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Modulation of cardiac pacemaker channels by tyrosine phosphorylation

Jianying Huang
West Virginia University

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MODULATION OF CARDIAC PACEMAKER CHANNELS BY TYROSINE PHOSPHORYLATION

by

Jianying Huang

**Dissertation submitted to the School of Medicine
at West Virginia University
in partial fulfillment of the requirements
for the degree of**

**Doctor of Philosophy
in
Cellular & Integrative Physiology**

Approved by

Han-Gang Yu, Ph.D., Committee Chairperson

Jefferson C. Frisbee, Ph.D.

Stanley M. Hileman, Ph.D.

William T. Stauber, Ph.D.

William F. Wonderlin, Ph.D.

Department of Physiology & Pharmacology

Morgantown, West Virginia

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**Keywords: pacemaker channel, funny current, tyrosine phosphorylation, HCN,
Src kinase, RPTP**

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Abstract

Modulation of Cardiac Pacemaker Channels by Tyrosine Phosphorylation

Jianying Huang

Encoded by hyperpolarization-activated cyclic nucleotide-gated (HCN) channels, the cardiac pacemaker current I_f is a major determinant of diastolic depolarization in sinus node myocytes and has a key role in the origin of heart beat. My dissertation consists of two chapters, focusing on the modulation of I_f and HCN channels by tyrosine phosphorylation, which is maintained by a fine balance between tyrosine kinases and phosphatases. Chapter 1 aims to investigate the role of Src tyrosine kinases in the regulation of sinus node I_f and HCN channels; Chapter 2 explores the modulation of ventricular I_f and HCN channels by a family of receptor protein tyrosine phosphatases (RPTPs).

Chapter 1 contains two sections. In Section 1, Src-mediated tyrosine phosphorylation was utilized to restore the surface expression of HCN4-D553N, a trafficking-defective mutant identified in a patient with sick sinus syndrome manifested by sinus bradycardia. The corrected D553N channel exhibited biophysical properties comparable to the wild-type HCN4 channel, suggesting a therapeutic potential of tyrosine phosphorylation for the treatment of sinus bradycardia. This part of results has been published. In Section 2, Src tyrosine kinases were found essential in facilitating the gating of HCN4 channel and activation of sinus node I_f , as well as increasing heart rate following the activation of β -adrenergic receptors. In addition, these beneficial effects of Src-mediated tyrosine phosphorylation were independent of cAMP. The manuscript summarizing these results is under review.

Chapter 2 is composed of three sections. In Section 1, the tyrosine phosphatase RPTP α was found to exert dramatic inhibition on the activity of HCN2 channel via reducing its surface expression, which was mediated by tyrosine dephosphorylation. This work has been published. Sections 2 and 3 summarized unpublished data. In Section 2, another two tyrosine phosphatases, RPTP μ and RPTP ϵ were identified in cardiac ventricles and found to differentially regulate HCN2 channel. In Section 3, the results that RPTP ϵ inhibited 573X, a cAMP insensitive HCN4 mutant identified in patients with sick sinus syndrome and sinus bradycardia, confirm that tyrosine phosphorylation could affect HCN channel independently of cAMP.

In summary, my studies demonstrate that tyrosine phosphorylation plays a significant role in regulating the activity of I_f and HCN channels, in which novel modulators like RPTP and previously unrecognized mechanisms were identified, such as the cAMP-independent pathway mediating heart rate increase following the activation of β -adrenergic receptors.

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Glossary of Abbreviations

4-AP	4-aminopyridine
AC	Adenylyl cyclase
Ach	Acetylcholine
AP	Action potential
bpm	Beats per minute
cAMP	Cyclic adenosine monophosphate
CNBD	Cyclic nucleotide-binding domain
CNG	Cyclic nucleotide-gated
CT	Crista terminalis
DMEM	Dulbecco's modified Eagle's medium
ECG	Electrocardiogram
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
G α_i	Inhibitory α subunit of G protein
G α_s	Stimulatory α subunit of G protein
HCN	Hyperpolarization-activated cyclic nucleotide-gated
HEK	Human embryonic kidney
I _{Ca}	Dihydropyridine-sensitive Ca ²⁺ current
I _{Ca,L}	L-type Ca ²⁺ current
I _{Ca,T}	T-type Ca ²⁺ current
I _f	Funny current, cardiac pacemaker current
Ig	Immunoglobulin
I _K	Delayed rectifier potassium current
I _{K,ACh}	Acetylcholine-activated K ⁺ current
I _{Kir}	Inwardly rectifying K ⁺ current
I _{Kr}	Rapidly activating delayed rectifier potassium current
I _{Ks}	Slowly activating delayed rectifier potassium current
ISO	Isoproterenol
I _{to}	Transient outward potassium current
KB	Krafte-brühe
K _v	Voltage-gated potassium channel
MAM	Meprin-A5 antigen-PTP
Na _v	Voltage-gated potassium channel
nrPTP	Non-receptor type protein tyrosine phosphatase
PAO	Phenylarsine oxide
PBS	Phosphate buffered saline
PFA	Paraformaldehyde
PKC	Protein kinase C
PP2	4-amino-5-(4-chlorophenyl)-7-(<i>t</i> -butyl)pyrazolo[3,4-d]pyrimidine
PP3	4-amino-7-phenylpyrazol[3,4-d]pyrimidine
PTP	Protein tyrosine phosphatase
rAt	Right atrium

RPTP	Receptor protein tyrosine phosphatase
SAN	Sinus node
SD	Sprague-Dawley
TEA	Tetraethylammonium
$V_{1/2}$	Activation midpoint, the voltage at which the tail current amplitude reaches half maximum
V_{th}	Activation threshold, the voltage at which the first time-dependent inward current larger than 10 pA was detected
β_2AR	β_2 adrenergic receptors
δ	Initial delay of activation in HCN channels
τ	Time constant
τ_{act}	Activation time constant

General Introduction

1. Discovery of the electrical conducting system of the heart

The heart is considered the hardest working muscle of our body. Estimated from an averaged resting cardiac output of 5 liters per min in adults, the heart pumps out almost 3 ounces of blood at every heartbeat and ejects 1,900 gallons (7,200 liters) of blood daily. From birth to death, the heart manages to deliver around 3 billion beats in the average person's life. As a preeminent biological oscillator, the heartbeat even defines our perception of time.¹ The exploration of the mechanism underlying the mysterious beating of the heart can be traced back to over 100 years ago.

As early as the second century, a prominent Roman physician, surgeon and philosopher, **Claudius Galen** believed that “the power of pulsation has its origin in the heart itself” and the heart “would not have arisen if the organ had had the same nature as the muscles throughout the whole animal.” He noticed that “the heart, removed from the thorax, can be seen to move for a considerable time, . . . a definite indication that it does not need the nerves to perform its own function.”, thereby supporting the myogenic origin of the heartbeat.²⁻⁴ The neurogenic origin of the heartbeat, on the other hand, was supported by the discovery of the sympathetic and parasympathetic nerves and ganglia inside and outside the heart, as well as the effect of galvanic stimulation on the nerves and heart in the 1830s and 1840s.^{3, 5, 6} The heated debate, known as the myogenic versus the neurogenic controversy argued about whether the heartbeat was triggered by inherent excitation by the heart muscle itself or the external stimulus from nerves.^{3, 5, 6} It was not until the discovery of the electrical system of the heart in the 19th century that this debate was eventually resolved.

In 1839, **Jan Evangelista Purkinje** (1787-1869) discovered in the ventricular subendocardium of the sheep heart a net of gray, flat and gelatinous fibers which he initially considered to be cartilaginous.^{7, 8} In 1883, **Walter Gaskell** (1847-1914) recognized that the impulse of the heartbeat began in the sinus venosus. He wrote "...in the Vertebrata, ...that part of the heart from which the rhythmical contractions arise, viz. the sinus venosus".⁹ He also noticed that this impulse spread downwards to ventricles with decreasing rhythmic ability. He observed that "the power of independent rhythmical contraction decreases regularly as (we) pass from the sinus to the ventricle" and "the rhythmical power of each segment of the heart varies inversely as its distance from the sinus".⁹ Gaskell's work also supported the myogenic theory. He isolated a strip of tortoise ventricular muscle devoid of ganglions or nervous connection and showed that the strip continued to pulsate at a rate similar to the intact heart. He concluded, "The rhythmic capacity of every part of the heart depends not upon the presence of ganglion cells but rather upon the persistence of a primitive condition of heart muscle."¹⁰ In 1893, prompted by Gaskell's work, **Wilhelm His, Jr** (1863-1934) discovered a connective tissue sheet forming a bundle uniting atria and ventricles, "I have succeeded in finding a muscle bundle which unites the auricular and ventricular septal walls. . . . The bundle arises from the posterior wall of the right auricle near the auricular septum, in the atrioventricular groove; attaches itself along the upper margin of the ventricular septal muscle . . . proceeds on top of this toward the front until near the aorta it forks itself into a right and left limb..."^{11, 12} However, he did not prove that this bundle actually conducted the impulse of the heartbeat, "I cannot state with certainty whether this bundle actually conducts the impulses from the auricle to the ventricle, as I did not perform any experiments dealing with the severing of the bundle."^{11, 13} In 1906, **Sunao Tawara** (1873-1952) found a "complex knoten" of tissue at the proximal end of the His bundle, which were

known as the atrioventricular (AV) node.¹⁴ Tawara also contemplated the electrical conducting system of the heart. He believed that the impulse of the heartbeat traveled from the AV node to the bundle of His, divided into the right and left bundle branches, and terminated as the Purkinje fibers.^{4, 14, 15} Therefore, over 50 years after the discovery of Purkinje fibers, Tawara was the first to appreciate their conducting roles.⁴

In 1906, inspired by Tawara's discovery of "the Knoten" (i.e. AV node), **Arthur Keith** (1866 -1955) advised **Martin Flack** (1882-1931), a medical student, "to examine other regions of the heart for such peculiar musculature."¹⁶ One of the specific aims Keith and Flack proposed was "To seek in the sinus, auricle, and bulbus cordis for a differentiation in form and structure of a system of muscle fibres corresponding to that now known to exist in the ventricle: in short, to ascertain whether the musculature in which the heart-impulse is held to arise, and by which it is conducted, differs in form and structure from that which is mainly contractile in nature." They found that "There is a remarkable remnant of primitive fibres persisting at the sino-auricular junction in all the mammalian hearts examined. These fibres are in close connection with the vagus and sympathetic nerves, and have a special arterial supply; in them the dominating rhythm of the heart is believed to normally arise." They stated that these fibers "can be dissected out on the superior vena cava in the region corresponding to the right venous valve, and at the coronary sinus in the interval between it and the inferior vena cava and left auricle." Macroscopically, they described these fibers to "resemble those of the a.-v. bundle in being paler than the surrounding musculature, i.e. in being of the white variety." Microscopically, these fibers were "striated, fusiform, with well-marked elongated nuclei, plexiform in arrangement, and embedded in densely packed connective tissue-in fact of closely similar structure to the Knoten." Their landmark study completed the electrical conducting system of the heart, which originates from

the sino-auricular junction (i.e. the sinoatrial or sinus node), travels through the AV node, to the bundle of His and terminates at the Purkinje fibers.⁴

Beginning in 1909, using a twin string galvanometer that was invented by **Willem Einthoven** (1860-1927) in 1901, **Thomas Lewis** (1881-1945) observed in dogs that only the curves from the superior vena cava, i.e. near the sinoatrial node, were identical with the normal rhythm.¹⁷ Further, he noticed that the electrode over the SA node always had a primary negativity.¹⁷ Based on the understanding that the point where the contraction starts becomes electrically negative to the inactive points of muscle, Lewis concluded that “The SA nodal region is that in which the excitation wave has its birth”¹⁷ At this moment, the myogenic versus the neurogenic controversy was finally resolved, reaching the conclusion that the origin of the heartbeat was myogenic.⁴

2. Understanding the sinus node action potential: discovery of the cardiac pacemaker current in sinus node myocytes

Consistent with the findings of the pioneers in this field, the exact location of sinus node pacemaker tissue has been observed and confirmed by numerous researchers. It is located in the intercaval region where the superior vena cava enters the right atrium and extends towards the endocardial side of the crista terminalis.^{18, 19} Due to a relatively high content of collagen²⁰, isolation of cardiomyocytes from the dissected sinus node requires extra collagenase as compared to atrial or ventricular myocyte isolation, yielding multiple types of cells, including atrial myocytes, fibroblasts and adipocytes in addition to sinus node myocytes.¹⁸ Despite a wide range of observed heart rates in mammals, the gross morphology of isolated sinus node myocytes is relatively conserved among species, such as mouse^{21, 22}, rat²³, rabbit²⁴⁻²⁶, guinea pig^{27, 28}, pig²⁹,

dog³⁰ and human.^{31, 32} Generally, there are three typical shapes of sinus node myocytes, “spindle,” “elongated,” (i.e. with a longer longitudinal axis than spindle cells) and “spider-like” (i.e. with branched cytoplasm).^{19, 30, 33} The sinus node myocytes have a membrane capacitance ranging from 20 pF to 60 pF with a mean around 40 pF³⁴, which is much smaller compared to atrial or ventricular myocytes whose membrane capacitance can reach over 100 pF.³⁵

Figure 1. Typical action potentials recorded from sinus node myocytes (A) and ventricular myocytes (B) isolated from Sprague-Dawley rats. The sinus node action potential (A) is adapted from Supplemental Figure S2 in Section 2 of Chapter 1. The ventricular action potential is from Lin YC et al., 2012.³⁶

The pacemaking ability of the sinus node myocytes is indicated by the unique form of action potentials recording from them (Figure 1A). The action potential in the sinus node myocytes differs markedly from that in the working myocardium in that it does not have a resting membrane potential. Instead, the term “maximal diastolic potential” was used to describe the most negative voltage the sinus node action potential can reach, which is approximately -60 mV in rat under physiological conditions (Figure 1A), approximately 20 mV more positive compared to the resting potential of ventricular myocytes (Figure 1B). The other hallmark of sinus node action potential is the diastolic depolarization (also termed “the pacemaker potential”), a depolarizing phase that drives the membrane voltage at the end of repolarization

when the maximal diastolic potential is reached to the following action potential threshold (Figure 1A). The diastolic depolarization has been noticed since the 1940s³⁷ and the key breakthroughs revealing its underlying ionic mechanisms were made in the late 1970s and early 1980s, including the discovery of the hyperpolarization-activated “funny” current (I_f) in rabbit sinus nodes³⁸ and calf Purkinje fibers.^{39, 40} The name “funny” current originated from the unusual properties of I_f relative to other ion channels known at the time, including (1) mixed permeability to sodium and potassium ions, (2) activation by hyperpolarization, and (3) slow activation and deactivation kinetics.³⁸ Once activated, the funny current I_f helps shift the membrane potential of sinus node myocytes from the maximal diastolic potential to the threshold potential of the dihydropyridine-sensitive Ca^{2+} current (I_{Ca}), which predominantly controls the upstroke of the sinus node action potential⁴¹ that is much slower than that of the ventricular action potential (Figure 1B). Two distinct I_{Ca} components have been identified⁴²: the L-type Ca^{2+} current ($I_{Ca,L}$) and the T-type current ($I_{Ca,T}$). $I_{Ca,T}$ is also believed to participate in the late diastolic depolarization.⁴² The repolarization of the sinus node action potential is mostly contributed to by the delayed rectifier potassium current (I_K)⁴³, consisting of rapid (I_{Kr}) and slow (I_{Ks}) currents^{44, 45}, and the transient outward potassium current (I_{to}).^{46, 47} The Na^+ - Ca^{2+} exchanger and Ca^{2+} release from the sarcoplasmic reticulum were also believed to contribute to the pacemaker potential⁴⁸⁻⁵¹, given the name of “calcium clock”, in contrast to the theory of “membrane clock” in which the funny current I_f and $I_{Ca,T}$ are involved. Nonetheless, the important role of I_f in generating the diastolic depolarization and modulating heart rate is indisputable.

3. Hierarchic organization of cardiac pacemakers

The electrical conduction of the heart has been intensively studied since the 19th century. Briefly, the cardiac pacemaking impulse is initiated at the sinus node, transmitted to the atria, through the atrio-ventricular node to arrive at the bundle of His, and terminated at Purkinje fibers embedded in the ventricular myocardium.⁵²

The funny current I_f was discovered in the late 1970s and identified as an important contributor to cardiac pacing.^{38, 53} Opened by hyperpolarization near the end of repolarization, I_f contributes significantly to diastolic depolarization, which leads to the threshold of calcium channel activation and action potential firing.⁵⁴ I_f is a time- and voltage-dependent inward current. It has several unusual features: (1) While most voltage-gated ion channels are activated by membrane depolarization, I_f channels are activated by hyperpolarization; 2) I_f channels pass both Na^+ and K^+ , with more Na^+ in and less K^+ out near the maximum diastolic potential, generating an inward current. I_f channels also pass tiny amounts of Ca^{2+} ions^{55, 56}; 3) I_f channels gate slowly, and their activation kinetics range from hundreds of milliseconds to seconds; 4) Pharmacologically, I_f is relatively insensitive to Ba^{2+} , a universal K^+ channel blocker, but can be abolished by a low concentration of Cs^+ (1-4 mM) or its specific blocker ZD7288 (3 μM).

I_f has been found in cardiac regions outside the sinus node, including atrium⁵⁷, AV node⁵⁸, and Purkinje fibers^{39, 40}. However, under physiologic conditions, these regions have much slower intrinsic pacing rates, and thus are overridden by the sinus node.⁵⁹ An association between the voltage-dependent activation of I_f and the pacemaker activities in these regions has been recognized. In the sinus node, the most negative potential (i.e. maximum diastolic potential) at the end of repolarization is around -60 to -70 mV and, I_f begins to activate around -50 mV.³⁸ In the secondary pacemaker Purkinje fibers, the maximal diastolic depolarization is near -85 mV and, I_f begins to activate around -80 mV.^{60, 61} In working ventricles that do not pace under

physiologic conditions, I_f has not been found in the physiologic voltage range for pacemaker activity (i.e. -50 mV to -100 mV). Instead, ventricular I_f activates at extremely negative potentials (i.e. -120 mV) that are outside the physiologic voltage range in guinea pig, canine, and rat ventricular myocytes.⁶²⁻⁶⁴ Therefore, there is an approximately 70 mV difference in the activation threshold of I_f between the primary pacemaker, sinus node, and the working ventricles. However, the molecular basis underlying this marked variation in the voltage dependence of I_f activation remains elusive.

In addition to the distinct biophysical properties in different cardiac regions, I_f is also subjected to developmental changes. For example, pacemaker activity is absent in adult mammalian ventricles, but present in cultured newborn ventricles.⁶² In rat neonatal ventricular myocytes, I_f was found to activate around -70 mV, and shift beyond the physiological voltage range (-113 mV) in adult rat ventricular myocytes.⁶² On the other hand, the effect of aging on I_f was investigated in both normotensive Wistar-Kyoto rats and spontaneously hypertensive rats.⁶⁵ ⁶⁶ Surprisingly, a prominent diastolic depolarization phase was observed in the action potential of the left ventricular myocytes from old (18 to 20 or 24 months old) rats.^{65, 66} In addition, the occurrence of I_f increased with aging. I_f was recorded in less than 20% of ventricular myocytes from young rats but greater than 90% from old rats.^{65, 66} Interestingly, abnormal pacemaker activity appears in adult ventricles under pathological conditions. For example, the activation threshold of I_f was shifted to more positive potentials and back to physiological voltages in ventricular myocytes from human failing hearts^{67, 68} and rat hypertrophied hearts.⁶⁹ Other than the depolarization shift of the activation threshold, the activation curve of I_f was shifted significantly to more positive potentials due to an increased I_f current density in hypertrophied or failing hearts.⁶⁷⁻⁶⁹ Moreover, the density of I_f currents was found to relate linearly to the severity

of cardiac hypertrophy.⁶⁶ Such supra-physiological activity of I_f has been recognized as a contributor to the increased propensity of the hypertrophied or failing hearts for arrhythmias.⁶⁶⁻⁶⁸ However, it is unknown what causes the shift of the voltage dependence of I_f activation either during development or in disease states.

4. The role of funny current (I_f) in autonomic regulation of heart rate

The sinus node region is richly innervated by the autonomic nervous system.⁷⁰ Isoproterenol, a β -adrenergic receptor agonist that stimulates sympathetic nerve activity, increases heart rate via facilitating I_f , and acetylcholine, a muscarinic receptor agonist which mimics parasympathetic activation, decreases heart rate by inhibiting I_f .^{38, 52, 71} Such autonomic regulation of heart rate is due to either a depolarizing or hyperpolarizing shift of the I_f activation curve and is mediated by changes in intracellular cAMP concentration.^{33, 60, 72-75} Briefly, isoproterenol increases the intracellular cAMP level through the classic $G\alpha_s$ -adenylyl cyclase-cAMP pathway, which increases the degree of steady-state current, thereby increasing the current available during diastolic depolarization to speed up this process.⁷⁶ Mechanistic studies have revealed that the effect of cAMP on I_f is through direct binding of cAMP to I_f channels⁷⁷ and cAMP-mediated phosphorylation.^{61, 78} In contrast, acetylcholine decreases cAMP concentration via $G\alpha_i$, resulting in a slower development of the diastolic depolarization as a consequence of a reduction of I_f by shifting its activation to more negative potentials.^{70, 79} In addition to I_f , the acetylcholine-activated K^+ current ($I_{K,ACh}$) was also involved in the negative chronotropic action of vagal activity.⁸⁰ However, acetylcholine inhibited I_f at a concentration 20-fold lower than that required to activate $I_{K,ACh}$,⁸¹ suggesting a dominant role of I_f in slowing heart rate by low acetylcholine doses (i.e. moderate vagal activity).

5. Biophysical properties of funny current (I_f)

I_f was named due to its “funny” characteristics, and the most unusual feature is its activation upon membrane hyperpolarization, unlike most other channels that are activated by depolarization. Once activated, I_f allows Na^+ , K^+ and tiny amounts of Ca^{2+} ions to pass, generating slow inward currents due to its slow kinetics of activation and deactivation. Pharmacologically, I_f is relatively insensitive to Ba^{2+} , a universal K^+ channel blocker, but can be inhibited by Cs^+ or its specific blocker ZD7288. Following its discovery in sinus node, I_f was also identified in Purkinje fibers^{39, 40, 78}, atrial^{57, 82-84} and ventricular myocytes⁶³⁻⁶⁵ among a variety of species. Moreover, the observation that I_f in various cardiac regions have different biophysical properties further supported the hierarchic organization of cardiac pacemakers.

5.1. Voltage dependence

In the sinus node across species including mouse, cat, rabbit, porcine and human, I_f currents start to activate when the membrane potential reaches -50 mV ^{22, 32, 57, 85, 86}, and this is called its activation threshold, which lays well within the voltage range for diastolic depolarization.^{33, 38} Once activated, I_f channels generate slow inward currents to reach the steady-state with a time constant (τ) around hundreds of milliseconds.^{32, 87} The voltage dependence of activation or the activation curve of I_f is S-shaped after fitting with Boltzmann functions.³³ The voltage where I_f reaches half of the maximal activation, the activation midpoint ($V_{1/2}$), can be obtained from the activation curve, and is approximately -70 to -80 mV in sinus node myocytes.⁸⁸ In contrast, I_f activates at more negative voltages in ventricular myocytes with the activation threshold being around -120 mV and an activation midpoint of -150 mV .^{63, 64}

Interestingly, the voltage dependence of I_f in the working myocardium changes during development and in pathological conditions. For example, the activation threshold was shifted to

around -70 mV in ventricular myocytes from rat neonates.⁶² In hypertensive rats, I_f was found fully activated at -120 mV and the activation midpoint was shifted towards depolarization by 60 mV.⁶⁵

5.2. Ion selectivity

The funny channel I_f generates a mixed inward cation current carried by K^+ and Na^+ ions with Na^+ predominating at the resting membrane potential which is close to the equilibrium potential of K^+ .⁸⁹ The ratio of the Na^+ to K^+ permeability ($P_{Na} : P_K$) of I_f is about 0.27.^{40, 90}, which can be increased with an elevated external K^+ concentration.⁹⁰ In addition, the conductance of I_f also increases with an increased extracellular K^+ concentration.⁴⁰ In contrast, I_f conducts little Na^+ in the absence of external K^+ ions, indicating that K^+ affects the permeation of Na^+ .⁸⁹ More recently it was discovered that I_f channels also pass tiny but significant amounts of Ca^{2+} ions accounting for about 0.5% of the inward currents passing I_f ^{55, 56}.

5.3. Pharmacological profile and clinical application

Similar to the inwardly rectifying K^+ currents (I_{Kir}), the funny current I_f can be blocked by Cs^+ ions at millimolar concentrations.⁹¹⁻⁹³ In contrast to I_{Kir} , I_f is insensitive to millimolar concentrations of Ba^{2+} and tetraethylammonium (TEA).⁸⁹ I_f is not sensitive to 4-aminopyridine (4-AP) either, a blocker of voltage-gated K^+ channels.⁹⁴

A family of specific organic blockers for I_f has been intensively investigated to develop a new generation of bradycardic agents since I_f activity is directly linked to heart rate modulation. Heart rate reduction was long believed to be a treatment for coronary heart diseases and angina pectoris^{95, 96}, however, the widely-used β -blockers or Ca^{2+} channel antagonists have non-specific negative inotropy effects in addition to their negative chronotropic actions^{53, 97} Ivabradine was identified as a pure heart rate lowering drug without negative inotropy effects.⁹⁸ It specifically

blocks I_f at micromolar concentrations by binding to the intracellular sites of the channel, decelerating the development of diastolic depolarization phase in sinus node, and eventually reducing the heart rate.⁵³ Ivabradine was found to be effective in preventing exertional angina and underlying ischaemia for patients with chronic stable angina pectoris.^{53, 99} Moreover, adverse effects of ivabradine were mild due to its specific negative chronotropic action.^{97, 100-102} Two separate clinical trials have confirmed that the antianginal efficacy of ivabradine was not inferior to that of the Ca^{2+} channel antagonist amlodipine or the β -blocker atenolol.^{102, 103} In fact, the resting and maximal heart rates were reduced to a greater extent in the ivabradine-treated group as compared to the amlodipine group.¹⁰³ The ongoing clinical trial BEAUTIFUL has been dedicated to assess the benefits of ivabradine with alternative names of Procoralan[®], Coralan[®], Corlentor[®] or Coraxan[®] in over 10,000 patients suffering from coronary artery disease accompanied by left ventricular dysfunction. The BEAUTIFUL trial involves 781 centers in 33 different countries with a follow up duration averaging 19 months and 35 months at maximum. The BEAUTIFUL trial has already shown that ivabradine significantly reduces the risk of coronary events by 22%, fatal and nonfatal myocardial infarction by 36%, and coronary revascularization by 30%.¹⁰⁴⁻¹⁰⁷

6. Molecular determinants of funny current (I_f): hyperpolarization-activated cyclic nucleotide-gated (HCN) channels

6.1. Isoforms of HCN channels and their distributions

A family of genes encoding hyperpolarization-activated channels was identified in 1998, named as HCN channels.^{94, 108, 109} Four family members have been identified in brain, and three of them (HCN1, HCN2, HCN4) are present in heart.¹¹⁰ Despite similar structural features, these

three cardiac isoforms of HCN channels are expressed non-uniformly.¹¹⁰ Abundant HCN4 and low levels of HCN2 mRNA transcripts are expressed in the sinus node, and much higher levels of HCN2 than HCN4 transcripts are present in the ventricle. HCN1 transcripts are expressed at low levels in the sinus node and largely absent in ventricles. Moreover, the increased mRNA abundance of HCN is associated with an increased I_f in disease states.^{67-69, 111} For example, the mRNA abundance of HCN2 and HCN4 is increased in hypertrophied heart.⁶⁹ In the ventricle of end-stage heart failure HCN4 mRNA levels are elevated by three-fold.¹¹¹

6.2. Structural features of HCN channels

Figure 2. Schematic topology of HCN channels. The cytosolic tail is constructed by Swiss-Pdbviewer 3.7. HCN4 mutations identified in patients with familial sick sinus syndrome are highlighted by green circles.

The HCN channels structurally resemble the voltage-gated potassium channel superfamily.¹¹² Like potassium channels, HCN channels are tetramers. Each monomer contains six transmembrane domains (S1-S6) with both N- and C- termini located on the cytosolic side of the plasma membrane (Figure 2). The S4 segment has positively charged amino acids, acting as the voltage sensor for HCN channels. The pore-forming region between S5 and S6 contains the same selective sequence GYG as potassium channels. However, compared to potassium channels, HCN channels only have moderate selectivity on cations. They are permeable to mainly K⁺ and Na⁺ ions, and also to Ca²⁺ ions in small amounts.

