Published in final edited form as:

Am J Physiol Cell Physiol. 2008 January ; 294(1): C355–C362. doi:10.1152/ajpcell.00236.2007.

Src Tyrosine Kinase Alters Gating of Hyperpolarization-Activated HCN4 Pacemaker Channel through Tyr⁵³¹

Chen-Hong Li^{*}, Qi Zhang^{*}, Bunyen Teng, S. Jamal Mustafa, Jian-Ying Huang, and Han-Gang Yu

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

Abstract

We recently discovered that the constitutively active Src tyrosine kinase can enhance the HCN4 channel activity by binding to the channel protein. To investigate the mechanism of modulation by Src of HCN channels, we studied the effects of a selective inhibitor of Src tyrosine kinase, PP2, on HCN4 and its mutant channels expressed in HEK293 cells using whole-cell patch clamp technique.

We found that PP2 can inhibit HCN4 currents by negatively shifting the voltage dependence of channel activation, decreasing the whole-cell channel conductance, and slowing activation and deactivation kinetics. Screening putative tyrosine residues subject to phosphorylation yielded two candidates: Tyr⁵³¹ and Tyr⁵⁵⁴. Substituting HCN4-Tyr⁵³¹ with phenylalanine largely abolished the effects of PP2 on HCN4 channels. Replacing HCN4-Tyr⁵⁵⁴ by phenylalanine did not abolish the effects of PP2 on voltage-dependent activation, but did eliminate PP2-induced slowing of channel kinetics. The inhibitory effects of HCN channels associated with reduced Src tyrosine activity is confirmed in HL-1 cardiomyocytes. Finally, we found that PP2 can decrease the heart rate in a mouse model.

These results demonstrate that Src tyrosine kinase enhances HCN4 currents by shifting their activation to more positive potentials and increasing the whole-cell channel conductance as well as speeding the channel kinetics. The tyrosine residue that mediates most of Src actions on HCN4 channels is Tyr⁵³¹.

Keywords

Src tyrosine kinase; HCN4 pacemaker channel; PP2

Introduction

Tyrosine kinase phosphorylation represents a novel regulatory mechanism of ion channels (12). Generated by the Hyperpolarization-activated, Cyclic Nucleotide-gated (HCN) channels (15,21,29), the cardiac pacemaker current, i_f , is a major contributor to the autonomous regulation of cardiac pacemaker activity in heart (1,6). We have previously reported shown that the reduced tyrosine kinase activity inhibits i_f in sinoatrial (SA) node and ventricular

Address correspondence to: Han-Gang Yu, PhD, Center for Interdisciplinary Research in Cardiovascular Sciences, Department of Physiology and Pharmacology, West Virginia University School of Medicine, One Medical Center Drive, Morgantown, West Virginia, 26506, Tel.: 304-293-2324; Fax: 304-293-5513; hyu@hsc.wvu.edu.

^{*}equal contributors

Disclosures

None.

myocytes as well as in HCN channels expressed in *Xenopus* oocytes (39,44). Recently, we discovered that a constitutively active Src tyrosine kinase can shift the HCN4 channel activation to more positive potentials and speeds its activation near diastolic potentials, while a negative dominant Src slows the HCN4 activation without apparent effect on the voltage dependence of HCN4 activation (3,4). The effects of Src on HCN4 channel properties are mediated by phosphorylation via a possible binding to the HCN4 channel protein. A recent study reported that inhibition of Src by PP2 only slows HCN4 channel activation kinetics without affecting the voltage dependence of channel activation (46). Moreover, the phosphotyrosines that mediate acceleration of activation kinetics by Src were tracked to HCN4-Tyr⁵⁵⁴ (46).

In this report, we searched tyrosine phosphorylation database (NetPhos) and point-mutated those tyrosine residues that are likely to be phosphorylated by tyrosine kinases. We then used PP2, a selective inhibitor of Src tyrosine kinase family, to study its effects on these mutant channels.

