SPANDIDOS PUBLICATIONS

MOLECULAR MEDICINE REPORTS 4: 569-573, 2011

# Spironolactone diminishes spontaneous ventricular premature beats by reducing HCN4 protein expression in rats with myocardial infarction

TAO SONG*, JING YANG*, YUAN YAO, HAITAO LI, YONGJUN CHEN, JUN ZHANG and CONGXIN HUANG

Department of Cardiology, Renmin Hospital of Wuhan University, and
Cardiovascular Research Institute,Wuhan University, Wuhan 430060, P.R. China

Received December 28, 2010; Accepted March 7, 2011

DOI: 10.3892/mmr.2011.455

**Abstract.** Hyperpolarization-activated current (I*f*) is the major ionic current contributing to the spontaneous diastolic depolarization of cardiac sinus node pacemaker cells. It is mediated by hyperpolarization-activated and cyclic nucleotide-gated (HCN) channels. However, several observations support a potential role of HCN channels in the arrhythmogenesis of working myocardium under pathological conditions. Spironolactone, a classic aldosterone blocker, has been proved to prevent spontaneous ventricular arrhythmias after myocardial infarction (MI). Here, we examined the effect of spironolactone on the expression of HCN channels and ventricular premature beats (VPBs) using a rat MI model. Sprague-Dawley rats were divided into a sham-operated group and MI groups treated with intragastric administration of saline or spironolactone (80 $\mu$g/kg/day) for 7 days immediately after ligation of the left coronary artery. Compared to the sham group, HCN2 and HCN4 protein levels were increased in MI rats. Treatment with spironolactone prevented the MI-induced increase of HCN4 protein levels (1.47±0.16 vs. 1.81±0.21, P<0.05). MI rats exhibited a marked increase of VPBs compared to the sham group (104±17 vs. 3±1 VPBs/h, P<0.05). This the increase was reduced by spironolactone (55±14 vs. 104±17 VPBs/h, P<0.05). Moreover, I*f* current inhibitor (ivabradine, 0.5 mg/kg) further decreased the occurrence of VPBs in the control and spironolactone groups to the same level (54±13 vs. 49±8 VPBs/h, P>0.05). In conclusion, spironolactone may prevent ischemia-induced VPBs by reducing HCN4 protein expression to basal levels.

*Correspondence to:* Dr Congxin Huang, Cardiovascular Research Institute, Division of Cardiology, Renmin Hospital of Wuhan University, 238 Jiefang Road, Wuchang, Wuhan 430060, P.R. China
E-mail: huangcongxing@yahoo.cn

*Contributed equally

*Key words:* myocardial infarction, hyperpolarization-activated cyclic nucleotide-gated channel (HCN), HCN4, arrhythmia, spironolactone

## Introduction

Hyperpolarization-activated current (I*f*) is a mixed $Na^+/K^+$ inward current activated by hyperpolarization and encoded by a family of genes termed hyperpolarization-activated cyclic nucleotide-gated channel (HCN) genes (1). In addition to its activity in sinoatrial (SA) node cells, I*f* has been considered to produce automatic activity in other cardiac regions, such as purkinje fibers, atrioventricular (AV) nodes, atria and ventricles (2). Latent pacemakers play a compensatory role in pacemaking when SA or AV node function is impaired. However, excessive activation of I*f* in cardiac regions can lead to enhanced automaticity from the ectopic focus, resulting in atrial and ventricular arrhythmias (3).

Four genes encoding the HCN channels (HCN1-HCN4) have been identified as functionally expressed (4,5). HCN2 and HCN4 are the predominant HCN transcripts in the adult heart (6). Previous studies have found that the expression of the HCN2 and/or HCN4 genes in ventricular cells undergoes dynamic changes in several heart diseases, such as heart failure (HF), hypertrophic cardiomyopathy (HCM), atrial fibrillation (AF) and acute myocardial infarction (AMI). Therefore, it has been postulated that the HCN/I*f* channels may contribute to arrhythmogenesis in disease states (7-11).