A consensus cyclic nucleotide-binding domain (CNBD) formed by approximately 120 amino acid residues is found in the C-terminal regions of HCN channels. The CNBD has been found to inhibit the voltage-dependent activation of the channels, and cAMP binding releases this inhibition. Structurally, the CNBD is composed of an initial alpha-helix (A in Figure 2), followed by an eight-stranded anti-parallel β -roll, a short α -helix (B in Figure 2), and a long C-helix (C in Figure 2). The binding pocket for cAMP is formed by residues at the interface between the β -roll and the C-helix (Figure 2).^{113, 114} The CNBD was also identified as a determinant for the surface expression of HCN2 channels.^{115, 116} Further, a four-amino acid motif (EEYP) in the B-helix of HCN2 was identified to play an important role in its surface expression by promoting the translocation of the channel from the endoplasmic reticulum.¹¹⁷ Interestingly, in contrast to HCN2, the CNBD does not seem to be critical in the surface expression of HCN4 channels. For example, HCN4 mutants lacking the entire CNBD (i.e. HCN4-573X) or harboring a truncated and dysfunctional CNBD (i.e. HCN4-695X) were both identified in patients with severe sinus bradycardia.^{118, 119} These two HCN4 mutants, however, were normally trafficked to the plasma membrane and expressed functional currents.^{118, 119}

The C-linker, another unique structural element consisting of about 80 amino acid residues that connects the CNBD with the S6 segment of the channel core, is believed to mediate

the interactions between the four monomers assembling the functional HCN channels.¹¹³ The C-linker consists of six α -helices (Figure 2, A'-F'). In addition, abundant tyrosine residues are found in the C-linker, illustrated by their protruding side chains on A' and B' helices (Figure 2). We and others have identified that tyrosine residues Y531 and Y554 located in the C-linker are responsible for the facilitation of HCN4 channels by Src-mediated tyrosine phosphorylation.^{120,}

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6.3. Sick sinus syndrome and HCN4 channelopathies

Sick sinus node syndrome is a collection of conditions usually resulting from a dysfunctional sinus node.¹²² As the sinus node is the origin of the heart beat, sick sinus node patients typically have an abnormal cardiac electrical activity, manifested by symptoms such as severe sinus bradycardia, sinus pauses or arrest, sinus node exit block and abnormal heart rate adaptation to exercise or stress.¹²³ The sick sinus node syndrome is usually associated with atrial fibrillation, the most common type of cardiac arrhythmias, including chronic atrial tachyarrhythmias, alternating periods of atrial bradyarrhythmias and tachyarrhythmias (i.e. the “bradycardia-tachycardia syndrome”).¹²³ The sick sinus node syndrome can occur at all ages, including in the newborn¹²⁴, however, its incidence increases exponentially with age mostly due to age-dependent degenerative fibrosis of the sinus node.^{123, 124} Statistics have revealed that sick sinus node patients account for 1 in every 600 cardiac patients older than 65 years.¹²² It is worth noting that bradyarrhythmias, which are the major manifestation of the sick sinus node syndrome, are responsible for almost half of sudden deaths in the hospital.¹²⁵ Despite this large population of patients, there is currently no pharmacological treatment for this disease and the only treatment available is to implant an electronic pacemaker. As a result, the sick sinus node syndrome accounts for approximately half of pacemaker implantations in the United States.¹²⁴

The HCN4 channel is the predominant isoform used to generate I_f in the sinus node. The significant role of HCN4 channels in cardiac pacing has been confirmed in transgenic mouse models. Stieber in 2003 demonstrated that HCN4 is highly expressed in the cardiac region of mouse embryos where the early sinus node develops.¹²⁶ Dramatically, global or cardiac-specific knockout of the HCN4 gene resulted in deaths between embryonic days 9.5 and 11.5.¹²⁶ In the HCN4-deficient mutant embryo, the amplitude of I_f was reduced by 85% corresponding to a failure in generating diastolic depolarization and the heart contracted significantly slower with irresponsiveness to cAMP as compared to the control embryo.¹²⁶

In the past decade, a total of six HCN4 mutants have been identified in patients with familiar sick sinus node syndrome^{118, 119, 127-130}, supporting the relevance of HCN4 to cardiac impulse generation in the sinus node. As illustrated in Figure 2, four of the six mutations occur in the C-terminus, with two at the C-linker (i.e. D553N, 573X) and another two at the CNBD (i.e. S672R, 695X); the remaining two mutation sites are in the pore-forming region (i.e. G480R, A485V).

In comparison to the wild-type HCN4, all of the mutants exhibited depressed gating properties, two of which (i.e. 573X and 695X) are due to cAMP insensitivity. The mutant 573X was identified in a 66-year-old woman who was admitted to a community hospital with a fractured nasal bone after a severe syncope.¹¹⁸ She had marked sinus bradycardia (41 beats per minute or bpm) and intermittent atrial fibrillation. The activity of 573X could not be increased by cAMP because this mutation causes the loss of entire CNBD due to a premature termination of translation occurring between the helices C' and D' in the C-linker (Figure 2), which was caused by a heterozygous 1-bp deletion in exon 5 of the human HCN4 gene. Similarly, the mutant 695X cannot be stimulated by cAMP due to a truncated CNBD resulting from a heterozygous insertion

of 13 nucleotides in exon 6 of the human HCN4 gene.¹¹⁹ The mutant 695X was identified in a family involving eight living carriers, all of whom showed severe sinus bradycardia with a mean resting heart rate of 45.9 ± 4.6 bpm. Although these patients have low basal heart rate, their heart rate could increase during exercise or when challenged with a β -agonist. However, such adrenergic stress on the mutant carriers was more likely to trigger distinctive episodes of ventricular arrhythmias, such as ventricular premature beats and ventricular bigeminy.

The dysfunction of mutants D553N, G480R and A485V is due to their reduced number of channels on the plasma membrane. D553N, a missense mutation in exon 5 of the HCN4 gene, was identified in a family involving three living carriers who showed similar clinical symptoms, such as recurrent syncope, QT prolongation in electrocardiograms, and polymorphic ventricular tachycardia, torsade de pointes.¹²⁷ This mutation occurs at helix B' of the C-linker (Figure 2). *In vitro* studies revealed depressed currents of the mutant channel associated with a reduced membrane expression of the channel protein. Mutants G480R and A485V have their mutation sites in the intra-membrane loop of the pore-forming region (Figure 2). G480R, a missense mutation in exon 4 of the human HCN4 gene was identified in eight family members showing asymptomatic sinus bradycardia.¹²⁸ Expression of G480R in *Xenopus* oocytes and human embryonic kidney (HEK) 293 cells revealed that mutant channels had reduced current density and were activated at more negative potentials as compared to wild-type channels. It has also been shown that the total and surface protein contents of G480R were reduced, accounting for the reduced currents of the mutant channels. A very similar HCN4 mutant (A485V) with reduced number of channel proteins on the cell surface was found in three unrelated families with familial sinus bradycardia.¹²⁹ The residue A485 is located at the channel pore and its mutation

into valine was identified in most of their bradycardiac relatives, including 19 individuals, but not in 150 controls. The pattern of autosomal-dominant inheritance was suggested.

Like mutant 695X, the hHCN4 mutation associated with familial sinus bradycardia, S672R, is located in the CNBD (Figure 2). S672R is a missense mutation in exon 7 of the human HCN4 gene.¹³⁰ A total of 15 family members carried this particular mutant, which was absent in 746 chromosomes from unrelated persons. Carriers of S672R had asymptomatic sinus bradycardia with a mean heart rate of 52.2 ± 1.4 bpm. Unlike the cAMP insensitivity of 695X, the S672R mutation did not affect cAMP-induced channel activation. In addition, S672R had a normal current expression in contrast to the reduced current density of D553N, G480R and A485V mutants. However, the activation of this mutant was shifted towards hyperpolarization accompanied by deceleration of deactivation kinetics, mimicking the actions of a low concentration (i.e. 10 to 30 nM) of acetylcholine on HCN4 channels, which contributed to bradycardia in the mutant carriers.

Clearly, the cAMP pathway offers no therapeutic strategy to rescue the normal gating of these mutants. Therefore, a new mechanism of modulating HCN4 channel activity independent of cAMP would benefit the development of therapeutic approaches to bradycardia. In my studies, tyrosine phosphorylation of HCN channels was explored in an attempt to discover a novel mechanism.

7. Emerging roles of tyrosine phosphorylation and dephosphorylation in cardiac electrophysiology

7.1. Tyrosine phosphorylation and dephosphorylation of cardiac ion channels

Cardiac electrical properties are directly controlled by gating of a variety of ion channels and activities of pumps. Tyrosine phosphorylation has been implicated in the modulation of K^+ , Na^+ , and Ca^{2+} channels as well as cyclic nucleotide-gated cation channels.¹³¹ It also plays an important role in regulating the activities of the Na^+/K^+ pump.¹³² The modulation of Shaker family voltage-gated potassium (K_v) channels by tyrosine phosphorylation has been firmly established. The delay rectifier K^+ ($K_v1.3$) current is reduced by activation of epidermal growth factor receptor (EGFR) or v-Src.¹³³ The inhibition is reversed by protein tyrosine kinases inhibitors. Associated with the inhibition of the current is an elevated tyrosine phosphorylation of the channel protein. A similar inhibitory effect of Src was reported for a human K^+ channel, hK_v1.5.¹³⁴

Tyrosine phosphatases perform opposing actions to tyrosine kinases. The receptor protein tyrosine phosphatase (RPTP) α was found associated with potassium channels, controlling their phosphorylation and activity in response to neurotransmitters.^{135, 136} It was discovered that lack of RPTP α increased phosphorylation and activity of K_v channels in Schwann cells.¹³⁷ It has also been reported that RPTP β increases Na^+ current by directly interacting with α - and β - subunits of the voltage-gated sodium (Na_v) channel.¹³⁸ RPTP μ is found to regulate K_v1.5 mRNA expression in cardiac myocytes.¹³⁹ PTPH1, a non-receptor protein tyrosine phosphatase is able to shift the voltage-dependence of $Na_v1.5$ channel towards hyperpolarization in HEK293 cells while an inactive PTPH1 does the opposite.¹⁴⁰

7.2. Tyrosine phosphorylation and dephosphorylation of funny current (I_f) and HCN channels

Accumulating evidence has shown that tyrosine phosphorylation may represent a novel regulatory mechanism of HCN channels. Epidermal growth factor (EGF) increased I_f through tyrosine phosphorylation.¹⁴¹ Genistein, a tyrosine kinase inhibitor, reduced I_f in rat ventricle and

caused a negative shift of its voltage dependence.^{141, 142} Previously, our lab discovered that a constitutively active Src tyrosine kinase increased the activity of HCN4 channel by shifting its voltage-dependent activation to more positive potentials and speeding its activation near the maximal diastolic potential.¹⁴³ Further, we demonstrated that in the same cell PP2, a selective inhibitor of Src tyrosine kinase, decreased HCN4 currents by negatively shifting the voltage-dependent activation of the channel and reducing the channel conductance.¹²⁰ We and others found that Src kinases phosphorylated key tyrosine residues (Y531 and Y554) in the C-linker of HCN4 channels.^{120, 121} Moreover, Src and HCN4 proteins are likely associated with each other.¹⁴³

Compared to tyrosine kinase, no research has been reported for the specific modulation of I_f and HCN channels by tyrosine phosphatases until our findings that RPTP α inhibited the activity of HCN2 channels dramatically.¹⁴⁴ We demonstrated that this inhibition was due to the reduced surface expression of the functional channels, a direct outcome of the association between HCN2 and RPTP α proteins, leading to tyrosine dephosphorylation of HCN2 channels.

7.3. Receptor protein tyrosine phosphatases (RPTPs)

Protein phosphorylation, which is maintained by balanced effects of kinases and phosphatases, has been involved in the regulation of intracellular communication, signal transduction and cell division. There are two general types of phosphorylation, tyrosine phosphorylation, which is the focus of my studies, and serine/threonine phosphorylation.

Participating in many signaling pathways controlling cell growth, differentiation and motility, tyrosine phosphorylation has been intensively studied over the past decade.^{145, 146} Compared to tyrosine kinases, however, little is known about tyrosine phosphatases, whose effects have been shown not simply to “reset the clock” by dephosphorylation.^{146, 147} There are

two classic categories of protein tyrosine phosphatases (PTPs): receptor type RPTPs and non-receptor type nrPTPs. Additionally, two types of PTPs have also been characterized, namely dual specificity PTPs¹⁴⁸ and low molecular weight PTPs.¹⁴⁹ RPTPs are the focus of my study. On the structural basis of distinct extracellular and intracellular domains, RPTPs are grouped into eight subfamilies designated type I/VI, IIa, IIb, III, IV, V, VII, VIII.¹⁵⁰ RPTP μ belongs to the type IIb subfamily, while RPTP α and RPTP ϵ are members of the type IV subfamily with the shortest extracellular domain among all RPTPs.¹⁵⁰ RPTP α , RPTP ϵ and RPTP μ share similarity of their intracellular domains, which bear duplicated catalytic domains, D1 and D2.¹⁵⁰

The activity of RPTPs can be regulated by post-translational modifications, such as glycosylation, phosphorylation, and oxidation. RPTP α contains eight putative sites for N-linked glycosylation, which is responsible for its two major forms: 98 kD and 114 kD in human, or 100 kD and 130 kD in mice.¹⁵¹⁻¹⁵³ In addition to N-linked glycosylation, RPTP α also possesses seryl and threonyl side chains that could be O-glycosylated. O-linked glycosylation is thought to explain the discrepancy between the predicted size of 88 kD and the observed size of 98 kD.¹⁵¹ RPTPs themselves can also be phosphorylated on tyrosine residues and serve as a docking site for SH2 domain-containing proteins, playing an important role in assembling supramolecular signaling complexes. For example, RPTP α was found to be constitutively phosphorylated on Tyr789 in the D2 region.¹⁴⁷ Upon phosphorylation by protein kinase C (PKC δ), the SH2 domain-containing protein Grb2 that associates with RPTP α is exchanged for another SH2 domain-bearing partner, pp60src.^{154, 155} Oxidative stress that deprotonates cysteine residues in the catalytic sites of RPTPs is another universal regulatory mechanism in this family.¹⁵⁶⁻¹⁵⁸ Oxidation may impede the phosphatase activity of RPTPs, thereby disrupting the balance between protein tyrosine kinases and phosphatases.^{158, 159}

8. Specific aims

There are two specific aims of my project, investigating the modulation of cardiac pacemaker channels by Src-family tyrosine kinases and receptor protein tyrosine phosphatases (RPTPs), respectively.

Specific aim 1 is summarized in Chapter 1 which consists of two sections. In Section 1, Src-family kinases including Src, Yes and Fyn were utilized to rescue an HCN4 mutant identified in a patient with sick sinus syndrome, D553N. This work was performed in HEK293 cells. In Section 2, the role of Src-mediated tyrosine phosphorylation in β -adrenergic modulation of heart rate was explored from the single cell to the whole animal. The interplay between isoproterenol (ISO) and PP2 was intensively studied on HCN4 channels in HEK293 cells, I_f in sinus node myocytes, the pacing rate of dissected sinus node tissue and the heart rate in whole rat *in vivo*, respectively.

Specific aim 2 is summarized in Chapter 2, divided into three sections. Sections 1 and 2 are to investigate RPTPs, including α , ϵ and μ isoforms, as potential modulators of HCN2 and HCN4 channels in HEK293 cells. In addition, changes of RPTP α protein content in cardiac ventricles were linked to the altered activity of ventricular I_f during development. In Section 3, a cAMP insensitive HCN4 mutant identified in a patient with sinus bradycardia, 573X, was examined in HEK293 cells to demonstrate that RPTP ϵ could modulate HCN4 independently of cAMP.

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Chapter 1 Modulation of Cardiac Pacemaker Channels by Tyrosine Kinases

Section 1

Rescue of a Trafficking Defective Human Pacemaker Channel via a Novel Mechanism: Roles of Src, Fyn, and Yes Tyrosine Kinases

I performed the biochemistry experiments and contributed to the experimental design and writing in this manuscript.

Section 2

Src Kinases Required for β -Adrenergic Stimulation of Heart Rate

I performed the majority of the experiments except the electrocardiogram recordings and contributed to the experimental design and writing in this manuscript.

Rescue of a Trafficking Defective Human Pacemaker Channel via a Novel Mechanism

ROLES OF Src, Fyn, AND Yes TYROSINE KINASES^{*,§}

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Yen-Chang Lin^{‡§1}, Jianying Huang^{‡§1}, Hong Kan^{‡¶}, Jefferson C. Frisbee^{‡§}, and Han-Gang Yu^{‡§2}

From the [‡]Center for Cardiovascular and Respiratory Sciences and the Departments of [§]Physiology and Pharmacology and [¶]Cardiology, West Virginia University School of Medicine, Morgantown, West Virginia 26506

Therapeutic strategies such as using channel blockers and reducing culture temperature have been used to rescue some long QT-associated voltage-gated potassium Kv trafficking defective mutant channels. A hyperpolarization-activated cyclic nucleotide-gated HCN4 pacemaker channel mutant (D553N) has been recently found in a patient associated with cardiac arrhythmias including long QT. D553N showed the defective trafficking to the cell surface, leading to little ionic current expression (loss-of-function). We show in this report that enhanced tyrosine phosphorylation mediated by Src, Fyn, and Yes kinases was able to restore the surface expression of D553N for normal current expression. Src or Yes, but not Fyn, significantly increased the current density and surface expression of D553N. Fyn accelerated the activation kinetics of the rescued D553N. Co-expression of D553N with Yes exhibited the slowest activation kinetics of D553N. Src, Fyn, and Yes significantly enhanced the tyrosine phosphorylation of D553N. A combination of Src, Fyn, and Yes rescued the current expression and the gating of D553N comparable with those of wild-type HCN4. In conclusion, we demonstrate a novel mechanism using three endogenous Src kinases to rescue a trafficking defective HCN4 mutant channel (D553N) by enhancing the tyrosine phosphorylation of the mutant channel protein.

Defective trafficking leading to the reduced surface expression of ion channels is one of the mechanisms responsible for a loss-of-function of the ion channel on the plasma membrane (1). Several methods have been developed to rescue the voltage-gated potassium Kv trafficking defective channels: reducing the culture temperature, applying the channel blockers, altering the molar ratio of glycerol, and using the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase inhibitor thapsigargin (2–6).

Hyperpolarizing-activated cyclic nucleotide-gated (HCN)³ pacemaker channels generate time- and voltage-dependent

inward currents, named I_h in neurons or I_f in the heart (7). They are important in various cell functions including excitability, synapse transmission, and rhythmic activity (7). The most well studied regulation of I_f is its response to autonomic stimulation. β -Adrenergic receptor activation increases and acetylcholine receptor activation decreases the intracellular cAMP levels, which in turn increases/decreases I_f by binding to the cyclic nucleotide-binding domain of the HCN channels, respectively (7). Other important mechanisms for the modulation of I_f /HCN channels have recently been found including β -subunit (8), lipids (9, 10), and p38 mitogen-activated protein kinase (11).

Accumulating evidence has revealed tyrosine phosphorylation as an important mechanism for modulation of HCN channel properties (12–16). An acute increase in tyrosine phosphorylation of I_f or HCN channels increases the channel activity, including an increase in the current amplitude, a positive shift of the voltage-dependent activation, an acceleration of activation kinetics, and an increase in whole cell conductance (12–15). Recently, we discovered that the cell surface expression of HCN2 channels can be remarkably inhibited by tyrosine dephosphorylation mediated by receptor-like protein tyrosine phosphatase α (RPTP α) and increased by tyrosine phosphorylation via Src kinase after long term treatment (17).

D553N, a missense HCN4 mutant, was recently identified in a patient with cardiac arrhythmia associated with depressed HCN gating properties (18). Functional and structural assays revealed that D553N expresses little ionic currents, which is possibly due to the defective channel trafficking so that the channels cannot reach the plasma membrane for normal functions (18).

The Src kinase family has nine members (19). They are closely related and share the same regulatory function. Three of them, Src, Fyn, and Yes, are ubiquitously expressed in a variety of tissues including neurons and myocytes (19, 20). Without stimulation, they are inactive. However, mutation of key tyrosine residue results in the constitutively active form of the kinase, SrcY529F, FynY531F, and YesY537F, respectively (15, 21, 22). Using these Src kinases, we show in this report a novel approach that can restore the surface expression of D553N for normal current expression via tyrosine phosphorylation.

EXPERIMENTAL PROCEDURES

cDNA Plasmids—The human version of HCN4-pcDNA1.1 was kindly provided by Dr. U. B. Kaupp and subcloned into pcDNA3.1 vector. HCN4-D553N-pcDNA3 was made by sub-

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¹ Both authors contributed equally to this work.

² To whom correspondence should be addressed: One Medical Center Dr., Rm. 3034/HSN, Dept. of Physiology, West Virginia University, Morgantown, WV 26506. Tel.: 304-293-2324; Fax: 304-293-5513; E-mail: hyu@hsc.wvu.edu.

³ The abbreviations used are: HCN, hyperpolarizing-activated cyclic nucleotide-gated; RPTP, receptor-like protein tyrosine phosphatase; PBS, phosphate-buffered saline.

stituting aspartic acid with asparagine using PCR. HCN4-D553N-DsRed was made by subcloning HCN4-D553N into the DsRed vector. RPTP α was a generous gift from Dr. Jan Sap (University of Copenhagen, Copenhagen, Denmark). The constitutively active form of Fyn, FynY531F, in pcDNA3.1 vector, was kindly provided by Dr. Shigeru Kanda (Nagasaki University, Nagasaki, Japan). The constitutively active form of Yes (YesY537F) was kindly provided by Dr. Arkadiusz Welman (Edinburgh Cancer Research Center), and we subcloned it into the pcDNA3.1 vector. Src529 (SrcY529F) was purchased from Upstate Biotechnology (Millipore). For simplicity, we also use Src, Fyn, and Yes and Src529, Fyn531, and Yes537 in the text for interchangeable use with the constitutively active form of each kinase: SrcY529F, FynY531F, and YesY537F.

Cell Culture and Plasmids Transfection—HEK293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen), supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 g/liter streptomycin. Cells with 50–70% confluence in 6-well plates were used for plasmid transfection using Lipofectamine2000 (Invitrogen).

Cell Lysis, Immunoprecipitation, and Western Blot Analysis—Total protein extracts were prepared from cells transfected for 24–48 h using CelLytic cell lysis reagent (Sigma) supplemented with protease inhibitors. For membrane fraction preparations, we used a membrane protein extraction kit (Pierce). The protein concentration of the lysate was determined using the Bradford or BCA assay. Equal amounts of total protein (0.5–1 mg) were incubated with a specific antibody for 1 h at 4 °C, and protein A/G Plus-agarose (Santa Cruz) was then added and incubated overnight with gentle rocking. The beads were washed three times with cold PBS buffer and resuspended in 2 $\times$ sample buffer. The immune complexes were separated by SDS-PAGE and analyzed by Western blot using the specific antibody of interest. Total protein of 5–20 μ g/sample was subjected to SDS-PAGE using 4–12% gradient gels (Invitrogen) and then transferred to nitrocellulose membranes (Amersham Biosciences) and incubated with proper antibodies. After washing and incubating with horseradish peroxidase-conjugated secondary antibody, immunoreactive proteins were visualized with the SuperSignal[®] West Pico kit (Pierce).

Cell Surface Biotinylation—Cell surface biotinylation experiments were performed by following the manufacturer's instruction (Pierce). Briefly, HEK293 cells transfected with desired plasmids were first treated with EZ-link Sulfo-NHS-SS-Biotin to label cell surface proteins. The cells were subsequently lysed with lysis buffer containing protease inhibitor mixture (Sigma). The labeled proteins were then isolated with immobilized NeutrAvidin-agarose. After washing three times, the bound proteins were released by incubating with SDS-PAGE sample buffer containing 50 mM dithiothreitol and then analyzed by Western blotting. All of the protein experiments were repeated at least three times.

Whole Cell Patch Clamp Recordings—For recording I_{HCN4} , day 1 (24–30 h) up to day 4 (90–98 h) post-transfection HEK293 cells with green fluorescence were selected for patch clamp studies. The HEK293 cells were placed in a Lucite bath in which the temperature was maintained at 25 ± 1 °C by a temperature controller (Cell MicroControls). I_{HCN4} currents were

recorded using the whole cell patch clamp technique with an Axopatch-200B amplifier. The current amplitude of HCN4 or D553N current is defined as the amplitude of the onset time-dependent inward current elicited by the hyperpolarizing pulse, excluding the instant jump at the beginning of the pulse. The current density is the current amplitude divided by the cell capacitance measured in each cell studied. The pipettes had a resistance of 2–4 M Ω when filled with internal solution: 6 mM NaCl, 130 mM potassium aspartate, 2 mM MgCl₂, 5 mM CaCl₂, 11 mM EGTA, and 10 mM HEPES; pH was adjusted to 7.2 by KOH. The external solution contained 120 mM NaCl, 1 mM MgCl₂, 5 mM HEPES, 30 mM KCl, 1.8 mM CaCl₂; pH was adjusted to 7.4 by NaOH. The I_{to} blocker, 4-aminopyridine (2 mM), was added to the external solution to inhibit the endogenous transient potassium current, which can overlap with and obscure I_{HCN4} tail currents recorded at +20 mV. The data were acquired by CLAMPEX and analyzed by CLAMPFIT (pClamp 8; Axon).

The data are shown as the means $\pm$ S.E. The threshold activation of I_{HCN4} is defined as the first hyperpolarizing voltage at which the first time-dependent inward current can be observed. Student's t test was used for statistical analysis with $p < 0.05$ being considered statistically significant. Time constants were obtained by using Boltzmann best fit with one exponential function on current traces that reach steady state. HCN4 activates slowly, and the cells would not tolerate pulses sufficiently long to reach the steady state. We therefore used the following approach to obtain an accurate estimate of the steady state activation (15). The onset current traces were fitted with a single exponential function to 30–40 s to allow estimates of steady state current levels. The fitted current amplitudes were then divided by the driving force (the difference between test pulses and the reversal potential that was measured in each cell) to obtain the conductance at each test pulse. The activation curves were constructed by normalizing the conductance to its maximal value in response to the most negative test pulse.

Confocal Fluorescent Imaging of HEK293 Cells—HEK293 cells transfected with HCN4-DsRed or HCN4-DsRed-D553N were incubated on coverslips and fixed in 4% paraformaldehyde/PBS for 15 min and then washed with PBS (10 mM phosphate buffer, 150 mM NaCl, pH 7.4) for 5 min for three times, followed by blocking in 1% bovine serum albumin/PBS, pH 7.4, for 60 min. After washing six times in PBS, the coverslips were mounted on slide glasses using Fluoromount G (Southern Biotechnology). The cells were imaged by a LSM510 confocal microscopy using a Plan-Neofluar 40 $\times$ /0.75 objective or a Plan-Apochromat 63 $\times$ /1.4 Oil differential interference contrast M27 objective. For DsRed imaging, a 1.2-milliwatt 543-nm HeNe laser was used for excitation, and a 560–615-nm BP emission filter was used for emission.

RESULTS

Inhibition of HCN4 Current Expression by RPTP α —We have recently demonstrated that RPTP α can inhibit the surface expression of HCN2 channels via tyrosine dephosphorylation (17). Given the high structural homology between HCN2 and HCN4 (>80%) (7), it was expected that RPTP α may also inhibit the surface expression of HCN4. Fig. 1 shows a typical current

FIGURE 1. RPTP α on HCN4 current expression in HEK293 cells. A, HCN4 current expression in response to 15-s hyperpolarizing pulses from -65 to -135 mV in 10-mV increments. Tail currents were recorded at $+20$ mV. B, in a cell co-transfected with RPTP α after 1 day of incubation, HCN4 current expression in response to 10-s hyperpolarizing pulses from -65 to -135 mV in 10-mV increments. C, in a cell co-transfected with RPTP α after 2 days of incubation, HCN4 current expression in response to 10-s hyperpolarizing pulses from -65 to -135 mV in 10-mV increments. D, HCN4 current expression in HEK293 cells co-expressed with the empty vector, pRK5.

expression of HCN4 expressed in HEK293 cells (Fig. 1A). The expression was dramatically suppressed across the test voltages (-65 to -135 mV) when HCN4 was co-expressed with RPTP α after 1 day of transfection (Fig. 1B). The reduction in current expression was associated with a negative shift in threshold activation (Figs. 1, A and B, arrows). After 2 days of transfection, RPTP α almost eliminated the current expression of HCN4 (Fig. 1C). The effect of RPTP α on HCN4 current expression is similar to that on HCN2 current expression (17). As a control, the empty vector, pRK5 (used to subclone RPTP α), did not affect the current expression of HCN4 (Fig. 1D). Each of these results was confirmed in an additional 5–7 cells.

Inhibited Surface Expression and Reduced Tyrosine Phosphorylation of HCN4 by RPTP α —Wondering whether HCN4 current inhibition of RPTP α is due to the suppressed membrane expression of the channel proteins, we examined the HCN4 channel membrane preparation (Fig. 2A). The left triplet shows HCN4 expression. The split bands indicate unglycosylated and glycosylated forms, similar to HCN2 membrane expression (17). The glycosylated form of HCN4 was significantly inhibited by RPTP α (middle triplet) and enhanced by Src529 (a constitutively active form of Src) (right triplet).