Materials and Methods

Cell Culture and Transfection

HEK293 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen), supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin. Cells with 90–95% confluence in 6-well plate were used for plasmid transfection using Lipofectamine2000 (Invitrogen), and the plasmid phrGFP (Stratagene) was co-transfected with the plasmids containing HCN genes to guide the selection of cells expressing HCN channels for patch clamp experiments.

HL-1 cardiomyocytes were kindly provided by Dr. W.C. Claycomb (Louisiana State University Health Science Center, New Orleans). Cells were maintained in "Claycomb Medium" (38), supplemented with 10 % fetal bovine serum (SAFC Biosciences), 2 mM L-glutamine, 100 μ M norepinephrine and 100U/ml-100ug/ml penicillin-streptomycin. The medium was changed every 24 hrs. Cells were passaged from 1: 2 split by 0.05% trypsin/EDTA. The digestion was stopped by adding soybean trypsin inhibitor. All cultures were grown at 37 °C, in an atmosphere of 5 % CO₂ and 95 % air at a relative humidity of approximately 95 %.

Plasmids Preparation and Site-directed Mutagenesis

Human HCN4 (hHCN4) cDNA was originally provided as a gift by Dr. U.B. Kaupp (Institut für Biologische Informationsverarbeitung, Germany). Two single HCN4 mutants, designated Y531F and Y554F, were generated respectively by replacing tyrosine residues at the positions 531 and 554 with phenylalanine using Stratagene QuikChange II XL Site Directed Mutagenesis Kits (Stratagene). Two pairs of oligonucleotide primers were used for mutagenesis: (HCN4-Y531F), F1: 5'-CTGCTC CACCTGCTTGAACCTTTTCCTGGTACTGGCG-3', R1: 5'-CGCCAGTACCAGGAAAAGTTCAA GCAGGTGGAGCAG-3'; (HCN4-Y554F), F1: 5'-GCAGCGCATCCACGACTTCTACGAGCAC CGCTACC-3'; R1: 5'-GGTAGCGGTGCTCGT AGAAGTCGTGGATGCGCTGC-3'. All mutations were verified by DNA sequencing analysis.

Protein extraction, Immunoprecipitation, and Western Blot Analysis

Total protein extracts were prepared from transfected cells after 18–48 hrs incubation with CytoBuster Protein Extraction Reagent (Novagen). The protein concentration of the lysate was determined using the Bradford method. Equal amounts of total protein (1.0g) were incubated with a specific antibody for 1hr at 4°C, and protein A/G PLUS-agarose (Santa Cruz) was then

added and incubated overnight with gentle rock. The beads were washed extensively with cold PBS buffer, and resuspended in 2xlaemmli sample buffer. The immune complexes were separated by SDS-PAGE and analyzed by Western blot using an anti-HCN4 polyclonal antibody (Alomone), an anti-Src monoclonal antibody (GD11, Upstate Biotechnology) and an anti-active Src (Clone 28, Biosource), respectively. For quantification of bands, the X-ray films were scanned using a densitometer (Molecular Device) and signals were analyzed using ImageQuanT software. All protein experiments were repeated at least three times.

Drugs

Small molecule, 4-amino-5-(4-chlorophenyl)-7-(*t*-butyl)pyrazolo[3,4-*d*]pyrimidine (PP2), also known as AG 1897 (17), has been widely used in identifying the substrates of Src kinase family members (7,17,28). Its inactive analog, 4-amino-7-phenylpyrazol[3,4-*d*]pyrimidine (PP3), is used as a negative control to confirm the action of PP2 (7).

Heart Rate Measurement

Experiments for the isolated heart were performed as previously described (23) using a murine model that has been fully characterized (19). Animals were anesthetized with intraperitoneal sodium pentobarbital (50 mg/kg). The heart was superfused with a modified Krebs-Henseleit buffer (mM: NaCl 120, NaHCO₃ 25, KCl 4.7, KH₂PO₄ 1.2, CaCl₂ 2.5, MgSO₄ 1.2, glucose 15, and EDTA 0.05). The perfusate was equilibrated with 95% O₂-5% CO₂ at 37°C, giving a pH of 7.4. Heart rate was recorded on a PowerLab multi-channel data acquisition system and processed using PowerLab Chart 5, ADInstruments).