Two landmark clinical trials (RALES and EPHESUS) found that low doses of aldosterone blocker (AB) led to a dramatic reduction in mortality rate (12,13). Recent studies have also shown the usefulness of AB for post-myocardial infarction patients (14). However, the mechanisms of AB are not completely understood. Researchers have suggested that the reduction of SCD by AB may be relevant to the decrease in ventricular arrhythmias (15).

Accordingly, we hypothesized that AB may exert an anti-arrhythmic effect in post-MI patients by targeting the HCN channels (HCN2 and/or HCN4). Ventricular premature beats are the most common spontaneous ventricular arrhythmias observed in patients after MI. In this study, we examined the effect of spironolactone, a classic aldosterone blocker, on the expression of HCN channels and on VPBs. Moreover, a highly selective I*f* current inhibitor (ivabradine) was used to determine whether the reduction of ventricular premature beats (VPBs) is caused by decrease in the expression of the HCN channels.

## Materials and methods

*Experimental model*. Adult male Sprague-Dawley rats (200-250 g) were used in this study. Animals were anesthetized with an intraperitoneal injection of 3% pentobarbital sodium (30 mg/kg) and underwent left coronary artery ligation through a left thoracotomy to induce MI (n=65) by regional ischemia or sham coronary ligation (n=12). The surgery was performed with electrocardiogram monitoring. Successful ligation of the left coronary artery was verified by the color change in the ischemic area and ECG lead I and a VL S-T segment elevations after the occlusion. In sham-operated rats, a suture was tied loosely around the left coronary artery without ligating it.

Seven rats succumbed within 24 h of coronary ligation. All rats surviving 24 h after the surgery were randomly assigned to three groups: sham-operated rats (sham, n=12), MI rats receiving intragastric administration of saline for 7 days (control, n=30), and MI rats treated with 80 $\mu$g/kg/day spironolactone for 7 days (spironolactone, n=28). The experiments were conducted in accordance with the Guidelines for Animal Experimentation of the Institutional Animal Care and Use Committee of Wuhan University.

*Monitoring of spontaneous arrhythmias*. One week after MI, the rats were anesthetized again and continuously monitored from 6 to 8 p.m. Heart rate (HR) and VPBs were measured with a computer-based electrophysiology system (LEAD2000B; Jinjiang Ltd., Chengdu, China). Ten rats in the control group and 11 rats in the spironolactone group received a single intravenous injection of ivabradine via the tail vein (0.5 mg/kg). Thirty minutes later, electrocardiogram (ECG) readings were recorded for another hour.

*Tissue collection*. At the end of the experimental period, the heart of rats not treated with ivabradine were removed from the chest under pentobarbital anesthesia (100 mg/kg, i.p.). The infarcted region was visually identified by a mottled and pale appearance. The myocardium extending 0.5-1.0 mm from the infarct scar was considered to represent the border zone of the myocardial infarction. To avoid contamination of the non-infarcted left ventricular free wall with the infarcted border, a myocardial area extending 1-2 mm from the border zone area was excluded.

*Western blot analysis*. Membrane protein was extracted from tissue samples followed by tissue homogenization at 4˚C. Equal amounts of protein extracts in each group were fractionated on 8% SDS-polyacrylamide gels for HCN2 (6% for HCN4) and transferred electrophoretically to polyvinylidene difluoride membrane (Millipore). The membranes were blocked in blocking buffer (5% nonfat dry milk in TBS) for 2 h at room temperature, then incubated with primary antibodies (HCN2, 1:500; HCN4, 1:250; β-actin, 1:1,000; Boster Biotechnology Co., Ltd., Wuhan, China) overnight at 4˚C. Horseradish peroxidase-conjugated anti-rabbit and anti-goat immunoglobulin IgG (1:1,000; Zhongshan Jin Qiao Biotechnology Co., Ltd., Beijing, China) was used as the second antibody, applied for 1 h at 37˚C. Immunoreactivity was enhanced by a chemiluminescence kit (Beyotime, Inc., China) and then the blots were exposed to film. The density of the bands on the Western blots was quantified using a Bio-Rad imaging system (Bio-Rad, Hercules, CA, USA).