Using the phosphotyrosine-specific antibody 4G10, Fig. 2B shows that the tyrosine phosphorylation of HCN4 channel protein (middle lane) was significantly enhanced by Src529 (second lane from the right) but inhibited by RPTP α (left lane). These results suggested that the altered membrane expression of HCN4 is possibly caused by the increased or decreased tyrosine

FIGURE 2. Src/RPTP α on HCN4 membrane protein expression and tyrosine phosphorylation in HEK293 cells. A, immunoblots (IB) on membrane fraction for HCN4, HCN4+RPTP α , and HCN4+Src529. β -Actin was used as a loading control. B, tyrosine phosphorylation of HCN4 by Src529 and RPTP α . The total protein samples were first immunoprecipitated (IP) using 4G10 antibody and then immunoblotted using an HCN4 antibody. Weak phosphorylation signals of HCN4 and HCN4+RPTP α can be seen in long exposed film (1 min). The immunoblot of HCN4 (right lane) was used as a size marker.

FIGURE 3. Src/RPTP α on HCN4 cell surface expression in HEK293 cells. A, fluorescence imaging of HCN4-DsRed, HCN4-DsRed+RPTP α , and HCN4-DsRed+Src529. B, fluorescence (left panel) and bright field (right panel) images of cells transfected with DsRed alone.

phosphorylation of the channel protein by Src tyrosine kinase and RPTP α tyrosine phosphatase, respectively.

To further seek supporting evidence that reduced ionic current expression of HCN4 is caused by the suppressed surface expression of HCN4 channels, we tagged HCN4 with a fluorescent protein, DsRed, and examined the distribution of HCN4 using fluorescent confocal microscopy. Fig. 3A shows a typical fluorescent image of HCN4 expressed alone in a HEK293 cell

FIGURE 4. Src/Fyn/Yes kinases on current expression of D553N at -125 mV. Currents elicited by hyperpolarizing pulses in 10-mV increments are presented for HCN4 (A), D553N (B), D553N+Src529 (C), D553N+Fyn531 (D), D553N+Yes537 (E), D553N+Src529+Fyn531 (F), D553N+Src529+Yes537 (G), D553N+Fyn531+Yes537 (H), and D553N+Src529+Fyn531+Yes537 (I). The test potentials are labeled in the figures. The pulse durations were 15 s for A–C, E, and G; 12 s for F, H, and I; and 6 s for D. The holding potential was -10 mV.

(left panel). An approximately equal amount of HCN4 is distributed on the plasma membrane and in the cytosol, consistent with the membrane protein expression results (Fig. 2A). When co-expressed with RPTP α , most HCN4 channels are retained in the cytosol (middle panel of Fig. 3A). On the other hand, Src529 significantly enhanced the cell surface expression of HCN4 (Fig. 3A, right panel). As a control, Fig. 3B shows the fluorescence (left panel) and bright field (right panel) images of the empty DsRed vector expressed in HEK293 cells.

Rescuing D553N Current Expression by Src, Fyn, and Yes—D553N has been recently identified in a patient suffering from sinus node dysfunction, long QT, ventricular tachycardia, and torsade de pointes (18). *In vitro* studies of the mutant channel revealed defective surface expression on plasma membrane, leading to the loss of current expression (18). Given the facts that the HCN4 channel activity including the channel surface expression can be significantly enhanced by Src-mediated tyrosine phosphorylation and the ubiquitous expression of three Src kinase family members (Src, Fyn, and Yes), we set forth to test the hypothesis that the current expression of the defective trafficking D553N can be restored by constitutively active forms of Src kinases (Src529, Fyn531, and Yes537).

Fig. 4 provides a typical set of current recordings under different conditions. The current expression of wild-type HCN4 is shown in Fig. 4A, as compared with the loss of current expres-

sion for D553N (Fig. 4B). Fig. 4 (C–E) shows the effects of individual Src, Fyn, and Yes on D553N current expression, respectively. Fig. 4 (F–H) also shows the effects of Src+Fyn, Src+Yes, and Fyn+Yes on D553N current expression, respectively. Fig. 4I shows the overall effects of combined Src+Fyn+Yes on D553N current expression.

For effective comparison of the actions of Src kinases on D553N to HCN4, we calculated the current density and the activation kinetics at -125 mV, which is near the fully activated voltage. Current density at the voltage in which all channels are open is directly related to our central interest of evaluating whether Src kinases can rescue the surface expression of D553N. The current densities under different conditions are shown in Fig. 5A.

Src and Yes, but not Fyn, can significantly rescue D553N current expression. Different combinations of three kinases all enhanced the current expression of D553N (Fig. 5A, asterisk). All three Src kinases expressed together (Fig. 4I) can restore $\sim 68\%$ current expression of D553N as compared with the wild-type HCN4 expression (Fig. 5A, dark bars, HCN4: 37.15 ± 3.21 pA/pF, $n = 7$; D553N+Src/Fyn/Yes: 25.25 ± 2.17 pA/pF, $n = 10$) (Fig. 5A).

The effects of Src/Fyn/Yes kinases on the current activation kinetics are also different (Fig. 5B). The time constants for activation kinetics were obtained by fitting the onset current with one-exponential function at -125 mV under different conditions. Fyn accelerated but Yes slowed the activation kinetics of D553N, whereas Src had no effects (p^* values). The combinations of Src+Fyn and Fyn+Yes can accelerate D553N activation kinetics, whereas the combination of Src+Yes cannot. A combination of all three Src kinases can speed D553N activation kinetics at -125 mV (Fig. 5, B and D); the time constants are 4.07 ± 0.59 s ($n = 3$) for D553N and 1.24 ± 0.05 s ($n = 9$) for D553N/Src/Fyn/Yes, respectively ($p = 0.01$). It is worth pointing out that in 16 D553N transfected cells we studied only three cells that expressed time-dependent inward currents at -125 mV and that were used for calculating current density and activation kinetics shown in Fig. 5 (A and B). The other 13 cells expressed no currents at the test potentials ranging from -75 mV to -135 mV. As a comparison, we also showed the statistical analysis of comparing Src kinases on D553N to HCN4 indicated by p values in Fig. 5B.

To assess the overall functional rescuing effects of Src/Fyn/Yes on D553N gating, we examined the biophysical properties of D553N co-expressed with Src/Fyn/Yes (Fig. 4I) in compari-

FIGURE 5. Src/Fyn/Yes kinases on current density and gating of D553N compared with HCN4. A, current density measured at -125 mV under different conditions. An asterisk indicates a statistically significant difference in comparison with D553N current density. B, time constants of activation kinetics at -125 mV under different conditions. p values indicated statistical significance in comparing the effects of the kinases on D553N to HCN4 (white bar). p^* values indicated statistical significance in comparing the effects of the kinases on D553N to D553N alone (gray bar). C, activation curves of HCN4 (WT) and D553N+Src/Fyn/Yes. D, activation kinetics of HCN4 (WT) and D553N+Src/Fyn/Yes. All of the statistical results were from seven to nine cells for each group except for D553N (only three cells expressed little currents of 16 cells tested).

FIGURE 6. Src/Fyn/Yes kinases on tyrosine phosphorylation and surface expression of D553N. A, tyrosine phosphorylation of HCN4, HCN4+Src529, HCN4+Fyn531, and HCN4+Yes537, respectively. B, tyrosine phosphorylation of D553N, D553N+Src529, D553N+Fyn531, and D553N+Yes537, respectively. Samples for A and B were immunoprecipitated (IP) with 4G10 antibody prior to the detection by HCN4 antibody. C, Src/Fyn/Yes effects on surface expression of D553N using a biotinylation method. IB, immunoblot.

son with the wild-type HCN4 (Fig. 4A). The Boltzmann equation best fit from the averaged activation curves obtained from tail currents (see “Experimental Procedures” for details in the construction of the activation curve) showed a small depolarizing shift of D553N+Src/Fyn/Yes compared with HCN4 with no

altered slope factors(s). However, statistical analysis from the means of two groups yielded no significant difference ($V_{0.5_HCN4}$: -86.1 ± 1.5 mV, $n = 7$; $V_{0.5_D553N+Src/Fyn/Yes}$: -81.6 ± 1.4 mV, $n = 9$; $p = 0.0724$, s_HCN4 : 8.2 ± 0.2 mV, $n = 7$; $s_D553N+Src/Fyn/Yes$: 8.4 ± 0.2 mV, $n = 9$, $p = 0.577$) (Fig. 5C). On the other hand, the effects of Src kinases on D553N activation kinetics are complex. Compared with the wild-type HCN4, the averaged activation kinetics for D553N+Src/Fyn/Yes were slowed at the beginning of the activation curve, accelerated near the middle of the activation curve, but statistically indistinguishable at the potentials after the half-activation point (Fig. 5D) ($n = 7$ for HCN4, $n = 9$ for D553N+Src/Fyn/Yes).

Enhanced Tyrosine Phosphorylation and Membrane Surface Expression of D553N by Src, Fyn, and Yes Tyrosine Kinases—We have previously shown that Src-mediated tyrosine phosphorylation increases the HCN2 and HCN4 channel activity via shifting the activation curve

to depolarizing potentials (short term effect) and enhancing the cell surface expression (long term effect) (12, 14, 15, 17). We have now demonstrated that reduced tyrosine phosphorylation by RPTP α (Fig. 2B) can lead to the suppressed surface expression of HCN4 (Fig. 2A). To investigate whether tyrosine phosphorylation is involved in rescuing actions of Src, Fyn, and Yes on D553N current expression, we studied the tyrosine phosphorylation state of D553N by Src, Fyn, and Yes kinase, respectively. Fig. 6 shows the tyrosine phosphorylation of HCN4 (Fig. 6A) and D553N (Fig. 6B) by three Src kinases in HEK293 cells. HCN4 background phosphorylation was weak and significantly increased by Src529 and Fyn531, but not by Yes537. D553N background phosphorylation was barely detectable but dramatically increased by Src529, Fyn531, and Yes537. It was noticed that Src529 increased most significantly the phosphorylation of the wild-type HCN4, but Fyn531 induced the highest phosphorylation of D553N channel proteins.

We next examined the effects of Src kinases on D553N surface expression using biotinylation approach (see “Experimental Procedures” for details). Fig. 6C shows that the surface expression of D553N was barely detected. Src, Fyn, and Yes each significantly enhanced the surface expression to various degrees. Yes had a larger effect than Src and Fyn in promoting the surface expression of D553N. The HCN4 wild-type surface expression (Fig. 6C, second lane from the left) was used as a positive control.

To seek further supporting evidence for rescuing surface expression of D553N by three Src kinases, we constructed the

FIGURE 7. Src/Fyn/Yes kinases on D553N fluorescence imaging. Fluorescence images of cells transfected with HCN4 (A), D553N (B), DsRed vector (C), D553N+Src529 (D), D553N+Fyn531 (E), and D553N+Yes537 (F). All of the results were repeated in an additional eight to ten cells.

D553N-DsRed fusion protein and performed the confocal imaging experiments. Fig. 7 shows that most wild-type HCN4 is expressed on the cell surface (Fig. 7A). By contrast, most D553N cannot reach the cell surface (Fig. 7B). Co-expression with either Src (Fig. 7D) or Yes (Fig. 7F) can significantly enhance the surface expression of D553N. However, Fyn has been much less significant on D553N surface expression, as evidenced by a significant amount of D553N remaining in the cytosol (Fig. 7E). DsRed vector itself was uniformly expressed across the cell (Fig. 7C) and served as a negative control. These results, combined with the protein biochemistry analysis, collectively provide the cellular evidence for the functional rescue of D553N current expression at the plasma membrane by Src/Fyn/Yes kinases.

DISCUSSION

Defective trafficking of mutant channels represents an important mechanism for Kv channels causing long QT2 (1). Studying long QT related Kv channel modulation has led to the findings that lower temperature and channel blockers can restore the surface and ionic current expression of the defective trafficking mutant channels (1). In this work, we showed for the first time that by modulating the Src/Fyn/Yes kinase activity, a human HCN4 trafficking defective mutant D553N (also linked to long QT (18)) can be rescued for normal surface and current expression. The corrected D553N exhibited the gating properties comparable with those of the wild-type HCN4 channels.

Enhanced tyrosine phosphorylation increased the activity of the cardiac pacemaker current, I_p , in the sinoatrial node cells (13). Using genistein, a nonspecific tyrosine kinase inhibitor, we found a differential modulation of tyrosine phosphorylation for HCN1, HCN2, and HCN4 expressed in *Xenopus* oocytes; genistein had no effects on HCN1 but reduced HCN2 or HCN4 current expression (12). In the case of HCN2, there was also a

negative shift in the voltage dependence of activation that accompanied the current reduction. These studies represent the *acute* effects of altered tyrosine phosphorylation of HCN channel proteins on the gating properties of I_p .

To investigate the *long term* effects of specific tyrosine kinases on HCN channels in a mammalian background, we studied the effects of Src kinase on HCN4 channel (the main isoform in the heart) expressed in HEK293 cells. We focused on the Src kinases for two reasons: it mediates epidermal growth factor receptor signaling (19) and Src homology 3 domain was initially used to clone the first HCN channels (23). We found that Src associated with and phosphorylated the HCN4 channel proteins, leading to the enhanced HCN4 current density near diastolic potentials (15). This was the first direct evidence showing that 1) HCN4 channels can be phosphorylated by Src-mediated tyrosine kinases and 2) long term effects of tyrosine phosphorylation of HCN4 channels can induce the changes in the current density, which directly correlates with the number of functional channels expressed at the plasma membrane. Accompanying the increased current density were the accelerated activation kinetics and a positive shift in the voltage-dependent activation, which has been typically observed in the short term modulation of HCN4 channels. These conclusions were confirmed by the subsequent investigation on the major tyrosine residues that mediate Src actions (14). Using PP2, a selective inhibitor of Src kinase family, we found that the reduced Src kinase activity can indeed shift the voltage-dependent activation to hyperpolarizing potentials, an effect mediated by HCN4 Tyr⁵³¹ (14). Another tyrosine residue, HCN4 Tyr⁵⁵⁴ previously reported by others (16), also contributed to the slowing of activation kinetics by PP2 (14). Work on the action of PP2 on HCN4 also resulted in two surprising observations. First, the PP2-induced negative shift of HCN4 voltage-dependent activation is not in agreement with our previous results with genistein. At least two factors can contribute to this discrepancy: mammalian cell (HEK293) *versus* amphibian (*Xenopus* oocytes) background and general (genistein) *versus* selective (PP2) inhibition of tyrosine kinases. Second, we found that PP2 also reduced the whole cell channel conductance (supplemental Fig. 3 in Ref. 14). These results implied that even the short term effect of altered tyrosine phosphorylation may affect the number of functional channels at the plasma membrane.

More recently in the investigation of the potential role the tyrosine phosphatase might play in the modulation of HCN channel function, we found the dramatic inhibition of HCN2 current expression by RPTP α (17). The inhibited HCN2 current expression was due to the reduced surface expression of HCN2 channels via association between RPTP α and the HCN2 channel proteins, resulting in the channel dephosphorylation (17). The work demonstrated a previously unrecognized feature of HCN channel modulation by tyrosine phosphorylation: the tyrosine phosphorylation state of HCN channel proteins represents one important regulatory mechanism for the cell surface expression of the functional channels, which directly determines the current expression of functional HCN channels. This feature may be utilized to enhance the surface and ionic current expression of HCN mutant channel that cannot reach the plasma membrane for normal function.

TABLE 1**Relative roles of Src, Fyn, and Yes on D553N surface/current expression and activation kinetics**

–, no or little expression or little effect or decrease; +, increase; ++, significant increase or acceleration.

	Surface expression ^a	Current density	Activation kinetics
D553N	–	–	–
+Src	++	++	–
+Fyn	+	–	++
+Yes	++	++	–

^a Combined results of biotinylation and fluorescence imaging.

Indeed, the evidence presented in this work showed that the enhanced tyrosine phosphorylation mediated by Src kinases can rescue the surface expression of D553N for normal channel function. What was unexpected, however, is the finding that three Src kinases that were ubiquitously expressed in the heart have different functional effects on D553N channel activity. Subsequent phosphorylation studies showed that all three kinases significantly enhanced phosphorylation of D553N with the order of potency: Fyn531 > Src529 > Yes537 (Fig. 6B). In comparison, the wild-type channel background phosphorylation was increased mostly by Src529, to moderate degree by Fyn531, and nearly unaffected by Yes537. In agreement with the previous studies, the Src kinase-mediated tyrosine phosphorylation is associated with the acceleration of channel activation kinetics (14–16). These differential effects by Src/Fyn/Yes on enhancing D553N expression and function are summarized in Table 1.

The differential phosphorylation of both wild-type HCN4 and D553N channels by three Src kinases suggested that different tyrosine residues are involved in mediating each of the kinases. These results also suggested a possibility that D553N may undergo a protein misfolding that prevents the nearby tyrosine residues from being phosphorylated. Association of Src tyrosine kinases appears to partially correct the protein folding that leads to the exposure of key tyrosine residues for phosphorylation.

To understand the mechanism by which tyrosine phosphorylation used to restore the surface expression of D553N, we proposed a model utilizing the three-dimensional crystal structure of the C-linker region of HCN2 for the following three reasons. First, D553N mutation occurred in the C-linker. Second, there is a high homology (91.6%) between HCN2 and HCN4 in the C-linker (supplemental Fig. S1). Third, HCN2 is the only protein in the HCN family whose crystal structure of the C-linker has been solved (24). HCN4 Asp⁵⁵³ corresponds to HCN2 Asp⁴⁷⁵. Among many potential mechanisms responsible for the defective trafficking of HCN4-D553N, protein misfolding is an attractive one. We hypothesized that there may exist a potential electrostatic interaction between Asp⁴⁷⁵ and Lys⁴⁷² of the B' helix. The negatively charged Asp⁴⁷⁵ is spatially close to the positively charged Lys⁴⁷², similar to the relative spatial locations of Lys⁴⁷² and Glu⁵⁰² of the D' helix, which have been demonstrated to form a salt bridge critical in maintaining the local folding of C-linker (24) (supplemental Fig. S2). The putative D475N (equivalent to D553N in HCN4) mutation can cause the loss of a negative charge, which may change the intersubunit interaction between Lys⁴⁷² and Glu⁵⁰² to alter local

folding of the C-linker structure. We noted that Lys⁴⁷² is changed to Arg⁵⁵⁰ in HCN4. With a guanyl group, Arg⁵⁵⁰ is more capable than Lys⁴⁷² in forming multiple electrostatic interactions with nearby residues having negative side chains. Therefore, in HCN4-D553N mutant, the potential salt bridge of Asp⁵⁵³ with Arg⁵⁵⁰ could be disrupted, which affected the interaction between Arg⁵⁵⁰ and Glu⁵⁸⁰ (Glu⁵⁰² in HCN2) that is critical in intersubunit contacts (24). Furthermore, the Src kinase-mediated tyrosine phosphorylation at Tyr⁵⁵⁴ residue near Asp⁵⁵³ (supplemental Fig. S1) could introduce a negatively charged phosphate group, which could mimic the effect of Asp⁵⁵³ to rebuild the salt bridge interaction between Arg⁵⁵⁰ and Glu⁵⁸⁰, consistent with the previous studies demonstrating the importance of Tyr⁵⁵⁴ (14, 16). For wild-type HCN4, the existing negative charge on Asp⁵⁵³ might repel the entry of a phosphate group and limit the phosphorylation on certain nearby tyrosine residues. It can explain why the differential modulation by Src, Fyn, and Yes is different between the wild-type HCN4 and the D553N mutant channels (Fig. 6, A and B).

While presenting a novel mechanism to correct the surface expression of a trafficking defective HCN4 mutant channel, we left at least three questions unanswered. First, what are the tyrosine residue(s) in HCN4 channel proteins that mediate the actions of Fyn and Yes? The same tyrosine residues (such as Tyr⁵³¹ and Tyr⁵⁵⁴) are unlikely to be used by all three kinases. Fyn, not Src, accelerated D553N activation kinetics (Fig. 5B). Fyn may target the tyrosine residues in or near A' and B' helices of the C-linker. On the other hand, Yes may phosphorylate different tyrosine residues that are located outside of the C-linker, which can explain its lack of acceleration in the activation kinetics. Second, what is the correlation, if any, among the surface expression and activation kinetics and the tyrosine phosphorylation state of HCN4 channels? Fyn showed significant tyrosine phosphorylation and acceleration of the channel activation kinetics but little effect on promoting the channel surface expression. Yes did not exert the highest phosphorylation in comparison with Src and Fyn (Fig. 6B), slowed the channel activation kinetics, but exhibited the most potency of promoting the cell surface expression of D553N (Figs. 6C and 7F and Table 1). It might involve other unknown proteins yet to be identified. Third, will an increase in the endogenous Src/Fyn/Yes kinase activity in myocytes help promote the surface expression of D553N *in vivo*? Addressing these questions represents our future research endeavors, leading to the discovery of an effective endogenous regulatory mechanism to correct cardiac arrhythmias caused by HCN channel mutants.

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Supplemental Data

Rescue of a Trafficking Defective Human Pacemaker Channel via a Novel Mechanism:
Roles of Src, Fyn, Yes tyrosine kinases

Yen-Chang Lin^{1,2,*}, MS; Jianying Huang^{1,2,*}, BS; Hong Kan^{1,3}, MD, PhD; Jefferson C.
Frisbee^{1,2}, PhD; Han-Gang Yu^{1,2}, PhD

¹Center for Cardiovascular & Respiratory Sciences, ²Department of Physiology and
Pharmacology, ³Department of Cardiology, West Virginia University School of
Medicine, Morgantown, WV 26506

Supplemental Figure (SF) Legends:

SF1: Upper panel: Sequence alignment of C-linker regions between mouse HCN2 (mHCN2) and human HCN4 (hHCN4). The localization of α -helices as deduced from the crystal structure of HCN2 is indicated by bars on top of the sequences. Lower panel: The ribbon diagram of mHCN2 C-terminus structure including C-linker and cyclic nucleotide binding domain (CNBD). Conserved aspartic acid residues in HCN2 and HCN4 are highlighted in green. Surrounding tyrosine residues that can potentially affect the local electrostatic interaction are highlighted in grey. The ribbon diagram was constructed in the software DeepView/SwissPdbViewer (<http://spdbv.vital-it.ch/>).

SF2: Structural model of a portion of the C-linker region of two neighboring HCN2 subunits showing the interaction between the negatively-charged carbonyl group of D475 (corresponding to HCN4-D553) and the positively-charged amino group of K472 (corresponding to HCN4-R550). For clarity only the side chains of D475, K472, E502 and the surrounding tyrosine residues are shown. Oxygen atoms are highlighted in red and nitrogen atoms in blue. The A' and B' helices are from one subunit while the C' and D' helices are from its neighboring subunit of the channel tetramer. The dotted line marks

a potential salt bridge formed between D475 and K472. The model was produced using the software DeepView/SwissPdbViewer (<http://spdbv.vital-it.ch/>).

SF1

SF2

Src Kinases Required for β -Adrenergic Stimulation of Heart Rate

Huang: Src modulation of heart rate via HCN4

Jianying Huang, BS¹, Yen-Chang Lin, PhD^{1,*}, Hong Kan, MD, PhD², Vincent Castranova, PhD²,
Han-Gang Yu, PhD¹

¹Center for Cardiovascular and Respiratory Sciences, Department of Physiology and Pharmacology, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV 26506; ²Pathology and Physiology Research Branch, National Institute for Occupational Safety and Health/CDC, Morgantown, WV 26506

*: currently at NIOSH/CDC, Morgantown

Corresponding author: Han-Gang Yu, Center for Interdisciplinary Research in Cardiovascular Sciences, Department of Physiology and Pharmacology, West Virginia University School of Medicine. Morgantown, WV

Tel: 304-293-2324, Fax: 304-293-5513, Email: hyu@hsc.wvu.edu

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Abstract

Background:

The increase of heart rate through β -adrenergic receptors is via increasing intracellular cAMP concentrations that facilitate hyperpolarization-activated cyclic nucleotide-gated (HCN) 4 channels. Our recent findings that Src kinases modulate HCN4 initiated a hypothesis that stimulation of heart rate by isoproterenol (ISO) requires Src kinases activity.

Methods and Results:

In Human Embryonic Kidney (HEK) 293 cells, a selective Src kinases inhibitor, PP2, reversed or prevented the enhancement of HCN4 by ISO. Additionally, PP2 inhibited 573X, a cAMP insensitive human HCN4 mutant, by negatively shifting the activation threshold and midpoint as well as decelerating activation kinetics. In isolated sinus node myocytes, PP2 decreased the hyperpolarization-activated “funny” current (traditionally called cardiac pacemaker current), I_f , by negatively shifting the activation curve and decelerating activation kinetics in contrast to ISO-induced stimulatory effects. The spontaneous action potentials were also slowed by PP2. Moreover, ISO failed to increase I_f or accelerate spontaneous action potentials in the presence of PP2. In the dissected rat sinus node, PP2 decreased its spontaneous beating rate. Further, ISO-induced stimulation of sinus node beating rate was prevented by PP2. Finally, in rats *in vivo*, PP2 attenuated the dose-dependent stimulation of heart rate by ISO, and chronically reduced the heart rate. These desensitized responses to ISO by PP2 were probably due to its inhibition on the surface expression of HCN4 channels in sinus node myocytes.

Conclusions:

We herein demonstrate that the increase of heart rate through β -adrenergic receptors is attenuated by inhibiting Src kinases, possibly via suppressing HCN4/ I_f activity in sinus node

independently of cAMP. Therefore, Src-mediated tyrosine phosphorylation plays an important role in regulating cardiac pacemaker activity and it could be a precondition to the increase of heart rate through β -adrenergic receptors.

Key words: β -adrenergic stimulation, heart rate, cAMP, Src tyrosine kinases, pacemaker current

Introduction

The well-established adrenergic signaling pathway that mediates the regulation of heart rate is through β -adrenergic receptor activation, G-protein, adenylate cyclase, and cAMP.¹⁻³ The intracellular cAMP level increases upon agonist binding to the β -adrenergic receptors. cAMP increases I_f in the sinus node by shifting its voltage-dependent activation toward more positive potentials associated with acceleration of activation kinetics.² Activated near the end of sinus node repolarization, I_f contributes to the early depolarization in diastole (*i.e.* diastolic depolarization).² The amplitude and speed of I_f activation determine the slope of diastolic depolarization, which controls the heart rate.²

I_f is generated by HCN channels. Three isoforms (HCN1, HCN2, HCN4) are present in the heart.⁴ HCN4 is the prevalent isoform in the sinus node.⁴ cAMP acts on HCN4 by directly binding to the cyclic nucleotide binding domain (CNBD) of HCN channel proteins, which releases the inhibition of C-linker on the channel gating, leading to faster opening at more positive potentials.^{5,6} Therefore, cAMP sensitivity of HCN4 has been proposed as a key event for control of heart rate.^{7,8}

Recently, mutations of HCN4 channels have been identified in patients with sick sinus syndrome manifested by bradycardia.⁹ All of the mutants exhibited depressed gating properties in comparison to the wild-type HCN4. Except for S672R¹⁰, they activated at very negative potentials due to loss of cAMP sensitivity or generated little currents due to reduced surface expression.¹¹⁻¹⁴ For example, two cAMP-insensitive HCN4 mutants, 573X and 695X, were identified in patients with severe bradycardia without ischemia or other structural heart diseases.^{14,15} Clearly, the cAMP pathway offers no therapeutic strategy to rescue normal gating of these mutants. Therefore, a novel mechanism of modulating HCN4 channel activity

independent of cAMP would be essential in the context of a therapeutic approach to bradycardia in general and to patients with HCN4 mutations in particular.

We have previously shown that reduced general tyrosine kinase activity decreased sinus node I_f by inhibiting the gating of HCN channels.¹⁶ Exploration of specific intracellular tyrosine kinases involved in the modulation of HCN channels revealed Src kinases.¹⁷ Increased Src kinases activity rescued an HCN4 trafficking-defective mutant D553N by restoring its plasma membrane protein contents.¹⁸ Moreover, receptor-like tyrosine phosphatase alpha (RPTP α) exhibited powerful inhibition of HCN channels by decreasing the surface expression of channels.^{18, 19} These studies have indicated that the surface expression of HCN channels, which was positively correlated with Src-mediated tyrosine phosphorylation, would occur prior to and might be a requirement for their modulation by ISO or cAMP. Therefore, we hypothesized that the stimulation of cardiac pacemaker activity through β -adrenergic receptors by ISO may be blunted if the Src kinase activity is inhibited.

To test this hypothesis, we used PP2, a widely used selective inhibitor of Src family kinases²⁰ as a probe to study the effects of reduced Src tyrosine kinase activity on the ISO-induced stimulation of HCN4 and 573X channels in HEK293 cells, I_f and spontaneous action potentials in single sinus node myocytes, spontaneous beating rate of the dissected sinus node, and heart rate, respectively. Our results indicate that reduced Src tyrosine kinase activity does indeed inhibit the activity of HCN4 and 573X. In addition, reduced Src kinases activity suppresses sinus node I_f and action potential, decreases sinus node beating rate, and slows heart rate. Importantly, all stimulatory effects of ISO on pacemaker activity from the single cell to the whole animal are either blocked or attenuated when Src tyrosine kinase activity is inhibited.

Materials and Methods

Animals

Male Sprague-Dawley rats [Hla:(SD) CVF] from Hilltop Lab Animals (Scottsdale, PA), 6-7 weeks of age and free of viral pathogens, parasites, mycoplasmas, Helicobacter, and CAR bacillus were used for all experiments. The rats were housed in cages ventilated with HEPA-filtered air under controlled temperature and humidity conditions and a 12-hr light/12-hr dark cycle. Food (Teklad 7913) and tap water were provided ad libitum. The rats were allowed to acclimate to the facilities for one week before studies were performed. The animal facilities are specific pathogen-free and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International. The animal protocols in this study were reviewed and approved by the Animal Care and Use Committees of West Virginia University and the National Institute for Occupational Safety and Health.