Whole Cell Patch Clamp Recordings

After 24–96h transfection cells with green fluorescence were selected for patch clamp experiments. The HEK293 cells were placed in a Lucite bath in which the temperature was maintained at 20°C ± 1°C by a temperature controller (Cell MicroControls, VA). I_f/I_{HCN4} currents were recorded using the whole-cell patch clamp technique with an Axopatch-200B amplifier. The pipettes had a resistance of 2–4 MΩ 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 contains (mM) 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-AP (2mM), was added to the external solution to inhibit the endogenous transient potassium current, which can overlap with and obscure i_f tail currents recorded at +20mV. Data were acquired by CLAMPEX and analyzed by CLAMPFIT (pClamp 8, Axon). The current traces are presented after leak subtraction.

Due to slow activation kinetics of HCN4 (4) and slower activation of its mutant channels, currents elicited by hyperpolarizing pulses near the threshold activation of the channel (e.g., –75mV to –95mV) did not reach steady states under 15-second pulse conditions we used for current recordings of HCN4 channels expressed in HEK293 cells. We thus either applied 20 to 60 seconds pulses (exemplified in Supplemental Figure 1) or fit the current traces to the steady states. The results are the same. In the case of fitting the current traces to the steady state, we divided the amplitude of the steady state current trace by that measured from 15-second current amplitude and obtained a ratio (always greater than 1). We then multiplied this ratio to the corresponding tail currents which were then used to construct the activation curves, calculation of the deactivation kinetics, and the channel conductance. Dash lines in patch clamp recordings indicate zero currents.

Only effects of PP2 after at least 10 minutes perfusion/incubation and 15–20 minutes washout from the same cell were considered. Data are shown as mean ± SE. The data comparison was considered statistically significant if $p < 0.05$ (by *Student's* test).

Results

Inhibition of Src decreases HCN4 channel activity

Figure 1 provides a representative set of recordings on HCN4 expressed in a HEK293 cell in the presence (incubation) (1A) and absence (washout) (1B) of 10 μ M PP2 in the same cell. Holding at -10 mV, the membrane was hyperpolarized for 15 seconds to the potentials ranging from -65 mV to -135 mV in 10mV increment, and stepped back to $+20$ mV for 2 seconds to record tail currents for channel deactivation. After washing out PP2, HCN4 currents are significantly increased. The inhibition of HCN4 current by PP2 was further confirmed by recording the current at -135 mV in the same cell in control solution, in the presence of PP2 after 10 minutes incubation, and after washout of PP2 (Supplemental Figure 2A). The inhibitory effect of PP2 on HCN4 was due to the reduced phosphorylation, not due to the direct action on the channel, since 10 μ M PP3 (an inactive isoform of PP2) did not affect HCN4 current after at least 10 minutes incubation (Supplemental Figure 2B).

Figure 1C shows the activation curves constructed from the data presented in 1A and 1B. The Boltzmann's fit yielded a midpoint ($V_{1/2}$) of -97 mV and -80 mV in the presence and absence of PP2, respectively. Averaging over 8 cells, the mean $V_{1/2}$ is -100 ± 2 mV with PP2 and -88 ± 2 mV without PP2. The 12mV difference is statistically significant ($p=0.0025$). On the other hand, no significant difference in the slope of activation curves was observed ($s=9.6 \pm 0.6$ with PP2 and 9.0 ± 0.5 without PP2). It is noticeable that the negative shift of activation curve induced by PP2 is accompanied by a negative shift of voltage threshold activation (V_{th}). The time-dependent inward current began to activate at -65 mV without PP2 (arrow in 1B) and at -75 mV with PP2 (arrow in 1A). This negative shift of V_{th} is in agreement with the negative shift in the midpoint of activation without changing the slope of activation curve.

The maximal whole-cell channel conductance also is decreased in the presence of PP2 (compare 1A and 1B). By dividing the tail currents by the driving force (the reversal potential was measured in each cell) we calculated the maximal whole-cell channel conductance (G_{max}) to be 16.4 ± 2.1 nS ($n=8$) without PP2 and 9.5 ± 0.8 nS ($n=8$) with PP2. The inhibition of PP2 on G_{max} is statistically significant ($p=0.01$).