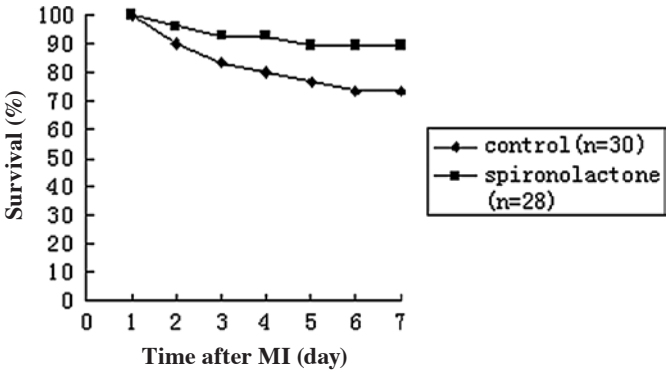


Figure 1. Survival curves in the spironolactone and control groups 7 days after MI. The survival rate in the spironolactone group (87.3%, 25/28) was significantly higher than that of the control group (73.3%, 22/30) (P<0.05).

Table I. Effect of spironolactone on heart rate and ventricular premature beats.

| | Groups | | |
|---|---|---|---|
| | Sham (n=12) | Control (n=22) | Spironolactone (n=25) |
| HR (beats/min) | 402±23 | 413±36 | 408±26 |
| VPBs/h | 3±1 | 104±17[a] | 55±14[a,b] |

Data are expressed as the mean ± SD. [a]P<0.05 vs. sham group, [b]P<0.05 vs. control group. HR, heart rate; VPBs, ventricular premature beats.

*Statistical analysis*. Data are expressed as the mean ± SD. The Kaplan-Meier method with a log-rank test was used for the survival analysis. One-way ANOVA was used to compare more than two groups, and the Newman-Keul's test was used to examine differences between individual groups. Statistical significance was defined as P<0.05.

## Results

*Experimental model*. The study was completed using 59 surviving animals, 22 in the control group, 25 in the spironolactone group and 12 in the sham group. The survival rate in the spironolactone group (87.3%, 25/28) was significantly higher than that of the control group (73.3%, 22/30) (P<0.05) (Fig. 1).

*Effects of spironolactone on heart rate and ventricular premature beats*. Compared to the sham group, the MI rats exhibited a significant increase in VPBs (104±17 vs. 3±1 VPBs/h, P<0.05). Spironolactone induced a marked decrease in VPBs in MI rats (55±14 vs. 104±17 VPBs/h, P<0.05), which was different from that of the sham group (55±14 vs. 3±1 VPBs/h, P<0.05). Spironolactone had no effect on HR in any of the groups (Table I). Ivabradine reduced HR

Table II. Effects of ivabradine on heart rate and ventricular premature beats in the control and the spironolactone groups.

| | Control group (n=10) | | Spironolactone group (n=11) | |
|---|---|---|---|---|
| | HR (beats/min) | VPBs/h | HR (beats/min) | VPBs/h |
| Before ivabradine treatment | 419±51 | 115±20 | 408±47 | 61±11[b] |
| After ivabradine treatment | 322±44[a] | 54±13[a] | 315±38[a] | 49±8[a] |

Data are expressed as the mean ± SD. [a]P<0.05 vs. rats before ivabradine treatment. [b]P<0.05 vs. the control group. HR, heart rate; VPBs, ventricular premature beats.