Cell culture and transfecting plasmids

HEK293 cells were grown on poly-D-lysine coated coverslips in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum, 100 IU/mL penicillin, and 100 g/L streptomycin. Cells with 50-70% confluence in 6-well plates were used for plasmid transfections (1-2 μ g for each plasmid) using Lipofectamine2000 (Invitrogen). HCN4 and 573X plasmids were fused with GFP for verification of expression and served as selection guidance for patch clamp studies.

Dissection of rat sinus node and isolation of sinus node myocytes

The heart was quickly removed from anesthetized Sprague-Dawley (SD) rats with sodium pentobarbital (50 mg/kg) and immersed in normal Tyrode solution containing heparin. A blunt end forceps was used to push against the ventricles to force out any remaining blood. New

Tyrode solution was constantly replaced and rinses continued until the heart was clear of blood. Ventricles were removed, and the sinoatrial region was exposed, dissected out, and placed in fresh Tyrode solution gassed with 100% O₂ at 37°C.

For isolation of sinus node myocytes, the sinoatrial region was digested in a Ca²⁺-free Tyrode solution containing 0.4 mg/ml Librase Blendyme 4 (Roche Applied Sciences) for approximately 20 min at 37°C. After digestion, the tissue was trimmed into strips of ~1 mm in width and 3-4 mm in length in Ca²⁺-free Tyrode solution. The digested tissue was then placed in Krafte-brühe (KB) solution. The sinus node myocytes were dissociated by gently puffing KB solution onto the tissue. Normal Tyrode solution contained (mM): NaCl, 140; KCl, 5.4; CaCl₂, 1.8; MgCl₂, 1; D-Glucose, 5.5; HEPES-NaOH, 5; pH 7.4. Krafte-brühe (KB) solution contained (mM): L-glutamic acid 50, KOH 80, KCl 40, MgSO₄ 3, KH₂PO₄ 25, HEPES 10, EGTA 1, taurine 20 and glucose 10; pH 7.2.

Figure S2 shows the morphology of rat sinus node (A) and myocytes isolated from it (B). Typical spindle-like or elongated spindle-like sinus node myocytes are shown in a-d. An atrial myocyte is also shown on the right for comparison (e). Regardless of different types of cells, we only selected cells with spontaneous action potentials (C) to study the effects of PP2 on sinus node I_f.

Whole-cell patch clamp studies of I_{H_{CN4}} and I_f

For recording I_{H_{CN4}}, day 1 up to day 3 post-transfected HEK293 cells with green fluorescence were selected for patch clamp studies. The HEK293 cells were placed in a Lucite bath with the temperature being maintained at 25 ± 1°C. For recording I_f, the isolated adult rat sinus node myocytes were placed in a Lucite bath with the temperature being maintained at 35 ± 1°C. The temperature was controlled by a temperature controller (Cell MicroControls, VA).

I_{HCN4} currents were recorded using the whole cell patch clamp technique with a MultiClamp-700B amplifier. The pipettes had a resistance of 2-4 M Ω when filled with internal solution (mM): NaCl 6, K-aspartate 130, MgCl₂ 2, CaCl₂ 5, EGTA 11, and HEPES 10; pH adjusted to 7.2 by KOH. The external solution contained (mM) NaCl 120, MgCl₂ 1, HEPES 5, KCl 30, CaCl₂ 1.8, and pH was adjusted to 7.4 by NaOH. The transient potassium current (I_{to}) blocker, 4-aminopyridine (4-AP) (2 mM), was added to the external solution to inhibit the endogenous transient potassium current, which can overlap with I_{HCN4} tail currents recorded at +40 mV.

Sinus node action potential and I_{f} currents were recorded using either the whole-cell or the amphotericin-B perforated patch clamp to avoid I_{f} rundown.²¹ Amphotericin-B was added to the internal solution to a final concentration of 240 $\mu\text{g/ml}$ on the day of use. The temperature of the chamber was maintained at $35 \pm 1^\circ\text{C}$. Action potentials were recorded in normal Tyrode containing (mM): NaCl 140, KCl 5.4, CaCl₂ 1.8, MgCl₂ 1, Glucose 5.5, Hepes 5, pH 7.4 adjusted by NaOH. The pipettes had a resistance of 6-8 M Ω when filled with internal solution composed of (mM) NaCl 10, K-aspartate 130, MgCl₂ 2, CaCl₂ 2, EGTA 5, Na₂-ATP 2, GTP (sodium salt) 0.1, creatine phosphate 5, pH 7.2 by KOH.

For I_{f} recordings, potassium channel blockers (2 mM 4-AP and 2 mM Ba²⁺) and calcium channel blockers (0.1 mM Cd²⁺, 2 mM Mn²⁺) were added to normal Tyrode to inhibit I_{to} and calcium currents, respectively, to avoid contaminating I_{f} deactivation at -30 mV. ATP, GTP and creatine phosphate were freshly prepared on the day of use.

Imaging of sinus node tissue and myocytes isolated from sinus node and atria

Images of isolated sinus node and atrial myocytes were obtained using AxioVision with ZEISS microscope (Axio Imager) equipped with an AxioCam HRc camera. All imaging experiments were performed at room temperature.

Electrocardiogram (ECG) recording in whole rat

Adult SD rats were anesthetized with inhaled 2% isoflurane mixed with oxygen at a flow rate of 2 liter/min. Isoproterenol (ISO), PP2 or PP3 (Sigma-Aldrich, St. Louis, MO) was administrated through a catheter (polyurethane, 3 French size) placed in the jugular vein in accordance with Animal Care and Use Committee of the National Institute for Occupational Safety and Health. The heart rate was recorded by a two-lead vector connected to a computerized cardiovascular continuous monitoring system (a PowerLab/4SP analog-to-digital converter, AD Instruments, Colorado Springs, CO) at a sampling rate of 1/1000 per sec. The results were averaged from five rats.

Immunofluorescence analysis of single sinus node myocytes

Isolated sinus node myocytes were placed on glass slides and left to settle for at least 1 hour before fixation in 4% paraformaldehyde (PFA) for 20 min at room temperature. For drug treatments, a final concentration of 10 μ M PP2 or 10 μ M PP3 in phosphate buffered saline (PBS) were incubated with semi-adhered myocytes for 10 min. PFA was removed and myocytes were washed three times with PBS. The cells were then permeablized with 0.5% Triton-X 100 in PBS for 2 min and rinsed with PBS three times. Then the cells were blocked for 30 min at room temperature using PBS containing 2% BSA. Primary antibody against the C-terminus of HCN4 (Abcam) was prepared by 1:100 dilution in the blocking solution, in which the cells were incubated over two nights at 4°C. After three times of washes with PBS, the secondary antibody (1:1000, Alexa Fluor 488, Invitrogen) was added and incubated for 1 hr in the dark at room

temperature. Following the final rinses with PBS and subsequently distilled water, the glass slides were coverslipped using 10 μ l Prolong Gold with DAPI (Invitrogen). This mounting media requires curing overnight in the dark at room temperature. The slides were then ready for examination using confocal microscopy (Carl Zeiss, LSM 510).

Data analysis

The whole-cell patch clamp data were acquired by CLAMPEX and analyzed by CLAMPFIT (pClamp 9, Axon). I_f/I_{HCN4} current amplitudes were determined by measuring the time-dependent inward currents that are sensitive to either 1 mM Cs^+ and/or 2 μ M ZD7288 (Figure 3A, inset). The activation curve (Figure 3B) was constructed on the relative membrane conductance by measuring the tail currents divided by the driving force ($E_{\text{test}} - E_{\text{rev}}$), which was then normalized to the maximal conductance. For current activation that did not reach steady-state (e.g., current traces in response to -60 mV, -70 mV, and -80 mV impulses in Figure 3), we fit the current traces to the steady-state using one exponential function¹⁷ and obtained the fitted current amplitude, which was used to construct the activation curve and calculate the activation kinetics (e.g., Figures 3B and 3C).

Dose-response curves for isoproterenol (ISO) were best fit by the Hill equation with a fixed Hill slope of 1, $f(x) = \text{bottom} + [\text{top} - \text{bottom}] / [1 + 10^{\log EC50 - x}]$, where x represents different doses of ISO injected into the rat. EC50 is the dose of ISO that increases heart rate by 50% of range between *bottom* (basal heart rate) and *top* (heart rate corresponding to the maximal dose of ISO). Data were acquired and analyzed by LabChart 7 (ADInstruments).

Data are shown as mean $\pm$ SEM. Student's t-test and one-way ANOVA (for more than two groups) were used for statistical analysis. $P < 0.05$ was considered as statistically significant.

Drugs

Small molecule, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), also known as AG 1897²², has been widely used in identifying the substrates of Src kinases family members^{22, 23}. Its inactive structural analog, 4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine (PP3), is used as a negative control to confirm the action of PP2²³.

Results

PP2 reversed and prevented ISO-induced enhancement of HCN4

To test the hypothesis that the stimulation of HCN4 by ISO is inhibited by reducing Src kinase activity, we studied the effects of ISO on HCN4 in the absence and presence of PP2, a selective inhibitor for Src kinases.²⁰ We first validated the β -adrenergic signaling pathway in HEK293 cells^{24, 25}, shown in Figure S1 and Table 1 that ISO was able to stimulate HCN4.

Figure 1 shows that the current expression of HCN4 (A) was inhibited by PP2 (10 μ M) (B) including a negative shift of activation threshold (V_{th} , the voltage at which the first time-dependent inward current larger than 10 pA was detected) (current trace in gray). In the presence of PP2, however, ISO (0.1 μ M) was unable to induce a positive shift of HCN4 activation (C) in contrast to Figure S1. After washout of PP2, ISO was able to shift the HCN4 activation to more depolarizing voltages as indicated by the positive shift in activation threshold (D). The activation threshold of HCN4 in this cell was -70 mV (A), negatively shifted to -80 mV by PP2 (B), remained at -80 mV in the presence of both ISO and PP2 (i.e. PP2 + ISO) (C), and positively shifted to -60 mV with ISO after washout of PP2 (D). On average, the activation threshold of HCN4 was positively shifted by 7.6 mV by ISO, and negatively shifted by 11.6 mV by PP2 and 16.6 mV by PP2 + ISO, respectively (Table 1).

Along with the shift in activation threshold was the activation midpoint ($V_{1/2}$, the voltage at which the tail current amplitude reaches half maximum). The HCN4 tail current corresponding to activation at -90 mV (gray line) was near half activation (1E). PP2 negatively shifted the activation midpoint close to -100 mV (1F). While PP2 remains in the bath, ISO was unable to positively shift the activation midpoint (1G). After PP2 washout, ISO succeeded to shift the activation midpoint towards -80 mV (1H). The average activation midpoint was positively shifted by 4.9 mV by ISO, and negatively shifted by 6.3 mV by PP2 and 13.7 mV by PP2 + ISO, respectively (Table 1). PP2 also slowed HCN4 activation kinetics regardless of ISO. At -120 mV, ISO accelerated the activation kinetics by 44% on average, and PP2 decelerated the activation kinetics by 67% by itself, and by 29% in the presence of ISO (Table 1).

PP2 inhibited HCN4-573X

To address the question whether the inhibitory effects of PP2 on ISO's modulation of HCN4 is cAMP-independent, we examined the effects of PP2 on HCN4-573X, an HCN4 mutant that lacks CNBD and therefore cAMP's regulation.¹³ Figure 2 shows that in a HEK293 cell expressing 573X, PP2 shifted the activation threshold (in gray) from -65 mV (A) to -75 mV (B), which was reversible after washout of PP2 (C). The averaged activation threshold of 573X was negatively shifted by 18.3 mV by PP2 ($V_{th_control}$: -55.0 ± 2.9 mV, V_{th_PP2} : -73.3 ± 3.1 mV, $n = 8$). The activation midpoint was also negatively shifted by PP2 ($V_{1/2_control}$: -81.7 ± 4.3 mV, $V_{1/2_PP2}$: -92.7 ± 4.8 mV, $n = 6$). In addition, at -125 mV, PP2 slowed the activation kinetics of 573X by 75% ($\tau_{act_control}$: 1286 ± 216 ms, τ_{act_PP2} : 2260 ± 268 ms, $n = 6$). These results confirmed our observation that PP2 also prevented the enhancement of HCN4 by cAMP in

HEK293 cells (Figure S1-D) and indicated that the inhibition of HCN4 by PP2 is cAMP independent.

PP2 decreased rat sinus node I_f

HCN4 is the prevalent isoform in the sinus node and a major contributor to sinus node pacemaker activity across species.^{4, 26} Our findings in HEK293 cells that reduced Src kinase activity blunted the stimulation of HCN4 by ISO suggested a possible similar effect of PP2 on the native cardiac pacemaker current, the sinus node I_f .

A representative I_f recorded in an isolated rat sinus node myocyte (Figure S2-B) is shown in Figure 3A. The inset shows that the current is relatively insensitive to 2 mM Ba^{2+} , but can be blocked by 1 mM Cs^+ or 2 μ M ZD7288, which are typical characteristics of I_f .² The activation threshold and midpoint are -60 mV and -83 mV, respectively for this cell (A). The average midpoint voltage is -77.8 mV (B, Table 2). Since Ba^{2+} can partially inhibit I_f ,²⁷ the activation threshold is more positive in the absence of Ba^{2+} (Figure S3).

Figure 3C shows the activation kinetics of sinus node I_f , which are the fastest compared to I_f in other cardiac regions such as Purkinje fibers and ventricles.²⁸ These data show that I_f gating properties in the rat sinus node are comparable to those in sinus node of other species such as mouse²⁹, dog^{30, 31}, human³², and especially for the rabbit in which I_f has been mostly studied.⁴

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Figure 3D shows that PP2 (10 μ M) significantly decreased I_f measured at -100 mV (gray line). The inhibition of PP2 on I_f was confirmed by the recovery of current after washout of PP2. PP3, an inactive analog of PP2 did not inhibit I_f (gray line), suggesting the inhibition of I_f by PP2 is through suppression of Src kinase activity. In a different cell, Figure 3E shows representative

data for altered stimulation of I_f by ISO in the absence and presence of PP2, respectively. At -95mV, ISO increased the current amplitude by shifting the activation curve of I_f towards depolarization (3F). In the presence of PP2, however, ISO was unable to increase I_f , rather, the current was decreased (3E, gray line) due to a negative shift of activation (3F, gray line). On average, PP2 induced a hyperpolarizing shift of 10 mV in the activation midpoint of I_f , whereas ISO induced a depolarizing shift of 10.5 mV on its own and a hyperpolarizing shift of 16 mV in the presence of PP2 (Table 2). The activation threshold was shifted 18.5 mV more negative by PP2, 5.6 mV more positive by ISO, and 23.5 mV more negative by PP2 + ISO, respectively (Table 2).

The activation kinetics was also slowed by PP2 (Table 2). On average, PP2 slowed I_f activation kinetics at -100 mV by 21%, while ISO accelerated it by 21% (Table 2). However, instead of acceleration, ISO decelerated I_f activation kinetics at -100 mV by 58% when PP2 was co-administrated (Table 2).

PP2 prevented ISO-induced stimulation of pacemaker activity in sinus node myocytes

I_f contributes to the early diastolic depolarization of the sinus node action potential.² The observation that the stimulation of I_f by ISO was prevented by PP2 led to the anticipation that the pacemaker activity in sinus node myocytes would also be suppressed by PP2.

Figure 4 shows that indeed, the spontaneous action potentials in an isolated sinus node myocyte (A), were inhibited by perfusion of 2 μ M PP2 for 1-2 minutes (B) but not PP3 in the same experimental conditions (F). After washout of PP2, the action potentials were recovered (C). Further, ISO (10 nM) failed to speed the spontaneous action potentials in PP2-treated myocytes (D, E), in contrast to the stimulation by ISO in non-treated myocytes (G, H). Similar

results were observed in an additional five myocytes. Figure 4I compares the cycle lengths of action potentials in ISO- and PP3-treated myocytes from the baseline. ISO decreased the cycle length by 15.6% (baseline: 661.7 ± 17.6 ms, ISO: 558.8 ± 9.6 ms, $p = 0.005$, $n = 6$). PP3 had a tendency to slightly increase the cycle length, but the change was not statistically significant (baseline: 661.7 ± 17.6 ms, PP3: 680.8 ± 27.6 ms, $p = 0.56$, $n = 6$).

Our previous studies showed that in addition to shifting the activation curve to hyperpolarizing potentials, PP2 also reduced the membrane channel conductance of HCN4 by at least 50%.²⁰ Figure 1 also indicated a significant depression of current amplitude of HCN4 by PP2. Given HCN4 is the main HCN isoform encoding I_f in the sinus node⁴ and our results that PP2 significantly suppressed sinus node I_f (Figure 3D), we further examined whether a reduced I_f channel conductance might inhibit the early diastolic depolarization of sinus node action potential.

To test this idea, we used a mathematical model developed by Demir, Clark and Giles for pacemaker activity of sinus node myocytes which incorporates the cholinergic and adrenergic modulations.³³ When the conductance of I_f was decreased by 50% (Figure S4-D), the sinus node action potential was decelerated due to a slowed diastolic depolarization (Figure S4-A), mimicking the consequence of inhibition of I_f by PP2. As a comparison, application of ISO at 10 nM accelerated the action potential (Figure S4-B). Remarkably, ISO stimulation of the pacemaker activity was prevented when the I_f channel conductance was reduced by 50% (Figure S4-C), mimicking the consequence of co-administration of PP2 and ISO. The results from this mathematical model were consistent with our experimental findings (Figure 4).

PP2 prevented ISO-induced stimulation of sinus node rate and heart rate

The spontaneous pacemaker activity of a dissected sinus node was recorded in real time. Figure S5 shows that based on the average of five sinus nodes, PP2 at 5-10 μM decreased its beating rate by 25.2 % (Control: 111 ± 2 bpm, PP2: 83 ± 4 bpm, $p < 0.01$). ISO at 0.1 μM increased its beating rate by 17.1% (Control: 111 ± 2 bpm, ISO: 130 ± 3 bpm, $p < 0.01$). The stimulation of ISO on the sinus node beating rate was diminished in the presence of PP2 (Control: 111 ± 2 bpm, ISO + PP2: 107 ± 4 bpm, $p = 0.4$). The beating rate was not affected by PP3 (Control: 111 ± 2 bpm, PP3: 109 ± 2 bpm, $p = 0.45$).

Figure 5A shows that in a whole rat *in vivo* PP2 increased, whereas ISO significantly decreased the R-R intervals in the electrocardiograms (ECG). PP3 increased the R-R interval to a much less degree compared to PP2. On average, PP2 chronically reduced heart rate by 21.8% at a dose of 0.2 mg/kg (Control: 326.4 ± 7.7 bpm, PP2: 254.6 ± 10.3 bpm, $n = 5$) (5B). PP3 at the same dose decreased heart rate by 8.3% (Control: 326.4 ± 7.7 bpm, PP3: 299.0 ± 4.8 bpm, $n = 5$). As a positive control, ISO at 0.4 $\mu\text{g/kg}$ acutely increased heart rate by 15.3% (Control: 326.4 ± 7.7 bpm, ISO: 376.0 ± 8.2 bpm, $n = 5$). All effects of PP2, PP3 and ISO were statistically significant ($p < 0.05$) (5B).

Figure 5C shows that ISO increases the heart rate in a dose-dependent manner ranging from 25 ng/kg (ISO25) to 400 ng/kg (ISO400) (top panel). After saline washout of ISO, PP2 at a relatively low dose (60 $\mu\text{g/kg}$) was applied, followed by the same doses of ISO (middle panel). PP2 eliminated the small increase of heart rate by ISO at doses of 25 ng/kg and 50 ng/kg, significantly attenuated the increase of heart rate by ISO at doses of 100 ng/kg, 200 ng/kg, and 400 ng/kg. In comparison, PP3 has little effect on the ISO-induced heart rate increase (bottom panel).

Figure 5D shows that the ISO dose-response curve on heart rate increase was shifted downward by PP2, indicating a reduced heart rate at every dose of ISO when PP2 was co-applied. In addition, the slope of the dose-response curve, which indicates the sensitivity to ISO on heart rate increase, was reduced in the presence of PP2. PP3, which bears a structural resemblance to PP2 but does not suppress Src kinase activity, however, had little suppression on the positive chronotropic effect of ISO. Based on an average of five rats, the effective concentration (EC50) for heart rate increase by ISO was 115 ± 5 ng/kg in the absence of PP2, 153 ± 4 ng/kg in the presence of PP2 ($p < 0.01$), and 125 ± 3 ng/kg in the presence of PP3 ($p = 0.053$).

PP2 resulted in intracellular retention of HCN4 channels in sinus node myocytes

We have previously identified that decreased tyrosine phosphorylation of HCN4 channels was correlated with reduced surface expression of the channel proteins.¹⁸ Therefore, the blunted response of sinus node I_f to ISO in the presence of PP2 was probably due to a decreased number of HCN4 channels on the plasma membrane. To test this hypothesis, we conducted immunofluorescent staining of HCN4 proteins in sinus node myocytes (Figure 6). HCN4 proteins were labeled with FITC-conjugated antibody. The nucleus was counterstained with DAPI. We found that the fluorescent intensity of HCN4 on the cell surface was much higher than the cytosol for control (6A). Pre-incubation of PP2 for 10 min, however, significantly altered the distribution of HCN4 proteins, indicated by an increased retention of HCN4 intracellularly (6B). As a negative control, PP3 did not cause the intensity of HCN4 to increase inside the cell (6C), rather, the two peaks in fluorescent intensity analysis suggested a similar localization of HCN4 proteins at the cell surface to the control (6A, 6C). The same results were obtained in an additional three to four cells. Our results suggested that PP2 caused a larger pool of HCN4

proteins to be retained intracellularly and hence exhibit a decreased number of functional channels on the plasma membrane, which would desensitize the response of sinus node I_f to ISO.

Discussion

The essential fight-or-flight response or sympathetic stimulation accelerates the heart rate by activating the β -adrenergic receptor signaling pathway. Direct binding of cAMP to the CNBD of HCN channels has been proposed to be a central physiological mechanism for β -adrenergic stimulation of heart rate.² This notion has been challenged by recent findings of two cAMP-insensitive HCN4 mutants, 573X and 695X, in patients with marked sinus bradycardia, since lack of cAMP action did not affect their positive chronotropic responses to β -adrenergic stimulation.^{7, 15}

We previously found that the tyrosine phosphorylation state of HCN channels is associated with not only the gating properties, but also the cell surface expression of the channels.^{18, 19} Src-mediated phosphorylation enhanced, while receptor-like protein tyrosine phosphatase (RPTP)-mediated dephosphorylation reduced, HCN currents and surface expression.^{18, 19} Further, three members of the Src-family kinases, Src, Fyn, and Yes, are ubiquitously expressed in a variety of tissues including the heart.³⁴ Using the constitutively active forms of these three Src kinases, we rescued a membrane trafficking defective human HCN4 mutant, D553N, for functioning at the cell surface.¹⁸ These findings formed the hypothesis that β -adrenergic stimulation of cardiac pacemaker activity via HCN4/ I_f channels requires Src kinases activity.

Inhibition of Src tyrosine kinases prevents enhancement of HCN4 activity by ISO

We used PP2 as a specific probe to explore the interplay between a reduced Src kinase activity and ISO on modulation of HCN4. We found that in the presence of PP2, the ability of ISO to stimulate HCN4 was diminished.

To further explore whether PP2 abolished the facilitation of HCN4 by ISO through the cAMP pathway, we tested the effects of PP2 on an HCN4 mutant (573X) lacking CNBD and the distal C-terminal region, which was identified in a patient suffering from severe sinus bradycardia (41 bpm) and intermittent atrial fibrillation.¹³ We found that PP2 retained its inhibition on the gating of the mutant channel by shifting the activation to a more negative potential and decelerating the activation kinetics. This result suggests that PP2 desensitizes ISO's modulation of HCN4 independently of the cAMP pathway. In addition, it indicates that Src phosphorylation sites are in the regions excluding the CNBD and the distal C-terminal region, in agreement with our and other's previous findings that the main sites (Y531 and Y554) that mediate Src enhancement of HCN4 are in the C-linker.^{20, 35}

Reduced Src kinases activity decreases and prevents ISO-induced enhancement of rat sinus node I_f and spontaneous action potentials

We chose the rat as an animal model to study the effects of PP2 on sinus node pacemaker activity for technical feasibility and animal welfare concerns as well as the potential for future transgenic studies. Indeed, PP2 decreased I_f current amplitude, slowed I_f activation kinetics, and shifted I_f activation curve to more negative potentials. Further, ISO-induced stimulation of I_f was completely lost in the presence of PP2.

It is worth emphasizing that in isolated rat sinus node myocytes, the activation threshold of I_f is well within the early diastolic depolarization of the action potential. The maximal

diastolic depolarization is about -60 mV (Figure 4), and I_f began to activate at -45 mV (Figure S3). More importantly, only a small I_f (about 1 pA) is needed to initiate the early diastolic depolarization due to the high membrane resistance.¹

It is not surprising that PP2 can suppress the spontaneous action potential in the sinus node, since I_f controls the early diastolic depolarization in the sinus node. What was surprising was that in the presence of PP2, ISO was unable to accelerate the sinus node action potential (Figure 4E). Both T-type and L-type calcium currents contribute to the late diastolic depolarization and firing of sinus node action potential, respectively.³⁶ ISO increases $I_{Ca,L}$, but not $I_{Ca,T}$ in the sinus node³⁶. Although ISO is unable to increase I_f in the presence of PP2, it should not affect the increase of $I_{Ca,L}$ that could speed the firing of sinus node action potentials. Probably, PP2 may also inhibit the calcium channels that are important contributors to the late diastolic depolarization and firing of action potentials in the sinus node.

Reduced Src kinases activity attenuates increase of sinus node beating rate and heart rate by ISO

In the dissected sinus node, PP2 slowed the spontaneous beating rate and blunted the rate increase by ISO. These results are in agreement with the inhibition of sinus node I_f and spontaneous action potentials by PP2. Additionally, PP2 decreased the heart rate and suppressed the positive chronotropic effect of ISO. While we cannot exclude the possibility that the negative chronotropic effects of PP2 were due to its actions on neuronal modulation of the sinus node, our collective evidence on HCN4, sinus node I_f , and sinus node beating rate supports an indispensable role for depressed HCN4 activity.

The previous study showed that the protein phosphatase inhibition increased I_f independently of cAMP and potentiated the β -adrenergic stimulation of I_f .³⁷ Consistently, we demonstrated that inhibition of Src kinases attenuated the β -adrenergic stimulation of I_f . Moreover, this is the first time that the important role of tyrosine phosphorylation on I_f modulation was investigated from the single cell to the whole animal. The question remains, however, as to what constitutes the underlying mechanism by which the altered state of tyrosine phosphorylation influences the sensitivity of I_f to β -adrenergic modulation.

Reduced Src kinase activity results in intracellular retention of HCN4 proteins in sinus node myocytes

Immunofluorescent staining was performed in order to seek the underlying mechanism for the desensitized response to ISO from single cell to whole animal. We have previously demonstrated a positive correlation between tyrosine phosphorylation state and surface expression of HCN channels in mammalian cells (HEK293)^{18, 19}, however, no studies have been done in sinus node myocytes. In the present work, we found that exposure to PP2, and not PP3, leads to a redistribution of HCN4 proteins in sinus node myocytes, suggesting that the protein retention was associated with a reduced tyrosine phosphorylation state. Since the effects of PP2 occurred in 10 min, which excluded the possibility of *de novo* synthesis of channel proteins, the increased intracellular retention of HCN4 was associated with a reduction of the functional channels on the plasma membrane. Thus, the reduced surface expression of HCN4 channels in sinus node myocytes treated acutely with PP2 well explained the blunted response of sinus node I_f to ISO and offered mechanistic insights into the ISO desensitization for the sinus node beating rate and heart rate.

A new mechanism for heart rate modulation by β -adrenergic receptors

We have shown that β -adrenergic receptor activation requires Src kinase activity, thus Src would probably be one of the downstream targets of the β -adrenergic receptor signaling pathway. Indeed, Src has been identified as a direct target of $G\alpha_s$ and $G\alpha_i$ subunits.^{38, 39} The $G\alpha_s$ and $G\alpha_i$ subunits bind to the catalytic domain and change the conformation of c-Src *in vivo*, leading to its activation. More recently, Src has been found to be bound to and directly activated by β_2 adrenergic receptors (β_2 AR).^{40, 41} In the sinus node, β_2 AR is the dominant isoform that mediates the modulation of heart rate.⁴² These findings suggest that upon activation of β_2 AR, Src may act on its own targets independent of the G-proteins/adenylyl cyclase/cAMP pathway. The diminished stimulation by ISO in the presence of PP2 on I_{HCN4} , sinus node I_f , action potentials, sinus node beating rate, and heart rate, respectively, supports the notion that ISO can increase heart rate directly via Src-mediated stimulation of HCN4 activity, which is independent of cAMP.