We next examined the effects of PP2 on HCN4 activation and deactivation kinetics. The current traces at -135 mV and $+20$ mV were fit with one-exponential function to obtain the time constants for current activation at -135 mV and deactivation at $+20$ mV, respectively. The mean activation time constants at -135 mV are 1.59 ± 0.42 sec ($n=8$) without PP2 and 2.39 ± 0.31 sec ($n=8$) with PP2; and the mean deactivation time constants at $+20$ mV are 0.25 ± 0.07 sec ($n=8$) without PP2 and 0.41 ± 0.09 sec ($n=8$) with PP2. The average slowing effects of PP2 on both activation and deactivation are statistically insignificant ($p=0.16$ for activation and $p=0.22$ for deactivation time constants), although the slowing effect was apparent in each cell tested. If we calculate the percentage of PP2 slowing of activation and deactivation kinetics in each cell, we have the average slowing of $53.4 \pm 20\%$ ($n=8$) on current activation at -135 mV and $53.5 \pm 15\%$ ($n=8$) on deactivation at $+20$ mV (see Supplemental Figure 3B).

The voltage dependence of HCN4 activation time constants is shown in Fig. 4A. The slowing effects of PP2 on HCN4 are statistically significant near diastolic potentials (-75 mV, -85 mV, $p=0.04$ and 0.002 , respectively) but less significant at the rest of voltages tested, even the slowing is apparent for each cell studied.

Y531, not Y554, mediates Src modulation of HCN4 current

To unveil the molecular mechanism that underlies the effects of Src tyrosine kinases on HCN4 channel properties, we searched the tyrosine residues in the C-linker (between S6 and CNBD) region that are likely to be phosphorylated (see a schematic diagram in Supplemental figure

4). We substituted Tyr⁵³¹ with phenylalanine to obtain the Y531F mutant. Figure 2 shows in a HEK293 cell the Y531F currents in the presence (2A) and absence (washout) (2B) of PP2. The onset and tail currents were elicited by the same voltage protocol used in Fig. 1. PP2 still decreased the current amplitudes at all test voltages. The G_{max} is decreased from 3.1 nS (2B) to 2.5 nS by PP2 (2A). However, the V_{1/2} and the slope of activation are unchanged (PP2: V_{1/2} = -96 mV, s = 8.7; Washout PP2: V_{1/2} = -98 mV, s = 8.6). Averaging over 6 cells, the mean G_{max} is 6.2 ± 1.4 nS in control and 5.1 ± 1.6 nS in the presence of PP2 (p = 0.6). The percent inhibition of G_{max} is 11.0 ± 2.6%. The averaged V_{1/2} and slope are -95.3 ± 2.7 mV and 10.3 ± 1.0 in control and -97 ± 3.8 mV and 12.0 ± 1.0 in the presence of PP2 (p = 0.46). The Y531F channel activation and deactivation kinetics are also unchanged by PP2: τ_{act}(-135 mV) = 2.24 ± 0.24 sec in control, τ_{act}(-135 mV) = 2.10 ± 0.18 sec in the presence of PP2 (p = 0.66); τ_{deact}(+20 mV) = 0.324 ± 0.054 sec (n = 6) in control, τ_{deact}(+20 mV) = 0.321 ± 0.052 sec (n = 6) in the presence of PP2 (p = 0.97). The percent changes in time constants by PP2 are summarized in Supplemental Figure 3C. These data support the role of Tyr⁵³¹ in mediating the increased channel activity of HCN4 by Src tyrosine kinase.