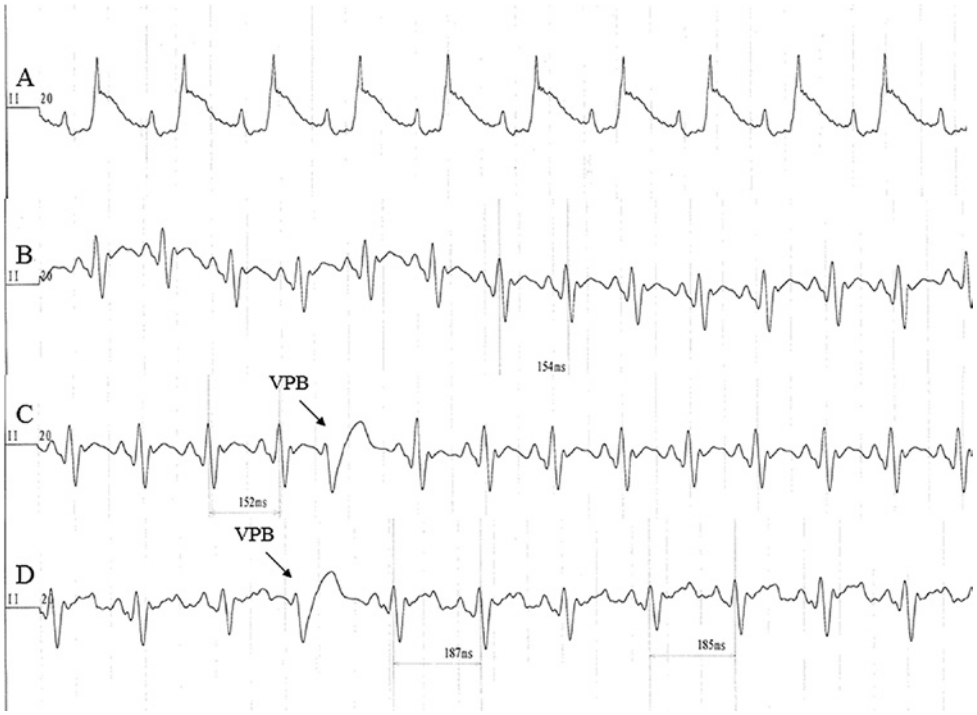


Figure 2. Electrocardiograms of ventricular premature beats (VPBs). (A) Model of acute myocardial infarction; (B) sham group; (C) spironolactone group; (D) spironolactone group after receiving a single intravenous injection of ivabradine via the tail vein.

in all the groups, and moreover induced a marked decrease in VPBs in the control and the spironolactone groups (Table II, Fig. 2). Before treatment with ivabradine, VPBs were more frequent in the control group compared to the spironolactone group (115±20 vs. 61±11 VPBs/h, P<0.05). However, the VPBs of both groups markedly decreased to the same level (54±13 vs. 49±8 VPBs/h, P>0.05) after treatment with ivabradine.

*Effect of spironolactone on HCN2 and HCN4 protein expression*. Fig. 3 shows a representative blot and quantitative results in which HCN2 and HCN4 band intensities were normalized to β-actin. Quantitative densitometric analysis revealed that the level of HCN2 protein was up-regulated by 1.37- and 1.31-fold (Fig. 3B) and the level of HCN4 protein was significantly up-regulated by 1.81- and 1.47-fold in the control and the spironolactone groups, respectively as compared to the sham group (all P<0.05). However, the increase in HCN4 values was significantly lower in the spironolactone-treated rats than in the control group (1.47±0.16 vs. 1.81±0.21, P<0.05) (Fig. 3C).

## Discussion

In this study, we confirmed that HCN2 and HCN4 protein levels improved in the ischemic left ventricular myocardium of rats 1 week after MI (11). The environment of the ventricular cells changed after AMI: aldosterone increased after rennin-angiotensin-aldosterone system (RASS) activation (16), the β2-adnephrin level was increased due to enhanced adrenergic nerve activity (17), and the delivery of endothelin-1 increased from injured blood vessel endothelium cells. These stimulating factors enhanced the protein expression of HCN2 and HCN4. Furthermore, we found that spironolactone reduced the expression of HCN4 protein after MI. This is in agreement with a study of Muto *et al*, which found that aldosterone at physiological concentrations up-regulates HCN channel expression, and that the increased expression of the HCN channels is completely blocked by spironolactone (16). Therefore, we concluded that spironolactone plays an essential role in reducing HCN channel expression after MI.