The collective evidence presented in this work suggests a novel mechanism for β -adrenergic stimulation of HCN4, illustrated in Figure 7. Circle 1 illustrates the established increase of HCN4 current by β -adrenergic stimulation via cAMP mechanisms (gray line). Circle 2 indicates the contribution of Src, activated either by $G\alpha_s$ and/or by β_2 AR. The inhibited Src kinases activity reduces (dashed gray line) and prevents the cAMP-mediated (gray line) increase of HCN4 current. This notion is supported by the decreased current in the presence of PP2 (dashed gray line) despite a lack of cAMP action (gray line) on 573X, illustrated in circle 3.

Conclusion

In the present work, we demonstrated an important role of Src kinase activity in β -adrenergic receptor signaling towards heart rate acceleration. Different from the cAMP-mediated mechanism, Src-mediated tyrosine phosphorylation can be potentially used to promote the cell surface expression of HCN4 channels, a novel strategy for pharmacological treatments for bradycardia in general, and for sick sinus syndrome caused by membrane defective or cAMP insensitive HCN4 mutants in particular.

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Disclosures

The authors report no conflicts.

Disclaimer

The opinions expressed in this manuscript are those of the authors and do not necessarily represent the views of the National Institute for Occupational Safety and Health, Center for Disease Control and Prevention of the USA.

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Figure Legends

Figure 1: PP2 reverses and prevents enhancement of HCN4 by ISO. (A) HCN4 currents in a HEK293 cell elicited by 10-sec hyperpolarizing pulses as indicated. (B) HCN4 currents in the presence of PP2. (C) HCN4 currents in the presence of PP2 and ISO. (D) HCN4 currents in the presence of ISO alone after washout of PP2. (E) Enlarged HCN4 tails from A. (F) Enlarged HCN4 tails from B. (G) Enlarged HCN4 tails from C. (H) Enlarged HCN4 tails from D. Current traces in gray correspond to the activation threshold (A-D) or voltages close to the activation midpoint (E-H).

Figure 2: PP2 inhibits HCN4-573X. In response to 10-sec hyperpolarizing pulses from -55 mV to -125 mV in 10 mV increments, currents were detected in a HEK293 cell expressing HCN4-573X (A), after 5-10 min perfusion of PP2 (10 μ M) (B), and after PP2 washout (C). Currents in gray indicate the activation threshold.

Figure 3: PP2 decreases and prevents stimulation of I_f by ISO (A) I_f currents elicited by 1-sec pulses from -50 mV to -120 mV in 10 mV increments. The holding potential was -30 mV. The inset shows that I_f current was not blocked by 2 mM Ba^{2+} , but by 1 mM Cs^+ or 2 μ M ZD7288. (B) I_f activation curve constructed from tail currents averaged from seven cells. (C) I_f activation kinetics averaged from seven cells. (D) At -100 mV, I_f (dark line, control) was unaffected by PP3 (gray line) but significantly decreased by PP2 (gray line). Washout of PP2 increased I_f (dark line). (E) At -95 mV, I_f (control) was increased by ISO, but decreased in the presence of ISO and PP2 (gray line). (F) activation curves for I_f (Control), in response to ISO and ISO + PP2 (gray line), respectively.

Figure 4: PP2 slows and prevents the stimulation of pacemaker activity by ISO in isolated sinus node myocytes. (A) spontaneous action potentials (APs) recorded from a sinus node myocyte. (B) PP2 at 2 μ M reduced the number of APs that were spontaneously fired. (C) Washout of PP2 partially recovered the number of APs. Similar experiments were repeated in an additional four cells. Dash lines indicate 0 mV. (D) APs recorded from a different sinus node myocyte pretreated with 2 μ M PP2 for 2-4 min. (E) In the presence of PP2, ISO (10 nM) failed to enhance the pacemaker activity. Similar experiments were repeated in an additional three cells. (F) APs recorded from a different cell incubated with PP3 (2 μ M) for 5 minutes. Similar results were obtained in an additional four cells. (G) APs recorded at the baseline, (H) APs recorded after perfusion of 10 nM ISO. (I) cycle lengths of baseline, in response to ISO and PP3, respectively.

Figure 5: PP2 reduces heart rate. (A) lead II ECG traces in the absence (basal) and presence of ISO, PP2, and PP3, respectively. (B) mean heart rate in the absence (Control) and presence of PP2, PP3, and ISO, respectively. * indicates statistical significance in comparison to control, n = 5 for each group. (C) Top panel: heart rate responses to various doses of ISO (ISO25: 25 ng/kg; ISO50: 50 ng/kg; ISO100: 100 ng/kg; ISO200: 200 ng/kg; ISO400: 400 ng/kg). Middle panel: heart rate responses to various doses of ISO in the presence of PP2 (60 μ g/kg). Bottom panel: heart rate responses to various doses of ISO in the presence of PP3 (60 μ g/kg). Similar results were reproduced in an additional four rats. (D) ISO dose-response curves in the absence (dark line) and presence of PP2 and PP3, respectively. The curves were fit by Hill equation with a fixed Hill slope.

Figure 6: PP2 leads to intracellular retention of HCN4 proteins in sinus node myocytes. (A)

Immunofluorescent staining of HCN4 channels for control (A), after 10-min incubation with 10 μ M PP2 (B) or 10 μ M PP3 (C). HCN4 proteins were labeled with FITC-conjugated antibody. The nucleus was counterstained with DAPI. The fluorescent intensity was plotted below each image. All of the results were repeated in an additional three to four cells.

Figure 7: Schematic diagram of a postulated new mechanism for Src-mediated enhancement of HCN4 activity upon activation of β -adrenergic receptors. The facilitation of

HCN4 channel by β -agonists is through the established mechanism of direct binding of cAMP to the CNBD of the channel (solid arrow). **Circle 1:** cAMP increases HCN4 current (gray line).

Circle 2: As Src kinases activity is inhibited by PP2 (dashed gray line), cAMP-mediated stimulation of HCN4 is blunted (gray line). **Circle 3:** PP2 remains effective in inhibiting 573X activity (dashed gray line), despite its cAMP irresponsiveness (gray line). β_2 AR: β_2 -adrenergic receptors, AC: adenylyl cyclase, CNBD: cyclic nucleotide-binding domain.

Table 1: Effects of PP2 and ISO on HCN4 gating properties

	V_{th} (mV)	$V_{1/2}$ (mV)	τ -act (-120 mV) (ms)
Control	-66.7 ± 3.6 (n = 15)	-87.2 ± 3.5 (n = 10)	2439 ± 110 (n = 10)
PP2*	-78.3 ± 2.8 (n = 9)	-93.5 ± 2.9 (n = 7)	4075 ± 305 (n = 7)
ISO	-59.1 ± 4.4 (n = 7)	-82.3 ± 2.3 (n = 7)	1356 ± 127 (n = 7)
PP2* + ISO	-83.3 ± 3.3 (n = 6)	-100.9 ± 2.3 (n = 6)	3135 ± 173 (n = 6)

*: PP3 did not have the effects of PP2 shown in the table.

Table 2: Effects of PP2 and ISO on rat sinus node I_f

	V_{th} (mV)	$V_{1/2}$ (mV)	τ -act (-100mV) (ms)
Control	-59.4 ± 4.8 (n = 11)	-77.8 ± 2.3 (n = 9)	416.3 ± 20.9 (n = 11)
PP2*	-77.9 ± 3.9 (n = 8)	-87.8 ± 2.3 (n = 6)	502.3 ± 48.1 (n = 6)
ISO	-53.8 ± 4.0 (n = 9)	-67.3 ± 2.3 (n = 7)	328.8 ± 22.0 (n = 7)
PP2*+ISO	-82.9 ± 3.6 (n = 6)	-93.8 ± 1.5 (n = 6)	658.0 ± 36.1 (n = 6)

*: PP3 did not have the effects of PP2 shown in the table.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Supplemental Data

Src Kinases Required for β -Adrenergic Stimulation of Heart Rate

Jianying Huang, BS¹, Yen-Chang Lin, PhD^{1,*}, Hong Kan, MD, PhD², Vincent Castranova, PhD², Han-Gang Yu, PhD¹

¹Center for Cardiovascular and Respiratory Sciences, Department of Physiology and Pharmacology, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV 26506; ²Pathology and Physiology Research Branch, National Institute for Occupational Safety and Health/CDC, Morgantown, WV 26506

Supplemental Figure Legends

Figure S1: Enhancement of HCN4 by ISO in HEK293 cells. (A) HCN4 currents were generated by 10-sec hyperpolarizing pulses. The holding potential was -10 mV. Tails were recorded at +40 mV. (B) HCN4 currents in response to ISO under the same voltage protocol as A. (C) HCN4 activation curves in the absence (dark line) and presence of ISO (gray line). (D) effects of 8-Br-cAMP (cAMP, 0.1 mM) and PP2 (10 μ M) on HCN4 current expression in HEK293 cells. cAMP increased, while PP2 decreased, HCN4 current amplitude. In the presence of PP2, however, cAMP failed to increase HCN4 current amplitude. The holding potential was -10 mV. The currents were recorded at -105 mV at $25 \pm 1^\circ\text{C}$. Similar results were obtained in an additional 8 cells.

Figure S2: Dissected rat sinus node and isolated sinus node myocytes. (A) location of sinus node (SAN) in the right atrium (rAt). CT: crista terminalis. (B) typical spindle-like (a) and elongated SAN cells (b-d) as well as an atrial myocyte (e). Bar scale: 10 μ m. (C) action potential recorded from an isolated SAN cell.

Figure S3: I_f recorded in a sinus node myocyte in the bath solution without Ba^{2+} . I_f began to activate at -45 mV, and increased its amplitude at more hyperpolarizing pulses. The holding potential was -30 mV. The pulse duration was 1 sec.

Figure S4: Modeling the PP2 inhibition of diastolic depolarization in the sinus node. (A) A 50% decrease in I_f channel conductance that mimics PP2 inhibition on HCN4 slowed down the action potential rate by decelerating the diastolic depolarization. (B) ISO at 10 nM increased the action potential rate by accelerating the diastolic depolarization. (C) ISO was unable to accelerate the action potential with a half reduction of I_f conductance by PP2. (D) I_f current amplitude was decreased in half when the channel conductance was reduced by 50%.

Figure S5: PP2 decreases sinus node beating rate. Mean sinus node beating rate in the absence of modulation agents (Control), in the presence of PP2, ISO, ISO + PP2, or PP3, respectively. * indicates statistical significance in comparison to control. n = 5 for each group.

Figure S1

Figure S2

Figure S3

Figure S4

Figure S5

Chapter 2 Modulation of Cardiac Pacemaker Channels by Tyrosine Phosphatases

Section 1

Novel Mechanism for Suppression of Hyperpolarization-activated Cyclic Nucleotide-gated Pacemaker Channels by Receptor-like Tyrosine Phosphatase- α

I performed the biochemistry experiments and contributed to the experimental design and writing in this manuscript.

Section 2

Differential Modulation of HCN2 by RPTP μ and RPTP ϵ

I performed all the experiments and wrote this manuscript.

Section 3

Reduced Tyrosine Phosphorylation Inhibits HCN4-573X Channel Independent of cAMP Signaling

I performed all the experiments and wrote this manuscript.

Novel Mechanism for Suppression of Hyperpolarization-activated Cyclic Nucleotide-gated Pacemaker Channels by Receptor-like Tyrosine Phosphatase- α *

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Jianying Huang, Aijie Huang, Qi Zhang, Yen-Chang Lin, and Han-Gang Yu¹

From the Center for Interdisciplinary Research in Cardiovascular Sciences, Department of Physiology and Pharmacology, West Virginia University School of Medicine, Morgantown, West Virginia 26506

We have previously reported an important role of increased tyrosine phosphorylation activity by Src in the modulation of hyperpolarization-activated cyclic nucleotide-gated (HCN) channels. Here we provide evidence showing a novel mechanism of decreased tyrosine phosphorylation on HCN channel properties. We found that the receptor-like protein-tyrosine phosphatase- α (RPTP α) significantly inhibited or eliminated HCN2 channel expression in HEK293 cells. Biochemical evidence showed that the surface expression of HCN2 was remarkably reduced by RPTP α , which was in parallel to the decreased tyrosine phosphorylation of the channel protein. Confocal imaging confirmed that the membrane surface distribution of the HCN2 channel was inhibited by RPTP α . Moreover, we detected the presence of RPTP α proteins in cardiac ventricles with expression levels changed during development. Inhibition of tyrosine phosphatase activity by phenylarsine oxide or sodium orthovanadate shifted ventricular hyperpolarization-activated current (I_h , generated by HCN channels) activation from nonphysiological voltages into physiological voltages associated with accelerated activation kinetics. In conclusion, we showed a critical role RPTP α plays in HCN channel function via tyrosine dephosphorylation. These findings are also important to neurons where HCN and RPTP α are richly expressed.

Activated by membrane hyperpolarization, the HCN2² channels are important to rhythmic activity in neurons and myocytes (1, 2). Accumulating evidence has also suggested an important role of tyrosine phosphorylation in modulating HCN channels (3, 4). We have recently shown that increased tyrosine phosphorylation state of HCN4 by activated Src tyrosine kinase can enhance the gating of HCN4 channels (5). Given that the efficacy of tyrosine phosphorylation is determined by the dynamic balance of tyrosine kinases and tyrosine

phosphatases, reduced tyrosine phosphatase activity is speculated to increase HCN channel activity. Receptor-like protein-tyrosine phosphatases (RPTPs) are transmembrane phosphatases critical in cell growth and cell adhesion (6). One member of RPTPs, RPTP α , has been proposed to be a positive regulator of Src tyrosine kinases (7, 8). As a protein-tyrosine phosphatase, RPTP α should also be able to dephosphorylate phosphotyrosines of channel proteins. This work was designed to investigate whether RPTP α can inhibit the HCN channel activity by decreasing the tyrosine phosphorylation state of HCN channel proteins.

EXPERIMENTAL PROCEDURES

DNA Plasmids—Mouse HCN2 cDNA in an oocyte expression vector, pGH, was initially obtained from Drs. Bina Santoro/Steve Siegelbaum (Columbia University). We subcloned it into the EcoRI/XbaI sites of pcDNA3.1 mammalian expression vector (Invitrogen) for functional expression in mammalian cells. Mouse HCN2 with hemagglutinin (HA) tag containing sequence ²⁸⁴GISAYGITYPYDVPDYAI²⁸⁵ inserted in the extracellular loop between S3 and S4 was obtained from Dr. Michael Sanguinetti (University of Utah) (9). We subcloned it into the HindIII/XbaI sites of pcDNA3.1 vector for surface expression study. RPRP α inserted in pRK5 vector was kindly provided by Dr. Jan Sap (University of Copenhagen, Denmark). Src529 was obtained from Upstate Biotechnology, Inc./Millipore.

Cell Culture and Plasmid Transfections—HEK293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 g/liter streptomycin. Cells with 50–70% confluence in 6-well plate were used for HCN2, Src529, and RPTP α plasmid transfections using Lipofectamine2000 (Invitrogen).

Cell Lysis, Immunoprecipitation, and Western Blot Analysis—Total protein extracts were prepared from transfected cells after 24–48 h of incubation with CytoBuster protein extraction reagent kit (Novagen) that contains 0.1% SDS. For membrane fraction preparations (e.g. HCN2 Western blotting analysis in Fig. 3), we used a membrane protein extraction kit (Pierce). The protein concentration of the lysate was determined using the Bradford or BCA assay. Equal amounts of total protein (0.5–1 mg) were incubated with a specific antibody for 1 h at 4 °C, and protein A/G PLUS-agarose (Santa Cruz Biotechnology) was then added and incubated overnight with gentle rocking. The beads were washed extensively with cold PBS buffer and resuspended in 2× sample buffer. The immune complexes were sep-

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¹ To whom correspondence should be addressed. Tel.: 304-293-2324; Fax: 304-293-5513; E-mail: hyu@hsc.wvu.edu.

² The abbreviations used are: HCN, hyperpolarization-activated cyclic nucleotide-gated; RPTP α , receptor-like protein-tyrosine phosphatase- α ; EGFP, enhanced green fluorescent protein; PBS, phosphate-buffered saline; HA, hemagglutinin; PNGase, peptide:N-glycosidase; PAO, phenylarsine oxide.

arated by SDS-PAGE and analyzed by Western blot using the specific antibody of interest. Total protein of 5–20 μg per sample was subjected to SDS-PAGE using 4–12% gradient gels (Invitrogen), then transferred to nitrocellulose membranes (Amersham Biosciences), and incubated with proper antibodies (e.g. HCN2 from Alomone; α -actin and β -actin from Abcam). After washing and incubating with horseradish peroxidase-conjugated secondary antibody, immunoreactive proteins were visualized with the SuperSignal[®] West Pico kit (Pierce). All protein experiments were repeated at least three times, if not mentioned in the text. Quantification of immunoblots was performed using ImageQuant software (version 5.1, GE Healthcare).

De-glycosylation was carried out by following the manufacturer's instructions. Briefly, the membrane protein fraction sample was denatured in 0.5% SDS and 40 mM dithiothreitol at 100 °C for 10 min and transferred to a solution composed of 50 mM sodium phosphate, pH 7.5, and 1% Nonidet P-40. Peptide: *N*-glycosidase F (PNGase, New England Biolabs, 500–1000 units for up to 20 μg of glycoprotein) was then added, and the reaction was incubated for 3 h at 37 °C. Samples were then cleaned and concentrated using the PAGEprepTM protein clean up kit (Pierce).

Surface expression detection was carried out by using a HCN2-HA plasmid. An anti-HA tag antibody (Millipore) that recognizes YPYDVPDYA was used in Western blotting analysis of HCN2 surface expression in HEK293 cells.

Confocal Fluorescent Imaging of HEK293 Cells—HEK293 cells transfected with HCN2-EGFP or HCN2-HA were incubated on coverslips, fixed in 4% paraformaldehyde/PBS for 15 min, and then washed with PBS (10 mM phosphate buffer, 150 mM NaCl, pH 7.4) for 5 min three times, followed by blocking in 1% bovine serum albumin/PBS, pH 7.4, for 60 min. For immunofluorescence confocal imaging of HCN2-HA, cells were then incubated with an anti-HA tag (fluorescein isothiocyanate) antibody (2 $\mu\text{g}/\text{ml}$, Abcam) in 1% bovine serum albumin/PBS, pH 7.4, for 60 min. After washing six times in PBS, coverslips were mounted on slide glasses using Fluoromount G (Southern Biotechnology). Cells were imaged by LSM510 confocal microscopy using a Plan-Neofluar 40 $\times$ /0.75 objective or a Plan-Apochromat 63 $\times$ /1.4 Oil DIC M27 objective. Excitation wavelength and filters for EGFP and fluorescein isothiocyanate are 488 nm and argon laser/emission BP505–530 nm.

Cardiac Tissue and Myocyte Preparation—Adult rats (300–350 g) were euthanized by intraperitoneal injection of pentobarbital in accordance with the Institutional Animal Care and Use Committee protocols. The heart was excised, and ventricles were cut out and prepared for protein chemistry experiments. The ventricular myocytes were prepared using a collagenase dissociation protocol as described previously (5, 10). Briefly, the aorta was cannulated, and the heart was retrogradely perfused with oxygenated Ca^{2+} -free Tyrode solution at 37 °C for 5 min, followed by oxygenated Ca^{2+} -free Tyrode solution containing 0.6 mg/ml collagenase II (Worthington) for 20–30 min. The ventricles were minced into small pieces in KB solution, dispersed, and filtrated through a 200- μm mesh. The isolated myocytes were stored in KB solution at room temperature for 1 h before patch clamp experiments.

Whole-cell Patch Clamp Recordings of HCN2 Currents and I_f —For recording I_{HCN2} , day 1 (24–30 h) up to day 4 (90–98 h) post-transfection, HEK293 cells with green fluorescence were selected for patch clamp studies. The HEK293 cells were placed in a Lucite bath in which the temperature was maintained at 25 ± 1 °C by a temperature controller (Cell MicroControls, Virginia Beach, VA). For recording I_f , the freshly isolated adult rat ventricular myocytes were placed in a Lucite bath in which the temperature was maintained at 35 ± 1 °C by a temperature controller (Cell MicroControls).

I_{HCN2} currents were recorded using the whole-cell patch clamp technique with an Axopatch-200B amplifier. The pipettes had a resistance of 2–4 megohms when filled with internal solution (mM) as follows: NaCl 6, potassium aspartate 130, MgCl_2 2, CaCl_2 5, EGTA 11, and HEPES 10; pH adjusted to 7.2 by KOH. The external solution contained (mM) the following: NaCl 120, MgCl_2 1, HEPES 5, KCl 30, CaCl_2 1.8, and pH was adjusted to 7.4 by NaOH. The I_o blocker, 4-aminopyridine (2 mM), was added to the external solution to inhibit the endogenous transient potassium current, which can overlap with and obscure I_{HCN2} tail currents recorded at 20 mV. Data were acquired by CLAMPEX and analyzed by CLAMPFIT (pClamp 8, Axon Instruments).

I_f currents were recorded using the whole-cell patch clamp technique with an Axopatch-700B amplifier. The external solution contained (mM) the following: NaCl 140, KCl 5.4, CaCl_2 1.8, MgCl_2 1, and glucose 10, NiCl_2 0.1, BaCl_2 5, 4-aminopyridine 2, tetrodotoxin 0.03, pH 7.4. The pipettes had a resistance of 2–4 megohms when filled with internal solution composed of (mM) the following: NaCl 6, potassium aspartate 130, MgCl_2 2, CaCl_2 1, $\text{Na}_2\text{-ATP}$ 2, Na-GTP 0.1, HEPES 10, pH 7.2. Sodium (I_{Na}) and potassium (I_o) current blockers, tetrodotoxin and 4-aminopyridine, were added to the external solution to inhibit the sodium and transient potassium currents. During I_f recording, BaCl_2 was added to the external solution to block I_{K1} background potassium current, which can mask I_f current.

Data are shown as mean $\pm$ S.E. The threshold activation of I_f is defined as the first hyperpolarizing voltage at which the time-dependent inward current can be observed. Student's *t* test was used for statistical analysis with $p < 0.05$ being considered as statistically significant. Time constants were obtained by using Boltzmann best fit with one exponential function on current traces that reach steady state (e.g. in the presence of phenylarsine oxide) or on current trace that can be fit to steady state (e.g. in the absence of phenylarsine oxide, at -150 mV).

RESULTS

RPTP α Inhibition of HCN2 Currents in HEK293 Cells—Our recent discovery showed that increased tyrosine phosphorylation of HCN4 channel by activated Src tyrosine kinase can enhance the channel gating properties in HEK293 cells (5, 11). We wondered whether increased tyrosine dephosphorylation by RPTP α may inhibit the gating properties of HCN channels expressed in HEK293 cells.

Functional expressions of HCN2 after 2 days (44–50 h) of transfection are shown in Fig. 1. HCN2 currents were elicited by hyperpolarizing pulses detailed in the figure legends. Typical biophysical properties of the expressed channels such as the

FIGURE 1. RPTP α inhibition of HCN2 current expression. HCN2 currents were recorded 2 days after cell transfection. *A*, HCN2 currents in a HEK293 cell expressing HCN2 alone. Hyperpolarizing pulses (4 s) from -65 to -125 mV were applied. *B*, hyperpolarization-activated currents in a HEK293 cell co-transfected with HCN2 and RPTP α . Hyperpolarizing pulses (5 s) from -75 to -125 mV were applied. *C*, hyperpolarization-activated currents in a HEK293 cell co-transfected with HCN2 and RPTP α . Hyperpolarizing pulses (3 s) from -75 to -125 mV were applied. *D*, HCN2 currents in a HEK293 cell co-transfected with HCN2 and the empty pRK5 vector. Hyperpolarizing pulses (5 s) from -65 to -125 mV were applied. The tail currents were recorded at $+20$ mV. The holding potential was -10 mV. Arrows in *A* and *D* indicate the threshold activation of HCN2 currents.

threshold activation, activation kinetics, and current densities are comparable with those reported previously (5, 11, 12). Co-transfection with RPTP α , however, resulted in a dramatic inhibition (Fig. 1*B*) or a surprising loss of HCN2 currents (Fig. 1*C*). Similar results were reproduced in 10 additional cells for each HCN2 channel co-expressed with RPTP α . As part of control experiments, the empty pRK5 vector did not affect HCN2 expression from 1 to 4 days post-transfection. Fig. 1*D* shows the HCN2 recordings in a HEK293 cell co-transfected with HCN2 and pRK5 vector after 2 days. Similar results were obtained in an additional five cells.

Interestingly, we found the degree by which RPTP α inhibited HCN2 current expression changed with time. Fig. 2 shows HCN2 current expression recorded in HEK293 cells co-transfected with RPTP α after 1 day (24–30 h) (*A*), 2 days (44–50 h) (*B*), and 4 days (*C*) post-transfection. Compared with HCN2 alone (Fig. 1*A*), co-transfection with RPTP α reduced HCN2 current density (pA/pF) measured at -125 mV by 64% after day 1 (HCN2 = 38.9 ± 1.7 , $n = 11$; RPTP α = 1.37 ± 0.5 , $n = 10$), by 96% after day 2 (RPTP α = 1.5 ± 0.5 , $n = 10$), and by 80% after day 4 (RPTP α = 7.7 ± 0.7 , $n = 10$) (Fig. 2*D*). The time-dependent inhibition is not only significant as compared with the control, but also significant among groups (e.g. day 1 and day 2; day 2 and day 4). To understand the cellular mechanisms that mediate the dramatic inhibition of RPTP α on HCN2 channel function, we carried out protein biochemical studies on HCN2 channels.

RPTP α Dephosphorylation of HCN2 Channels—We have previously shown that increased tyrosine phosphorylation of HCN4 channels by a constitutively active form of Src, Src529, increased channel conductance near diastolic potentials asso-

FIGURE 2. RPTP α induced time-dependent inhibition of HCN2 current expression. Hyperpolarizing pulses of 5 s ranging from -75 to -125 mV (*A* and *B*) or from 85 to -125 mV (*C*) in 10-mV increments were applied to elicit HCN2 currents in HEK293 cells co-transfected HCN2 with RPTP α after day 1 (*A*), day 2 (*B*), and day 4 (*C*). *D*, averaged HCN2 current density at -125 mV for the corresponding time periods.

ciated with accelerated activation kinetics (5, 11). Inhibition of HCN2 current expression by RPTP α led us to examine whether the tyrosine phosphorylation state of HCN2 channels might be decreased by RPTP α .

In HEK293 cells transfected with HCN2, 29 h after transfection the cell lysates were first immunoprecipitated by a phosphotyrosine-specific antibody, 4G10, followed by detection of HCN2 signals using an anti-HCN2 antibody. Fig. 3*A* shows that HCN2 channels were tyrosine-phosphorylated (2nd left lane). Immunoblot of HCN2 in Fig. 3*A*, 1st left lane, served as a positive control. The nontransfected cells and cells transfected with RPTP α alone were used as negative controls. The band close to 112 kDa is the glycosylated (mature) form expressing on the membrane surface, and the band near 100 kDa is the unglycosylated (immature) form that is not expressed on the membrane surface (13). We also confirmed the glycosylated signal by using PNGase, which can remove the *N*-glycosylation of HCN2 (Fig. 3*B*). In comparison with the untreated sample, PNGase treatment significantly decreased the upper band (*N*-glycosylated signal) and increased the lower band (un-*N*-glycosylated signal). The ratio of glycosylated over unglycosylated signals is decreased in the PNGase-treated sample. Nature of the third band is unknown. Tyrosine phosphorylation levels of HCN2 are much lower in cells co-expressing HCN2 and RPTP α than in cells co-transfected with HCN2 and Src529 (right panel of Fig. 3*A*). It is noted that the glycosylated HCN2 signal was significantly enhanced by Src529, whereas the unglycosylated signal was barely detectable.

To seek direct evidence for RPTP α -induced dephosphorylation on HCN2 channel, we examined immunoblots using 4G10 in cells expressing HCN2 alone, with Src529, with RPTP α , and with RPTP α in the presence of $1 \mu\text{M}$ phenylarsine oxide (PAO) (a non-specific tyrosine phosphatase inhibitor) for 40 min, respectively. Shown in Fig. 3*C*, location of predicated HCN2 signal is marked by arrows. Background tyrosine phosphorylation of HCN2 is low but

FIGURE 3. RPTP α induced tyrosine dephosphorylation and surface expression of HCN2 channels. A, HEK293 cells were nontransfected (NT), transfected with either HCN2 or RPTP α alone. Membrane fractions of the samples were immunoprecipitated (IP) using 4G10 followed by HCN2 signal detection using an anti-HCN2 antibody. The 1st left lane shows HCN2 immunoblots (IB). HCN2 was also co-transfected with Src529 and RPTP α , respectively, for comparison of tyrosine phosphorylation levels. B, Western blotting analysis for deglycosylation of HCN2 channel by PNGase. HCN2 signals were detected using the HCN2 antibody for PNGase-treated and untreated samples. C, 4G10 immunoblots in HEK293 cells transfected with HCN2, HCN2+Src529, HCN2+RPTP α , and HCN2+RPTP α -treated with PAO for 40 min, respectively. A separate blot in nontransfected sample served as a negative control. Actin signals were used as loading controls. D, time-dependent inhibition of HCN2 channel surface expression by RPTP α as follows: immunoblots of HCN2 alone, co-transfected with RPTP α for 29, 48, and 96 h, respectively. NT indicates a nontransfected sample serving as a negative control. The HCN4-transfected sample was also used for another control. E, immunoblots of HCN2 (left panel) or HCN2-HA (right panel) in HEK293 cells transfected with HCN2 (or HCN2-HA) alone, co-transfected with RPTP α or Src529. An anti-HCN2 antibody (left panel) or an anti-HA tag antibody (upper panel) was used. Nontransfected (NT) and nontagged HCN2 transfected cells were used as negative controls for HA antibody.

noticeable, which was significantly increased by Src529 and decreased by RPTP α , respectively. However, inhibited tyrosine phosphorylation of HCN2 by RPTP α can be reversed by treating cells with PAO. Signals were normalized to β -actins. In comparison with HCN2, Src529 increased HCN2 phosphorylation by about 4-fold (3.7 ± 0.8 , $n = 3$), whereas RPTP α decreased it by more than 2-fold (2.2 ± 0.6 , $n = 4$). Compared with HCN2 + RPTP α (Fig. 3C, 2nd left lane), cells treated with PAO (1st left lane) increased HCN2 phosphorylation by about 18-fold (17.7 ± 1.5 , $n = 3$). Nontransfected cells were used as a negative control (1st right lane of Fig. 2C). Combined with 4G10 immunoprecipitation results in Fig. 3A, these results strongly suggest that RPTP α can dephosphorylate HCN2 channel proteins expressed in HEK293 cells.

