L., Chartier, D., Melnyk, P., Hohnloser, S. H., and Nattel, S. 2003, J. Physiol., 551, 801.
13. Melnyk, P., Ehrlich, J. R., Pourrier, M., Villeneuve, L., Cha, T. J., and Nattel, S. 2005, Cardiovasc. Res., 65, 104.
14. Chen, Y. J., Chen, S. A., Chen, Y. C., Yeh, H. I., Chang, M. S., and Lin, C. I. 2002, Basic Res. Cardiol., 97, 26.
15. Chen, Y. C., Chen, S. A., Chen, Y. J., Tai, C. T., Chan, P., and Lin, C. I. 2004, J. Cardiovasc. Electrophysiol., 15, 567.
16. Chen, Y. C., Pan, N. H., Cheng, C. C., Higa, S., Chen, Y. J., and Chen, S. A. 2009, J. Cardiovasc. Electrophysiol., 20, 1039.
17. Namekata, I., Tsuneoka, Y., Takahara, A., Shimada, H., Sugimoto, T., Takeda, K., Nagaharu, M., Shigenobu, K., Kawanishi, T., and Tanaka, H. 2009, J. Pharmacol. Sci., 110, 111.

18. Jones, S. A., Yamamoto, M., Tellez, J. O., Billeter, R., Boyett, M. R., Honjo, H., and Lancaster, M. K. 2008, Circ. Arrhythm Electrophysiol., 1, 39.
19. Chang, S. H., Chen, Y. C., Chiang, S. J., Higa, S., Cheng, C. C., Chen, Y. J., and Chen, S. A. 2008, Life Sci., 83, 284.
20. Yu, M. C., Huang, C. F., Chang, C. M., Chen, Y. C., Lin, C. I., and Chen, S. A. 2011, Heart Vessels., 26, 101.
21. Honjo, H., Boyett, M. R., Niwa, R., Inada, S., Yamamoto, M., Mitsui, K., Horiuchi, T., Shibata, N., Kamiya, K., and Kodama, I. 2003, Circulation, 107, 1937.
22. Wongcharoen, W., Chen, Y. C., Chen, Y. J., Chang, C. M., Yeh, H. I., Lin, C. I., and Chen, S. A. 2006, Cardiovasc. Res., 70, 497.
23. Chen, Y. J., Chen, Y. C., Wongcharoen, W., Lin, C. I., and Chen, S. A. 2008, Br. J. Pharmacol., 153, 915.
24. Namekata, I., Tsuneoka, Y., Akiba, A., Nakamura, H., Shimada, H., Takahara, A., and Tanaka, H. 2010, Bioimages, 18, 11.
25. Takahara, A., Sugimoto, T., Kitamura, T., Takeda, K., Tsuneoka, Y., Namekata, I., and Tanaka, H. 2011, J. Pharmacol. Sci., 115, 176.
26. Tanaka, H., Nishimaru, K., Aikawa, T., Hirayama, W., Tanaka, Y., and Shigenobu, K. 2002, Br. J. Pharmacol., 135, 1096.
27. Coutu, P., Chartier, D., and Nattel, S. 2006, Am. J. Physiol. Heart Circ. Physiol., 291, H2290.
28. Seol, C. A., Kim, J., Kim, W. T., Ha, J. M., Choe, H., Jang, Y. J., Shim, E. B., Youm, J. B., Earm, Y. E., and Leem, C. H. 2008, Prog. Biophys. Mol. Biol., 96, 132.
29. Wang, T. M., Luk, H. N., Sheu, J. R., Wu, H. P., and Chiang, C. E. 2005, Int. J. Cardiol., 104, 59.
30. Lo, L. W., Chen, Y. C., Chen, Y. J., Wongcharoen, W., Lin, C. I., and Chen, S. A. 2007, Eur. J. Pharmacol., 571, 197.
31. Doisne, N., Maupoil, V., Cosnay, P., and Findlay, I. 2009, Am. J. Physiol. Heart Circ. Physiol., 297, H102.