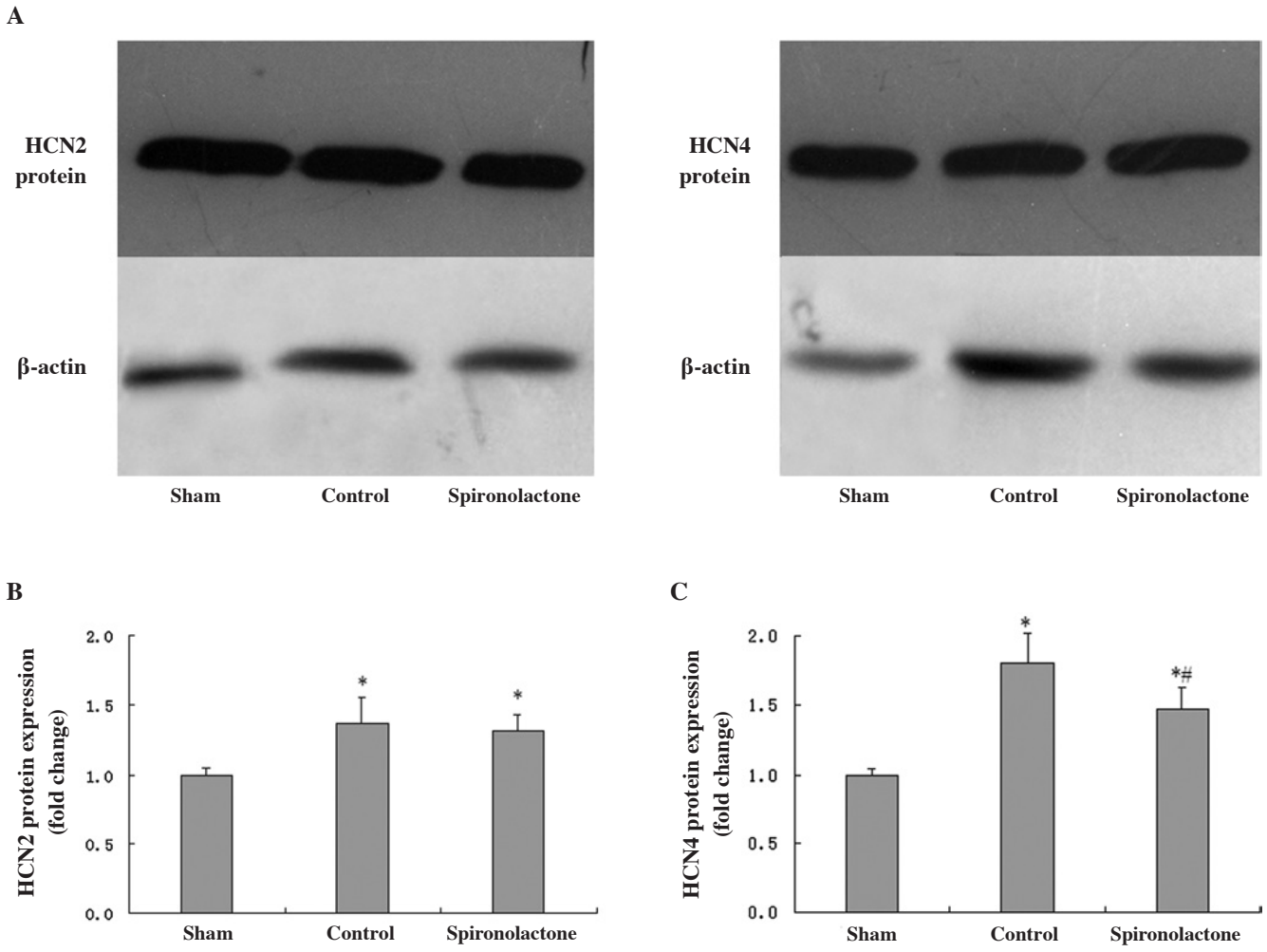


Figure 3. Western blot analysis of HCN2 and HCN4 after MI. (A) HCN2 and HCN4 protein levels were measured by Western blot analysis. (A) Original representative Western blots. (B and C) The blots were scanned and band intensities were quantified by densitometric analysis, performed using the ImageJ program. Expression of HCN2 and HCN4 protein were normalized to that of β-actin. Results are presented as fold changes relative to sham group. Data are expressed as the mean ± SD (n=12 to 14 for each group). *P<0.05 vs. sham group; #P<0.05 vs. control group.

HCN channel family genes, which figure prominently in physiological automaticity, are related to automatic pacing activity (18,19). Previous studies have found that the alteration of the pacemaker I*f* was related to arrhythmia in hypertrophic hearts (8,20); for example, Stillitano *et al* (21) found that I*f* is of larger amplitude and activates at less negative potential in failing ventricular myocytes. However, there have been no reports on the direct relationship between HCN channels and arrhythmia, although increasing evidence supports a potential role of the HCN channels in the development of arrhythmogenesis of the ventricular myocardium in the pathological heart (8-11). Thus, many researchers are examining the effect of ivabradine on arrhythmia after MI (22,23).

It is well known that ivabradine is a novel HR lowering agent prescribed in patients with stable angina pectoris (24). This selective and specific inhibitor of the cardiac pacemaker I*f* current reduces HR both at rest and during exercise, without altering myocardial contractility and relaxation, atrioventricular conduction and ventricular repolarization, in all animals (25,26). Moreover, several studies have found that ivabradine reduced VPBs and induced an increase in the ventricular fibrillation threshold after MI (22,23). However, these beneficial effects of ivabradine may be related to the reduction in HR.