RPTP α Inhibition of HCN2 Cell Surface Expression—To seek additional evidence for contribution of RPTP α -induced tyrosine dephosphorylation to the inhibition of HCN2 surface expression, we first utilized the time-dependent phosphatase activity of RPTP α . It was reported using SF9 cells that RPTP α activity changed with time as follows: significantly increased after 24 h, reached maximum after 48 h, and declined at 96 h (14). Although no reports have shown similar time-dependent changes in RPTP α activity in HEK293 cells, we observed a time-

dependent inhibition of HCN2 currents by RPTP α (Fig. 2) that implied a possible time-dependent RPTP α activity in HEK293. We examined HCN2 expression co-transfected with RPTP α after day 1 (29 h), day 2 (48 h), and day 4 (96 h), respectively (Fig. 3D). Compared with HCN2 alone, which served as a positive control, HCN2 co-transfected with RPTP α for 29 h began to increase the unglycosylated signal. HCN2 signals were barely detected after 48 h of transfection but readily detected after 96 h of transfection. Cells with nontransfection served as a negative control. Cells transfected with HCN4 were used as additional control for HCN2 antibody specificity. Similar results were obtained from an additional three experiments. These results provided a biochemical explanation for RPTP α -induced time-dependent inhibition of HCN2 currents (Fig. 2), which favored a mechanism that tyrosine dephosphorylation may be involved in retaining HCN2 in the cytoplasm by RPTP α in a time-dependent manner.

We then compared the effects of RPTP α on surface expression of either the HCN2 or the HA-tagged HCN2 channel proteins. As shown in Fig. 3E, after 24–30 h of transfection HCN2 (left panel) or HCN2-HA (right panel) channels expressed alone in HEK293 cells gave rise to three bands (one near 112 kDa, one near 100 kDa, and the nature of the third band unknown). Co-transfection of Src529 significantly increased the glycosylated form of HCN2 or HCN2-HA, whereas co-transfection with RPTP α decreased the glycosylated form of HCN2 or HCN2-HA. The specificity of the HA antibody was tested in nontransfected and nontagged HCN2-transfected cells (rightmost panel of Fig. 3E). β -Actins were used as loading controls. Taken together, these data strongly supported the notion that the surface expression of the HCN2 channel is associated with HCN2 channel tyrosine phosphorylation levels.

RPTP α Inhibition of Surface Fluorescence of HCN2 Channels—We next employed confocal laser scanning microscopy to study the surface expression of a HCN2-EGFP fusion protein and an HCN2-HA construct in HEK293 cells. As shown in Fig. 4A, HCN2 channels were normally expressed mostly on the membrane surface and some in the cytoplasm. Co-transfection with RPTP α retained most of the HCN2 in the cytoplasm (Fig. 4B). The empty EGFP vector, which served as a negative control, was expressed homogeneously in the cell (Fig. 4C). In addition, we used an anti-HA tag antibody to study the immunofluorescent imaging of the HCN2-HA channels. After 2 days

FIGURE 4. Confocal images of HCN2 surface expression in HEK293 cells. Fluorescent images of the HCN2-EGFP fusion protein are shown in the absence (A) and presence (B) of RPTP α . Immunofluorescent images of the HCN2-HA channels are shown in the absence (D) and presence (E) of RPTP α . EGFP vector is shown in C. Bright field images provided shapes of cells where the transverse scanning imaging was performed (A–E).

with RPTP α dramatically reduced the fluorescent signals (Fig. 4E). Similar results were obtained in the additional 8–10 cells for each experiment. Comparing the results of HCN2-EGFP with those of HCN2-HA clearly showed retention of HCN2 by RPTP α in the cytoplasm, which is consistent with the biochemical evidence shown in Fig. 3.

RPTP α Expression and Interaction with HCN2 Channels in Cardiac Myocytes—RPTP α has been previously detected at mRNA levels

in whole heart preparation (14), but its protein expression in heart has not been reported. To extend our findings to a relatively physiological context, we examined the protein expression of RPTP α in adult rat ventricles. As shown in Fig. 5A, RPTP α protein signals were indeed detected in adult rat ventricles by Western blot analysis using an anti-RPTP α antibody. A mouse version of RPTP α expressed in HEK293 cells was used as a positive size control which showed both a weak band at 100 kDa (p100) and a strong band at 130 kDa (p130). Both p100 and p130 bands are glycosylated forms of RPTP α (15). To increase the sensitivity, we immunoprecipitated the samples followed by Western blot using the same antibody. Using this method, the p130 band was also detected (*middle lane* in Fig. 5C) but at much lower levels compared with p100 in adult rat ventricles. The 66-kDa (p66) band in Fig. 5C is the truncated form of RPTP α that contains the phosphatase catalytic domains (16). It loses phosphatase activity after truncation. The nontransfected sample was used as a negative control. β -Actin and cardiac α -actin were used as loading controls for HEK293 cells and cardiac tissues, respectively. Six additional repeats of the same experiment confirmed the protein expression of RPTP α in cardiac ventricles.

The fact that HCN2 and HCN4 are the only two HCN isoforms present in cardiac ventricles with HCN2 being the prevalent one (17) and that RPTP α can dephosphorylate HCN2 channels in HEK293 cells led us to hypothesize that RPTP α may associate with HCN2 in cardiac ventricles. Using co-immunoprecipitation assay, Fig. 5B showed that HCN2 signals can be detected with an HCN2 antibody in the cell lysates immunoprecipitated using an RPTP α antibody (*left panel* of Fig. 5B). Immunoblot of HCN2 in HEK293 cells was used as a positive control. It needs to be pointed out that in the figure the HCN2 signals in HEK293 cells represents the glycosylated (112 kDa) and un-glycosylated (100 kDa) forms, whereas the strong band near 100 kDa in rat ventricle after RPTP α immunoprecipitation is the unglycosylated HCN2 (the glycosylated HCN2 signal was too weak to be detected). This is consistent with the inhibition of HCN2 surface expression by RPTP α . Sample immunoprecipitated with IgG served as a negative control. On the other hand, the band of RPTP α can be detected with an RPTP α antibody in the sample immunoprecipitated using an HCN2 antibody (*right panel* of Fig. 5B). All experiments were repeated at least three times. These data collectively suggested that RPTP α

FIGURE 5. RPTP α expression in rat ventricles. A, RPTP α protein detection in adult rat ventricles using an anti-RPTP α antibody (Santa Cruz Biotechnology). HEK293 cells transfected with mouse RPTP α (RPTP α) or nontransfected (NT) were used as positive and negative controls, respectively. IB, immunoblot. Actins (α -actin in cardiac tissues and β -actin in HEK293 cells) were used as loading controls. B, co-immunoprecipitation of RPTP α with HCN2 in adult rat ventricle. *Left panel*, the adult rat ventricular (Ven) samples were immunoprecipitated (IP) using an RPTP α antibody followed by HCN2 signal detection using an HCN2 antibody. Immunoblot of HCN2 expressed in HEK293 cells served as a positive control. *Right panel*, the adult rat ventricular samples immunoprecipitated using an HCN2 antibody followed by RPTP α signal detection using an RPTP α antibody. Sample immunoprecipitated with IgG followed by RPTP α signal detection was used as a negative control. C, samples from adult (43 days) and neonatal (1 day) rat ventricles (ARV and NRV, respectively) were immunoprecipitated and detected for RPTP α using the same RPTP α antibody. HEK293 cells with nontransfection (NT) were used as negative controls. HEK293 cells transfected with RPTP α alone was adult and neonatal rat ventricles.

(44–50 h) of transfection in HEK293 cells, HCN2 channels can be readily detected on the plasma membrane (Fig. 4D). Under the identical optical settings, co-transfection of HCN2-HA

FIGURE 6. **Reduced tyrosine phosphatase activity on I_f in adult rat ventricular myocytes.** I_f was recorded in adult rat ventricular myocytes in the absence (A) and presence of 1 μ M phenylarsine oxide (B) and 1 mM sodium orthovanadate (C). The pulse protocol used for A and B was shown in D. C, I_f was elicited by 6-s hyperpolarizing pulses ranging from -80 to -135 mV in 10-mV increments. The holding potential was -50 mV. Arrows indicate the threshold activation of I_f .

is indeed present and can interact with HCN2 channels in adult rat ventricles.

Altered RPTP α Expression during Development—Our early studies showed that the voltage-dependent activation of I_f is shifted to more negative potentials during development (10, 18). Given the suppression of HCN channel expression by RPTP α , we hypothesized that RPTP α protein expression may be lower in newborn than in adult rat ventricles. Fig. 5C demonstrated that although the major form of RPTP α (p100) can be readily detected in adult ventricles, it is barely detectable in neonatal (day 1) rat ventricles. p100 RPTP α protein levels were 7.3 ± 0.8 ($n = 3$) times higher in adult than in neonatal ventricles. Levels of p130 RPTP α were also higher, whereas the expression levels of p66 RPTP α were lower (but insignificantly) in adult ventricles (0.8 ± 0.3 , $n = 3$). The total RPTP α protein expression was higher in adult than in neonatal ventricles (3.9 ± 0.6 , $n = 3$). HEK293 cells without transfection were used as a negative control, and cells were transfected with RPTP α as a positive control. Using immunoprecipitation of the sample and Western blotting with the same RPTP α antibody, we showed that the endogenous RPTP α exists in HEK293 cells (Fig. 5C, rightmost lane), which cannot be visualized by immunoblot without prior immunoprecipitation (2nd left lane). Similar results were obtained in three additional experiments.

Reduced Tyrosine Phosphatase Activity Increased I_f Activity in Adult Rat Ventricular Myocytes—To seek physiological implication on the inhibition of HCN channel function by increased tyrosine phosphorylation activity, we studied the pacemaker current in adult rat ventricular myocytes. The pacemaker current, I_f , is a time- and voltage-dependent inward current, which is an important contributor to cardiac pacemaker activity in response to β -adrenergic and muscarinic acetylcho-

line receptor stimulation (19). In normal conditions, I_f activates at nonphysiological voltages (10, 20, 21). In response to enhanced tyrosine kinase activity, I_f activation in rat ventricle was shifted to depolarizing voltages associated with accelerated activation kinetics (4). Given that RPTP α expression is high in adult ventricular myocytes and phenylarsine oxide can inhibit RPTP α -induced tyrosine dephosphorylation (Fig. 3C), applying phenylarsine oxide was hypothesized to increase I_f activity in adult ventricular myocytes.

Fig. 6A shows a typical I_f recording from an adult rat ventricular myocyte. Holding the membrane at -50 mV, hyperpolarizing pulses for 4.5 s were applied from -70 to -150 mV in 10-mV increments and stepped further to -150 mV for recording tail currents (pulse protocol shown in Fig. 6D). In another myocyte incubated with 1 μ M PAO

for 10–15 min, the same pulse protocol was applied. The threshold activation of I_f in the absence of phenylarsine oxide was around -120 mV in this myocyte (arrow in Fig. 6A), similar to our previous results (4, 10, 18). The threshold activation of I_f in the presence of PAO, however, was surprisingly shifted to a much more depolarized potential around -80 mV (arrow in Fig. 6B). Averaging over six myocytes, the threshold activation of I_f in the presence of PAO was -78 ± 7 mV and -113 ± 4 mV in the absence of PAO ($p < 0.01$). This is a nearly 40-mV positive shift of I_f threshold activation in response to the acute effect of reduced tyrosine phosphatase activity.

Because of extremely negative activation of I_f in adult ventricular myocytes, the same pulse protocol was able to make I_f to the steady states in the presence, but not in the absence, of PAO, indicating that PAO can induce much faster I_f activation. At -150 mV (at which Boltzmann best fit with one exponential function could be readily performed on the current trace in the absence of PAO), activation kinetics were 2123 ± 607 ms in the absence of PAO ($n = 6$) and 741 ± 192 ms in the presence of PAO ($n = 7$) ($p < 0.05$). These results are in agreement with the increased I_f channel activities induced by increasing tyrosine kinase activity in our previous studies (4).

To verify that the enhanced I_f activity (depolarized threshold activation associated with faster activation kinetics) by PAO was indeed because of the reduced tyrosine phosphatase activity, we used another inhibitor, sodium orthovanadate, which is structurally different from PAO. Shown in Fig. 6C, in myocytes incubated with 1 mM sodium orthovanadate for 30–40 min, I_f was elicited by 6 s of hyperpolarizing pulses from -80 to -130 mV in 10-mV increments. The threshold activation of I_f was -90 mV in this myocyte (arrow in Fig. 6C). The averaged I_f threshold activation was -93 ± 6 mV ($n = 3$), which is signif-

icantly shifted to more positive potentials as compared with control ($p < 0.05$). These data are consistent with those obtained from PAO-treated myocytes.

DISCUSSION

In this study, we provided evidence showing dramatic inhibitory effects of tyrosine dephosphorylation by RPTP α on HCN2 channels. Two mechanisms are likely involved as follows: tyrosine dephosphorylation and membrane trafficking of HCN channels. Both are mediated by RPTP α .

In HEK293 cells co-expressing HCN2 channels with RPTP α for 2 days yielded surprising inhibition or even elimination of the current expression. There are two plausible explanations as follows: the channels were retained in the cytoplasm leading to little or no expression of functional channels on plasma membrane, or gating properties of the channels on the plasma membrane were inhibited. We performed Western blot analysis on the membrane fraction of cells and revealed two known HCN2 signals. One around 100 kDa is the unmodified form with the predicted molecular weight, and the other near 112 kDa is the glycosylated form of HCN2. The constitutively active Src, which increases the tyrosine phosphorylation level of the channels, increased the surface expression of the channels. On the other hand, RPTP α , which decreases the tyrosine phosphorylation level of the channels, retained most channels in the cytoplasm. This is also evidenced by the association of HCN2 with RPTP α in which stronger unglycosylated HCN2 bands were detected in samples immunoprecipitated by RPTP α antibody (Fig. 5B).

It is surprising that the HCN2 channel expression was largely blocked by RPTP α after 2 days of transfection and reappeared after 4 days of transfection (Fig. 2B and Fig. 3D). It offers a likely explanation to no measurable or much smaller time-dependent inward currents in HEK293 cells co-transfected by HCN2 with RPTP α for the same time periods (Fig. 1, B and C). It is worth noting that whole-cell patch clamp technique applied to individual cells is more sensitive than Western blotting, which obtains the average result from batch of cells. Therefore, after transfection for 2 days we were able to detect small current expression in some cells, but not in protein expression.

Protein-tyrosine phosphatases, like protein-tyrosine kinases, play a critical role in the regulation of physiological events (7, 8). RPTP α has a short extracellular domain (about 123–150 amino acids long) that contains eight potential *N*-glycosylation sites (15, 22). Following a transmembrane region, there are two tandem domains having phosphatase catalytic activity. RPTP α is expressed in two isoforms differing by nine residues (22) that are highly glycosylated, p100 and p130 on SDS-PAGE (15). The p100 form contains only *N*-linked glycosylation, whereas p130 contains both *N*-linked and *O*-linked glycosylation (15). Both forms have the similar enzymatic activities (15).

We found that RPTP α expression is cell-specific. In HEK293 cells, a strong signal at 130 kDa is detected, which is the predominant signal that has been frequently observed in previous studies (15). In adult rat ventricles, however, we found that the prevalent form of RPTP α is p100 (a precursor of p130 (15)). We also detected a p66 form of RPTP α in cardiac ventricles after increasing the blotting sensitivity by immunoprecipitating the

samples followed by signal detection using the same antibody (Fig. 5C). Previous study reported that p66 is the N-terminal region truncated form of RPTP α that is catalyzed by calpain (a calcium-dependent proteolytic enzyme) and loses the phosphatase enzymatic activity (16). It may not be coincident that calpain-1 expression levels in heart are decreased during development (23), which leaves more active isoforms (100 and 130 kDa) of RPTP α in adult ventricle. The physiological relevance of p66 RPTP α is currently unknown.

To investigate the physiological implications of RPTP α suppression on HCN2 channels with regard to modulation of cardiac pacemaker activity, we examined the levels of RPTP α protein expression in neonatal (which exhibit spontaneous pacemaker activity) and adult rat ventricles (which do not have spontaneous pacemaker activity under physiological conditions). We found higher RPTP α levels in adult than in neonatal ventricles, which is in parallel to the physiological activation of neonatal ventricular I_f and nonphysiological activation of adult ventricular I_f .

Ample evidence has already recognized a close association between the voltage-dependent activation of I_f and the pacemaker activities in different heart regions (3, 19–21, 24). The threshold activation of I_f is tissue-specific across the heart regions as follows: from around -50 mV in the sinoatrial node to -110 – -120 mV in ventricles (3, 18, 20, 21). Voltage-dependent activation of I_f also changes with development and pathologic conditions. In neonatal rat ventricles, I_f activates around -70 mV and shifts to more negative potentials beyond the physiological voltage range in adult ventricle (-113 mV) (18). Hypertrophied or failing heart increases I_f current density and shifts its activation to physiological voltages (25, 26). The enhanced I_f under pathological conditions has been implicated in atrial and ventricular arrhythmias (25, 26). It is currently unknown how developmental and pathological conditions cause the shift of I_f voltage-dependent activation.

PAO is a phosphotyrosine phosphatase inhibitor that cross-links vicinal thiol ($-\text{SH}/-\text{OH}$ and $-\text{SH}/-\text{CO}_2\text{H}$) groups, thereby inactivating phosphatases possessing *X*-Cys-*X*-Cys-*X* motifs, and it does not affect tyrosine kinases (27). On the other hand, the vanadate (VO_4^{3-}) ion binds irreversibly to the active sites of tyrosine phosphatases, likely acting as a phosphate analogue (28). Therefore, Na_3VO_4 is a competitive inhibitor. In adult rat ventricular myocytes perfused with either phenylarsine oxide for 10–15 min or sodium vanadate for 30–40 min, we recorded an I_f within physiological voltages associated with faster activation kinetics, which is comparable with neonatal ventricular I_f . Because PAO and Na_3VO_4 inhibit tyrosine phosphatase activity by different mechanisms, the significantly enhanced I_f activity favored a reduced tyrosine phosphatase activity. Both inhibitors, however, are not selective to RPTP α , and we cannot exclude the potential contribution of tyrosine phosphatases other than RPTP α to the altered I_f . Because the effects occurred less than an hour, increased membrane trafficking of HCN channels may not be the main mechanism. Rather, the enhanced HCN2 channel activity because of increased tyrosine phosphorylation is the favorable underlying mechanism.

Our data have shown a critical role that RPTP α plays in the tyrosine dephosphorylation of HCN2 channels. The short-term

effect (10–40 min), which likely involves the tyrosine dephosphorylation, is the reduced I_f activity in cardiac myocytes. Since the discovery of I_f in adult mammalian ventricular myocytes 15 years ago (20), it is the first time that we are able to shift the ventricular I_f activation from nonphysiological voltages to physiological potentials by acutely inhibiting the endogenous tyrosine phosphatase activity.

The long-term effects (days) include the inhibition of HCN channel surface expression and possibly channel biosynthesis. Recently, HCN4 mutants have been linked to the bradycardia and long-QT arrhythmias (29–32). The common cellular mechanism was retaining membrane trafficking caused by the truncated HCN4 protein lacking cyclic nucleotide binding domain (31), D553N in the C-linker between S6 and cyclic nucleotide binding domain (32), and G480R in the channel pore region (29). The evidence we presented in this work provided a novel mechanism that may be used to enhance the surface expression of mutant HCN channels for effecting normal cardiac pacemaker activity.

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Differential Modulation of HCN2 by RPTP μ and RPTP ϵ

Jianying Huang and Han-Gang Yu

Center for Cardiovascular and Respiratory Sciences, Department of Physiology and Pharmacology, Robert
C. Byrd Health Sciences Center, West Virginia University, Morgantown, WV 26506

Abstract

We have previously shown that hyperpolarization-activated cyclic nucleotide-gated (HCN) 2 channels can be modulated via a new mechanism - tyrosine dephosphorylation by a receptor protein tyrosine phosphatase (RPTP) α . We report here that we detected the protein of two other RPTPs, RPTP μ and RPTP ϵ , in rat and human heart. Since the isoform-specific effects of RPTPs on ion channels have been recently discovered in non-cardiac tissues (such as Schwann cells) and the roles of RPTP μ and RPTP ϵ are unknown in cardiac functions, we investigated the modulation of these two tyrosine phosphatases on HCN2 channel properties.

Unlike RPTP α , RPTP μ did not suppress current expression of HCN2 when expressed in human embryonic kidney (HEK) 293 cells. Instead, RPTP μ significantly decreased the slope factor for HCN2 channels and made the activation curve steeper without affecting the activation midpoint. However, the activation kinetics of HCN2 was not significantly altered. RPTP μ -C1095S, a mutant of RPTP μ without its enzymatic activity, surprisingly inhibited the current expression of HCN2, leading to a smaller current density measured at -120 mV that was associated with a more negative activation threshold.

RPTP ϵ , on the other hand, significantly suppressed the current expression of HCN2, like RPTP α . In contrast, the “substrate-trapping” mutant of RPTP ϵ , D302A, failed to inhibit HCN2 current expression. We also detected in cardiac ventricles the protein content of its cytosolic form, cyt-PTP ϵ , which appeared to be the predominant form of PTP ϵ in heart. This non-receptor type of PTP ϵ retained the enzymatic activity of

RPTP ϵ and inhibited current expression of HCN2 to the same degree as RPTP ϵ . In addition, both RPTP ϵ and cyt-PTP ϵ decelerated the activation kinetics of HCN2.

In conclusion, we showed that although both were present in the heart, RPTP μ and RPTP ϵ differentially modulated biophysical properties of HCN2 channels *in vitro*, indicating a non-redundancy in the regulatory roles of RPTPs. Further, the marked difference in the modulation of HCN2 channels by these two RPTPs and their corresponding catalytically inactive mutants suggested a possible mechanism via tyrosine phosphorylation. However, the underlying mechanism by which RPTP μ and RPTP ϵ differentially modulate HCN2 activity remains elusive and requires further investigation.

Key words: HCN2, RPTP μ , RPTP ϵ , tyrosine phosphorylation

Introduction

The funny current generated by hyperpolarization-activated cyclic nucleotide-gated (HCN) channels is an important contributor to rhythmic activity in neurons and myocytes.¹⁻³ There are four isoforms of HCN channels (HCN1-4) encoding the native cardiac pacemaker current or the funny current, I_f , and HCN2 is the predominant isoform in cardiac ventricles.⁴ HCN gene expression was found elevated in end-stage heart failure and cardiac hypertrophy, accounting for an enhanced I_f activity which was arrhythmogenic in such conditions.⁵⁻⁸

Activated upon membrane hyperpolarization, HCN channels allow passages of mixed cations, including both K^+ and Na^+ ions, and a much smaller fraction of Ca^{2+} ions. HCN channels are tetramers and each monomer contains six transmembrane domains (S1-S6) with both N- and C- termini located on the cytosolic side of the plasma membrane. In the C-terminus of HCN channels, a consensus cyclic nucleotide-binding domain (CNBD) is responsible for the enhancement of channel gating upon direct binding of cAMP.

Accumulating evidence has suggested an important role of tyrosine phosphorylation in modulating HCN channels.^{9, 10} We and others have demonstrated that increased tyrosine phosphorylation state of HCN channels by activated Src tyrosine kinase facilitated the channel gating by shifting the activation threshold and midpoint towards depolarization, as well as accelerating activation kinetics.¹¹⁻¹³ In addition, the key tyrosine residues mediating the regulation of HCN channels by Src kinases have been identified in the C-linker, which is the structural element linking the last transmembrane domain of HCN channels (S6) with the CNBD.

Since the tyrosine phosphorylation state of HCN channels is determined by a fine balance between tyrosine kinases and tyrosine phosphatases, we have also studied the modulation of HCN channels by tyrosine phosphatases. We found that RPTP α , a type IV member of receptor protein tyrosine phosphatases (RPTPs), dramatically inhibited HCN2 currents in HEK293 cells, an effect accompanied by a significant reduction of the tyrosine phosphorylation state on the channel proteins.¹⁴ In ventricular myocytes, suppressing tyrosine phosphatase activity by phenylarsine oxide or sodium orthovanadate enhanced I_f activity by positively shifting its activation threshold from non-physiological into physiological voltages and accelerating the activation kinetics.¹⁴ More recently, we have successfully rescued the function of a trafficking-defective HCN4 mutant D553N that was identified in a patient with sinus bradycardia by enhancing the Src-mediated tyrosine phosphorylation *in vitro*.¹⁵ Our studies implied a positive relationship between the degree of tyrosine phosphorylation and the surface expression of HCN channels.^{14, 15}

Receptor protein tyrosine phosphatases (RPTPs) are transmembrane phosphatases critical in many signaling pathways mediating cell growth, differentiation and motility, as well as regulating the expression and function of ion channels.^{14, 16-23} All RPTPs consist of an extracellular domain, a single membrane-spanning domain, and one or two catalytic domains in the cytosol.²⁴ In RPTPs bearing two enzymatic domains, the membrane-proximal D1 domain is usually active while the membrane-distal D2 domain often plays a regulatory role with little dephosphorylation capability.²⁵⁻²⁹ While the cytoplasmic domains of RPTPs are conserved across all RPTP subfamilies, the extracellular domains vary greatly in length and structure. Depending on the structure of the extracellular domain, RPTPs are classified into eight subfamilies, including type I/VI, IIa, IIb, III, IV,

V, VII, and VIII.²⁴ For example, type IV RPTPs like RPTP α and RPTP ϵ have the shortest extracellular domains among all RPTPs members, and their extracellular domains are heavily glycosylated. In contrast, type IIb RPTPs such as RPTP μ have an extracellular meprin-A5 antigen-PTP (MAM) domain, a single immunoglobulin (Ig)-like domain, and multiple fibronectin type III repeats. However, the functions of the diverse extracellular domains of RPTPs are unclear.

Previous to our studies that RPTP α inhibited HCN2 channels¹⁴, it was shown that RPTP α and RPTP ϵ could also decreased the current amplitude of voltage-gated potassium (K_v) channels in Schwann cells.²⁰ Moreover, RPTP α inhibited K_v channels more strongly than RPTP ϵ due to a constitutive association with the channel proteins. RPTP α also activated Src in sciatic nerve extracts, but RPTP ϵ did not. Clearly, the regulation of RPTP α and RPTP ϵ on K_v channels was not redundant although both tyrosine phosphatases belong to the type IV subfamily. Therefore, as a continuation of our previous studies on RPTP α and HCN2, we would like to further examine the effects of RPTP ϵ on HCN2 channels overexpressed in HEK293 cells.

RPTP μ , a type IIb member of RPTPs, has a much longer extracellular domain and interacts with the cadherin/catenin system.^{30, 31} Additionally, the surface expression of RPTP μ was regulated by cell-cell contact^{32, 33}, indicating that RPTP μ may contribute to cell-cell adhesion. Moreover, RPTP μ was found to regulate the mRNA abundance of K_v1.5 in cardiac myocytes²², suggesting the involvement of RPTP μ in abnormal electrical activity under pathological conditions associated with the loss of cell-cell interactions in heart. However, despite these evidences supporting a regulatory role for RPTP μ in heart, there is currently no report on the protein expression of RPTP μ in cardiac tissue.

To the best of our knowledge, no research has been conducted to elucidate the modulation of HCN channels by RPTPs except our initial findings on the regulation of HCN2 channels by RPTP α .¹⁴ This work was designed to examine the regulation of HCN2, the principle isoform present in cardiac ventricles, by RPTP μ and RPTP ϵ , respectively, in addition to validating the existence of RPTP μ and RPTP ϵ proteins in cardiac tissues.