32. Maupoil, V., Bronquard, C., Freslon, J. L., Cosnay, P., and Findlay, I. 2007, Br. J. Pharmacol., 150, 899.
33. Kalifa, J., Jalife, J., Zaitsev, A. V., Bagwe, S., Warren, M., Moreno, J., Berenfeld, O., and Nattel, S. 2003, Circulation, 108, 668.
34. Lee, S. H., Chen, Y. C., Chen, S. Y., Lin, C. I., Chen, Y. J., and Chen, S. A. 2008, Eur. J. Clin. Invest., 38, 17.
35. Seol, C. A., Kim, W. T., Ha, J. M., Choe, H., Jang, Y. J., Youm, J. B., Earm, Y. E., and Leem, C. H. 2008, Prog. Biophys. Mol. Biol., 97, 217.
36. Iwasaki, H., Takahara, A., Nakamura, Y., Satoh, Y., Nagai, T., Shinkai, N., and Sugiyama, A. 2009, J. Pharmacol. Sci., 110, 410.
37. Neuberger, H. R., Schotten, U., Verheule, S., Eijsbouts, S., Blaauw, Y., van Hunnik, A., and Allessie, M. 2005, Circulation, 111, 30.
38. Nouchi, H., Takahara, A., Nakamura, H., Namekata, I., Sugimoto, T., Tsuneoka, Y., Takeda, K., Tanaka, T., Shigenobu, K., Sugiyama, A., and Tanaka, H. 2008, Eur. J. Pharmacol., 597, 81.
39. Chen, Y. C., Chen, S. A., Chen, Y. J., Chang, M. S., Chan, P., and Lin, C. I. 2002, J. Am. Coll. Cardiol., 39, 366.
40. Lee, S. H., Chen, Y. C., Chen, Y. J., Chang, S. L., Tai, C. T., Wongcharoen, W., Yeh, H. I., Lin, C. I., and Chen, S. A. 2007, Life Sci., 80, 1806.
41. Lin, Y. K., Lai, M. S., Chen, Y. C., Cheng, C. C., Huang, J. H., Chen, S. A., Chen, Y. J., and Lin, C. I. 2012, Clin. Sci. (Lond)., 122, 121.
42. Datino, T., Macle, L., Qi, X. Y., Maguy, A., Comtois, P., Chartier, D., Guerra, P. G., Arenal, A., Fernandez-Aviles, F., and Nattel, S. 2010, Circulation, 121, 963.
43. Aistrup, G. L., Villuendas, R., Ng, J., Gilchrist, A., Lynch, T. W., Gordon, D., Cokic, I., Mottl, S., Zhou, R., Dean, D. A., Wasserstrom, J. A., Goldberger, J. J., Kadish, A. H., and Arora, R. 2009, Cardiovasc. Res., 83, 481.
44. Hashimoto, N., Yamashita, T., and Tsuruzoe, N. 2006, Pharmacol. Res., 54, 136.
45. Machida, T., Hashimoto, N., Kuwahara, I., Ogino, Y., Matsuura, J., Yamamoto, W., Itano, Y., Zamma, A., Matsumoto, R., Kamon, J., Kobayashi, T., Ishiwata, N., Yamashita, T., Ogura, T., and Nakaya, H. 2011, Circ. Arrhythm Electrophysiol., 4, 94.

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

Iyuki Namekata and Hikaru Tanaka

Department of Pharmacology,
Toho University Faculty of
Pharmaceutical Sciences,
Funabashi, Chiba 274-8510, Japan.

Address for correspondence and proofs:
Hikaru Tanaka,
Department of Pharmacology,
Toho University Faculty of Pharmaceutical Sciences,
Miyama 2-2-1 Funabashi, Chiba 274-8510, Japan.
Telephone number: +81-474722092
Facsimile number: +81-474722113
E-mail: htanaka@phar.toho-u.ac.jp

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