In the present study, we found that VPBs occurred frequently in the MI rats, and that HCN4 protein levels were increased (Fig. 3C). Spironolactone significantly decreased the HCN4 protein levels and the occurrence of VPBs. Notably, ivabradine markedly decreased the VPBs of the spironolactone and the control groups to the same level (Table II), but the HR of both groups was similar. Based on previous results and those reported here, we concluded that the up-regulation of HCN4 protein in MI rats increased the ventricular ectopic impulse, and that the beneficial effect of spironolactone in reducing VPBs may be directly related to the reduction of HCN4 protein levels.

Previous studies have also reported that spironolactone leads to a dramatic reduction in the mortality rate (12-14). A subsequent study showed that the combination of VPB plus couplets is an independent indicator of the total mortality rate after acute MI (27). However, the mechanisms of this effect are unclear; several mechanisms may account for the reduction in mortality caused by spironolactone, such as attenuated sympathetic tone

(28,29) and improved parasympathetic activity (30,31), or direct inhibition of the MI-induced increase in T-type calcium current (32,33). In this study, we found a correlation between improvements in the survival rate (Fig. 1) and the reduction of VPBs (Table I) in MI rats treated with spironolactone. Thus, VPB reduction due to a decrease in HCN4 protein expression may be a mechanism by which spironolactone reduces mortality in MI rats.

In conclusion, spironolactone induces the restoration of HCN4 protein expression. This effect reduced VPBs, which may play an important role in reducing mortality after MI. The decrease in VPBs in the MI heart induced by spironolactone may be achieved, at least in part, by decreasing HCN4 protein expression.

## Acknowledgements

This study was supported by a grant from the National Natural Science Foundation of China (no. 30700312).