Materials and Methods

DNA Plasmids

Mouse HCN2 cDNA in an oocyte expression vector, pGH, was initially obtained from Drs. Bina Santoro/Steve Siegelbaum (Columbia University). We subcloned it into the EcoRI/XbaI sites of pcDNA3.1 mammalian expression vector (Invitrogen) for functional expression in mammalian cells. RPTP μ and its inactive point mutation C1095S encoded in pMT2 vector were kindly provided by Dr. Martijn Gebbink from University Medical Centre, Utrecht, the Netherlands. RPTP ϵ and its “substrate-trapping” mutant D302A, as well as the non-receptor type cyt-PTP ϵ containing a FLAG tag at their 3' end were cloned into the pcDNA3 expression vector. They were kindly offered by Dr. Ari Elson from the Weizmann Institute of Science, Rehovot, Israel.

Cell Culture and Plasmid Transfection

HEK293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 g/liter streptomycin. Cells with 50-70% confluence in 6-well plates were transfected with desired plasmids using Lipofectamine2000 (Invitrogen).

Cell Lysis and Western Blot Analysis

Total protein extracts were prepared from transfected HEK293 cells after 24-48 h using lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% Nonidet P-40) supplemented with a cocktail of protease inhibitors (Sigma). For membrane fraction preparations, we used a membrane protein extraction kit (Pierce). The protein concentrations of lysates were determined by the Bradford or BCA assay. Total protein of 5-20 μ g per sample was subjected to SDS-PAGE using 4-12% gradient gels (Invitrogen), then transferred to nitrocellulose membranes (Amersham Biosciences), and incubated with proper antibodies. The antibody against HCN2 and FLAG was purchased from Alomone and Sigma, respectively. Antibodies for α - and β -actin were purchased from Abcam. The antibody recognizing RPTP ϵ in rat was generously provided by Dr. Ari Elson from the Weizmann Institute of Science, Rehovot, Israel. The human RPTP ϵ antibody was purchased from Abgent. Anti-RPTP μ was purchased from Millipore. After washing and incubating with horseradish peroxidase-conjugated secondary antibody, immunoreactive proteins were visualized with the SuperSignal West Pico kit (Pierce). All protein experiments were repeated at least three times, if not mentioned in the text.

Cardiac Tissue Preparation

Adult rats (300-350 g) were euthanized by intraperitoneal injection of pentobarbital in accordance with the Institutional Animal Care and Use Committee protocols. The heart was excised; ventricles were cut out and stored in -80 °C after pre-treatment with liquid nitrogen. The frozen tissue was homogenized and the total proteins were extracted by the method described above.

Whole-cell Patch Clamp Recordings

For recording HCN2 currents, GFP plasmid was co-transfected with target plasmids for guidance. Fluorescent HEK293 cells 24-48h post-transfection were selected for patch clamp studies. The HEK293 cells were placed in a Lucite bath in which the temperature was maintained at 25 ± 1 °C by a temperature controller (Cell MicroControls, Virginia Beach, VA). HCN2 currents were recorded using the whole-cell patch clamp technique with a MultiClamp-700B amplifier. The pipettes had a resistance of 2-4 M Ω when filled with internal solution (mM) as follows: NaCl 6, potassium aspartate 130, MgCl₂ 2, CaCl₂ 5, EGTA 11, and HEPES 10; pH adjusted to 7.2 by KOH. The external solution contained (mM) the following: NaCl 120, MgCl₂ 1, HEPES 5, KCl 30, CaCl₂ 1.8, and pH was adjusted to 7.4 by NaOH. The I_{to} blocker, 4-aminopyridine (2 mM), was added to the external solution to inhibit the endogenous transient potassium current which can overlap with and obscure HCN2 tail currents recorded at +20 or +40 mV.

Data were acquired by CLAMPEX and analyzed by CLAMPFIT (pClamp 9.2, Axon Instruments). Data are shown as mean $\pm$ SEM. Student's *t* test was used for statistical analysis with *p* < 0.05 being considered as statistically significant. Time constants were obtained by using Boltzmann best fit with one exponential function on current traces that reach steady state or on current traces that can be fit to steady state.

Results

Existence of RPTP μ , RPTP ϵ , and cyt-PTP ϵ proteins in cardiac ventricles

Previous studies demonstrated the presence of RPTP μ , a type IIb receptor protein tyrosine phosphatase, in whole-heart preparation at RNA levels.³⁴ We performed the

Western blot analysis and showed for the first time the marked level of RPTP μ protein in both human (Figure 1A, lane 4) and adult rat (lane 5) heart ventricles, suggesting its possible physiological role in cardiac function. Human RPTP μ transfected (lane 2) and non-transfected (lane 3) HEK293 cells were used as a positive and a negative control, respectively. The predicted size of the peptide backbone of RPTP μ is 162 kD, and the mature protein is 195 kD after N-glycosylation at its extracellular domain.³⁵ Consistently, we demonstrated that the apparent size of the full-length protein of RPTP μ was between 150 kD and 250 kD (lanes 2, 4 and 5). We noticed a cleaved form of RPTP μ with a size of approximately 100 kD (lanes 2 and 5), which was also observed by others.³⁶ It is clear that RPTP μ in cardiac ventricles underwent more intensive proteolysis in rat (lane 5) than in human (lane 4), indicated by a stronger band around 100 kD in rat than in human.

The protein of RPTP ϵ , a type IV receptor protein tyrosine phosphatase, has been shown in brain and lung³⁷, however, no previous studies have been done in heart. In Figure 1B, we showed for the first time that the RPTP ϵ protein existed in rat (lane 15) and human (lane 16) cardiac ventricles. The rat brain lysate was used as a positive control (lane 14), revealing two major bands at 100 kD and 75 kD. In contrast, the predominant band in both rat (lane 15) and human (lane 16) heart appeared at approximately 75 kD and lower, suggesting a tissue-specific processing of this phosphatase.³⁷ Non-transfected HEK293 cells were used as a negative control (lane 7). A mouse version of RPTP ϵ tagged with FLAG was transfected into HEK293 cells, from which the cytosolic (lane 8) and the membrane fractions (lanes 9, 10) were extracted. It was shown that both the non-glycosylated (~80 kD) and glycosylated (~100 kD) proteins were exclusively located in the membrane fraction (lane 9, 10), and not present in the cytosol (lane 8). Similarly, the

cytosolic (lane 11) and membrane fractions (lanes 12, 13) separated from lysates of cyt-PTP ϵ transfected HEK293 cells were also examined. The protein cyt-PTP ϵ is translated from a shorter version of mRNA that originated from the same PTP ϵ gene as RPTP ϵ .³⁸ Lacking the extracellular domain but retaining the catalytic domain intracellularly, cyt-PTP ϵ possessed the same tyrosine phosphatase activity as RPTP ϵ .³⁸ However, different from the exclusive membrane localization of RPTP ϵ (lanes 8-10), cyt-PTP ϵ was found both in the cytosol (lane 11) and on the membrane (lanes 12, 13). We showed that the major form of PTP ϵ in cardiac ventricles was the non-receptor type, cyt-PTP ϵ (lanes 15, 16), while in brain (lane 14) the receptor type RPTP ϵ had a substantially higher expression level than the heart. In addition, the broad bands of cyt-PTP ϵ that appeared between 50 kD and 75 kD (lanes 11-16) may include another two proteins, p67 and p65, which were difficult to separate in SDS-PAGE.^{20, 39, 40} p67 is translated from either the receptor- or non-receptor-type of PTP ϵ mRNA by internal initiation of translation, and p65 is a form of RPTP ϵ cleaved by calpain.²⁹

β -Actin and cardiac α -actin were used as loading controls for HEK293 cells and cardiac tissues, respectively. Two additional repeats of the same experiment confirmed the existence of RPTP μ , RPTP ϵ , and cyt-PTP ϵ proteins in cardiac ventricles.

Modulation of HCN2 currents by RPTP μ

We have previously identified that increased tyrosine phosphorylation of HCN channels was positively related to enhanced channel function.^{11, 12} More recently, we discovered that RPTP α significantly inhibited HCN2 currents in HEK293 cells.¹⁴ Here

we tested the effects of another isoform of the receptor protein tyrosine phosphatases, RPTP μ , on HCN2 channels overexpressed in HEK293 cells (Figure 2).

HCN2 currents were elicited by hyperpolarizing pulses from -70 mV to -120 mV (Figure 2A). Typical biophysical properties of the expressed channels such as the activation threshold, activation kinetics, and current densities are comparable with those reported previously.^{13, 14} Unlike RPTP α , RPTP μ did not significantly alter the current density of HCN2 in HEK293 cells (Figures 2B, 4). Similar results were reproduced in five additional cells. On average, the current density of HCN2 with and without RPTP μ at -120 mV was 35.6 ± 6.1 pA/pF and 38.8 ± 6.4 pA/pF, respectively ($n = 6$, $p > 0.05$). In addition, the reversal potential of HCN2 channels was unchanged by RPTP μ (-20 mV, $n = 5$) (Figure 2E). Therefore, it is reasonable to postulate that HCN2 was not a substrate for RPTP μ . We then introduced a mutant of RPTP μ , C1095S, in our study. This point mutation at residue 1095 from cysteine to serine completely abolishes the catalytic activity of RPTP μ in mammalian cells.^{33, 36} Interestingly, RPTP μ -C1095S was able to suppress HCN2 currents significantly by reducing the current density at -120 mV from 38.8 ± 6.4 pA/pF to 8.0 ± 3.0 pA/pF ($n = 6$, $p < 0.05$) (Figures 2D, 4). The phenomena that the wild-type RPTP μ failed but the catalytically inactive RPTP μ -C1095S mutant succeeded in inhibiting HCN2 current expression *in vitro* indicated that this phosphatase was likely to regulate HCN2 channels through tyrosine phosphorylation.

Despite a direct effect on the current density, RPTP μ significantly reduced the slope factor of HCN2 channels from 8.81 ± 0.46 to 4.62 ± 0.61 ($n = 5$, $p < 0.05$), indicating an increased voltage sensitivity by RPTP μ . This further confirms a regulatory role of RPTP μ on HCN2 channels *in vitro*. In contrast to the significantly decreased slope

factor, the activation midpoint remained unaltered by RPTP μ (HCN2: -94 ± 3 mV, HCN2 + RPTP μ : -87 ± 3 mV, $n = 5$, $p > 0.05$). Additionally, the activation kinetics of HCN2 at -120 mV was not significantly altered by RPTP μ either (HCN2: 0.32 ± 0.06 sec, HCN2 + RPTP μ : 0.46 ± 0.07 sec, $n = 5$, $p > 0.05$).

Associated with a reduction in the current density, the activation threshold was also negatively shifted by the enzymatically inactive RPTP μ -C1095S mutant by 12.4 mV (HCN2: -85.6 ± 2.9 mV, HCN2 + RPTP μ -C1095S: -98 ± 3.7 mV, $n = 6$, $p < 0.05$). As a negative control, the empty vector of RPTP μ and RPTP μ -C1095S, pMT2, was co-transfected with HCN2 channels and neither inhibited the current density nor altered the gating properties of HCN2 channels (Figure 2C).

Modulation of HCN2 Currents by RPTP ϵ

Different from RPTP μ , RPTP ϵ , completely eliminated HCN2 currents in 15 out of 17 HEK293 cells (Figure 3B), and greatly inhibited the currents in the other two cells (Figure 3C). On average, co-transfection of RPTP ϵ significantly reduced the HCN2 current density at -120 mV from 38.8 ± 6.4 pA/pF ($n = 6$) to 2.5 ± 1.1 pA/pF ($n = 17$) ($p < 0.05$). Such dramatic suppression was also observed on HCN2 current expression by RPTP α .¹⁴ It is worth noting that RPTP α and RPTP ϵ are the only two members of the type IV receptor protein tyrosine phosphatases with an extracellular domain structure that is distinct from that of RPTP μ , a type IIb isoform.^{18, 24} To further demonstrate that the suppression of the HCN2 current density was due to the tyrosine dephosphorylation by RPTP ϵ , we examined the effects of a “substrate-trapping” mutant of RPTP ϵ , D302A, on HCN2 currents.³⁹ Remarkably, RPTP ϵ -D302A was unable to inhibit the HCN2 current

expression (Figure 3D). On average, the current density of HCN2 channel at -120 mV in the absence and presence of RPTP ϵ -D302A was 38.8 ± 6.4 pA/pF and 32.6 ± 9.3 pA/pF, respectively ($n = 6$, $p > 0.05$) (Figure 4). The significant contrast as to the modulation of HCN2 current expression between the wild-type RPTP ϵ and catalytically inactive mutant RPTP ϵ -D302A implied that the decreased tyrosine phosphorylation state of HCN2 channels was probably associated with the suppression of HCN2 currents by RPTP ϵ .

Next, we examined the effects of the non-receptor type PTP ϵ , cyt-PTP ϵ which appeared to be the major type of PTP ϵ in cardiac ventricles (Figure 1, lanes 15, 16). Given the identical enzymatic domains to RPTP ϵ , we expected a similar inhibition of HCN2 currents by cyt-PTP ϵ . Indeed, we detected a similar degree of suppression on HCN2 currents by cyt-PTP ϵ as compared to RPTP ϵ (Figure 4). In 11 out of 14 HEK293 cells co-transfected with cyt-PTP ϵ , no time-dependent inward current was recorded (Figure 3E), and in the other three cells, HCN2 currents were found substantially reduced (Figure 3F). At -120 mV the averaged current density was decreased significantly from 38.8 ± 6.4 pA/pF ($n = 6$) to 1.3 ± 0.8 pA/pF ($n = 13$) by cyt-PTP ϵ ($p < 0.05$) (Figure 4).

Accompanied by the depression of current expression, the activation kinetics of HCN2 was also significantly decelerated by RPTP ϵ and cyt-PTP ϵ . On average, the activation time constant at -120 mV was 0.32 ± 0.06 sec for HCN2 alone ($n = 6$), 1.33 ± 0.45 sec in the presence of RPTP ϵ ($n = 4$, $p < 0.05$), and 0.60 ± 0.06 sec when cyt-PTP ϵ was co-expressed ($n = 3$, $p < 0.05$).

Discussion

In the present work, we demonstrated for the first time the existence of two receptor protein tyrosine phosphatases (RPTPs), RPTP μ and RPTP ϵ , as well as the non-receptor type of PTP ϵ , cyt-PTP ϵ in rat and human ventricles. More importantly, we discovered that these tyrosine phosphatases differentially regulated the current expression and gating properties of HCN2 channels, the dominant HCN isoform responsible for generating ventricular I_f . The results that the catalytically inactive RPTPs modulated HCN2 channels to the opposite direction of the wild type RPTPs suggest tyrosine phosphorylation as an underlying mechanism.

In order to provide evidence for the possible physiological role of RPTPs in heart, we first assessed protein expression of RPTP μ , RPTP ϵ , and cyt-PTP ϵ in cardiac ventricles. We showed the existence of all three tyrosine phosphatases in human and rat ventricles. We also found a cleaved form of RPTP μ that was much more abundant in rat heart than human heart (Figure 1, lanes 4, 5), indicating that the proteolysis of RPTP μ was species-specific. There are two major forms generated from the same PTP ϵ gene, the receptor type RPTP ϵ and the non-receptor type cyt-PTP ϵ , both of which were detected in brain (lane 14). However, cyt-PTP ϵ appeared to be the principle form in heart where little RPTP ϵ was detected (lanes 15, 16), suggesting a tissue-specific expression of PTP ϵ proteins. RPTP ϵ and cyt-PTP ϵ have the same enzymatic activity.³⁸ The lack of the extracellular domain in cyt-PTP ϵ allowed it a more diffused cellular localization including both in the cytosol (lane 11) and on the membrane (lanes 12, 13).

Our previous studies suggested a positive correlation between an increased tyrosine phosphorylation state of HCN channels and an enhanced channel activity.^{11, 12} We also discovered that RPTP α significantly inhibited HCN2 currents in HEK293

cells.¹⁴ With the shortest extracellular domain, RPTP α has the simplest structure among all RPTPs members.^{18, 24} We therefore studied a more complicated RPTP in structure, RPTP μ , which belongs to the type IIb RPTPs. The extracellular domain of RPTP μ is much longer than RPTP α , containing a MAM domain, a single Ig-like domain, and multiple fibronectin type III repeats. Unlike RPTP α , RPTP μ did not significantly alter the current density or the reversal potential of HCN2 channels overexpressed in HEK293 cells (Figures 2B, 2E, 4). Surprisingly, the enzymatically inactive RPTP μ , C1095S, was able to suppress HCN2 currents significantly (Figures 2D, 4). It indicated that RPTP μ might regulate HCN2 channels via tyrosine phosphorylation. However, further studies including examination of the tyrosine phosphorylation state of HCN2 in the absence and presence of RPTP μ , respectively, would be essential to draw such a conclusion.

Despite a non-significant effect on the HCN2 current density, RPTP μ increased the voltage sensitivity of HCN2 by reducing the slope factor without changing the activation midpoint. RPTP μ -C1095S, on the other hand, shifted the activation threshold of HCN2 towards hyperpolarization. Since our previous studies suggested that HCN channel activity was positively related to the tyrosine phosphorylation state of the channel proteins^{11, 12, 14}, it is unexpected to observe that RPTP μ facilitated, while its catalytically-dead mutant 1095S, inhibited HCN2 channel activity. It is possible that RPTP μ and RPTP α might interact with various tyrosine residues on HCN2 channels, and increased phosphorylation on certain tyrosine residues could impair the channel activity. Such findings expand our understanding of the modulation of HCN channels by tyrosine phosphorylation and further studies are required to identify specific tyrosine residues subjected to various RPTPs.

Different from RPTP μ but similar to RPTP α , both RPTP ϵ and cyt-PTP ϵ almost completely abolished HCN2 currents in transfected HEK293 cells (Figures 3B, 3C, 3E, and 3F). In contrast, the “substrate-trapping” mutant of RPTP ϵ , D302A, restored the eliminated HCN2 current expression to the control level (Figures 3D, 4). This result implied that decreased tyrosine phosphorylation was probably associated with the suppression of HCN2 currents by RPTP ϵ and cyt-PTP ϵ . To determine whether the suppressed HCN2 currents were due to a decreased surface expression or a defective gating of the channel, it is necessary to perform additional studies to examine the membrane proteins of HCN2 in the presence of RPTP ϵ or cyt-PTP ϵ .

Since RPTP α and RPTP ϵ are the only two members of the type IV receptor protein tyrosine phosphatases with a highly homologous extracellular domain while RPTP μ is grouped into the type IIb isoform with a more complicated extracellular domain both in length and in structure^{18, 24}, we hypothesized that the extracellular domain of RPTPs was critical in differentially regulating HCN2 channels. Although RPTP ϵ and cyt-PTP ϵ inhibited HCN2 currents to a similar degree, they varied significantly in altering the activation kinetics of HCN2 channels. RPTP ϵ increased the activation time constant at -120 mV of HCN2 by approximately four fold while cyt-PTP ϵ , that lacks the extracellular domain, only slowed down the activation kinetics by two fold. Further studies such as swapping the extracellular domains of RPTP μ and RPTP α or RPTP ϵ and testing the actions of these chimeras on HCN2 could offer more details on the regulatory roles of extracellular domains of RPTPs.

We have previously demonstrated that RPTP α inhibited the HCN2 channel, the major isoform encoding ventricular I_f .¹⁴ Moreover, we found that the protein content of

RPTP α increased in adult rat ventricles as compared to neonatal ventricles, which paralleled the physiological activation of neonatal ventricular I_f and non-physiological activation of adult ventricular I_f . Therefore, additional studies on the developmental changes of RPTP μ , RPTP ϵ , and cyt-PTP ϵ would help illuminate the loss of spontaneous pacemaking activity in adult cardiac ventricles. It would also be interesting to investigate the alteration in the expression of these tyrosine phosphatases in diseases such as heart failure or cardiac hypertrophy when an enhanced ventricular I_f activity is detected and considered arrhythmogenic.⁵⁻⁸

In conclusion, we have demonstrated in this work for the first time the differential modulation of HCN2 channels by a variety of protein tyrosine phosphatases, including RPTP μ , RPTP ϵ , and cyt-PTP ϵ , whose protein contents were detected in cardiac ventricles. Our findings suggest that the extracellular domains of RPTPs might be a key structural determinant in regulating HCN2 channels. Moreover, different subfamilies of RPTPs may interact with various tyrosine residues on HCN2 channel proteins. Although an increased tyrosine phosphorylation of HCN2 channels is usually linked to an enhanced channel function, phosphorylating certain tyrosine residues could impair the channel activity. Further investigations, however, are required to uncover the underlying mechanism by which RPTP μ , RPTP ϵ , and cyt-PTP ϵ differentially modulate HCN2 activity.

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Figure Legends

Figure 1. RPTP μ (A) and RPTP ϵ (B) detected in cardiac ventricles. Lane-1, marker; 2, HEK293 cells transfected with human RPTP μ ; 3, non-transfected HEK293 cells; 4, human ventricle; 5, rat ventricle; 6, marker; 7, non-transfected HEK293 cells; 8, HEK293 cells transfected with mouse RPTP ϵ (cytosolic fraction); 9,10, HEK293 cells transfected with mouse RPTP ϵ (membrane fraction); 11, HEK293 cells transfected with mouse cyt-PTP ϵ (cytosolic fraction); 12, 13, HEK293 cells transfected with mouse cyt-PTP ϵ (membrane fraction); 14, rat brain; 15, rat ventricle; 16, human ventricle. β -actin served as a loading control for HEK293 cells, and α -actin for cardiac ventricles.

Figure 2. Effects of RPTP μ on HCN2 in transfected HEK293 cells. Current expression of HCN2 (A), HCN2 co-expressed with RPTP μ (B), HCN2 co-expressed with the empty vector (C) or the inactive mutant of RPTP μ , C1095S (D). The cell was held at -10 mV. Currents were elicited by hyperpolarizing pulses from -70 mV to -120 mV (A-C) or -130 mV (D) in 10 mV increments. Tail currents were recorded at +20 mV. Unchanged reversal potential (around -20 mV) of HCN2 co-expressed with RPTP μ was shown in (E). The reversal potential was obtained by measuring tail currents at test potentials ranging from -40 mV to 0 mV after a prepulse of -110 mV to activate the channel.

Figure 3: Effects of RPTP ϵ on HCN2 in transfected HEK293 cells. Current expression of HCN2 (A), HCN2 co-expressed with RPTP ϵ (B, C), with a “substrate-trapping” mutant (D302A) that loses the enzymatic activity (D), and HCN2 co-expressed

with the non-receptor form of RPTP ϵ , cyt-PTP ϵ (E, F). The cell was held at -10 mV. Currents were elicited by hyperpolarizing pulses from -70 mV (A, D) or -80 mV (B, C, F) to -130mV in 10 mV increments. The test potentials ranged from -90 mV to -160 mV in (E). Tail currents were recorded at +40 mV (A-D), or +20 mV (E, F).

Figure 4. Effects of RPTP μ and RPTP ϵ on current densities of HCN2 at -120 mV. *
indicates the significant difference compared to HCN2.

Figure 1

Figure 2

Figure 3

Figure 4

Reduced Tyrosine Phosphorylation Inhibits HCN4-573X Channel Independent of cAMP Signaling

Jianying Huang, Han-Gang Yu.

Center for Cardiovascular and Respiratory Sciences, Department of Physiology and Pharmacology, West Virginia
University, Morgantown, WV

Abstract

In the U.S., sick sinus node patients account for 1 in every 600 cardiac patients older than 65 years, with bradycardia being its major manifestation and responsible for nearly half of the sudden deaths in hospital. Despite the prevalence of this disease, there is currently no pharmacological treatment because the mechanistic etiology remains elusive.

Recently, a mutated cardiac pacemaker channel, HCN4-573X, was identified in a patient of sick sinus syndrome with bradycardia. Lacking the cyclic nucleotide binding domain (CNBD), HCN4-573X is irresponsive to cAMP. We have previously demonstrated that inhibition of Src tyrosine kinases activity significantly and reversibly suppressed the activity of HCN4-573X to the same degree as HCN4, indicating that the CNBD was not required for the modulation of the channel by tyrosine phosphorylation.

In this study, we further tested whether modulation of HCN4-573X channels by tyrosine phosphorylation was independent of cAMP using a type IV receptor protein tyrosine phosphatase (RPTP) ϵ . We found that RPTP ϵ completely abolished, while its enzymatically inactive mutant had no effect on, the current expression of HCN4-573X, suggesting that the reduced tyrosine phosphorylation of HCN4-573X was directly associated with the elimination of the whole cell current density of the channel.

We also showed that RPTP ϵ inhibited the current expression of HCN4 to a similar degree to that of HCN4-573X, confirming that the C-linker, not the CNBD was the major domain mediating tyrosine phosphorylation by which the current expression was regulated. Unexpectedly, the inactive mutant of RPTP ϵ also inhibited the current expression of HCN4.

In conclusion, we discovered that RPTP ϵ eliminated the current expression of both HCN4 and its cAMP insensitive mutant lacking the CNBD, HCN4-573X. Our data suggest that tyrosine

phosphorylation can regulate the HCN4 channel independently of cAMP, which provides a new target for future drug development to treat sinus bradycardia.

Key words: HCN4, 573X, RPTP ϵ , tyrosine phosphorylation, bradycardia, sick sinus syndrome

Introduction

Sick sinus node syndrome is a collection of conditions usually resulting from a dysfunctional sinus node.¹ Sick sinus node patients typically have an abnormal cardiac electrical activity, manifested by symptoms such as severe sinus bradycardia, sinus pauses or arrest, sinus node exit block and abnormal heart rate adaptation to exercise or stress.² Sick sinus node patients account for 1 in every 600 cardiac patients older than 65 years.¹ It is worth noting that bradyarrhythmias, which are the major manifestation of the sick sinus node syndrome, are responsible for almost half of the sudden deaths in hospitals.³ Despite its prevalence, there is no pharmacological alternative to the implantation of an electronic pacemaker, the only effective treatment of bradycardia at present, and sick sinus node syndrome accounts for approximately half of the pacemaker implantations performed in the United States.⁴ The lack of pharmacological treatments for sick sinus syndrome or bradycardia has been largely due to the unknown mechanisms that directly cause the disease.

The hyperpolarization activated, cyclic nucleotide-gated (HCN) channels encode the cardiac pacemaker current, I_f , responsible for early-diastolic depolarization in the sinus node, the primary pacing region of the heart.⁵ Among the four isoforms of HCN channel family, HCN4 is prevalently expressed in the sinus node.⁶ Global or cardiac-specific knockout of the HCN4 gene in mice resulted in deaths between embryonic days 9.5 and 11.5.⁷ In the embryo deficient of HCN4, the amplitude of I_f was reduced by 85%, corresponding to a failure in generating the diastolic depolarization.⁷ In addition, the heart contracted significantly slower than the control embryo with irresponsiveness to cAMP.⁷

Acceleration of heart rate through β -adrenergic receptors is mediated by cAMP signaling. One of the mechanisms is the direct binding of cAMP to the funny or HCN channel.⁸ The HCN

channel is a tetramer and each monomer consists of six transmembrane domains.⁸ It contains a cyclic nucleotide-binding domain (CNBD) in the C-terminal region immediately after the C-linker motif which links to the end of the last transmembrane domain (S6). Binding of cAMP to the CNBD releases the autoinhibition on the C-linker region and causes a positive shift of activation curve associated with acceleration of activation kinetics.⁸ This well-established mechanism central to the essential fight-or-flight response to increase heart rate has apparently failed in the treatment of bradycardia.

Recently, an HCN4 mutant, 573X was identified in a 66-year-old woman who was admitted to a community hospital with a fractured nasal bone after a severe syncope.⁹ She had marked sinus bradycardia (41 beats per minute) and intermittent atrial fibrillation. The mutant protein had a truncated C-terminus and lacked the CNBD due to a premature termination of translation resulting from a single nucleotide deletion. Since cAMP facilitated HCN4 channels by directly binding to the CNBD of the channel proteins, 573X was insensitive to increased intracellular cAMP levels in COS-7 cells.⁹ The cardiac-specific expression of 573X also resulted in a marked reduction in heart rate both at rest and during exercise in conscious mice.¹⁰

In the past decade, a total of six mutations including HCN4-573X (i.e. D553N, 573X, 695X, S672R, G480R, A485V) were identified in HCN4 channel proteins and linked to sick sinus syndrome and bradycardia.^{9, 11-15} Biophysical studies of these mutants revealed that cAMP failed to rescue or facilitate their activity. Thus a novel approach to modulating HCN4 channels is needed to enhance the channel activity independent of the cAMP signaling.

We have recently found that the Src-mediated tyrosine phosphorylation of HCN4 channels increased the channel activity.¹⁶ We also found that an increased tyrosine phosphorylation state of the HCN4 channel protein promoted the surface expression of

functional channels, and which has been used to successfully rescue a trafficking-defective HCN4 mutant, D553N, for normal gating.¹⁷ Our previous work on HCN4-573X mutant showed that a selective inhibitor of Src kinases, PP2 at 10 μ M for 5-8 minutes of perfusion, increased the activation time constant (τ -act) of 573X by 75% at -125 mV; prolonged perfusion time (15 minutes) increased τ -act by over 3-fold. PP2 also significantly inhibited the current density of 573X by 24% at -125 mV, shifted its activation threshold to a more negative potential by 18.3 mV, and shifted the activation midpoint to a more negative potential by 11 mV. Moreover, the effects of PP2 on these properties were reversible and similar to those on the wild-type HCN4, indicating that the CNBD is not required for modulating HCN4 channels through tyrosine phosphorylation.