## References

1. Biel M, Schneider A and Wahl C: Cardiac HCN channels: structure, function, and modulation. Trends Cardiovasc Med 12: 206-213, 2002.
2. Barbuti A and Difrancesco D: Control of cardiac rate by 'funny' channels in health and disease. Ann NY Acad Sci 1123: 213-223, 2008.
3. Stieber J, Hofmann F and Ludwig A: Pacemaker channels and sinus node arrhythmia. Trends Cardiovasc Med 14: 23-28, 2004.
4. Kaupp UB and Seifert R: Molecular diversity of pacemaker ion channels. Annu Rev Physiol 63: 235-257, 2001.
5. Ludwig A, Budde T, Stieber J, *et al*: Absence epilepsy and sinus dysrhythmia in mice lacking the pacemaker channel HCN2. EMBO J 22: 216-224, 2003.
6. Robinson RB and Siegelbaum SA: Hyperpolarization-activated cation currents: from molecules to physiological function. Annu Rev Physiol 65: 453-480, 2003.
7. Hoppe UC and Beuckelmann DJ: Characterization of the hyperpolarizationactivated inward current (If) in isolated human atrial myocytes. Cardiovasc Res 38: 788-801, 1998.
8. Cerbai E, Pino R, Porciatti F, Sani G, Toscano M, Maccherini M, Giunti G and Mugelli A: Characterization of the hyperpolarization-activated current, I(f), in ventricular myocytes from human failing heart. Circulation 95: 568-571, 1997.
9. Cerbai E, Sartiani L, DePaoli P, Pino R, Maccherini M, Bizzarri F, DiCiolla F, Davoli G, Sani G and Mugelli A: The properties of the pacemaker current I(f) in human ventricular myocytes are modulated by cardiac disease. J Mol Cell Cardiol 33: 441-448, 2001.
10. Lai LP, Su MJ, Lin JL, Tsai CH, Lin FY, Chen YS, Hwang JJ, Huang SK, Tseng YZ and Lien WP: Measurement of funny current I(f) channel mRNA in human atrial tissue: correlation with left atrial filling pressure and atrial fibrillation. J Cardiovasc Electrophysiol 10: 947-953, 1999.
11. Xia S, Wang Y, Zhang Y, Deng SB, Du JL, Wang XC and She Q: Dynamic changes in HCN2, HCN4, KCNE1, and KCNE2 expression in ventricular cells from acute myocardial infarction rat hearts. Biochem Biophys Res Commun 395: 330-335, 2010.
12. Pitt B, Zannad F, Remme WJ, Cody R, Castaigne A, Perez A, Palensky J and Wittes J: The effect of spironolactone on morbidity and mortality in patients with severe heart failure. N Engl J Med 341: 709-717, 1999.
13. Pitt B, Remme W, Zannad F, Neaton J, Martinez F, Roniker B, Bittman R, Hurley S, Kleiman J and Gatlin M: Eplerenone, a selective aldosterone blocker, in patients with left ventricular dysfunction after myocardial infarction. N Engl J Med 348: 1309-1321, 2003.
14. Pitt B and Pitt GS: Added benefit of mineralocorticoid receptor blockade in the primary prevention of sudden cardiac death. Circulation 115: 2976-2982, 2007.
15. Stambler BS, Laurita KR, Shroff SC, Hoeker G and Martovitz NL: Aldosterone blockade attenuates development of an electrophysiological substrate associated with ventricular tachyarrhythmias in heart failure. Heart Rhythm 6: 776-783, 2009.
16. Muto T, Ueda N, Opthof T, Ohkusa T, Nagata K, Suzuki S, Tsuji Y, Horiba M, Lee JK, Honjo H, Kamiya K, Kodama I and Yasui K: Aldosterone modulates I(f) current through gene expression in cultured neonatal rat ventricular myocytes. Am J Physiol Heart Circ Physiol 293: 2710-2718, 2007.
17. Graf EM, Heubach JF and Ravens U: The hyperpolarization-activated current If in ventricular myocytes of non-transgenic and beta2-adrenoceptor overexpressing mice. Naunyn Schmiedebergs Arch Pharmacol 364: 131-139, 2001.
18. DiFrancesco D: Pacemaker mechanisms in cardiac tissue. Annu Rev Physiol 55: 455-472, 1993.
19. Mitsuiye T, Shinagawa Y and Noma A: Sustained inward current during pacemaker depolarization in mammalian sinoatrial node cells. Circ Res 87: 88-91, 2000.
20. Cerbai E, Barbieri M and Mugelli A: Occurrence and properties of the hyperpolarization-activated current If in ventricular myocytes from normotensive and hypertensive rats during aging. Circulation 94: 1674–1681, 1996.
21. Stillitano F, Lonardo G, Zicha S, Varro A, Cerbai E, Mugelli A and Nattel S: Molecular basis of funny current (If) in normal and failing human heart. J Mol Cell Cardiol 45: 289-299, 2008.
22. Milliez P, Messaoudi S, Nehme J, Rodriguez C, Samuel JL and Delcayre C: Beneficial effects of delayed ivabradine treatment on cardiac anatomical and electrical remodeling in rat severe chronic heart failure. Am J Physiol Heart Circ Physiol 296: 435-441, 2009.
23. Vaillant F, Timour Q, Descotes J, Manati W, Belhani D, Bui-Xuan B, Tabib A, Bricca G and Chevalier P: Ivabradine induces an increase in ventricular fibrillation threshold during acute myocardial ischemia: an experimental study. J Cardiovasc Pharmacol 52: 548-554, 2008.
24. Borer JS, Fox K, Jaillon P and Lerebours G: Antianginal and antiischemic effects of ivabradine, an I(f) inhibitor, in stable angina: a randomized, double-blind, multicentered, placebo-controlled trial. Circulation 107: 817–823, 2003.
25. DiFrancesco D and Camm JA: Heart rate lowering by specific and selective I(f) current inhibition with ivabradine: a new therapeutic perspective in cardiovascular disease. Drug 64: 1757-1765, 2004.
26. Mulder P, Barbier S, Chagraoui A, Richard V, Henry JP, Lallemand F, Renet S, Lerebours G, Mahlberg-Gaudin F and Thuillez C: Long-term heart rate reduction induced by the selective I(f) current inhibitor ivabradine improves left ventricular function and intrinsic myocardial structure in congestive heart failure. Circulation 109: 1674-1679, 2004.
27. Drögemüller A, Seidl K, Schiele R, Schneider S, Gitt A, Gottwik M, von Leitner ER, Poppe C, Rettig-Stürmer G and Senges J: Prognostic value of non-sustained ventricular tachycardias after acute myocardial infarction in the thrombolytic era: importance of combination with frequent ventricular premature beats. Z Kardiol 92: 164-172, 2003.
28. Struthers AD: Impact of aldosterone on vascular pathophysiology. Congest Heart Fail 8: 18-22, 2002.
29. Weber KT, Sun Y, Wodi LA, Munir A, Jahangir E, Ahokas RA, Gerling IC, Postlethwaite AE and Warrington KJ: Toward a broader understanding of aldosterone in congestive heart failure. J Renin Angiotensin Aldosterone Syst 4: 155-163, 2003.
30. Weber KT: Aldosterone in congestive heart failure. N Engl J Med 345: 1689-1697, 2001.
31. Sun Y, Ramires FJ and Weber KT: Fibrosis of atria and great vessels in response to angiotensin II or aldosterone infusion. Cardiovasc Res 35: 138-147, 1997.
32. Lesouhaitier O, Chiappe A and Rossier MF: Aldosterone increases T-type calcium currents in human adrenocarcinoma (H295R) cells by inducing channel expression. Endocrinology 142: 4320-4330, 2001.
33. Rossier MF, Lesouhaitier O, Perrier E, Bockhorn L, Chiappe A and Lalevée N: Aldosterone regulation of T-type calcium channels. J Steroid Biochem Mol Biol 85: 383-388, 2003.