In this work, we seek further evidence to support our hypothesis that tyrosine phosphorylation could regulate HCN4 channels independently of cAMP by testing the effects of a type IV receptor protein tyrosine phosphatase, RPTP ϵ , on 573X.

Materials and Methods

DNA Plasmids

The human version of HCN4-pcDNA1.1 was kindly provided by Dr. U. B. Kaupp and subcloned into pcDNA3.1 vector. HCN4-573X is a generous gift from Dr. Dirk Isbrandt (University Medical Center Hamburg).

Cell Culture and Plasmid Transfection

Human Embryonic Kidney (HEK) 293 cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100

g/liter streptomycin. Cells with 50-70% confluence in 6-well plates were transfected with desired plasmids using Lipofectamine LTX (Invitrogen).

Whole-cell Patch Clamp Recordings

For recording I_{HCN4} and I_{573X} , day 1 (24-30 h) up to day 4 (90-98 h) post-transfection, HEK293 cells with green fluorescence were selected for patchclamp studies. The HEK293 cells were placed in a Lucite bath in which the temperature was maintained at 25 ± 1 °C by a temperature controller (Cell MicroControls). I_{HCN4} and I_{573X} currents were recorded using the whole cell patch clamp technique with a MultiClamp-700B amplifier. The current amplitude of HCN4 or 573X currents is defined as the amplitude of the time-dependent inward current elicited by the hyperpolarizing pulse, excluding the instant jump at the beginning of the pulse. The current density is the current amplitude divided by the cell capacitance measured in each cell studied. The pipettes had a resistance of 2-4 M Ω when filled with internal solution: 6 mM NaCl, 130 mM potassium aspartate, 2 mM MgCl₂, 5 mM CaCl₂, 11 mM EGTA, and 10 mM HEPES; pH was adjusted to 7.2 by KOH. The external solution contained 120 mM NaCl, 1 mM MgCl₂, 5 mM HEPES, 30 mM KCl, 1.8 mM CaCl₂; pH was adjusted to 7.4 by NaOH. The I_{to} blocker, 4-aminopyridine (2mM), was added to the external solution to inhibit the endogenous transient potassium current, which can overlap with and obscure I_{HCN} tail currents recorded at +40 mV. The threshold activation of I_{HCN4} and I_{573X} is defined as the first hyperpolarizing voltage at which the first time-dependent inward current greater than -10 pA can be observed. The data were acquired by CLAMPEX and analyzed by CLAMPFIT (pClamp9.2, Axon). The data were shown as the means $\pm$ SEM. Student's t test was used for statistical analysis with $p < 0.05$ being considered statistically significant.

Results

Properties of HCN4-573X expressed in HEK293 cells

We first studied the current expression of HCN4-573X transiently transfected into HEK293 cells. Like the wild type, 573X could not be activated upon membrane depolarization as no time-dependent currents were generated by depolarizing pulses from -20 mV to +50 mV (Figure 1A). In contrast, slow inward currents typical for HCN4 were elicited by hyperpolarizing pulses from -45 mV to -115 mV in 573X transfected cells (Figure 1B). Although 573X lacks the CNBD, its current expression was not significantly different from the wild type channel. On average, the whole cell current densities of HCN4 and 573X were 105 ± 27 pA/pF ($n = 8$) and 82 ± 17 pA/pF ($n = 9$), respectively ($p > 0.05$) (Figure 4). In addition, we examined the reversal potential of 573X that was defined as the voltage at which the net current passing through the channel was zero. As indicated from Figure 1C, the reversal potential of 573X was between -15 mV and -5 mV, slightly more positive than the wild type HCN4 that typically has a reversal potential of -20 mV in the same experimental condition.

Interestingly, we noticed a unique feature of 573X in that it did not have the initial delay of activation. As shown in Figure 1D, the initial delay (δ) of HCN4 current activated at -100 mV was approximately 200 ms, consistent with our previous report that HCN4 activated at -95 mV had an averaged delay of 185 ms.¹⁸ We have also demonstrated that this delay was significantly shortened by increased Src-mediated tyrosine phosphorylation of the HCN4 channel. However, it was very clear to us that no 573X currents contained the initial delay of activation, indicating a faster response to membrane hyperpolarization in generating inward currents.

Inhibition of HCN4 currents by RPTP ϵ

Previously, we have demonstrated that RPTP α , a type IV receptor protein tyrosine phosphatase, dramatically eliminated both HCN2 and HCN4 currents at all voltages within the activation range.^{17, 19} In the previous section, we showed that another type IV receptor tyrosine phosphatase, RPTP ϵ , was also able to completely abolish HCN2 currents. Given the high structural homology between HCN2 and HCN4 (~80%)²⁰, we anticipated that RPTP ϵ may inhibit HCN4 currents.

Figure 2A depicts the typical current expression of HCN4 expressed in HEK293 cells with an activation threshold of -70 mV. Typical biophysical properties of the expressed HCN4 channels such as the threshold activation, activation kinetics, and current densities are comparable with those reported previously.^{16, 17} Indeed, we found that RPTP ϵ markedly decreased HCN4 currents at all test voltages from -50 mV to -110 mV in 8 out of 21 HEK293 cells (Figure 2B). In the other 13 cells, no typical HCN4 currents could be observed at voltages ranging from -60 mV to -120 mV with RPTP ϵ co-expressed (Figure 2C). On average, the whole-cell current density of HCN4 at -125 mV where the channel was fully activated was 105 ± 27 pA/pF ($n = 8$), which was significantly reduced to 12 ± 3 pA/pF by RPTP ϵ ($n = 21$, $p < 0.0001$) (Figure 4).

Since the reduction in HCN4 current densities by RPTP ϵ was probably linked to a decreased tyrosine phosphorylation on the channel proteins, we further tested the effects of a “substrate-trapping” mutant of RPTP ϵ , D302A, on HCN4 currents (Figure 2D).²¹ It turned out that this inactive tyrosine phosphatase could inhibit HCN4 currents to the same degree as the functional RPTP ϵ (Figure 4). Averaged over 8 cells, the whole-cell current density in HCN4 and RPTP ϵ -D302A co-transfected cells was 14 ± 4 pA/pF, which was significantly lower than the

control (HCN4: 105 ± 27 pA/pF). The mechanism by which RPTP ϵ and its inactive form, D302A, suppressed the current expression of HCN4 channels was unclear.

Inhibition of HCN4-573X currents by RPTP ϵ

Previously, we and others identified that Src kinases increased the activity of HCN4 channels via tyrosine residues located in the C-linker, Y531 and Y554.^{16, 22} Consistent with this, in Section 2 of Chapter 1 we have shown that suppression of Src kinases activity by PP2 inhibited the activity of HCN4-573X, possibly via dephosphorylating the key tyrosine residues at the C-linker, given that 573X lacks the entire CNBD but retains the complete C-linker region.

To confirm that tyrosine phosphorylation could regulate HCN4 channels independently of cAMP, we co-transfected RPTP ϵ with 573X and found a complete abolishment of the time-dependent inward currents responding to hyperpolarizing pulses ranging from -40 mV to -130 mV (Figure 3B). In a total of 18 HEK293 cells, no current expression of 573X was detected. The average whole-cell current density was reduced from 82 ± 17 pA/pF ($n = 9$) for 573X alone to 2 ± 1 pA/pF for 573X in the presence of RPTP ϵ ($n = 18$, $p < 0.0001$) (Figure 4). Such an elimination of current expression supported the notion that tyrosine phosphorylation could modulate the activity of 573X in spite of its lack of the CNBD and resulting cAMP insensitivity.

To further demonstrate that the suppression of 573X current expression was due to tyrosine dephosphorylation by RPTP ϵ , we examined the effects of the enzymatically inactive form of RPTP ϵ , D302A, on 573X currents. Remarkably, we were able to observe the typical 573X currents in the presence of RPTP ϵ -D302A (Figure 3C). On average, the current density of 573X at -125 mV in the absence and presence of RPTP ϵ -D302A was 82 ± 17 pA/pF ($n = 9$) and 110 ± 26 pA/pF, respectively ($n = 10$, $p > 0.05$) (Figure 4). The phenomenon that 573X currents

were abolished by an active RPTP ϵ but unaffected by a catalytically inactive RPTP ϵ -D302A suggests that tyrosine phosphorylation may be a possible underlying mechanism to regulate the current expression of 573X independently of cAMP.

Discussion

In the present study, we investigated whether RPTP ϵ , a type IV receptor protein tyrosine phosphatase, could regulate HCN4 channels through tyrosine phosphorylation independently of cAMP. To test this hypothesis, we used a cAMP insensitive HCN4 mutant, 573X, which was identified in a patient with idiopathic sick sinus syndrome manifested by severe sinus bradycardia and atrial fibrillation. The mutant protein had a truncated C-terminus and lacked the entire cyclic nucleotide-binding domain (CNBD) that was the binding site for cAMP, which made this mutant an ideal candidate for studying cAMP independent pathways.

In Section 2 of Chapter 1, we showed that inhibition of Src tyrosine kinases significantly suppressed the activity of HCN4-573X to the same degree as the wild-type HCN4. Here we found that an active RPTP ϵ completely eliminated 573X currents (Figures 3B, 4). However, the catalytically inactive RPTP ϵ mutant D302A failed to exert any inhibitory effects (Figures 3C, 4), indicating that RPTP ϵ regulated HCN4-573X independently of cAMP and likely by the mechanism of tyrosine dephosphorylation. Further studies such as detection and comparison of the tyrosine phosphorylation states of 573X proteins in the absence and presence of RPTP ϵ will be essential to reveal the underlying mechanism.

Like 573X, we found that HCN4 currents were almost completely abolished by RPTP ϵ as well (Figures 2B, 2C, 4). Surprisingly, the inactive mutant RPTP ϵ -D302A was still able to inhibit HCN4 currents to the same degree as the active RPTP ϵ (Figures 2D, 4). Since the current

expression of 573X was unaffected by RPTP ϵ -D302A, it is possible that this “substrate-trapping” mutant of RPTP ϵ sequestered HCN4 channels via interaction with the CNBD. Our preliminary studies have revealed that in contrast to a prominent depolarizing shift of 10-20 mV of the activation curve for HCN4 channels, cAMP was unable to stimulate HCN4 channels when co-expressed with RPTP ϵ -D302A in HEK293 cells (data not shown), suggesting that this inactive RPTP ϵ could possibly occupy or block the binding site of cAMP, thereby inhibiting the current expression. Without evidence of a physical interaction between the CNBD and RPTP ϵ -D302A protein, however, it would be immature to draw such a conclusion at the current stage.

Besides the main study investigating the regulation of 573X by tyrosine phosphatase RPTP ϵ , we also identified an interesting biophysical property of 573X in that it did not have an initial delay of current activation, which is one of the characteristics of HCN4 currents.²³ We observed a delay of HCN4 activation for approximately 200 ms at -100 mV, which was completely missing in 573X currents (Figure 1D). It was long noticed that the funny current in cardiac myocytes as well as HCN4 currents undergo an early activation delay preceding their time-dependent components.²³⁻²⁷ The initial delay of activation was defined as the time required for a 5% increase in current magnitude from the start of the test-pulse.²³ The magnitude of delay is not a fixed value for I_f or HCN4 currents. It decreases as the hyperpolarizing pulse becomes more negative. Moreover, we have previously discovered an inverse relationship between the tyrosine phosphorylation state of HCN4 channel proteins and the initial activation delay.¹⁸ The delay of HCN4 channels in transfected HEK293 cells was reduced by 75% when co-expressed with a constitutively active Src tyrosine kinase, but prolonged by 2 fold when co-expressed with a dominant negative Src. The lack of delay in 573X suggests that the delay may possibly be

caused by a movement or conformational change of the CNBD as an early response to the hyperpolarizing pulses preceding the opening of the gate.

The delay becomes a significant factor in cardiac pacing because the time-scale of diastolic depolarization was 200-300 ms in humans and < 200 ms in rodents.²³ Therefore the long activation delay of HCN4 channels enabled the small currents generated during the delay period to dominate over the time-dependent current component on the time-scale of cardiac pacing. The lack of delays in 573X currents will actually lead to a larger inward current contributing to the early-diastolic depolarization in the sinus node as compared to the wild type HCN4, which eventually results in a faster heart rate if 573X had the same cAMP sensitivity as HCN4. Therefore, the “gain of function” due to a loss in the initial delay was reversed by the “loss of function” caused by cAMP insensitivity, contributing to the sinus bradycardia in the patient.

In summary, we found that a type IV receptor protein tyrosine phosphatase, RPTP ϵ , eliminated the current expression of a cAMP insensitive HCN4 mutant, 573X, to a similar degree to that of the wild type HCN4, implying that tyrosine phosphorylation may regulate HCN4 channels independently of cAMP. To conclude that RPTP ϵ indeed modulated HCN4 and 573X channels via tyrosine phosphorylation, further studies evaluating the tyrosine phosphorylation state of channel proteins are necessary. Nonetheless, our results that an active tyrosine phosphatase, but not its inactive form, inhibited 573X currents support our hypothesis that tyrosine phosphorylation could regulate HCN4 channels independently of cAMP, which has a clinical implication in designing a novel therapeutic strategy for the treatment of sick sinus syndrome and bradycardia. Therefore, increasing the tyrosine phosphorylation state of HCN4 channels could be potentially used to enhance the channel activity, thereby increasing the amount

of time-dependent inward current available at the early-diastolic depolarization in the sinus node and leading eventually to an increased heart rate.

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Figure Legends

Figure 1: Properties of 573X in HEK293 cells. Current expressions of 573X by depolarization (A) or hyperpolarization (B, C). (A) The cell was held at -30 mV. No time-dependent currents were elicited by depolarizing pulses of 6 sec from -20 mV to +50 mV in 10 mV increments. (B) The cell was held at -10 mV. Time-dependent inward currents were elicited by hyperpolarizing pulses of 10 sec from -45 mV to -115 mV in 10 mV increments. Tail currents were recorded at +40 mV. (C) The cell was held at -10 mV. The reversal potential was obtained by measuring tail currents at test potentials ranging from -45 mV to +15 mV after a prepulse of -110 mV for 10 sec to activate the channel. (D) The initial delay (δ) of HCN4 current activation at -100 mV was missing in 573X.

Figure 2: Inhibitory effects of RPTP ϵ on the current expression of HCN4 in HEK293 cells. Current expression of HCN4 (A), HCN4 co-expressed with RPTP ϵ (B, C), and HCN4 co-expressed with the enzymatically inactive mutant of RPTP ϵ , D302A (D). The cell was held at -10 mV. Currents were elicited by hyperpolarizing pulses of 10 sec from -60 mV to -130 mV (A), -50 mV to -110 mV (B), -60 mV to -120 mV (C), and -70 mV to -130 mV (D) in 10 mV increments. Tail currents were recorded at +40 mV.

Figure 3: Inhibitory effects of RPTP ϵ on the current expression of 573X in HEK293 cells. Current expression of 573X (A), 573X co-expressed with RPTP ϵ (B), and 573X co-expressed with the “substrate-trapping” RPTP ϵ mutant D302A (C). The cell was held at -10 mV. Currents were elicited by hyperpolarizing pulses of 10 sec (16 sec for B) from -60 mV (A), -40 mV (B) or -50 mV (C) to -130 mV in 10 mV increments. Tail currents were recorded at +40 mV.

Figure 4: Effects of RPTPε on current densities of HCN4 and 573X at -125mV. * indicates the significant difference compared to HCN4 or 573X.

Figure 1

Figure 2

Figure 3

Figure 4

General Discussion

My research focuses on the modulation of HCN channels and the funny current (I_f) by tyrosine phosphorylation. Two HCN channels that are the major isoforms encoding I_f in cardiac ventricles (i.e. HCN2) and sinus node (i.e. HCN4), respectively, were investigated. Tyrosine phosphorylation of HCN channels is maintained by a fine balance between tyrosine kinases and phosphatases. In my study, Src-family tyrosine kinases and receptor protein tyrosine phosphatases (RPTPs) were examined in detail in regulation of HCN2 and HCN4 channels.

An early study in rabbit sinus node myocytes revealed a regulatory role of tyrosine kinases on I_f . Epidermal growth factor (EGF) increased I_f amplitude in the diastolic range of potential and its maximal conductance, which was eliminated by genistein, a non-specific tyrosine kinase inhibitor. However, no change in the voltage-dependent activation of I_f was observed.¹ Further studies on individual cardiac isoforms of HCN channels expressed in *Xenopus* oocytes revealed a differential modulation of HCN1, HCN2, and HCN4 by tyrosine kinases. Inhibition of tyrosine kinase activity by genistein reduced HCN2 and HCN4 currents without affecting HCN1.² Genistein also caused a negative shift in the voltage dependence of activation for HCN2 channels.² These effects of EGF or genistein occurred in 5-10 min, typical of acute regulation of I_f or HCN channels that usually involves post-translational modification of channel proteins, such as altering their phosphorylation state.

The long term effects of tyrosine kinases on HCN channels were studied by co-expressing the constitutively active Src kinase (Src529) with HCN channels in HEK293 cells, a mammalian cell line. When Src529 phosphorylated HCN4 via an association with the channel protein, an enhanced whole-cell current density near diastolic potentials was observed.³ In addition to the increase of current density that directly correlates with the number of functional

channels on the plasma membrane, the gating properties of HCN4 channels were altered, demonstrated by the acceleration in activation kinetics and a positive shift in the voltage-dependent activation. Our further studies using PP2, a selective inhibitor of Src-family kinases, identified the key tyrosine residue (i.e. Y531) in mediating the facilitation of HCN4 channel by Src.⁴ We found that PP2 inhibited HCN4 currents by negatively shifting the voltage dependence of channel activation, decreasing the whole-cell channel conductance, and slowing activation and deactivation kinetics. In contrast, replacement of the Y531 residue by phenylalanine in HCN4 abolished sensitivity to a Src inhibitor. Another tyrosine residue in HCN4, Y554, previously reported by others in HCN2 channels⁵, also contributed to the deceleration of activation kinetics by PP2. The inhibitory effects of PP2 occurred after a 10-min perfusion, suggesting that even the short term effect of adjusting tyrosine phosphorylation state on HCN4 channels may affect the number of functional channels on the plasma membrane, probably via re-distribution between subcellular compartments rather than *de novo* synthesis of proteins.

In the past decade, a total of six HCN4 mutations were identified in patients with idiopathic sick sinus syndrome manifested in sinus bradycardia. *In vitro* expression of these mutants in mammalian cell lines revealed that the dysfunction of mutants D553N, G480R and A485V was due to their reduced surface expression.⁶⁻⁸ Therefore, we aimed to promote the surface expression of these mutants by Src tyrosine kinases in search of a pharmacological strategy for the treatment of sinus bradycardia that is currently lacking. Indeed, we successfully restored the membrane expression of D553N in HEK293 cells by overexpressing three Src-family tyrosine kinases that were constitutively active, Src529, Fyn531, and Yes537.⁹ The corrected D553N exhibited gating properties comparable with those of the wild-type HCN4 channels. Although these Src kinases are ubiquitously expressed in heart¹⁰, interestingly, their

effects on D553N channels were not identical. For example, Fyn531 has the greatest potency in enhancing the phosphorylation state of D553N while Yes537 the least. In agreement with the previous studies that the Src-mediated tyrosine phosphorylation was associated with the acceleration of channel activation kinetics³⁻⁵, Fyn531 accelerated D553N activation kinetics while Yes537 did not. On the other hand, Fyn531 had the least effect in enhancing surface expression and increasing the whole-cell current density of D553N, which were facilitated the most by Yes537. Therefore, the correlation among surface expression and Src-mediated tyrosine phosphorylation of HCN4 channels, if any, required further investigations. It is possible that other unknown proteins were involved besides Src tyrosine kinases. In addition, the differential phosphorylation by three Src kinases suggested that different tyrosine residues might be involved in mediating each of these kinases. We and others have previously identified the key tyrosine residues (i.e. Y531, Y554) mediating the actions of Src kinases on HCN4 channels at the C-linker^{4, 5}, which is a conserved domain among HCN isoforms and links the last transmembrane domain to the cyclic nucleotide-binding domain (CNBD). The C-linker was critical in inter-subunit interactions of HCN channels and was demonstrated to control coupling of ligand binding to channel gating in both the cyclic nucleotide-gated ion channel (CNG) and HCN channel.¹¹⁻¹⁵ It was speculated that the introduction of the bulky phosphate group by tyrosine kinases would weaken the electrostatic interactions between neighboring C-linkers, thereby accelerating the activation kinetics of the channel.^{16, 17} According to this model, Fyn may target the tyrosine residues at the C-linker. On the other hand, Yes may phosphorylate tyrosine residues located outside of the C-linker, which can explain its lack of acceleration in the activation kinetics.

Another HCN4 mutant related to sick sinus syndrome and sinus bradycardia, 573X, was also studied in order to illuminate the relationship between the cAMP signaling pathway and Src-mediated tyrosine phosphorylation in the regulation of HCN4 channels. The mutant 573X was identified in a patient suffering from severe sinus bradycardia (41 bpm) and intermittent atrial fibrillation.¹⁸ It has a truncated C-terminus lacking the entire CNBD. We found that inhibition of Src kinase activity by PP2 for 10-15 min significantly suppressed the activity of 573X, demonstrated by decreased whole-cell current density, hyperpolarization shift of the voltage-dependent activation, and deceleration of the activation kinetics. These data further confirm that the main tyrosine residues mediating the actions of Src on HCN4 channels are located at the C-linker, outside the CNBD.^{4,5}

It has been accepted that the facilitation of I_f via direct binding of cAMP to the CNBD of the channel protein is essential for heart rate increase following activation of β -adrenergic receptors.¹⁹ However, our data on 573X channels indicated the cAMP-independence of tyrosine phosphorylation in the regulation of HCN channels. Consistent with this, 573X knock-in mice remain responsive to isoproterenol (ISO), a classic β -agonist, suggesting that the cAMP-mediated regulation did not play an indispensable role in heart rate adaptation.²⁰ More recently, a new cAMP insensitive mutant of HCN4 channel, 695X, was discovered in patients with familial sick sinus syndrome and sinus bradycardia.²¹ Despite the cAMP insensitivity, the heart rates of these patients could still be increased by the β -agonist dobutamine or during exercise.²¹ Earlier studies have proven an involvement of Src tyrosine kinase in the signaling pathway of β -adrenergic receptor activation. Src was not only found to be bound to and directly activated by β -adrenergic receptors^{22, 23}, but also identified as a direct target of $G\alpha_s$ and $G\alpha_i$ subunits.^{24, 25} We therefore speculated Src-mediated tyrosine phosphorylation as the key process modulating

HCN4 channels when the cAMP pathway failed. Our hypothesis was supported by the collection of data from the single cell to the whole animal showing that the stimulation by ISO on I_{HCN4} in HEK293 cells, I_f and action potential in sinus node myocytes, spontaneous beating rate of dissected sinus node tissue, and heart rate was diminished in the presence of PP2. Similar to our previous findings, inhibition of Src kinase activity was directly associated with decreased whole-cell current density, hyperpolarization shifts of activation threshold and midpoint, as well as decelerated activation kinetics of HCN4 and sinus node I_f . Immunofluorescent staining of sinus node myocytes uncovered that PP2 triggered an internalization of HCN4 channels from the plasma membrane, which offered mechanistic insights for the insensitivity to β -agonist stimulation under the condition of decreased Src kinase activity. In summary, our study discovered a new mechanism for heart rate modulation by β -adrenergic receptors with the clinical relevance that increasing tyrosine phosphorylation has the potential for pharmacological treatment of bradycardia in general, and for sick sinus syndrome caused by membrane defective or cAMP insensitive HCN4 mutants in particular. The limitations of our work, however, lies in the nonspecificity of PP2 in inhibiting individual member of Src tyrosine kinases since differential regulation on HCN4 channels by Src, Fyn and Yes has been demonstrated before. Further studies are needed to identify specific kinase(s) involved downstream of β -adrenergic receptor activation.

As tyrosine phosphorylation level is regulated by tyrosine kinases and phosphatases, we explored the actions of tyrosine phosphatases on I_f as well by using the general tyrosine phosphatases inhibitors, phenylarsine oxide (PAO) and sodium orthovanadate.²⁶ Perfusion of PAO for 10-15 min in adult rat ventricular myocyte led to a nearly 40 mV shift of the activation threshold of I_f from approximately -120 mV to -80 mV. Similarly, another tyrosine phosphatases

inhibitor, sodium orthovanadate, shifted the action threshold of ventricular I_f to about -90 mV after an incubation period of 30-40 min. Inhibition of tyrosine phosphatase activity in ventricular myocytes is sufficient to shift the activation of I_f from the non-physiological to physiological range. It has been recognized for a long time that the voltage-dependent activation of I_f is shifted towards hyperpolarization from the primary pacing region of the heart, sinus node to Purkinje fibers, and to the working myocardium, including atrium and ventricle.^{1, 19, 27-29} The threshold activation of I_f is around -60 mV in the sinus node and -120 mV in ventricles.^{1, 27, 28, 30} Although the adult ventricular I_f is activated beyond the physiological range, the neonatal ventricular I_f is activated as positive as -70 mV.³⁰ Similar to neonatal ventricular myocytes, the voltage-dependent activation of I_f in disease conditions such as heart failure is also shifted towards depolarization, which is implicated in atrial and ventricular arrhythmias.^{31, 32} However, it is currently unknown how developmental and pathological conditions cause the shift of voltage-dependent activation of I_f , and our data indicate a significant role of tyrosine phosphatases. In addition to the depolarization shift of activation by inhibiting tyrosine phosphatase activity, the activation kinetics of I_f was also accelerated, consistent with our previous conclusion that enhanced tyrosine phosphorylation is correlated with a faster activation process.

In search of particular tyrosine phosphatases that regulate HCN channels, we investigated the family of receptor protein tyrosine phosphatases (RPTPs). Protein contents of three phosphatases RPTP α ²⁶, RPTP ϵ and RPTP μ were detected in cardiac ventricles. The type IV isoforms, RPTP α ²⁶, RPTP ϵ and its non-receptor form, cyt-PTP ϵ , dramatically abrogated HCN2 currents in HEK293 cells. Little time-dependent inward current could be elicited by hyperpolarization when RPTP α , RPTP ϵ or cyt-PTP ϵ was co-expressed with HCN2 channels individually. In contrast, their catalytically inactive mutants such as the “substrate-trapping”

mutant of RPTP ϵ , D302A, was unable to inhibit HCN2 currents, suggesting a positive correlation between the whole-cell current density and the tyrosine phosphorylation level of HCN2 channels. It is worth noting that in parallel to a negative shift in the voltage-dependent activation of ventricular I_f during development, the protein content of RPTP α in rat ventricles increased by approximately 4 fold from neonates to adults.²⁶ With the shortest extracellular domain, RPTP α and ϵ have the simplest structure in the RPTP family. We therefore studied a more complicated RPTP in structure, RPTP μ , which has a longer extracellular domain consisting of defined domains such as MAM, Ig-like and fibronectin type III repeats, characteristic of type IIb RPTPs.^{33, 34} Unlike RPTP α or ϵ , RPTP μ did not alter the whole-cell current density of HCN2 channels while its inactive mutant, C1095S, was able to suppress HCN2 currents significantly. Moreover, RPTP μ increased the voltage sensitivity of HCN2 channels, but RPTP μ -C1095S, on the other hand, shifted the voltage-dependent activation towards hyperpolarization. This result is unexpected since it implies that tyrosine dephosphorylation might result in an increased activity of HCN2 channels, which opposes our previous findings. However, in the study where Src, Yes, and Fyn rescued the trafficking-defective HCN4 mutant D553N, we already noticed that the kinases mediating tyrosine phosphorylation and regulating the whole-cell current density or surface expression of the channel were unlikely to be the same. Similar to the differential regulation by Src kinases on HCN4 channels, the differential regulation by RPTPs was also observed on HCN2 channels. It is possible that RPTP μ and RPTP α (or RPTP ϵ) interact with different tyrosine residues of HCN2 channels, and increased phosphorylation on certain tyrosine residues could impair channel activity. Furthermore, the extracellular domain of RPTPs may play an important role in mediating this differential regulation since RPTP α and RPTP ϵ modulated HCN2 channel in a similar manner, markedly different from RPTP μ .

We demonstrated that PP2 significantly suppressed the activity of 573X by inhibiting Src kinases. To confirm that tyrosine phosphorylation of HCN channels is indeed cAMP independent; we co-expressed RPTP ϵ and 573X in HEK293 cells. We found that the wild type RPTP ϵ completely eliminated 573X currents, but that the catalytically inactive RPTP ϵ -D302A failed to exert any inhibitory effects.

In conclusion, our studies on I_f in sinus node and ventricular myocytes as well as HCN2 and HCN4 channels overexpressed in cells of a mammalian background have shown clearly that changing tyrosine phosphorylation state could significantly alter channel activity by affecting the whole-cell current density, voltage-dependent activation and activation kinetics. Moreover, the specific tyrosine residues mediating the differential regulation by the families of Src kinases and RPTPs remain to be identified. Our work also discovered Src kinases as a novel regulator of I_f downstream of β -adrenergic receptor activation, suggesting an essential role of tyrosine phosphorylation in heart rate adaptation. More importantly, we found that tyrosine phosphorylation could regulate I_f independently of cAMP, which has a clinical implication in designing a novel therapeutic strategy for the treatment of sick sinus syndrome and bradycardia.

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