It has been recently reported that the Src tyrosine kinase can speed HCN4 channel activation and the effect is mediated by Tyr⁵⁵⁴ (46). To investigate the different findings, we constructed the Y554F mutant and examined the effects of PP2 on its channel properties. Figure 3 shows a representative set of current recordings of Y554F channels expressed in a HEK293 cell. The currents were elicited by 15-second hyperpolarizing pulses from -65 mV to -135 mV (3B) or -65 mV to -145 mV (3A). The activation curves were plotted for control and PP2 and normalized to G_{max} (3C). To our surprise, the Boltzmann fit yielded the V_{1/2} of -80 mV for control and -98 mV for PP2, respectively, with no significant change in slope factor (s_{Y554F} = 14, s_{PP2} = 17). The mean values of V_{1/2} and slope are -88 ± 3 mV and 12 ± 1 for control and -101 ± 1 mV and 14 ± 1 for PP2 (n = 5). The 13 mV negative shift in V_{1/2} induced by PP2 is statistically significant (p = 0.01), which is close to the 12 mV negative shift of the wild type channel activation induced by PP2 (Fig. 1). The different actions of PP2 on Y531F and Y554F currents recorded at -135 mV are illustrated in the Supplemental Figures 2C and 2D in which PP2 has little effects on Y531F, whereas it reversibly inhibits Y554F.

We next examined the effects of PP2 on Y554F maximal whole-cell channel conductance and kinetics. Averaged G_{max} of Y554F was also decreased by PP2: 29.4 ± 2.6 nS for control and 20.4 ± 2.1 nS for PP2 (n = 6, p = 0.03). The percent inhibition of G_{max} by PP2 is 31 ± 2% (n = 6) (Supplemental Figure 3A). The mean activation time constant at -135 mV is 1.70 ± 0.20 sec for control and 1.96 ± 0.30 sec for PP2 (n = 6, p = 0.48). The mean deactivation time constants at +20 mV are 268 ± 23 msec for control and 260 ± 33 msec for PP2 (n = 6, p = 0.84). While statistically insignificant by comparing the means, the slowing of kinetics in each cell induced by PP2 is consistent for all cells tested. The percent slowing is 16 ± 6% (n = 6) for activation and 12 ± 4% for deactivation (n = 6), respectively (Supplemental Figure 3C).

The voltage dependence of Y531F and Y554F activation time constants is shown in Fig. 4B. The slowing effects of PP2 on Y554F are statistically significant near the diastolic potential (-75 mV, p = 0.04) but less significant at the rest of voltages tested. On the other hand, no significant slowing of kinetics by PP2 was observed on Y531F at the entire voltages in all cells tested. Figure 4D shows the average time constants of WT, Y531F, and Y554F activation. The slowing effect of channel activation is more significant for Y531F (white bar) than Y554F (dark bar) in comparison to the WT (dark grey bar).

It is worth pointing out that the inhibitory effects of PP2 on the gating properties of HCN4 and Y554F cannot be revealed if we normalize the currents to their maximal amplitudes (Supplemental Figure 5B and 5C), while the slowing effect of PP2 remains apparent (Supplemental Figure 5A).

Src phosphorylation on multiple tyrosine residues of HCN4

Given the existence of multiple tyrosine residues in HCN4 protein that can be potentially phosphorylated by tyrosine kinases, we wondered whether HCN4 mutants may still be phosphorylated since phosphorylation on different sites can induce different or no functional effects (46), as evidenced by PP2 actions on Y531F and Y554F. Using a phosphotyrosine specific antibody (4G10) to immunoprecipitate the samples followed by detection with a HCN4 antibody, figure 4A shows that tyrosine residues of Y531F and Y554F mutant channels expressed in HEK293 cells are still phosphorylated. Cells with no transfection (NT) or transfected with HCN4 used for direct immunoprecipitation using the same antibody are shown as controls (left two lanes of 5A). HCN4 Western analysis often shows two bands, non-glycosylated (near 130kD) and glycosylated (near 150kD) forms, using co-IP protocol (22). The specificity of HCN4 antibody has been previously tested (4).

PP2 inhibition of phosphorylation of HCN4 tyrosines

We have shown that PP2 inhibits HCN4 current on the assumption that the effect is mediated by inhibiting the Src kinase activity, which is indirectly supported by enhanced phosphorylation of HCN4 tyrosine by Src (4). To seek direct biochemical evidence, we show in figure 5B that in HEK293 cells expressing HCN4 channels the tyrosine phosphorylation of HCN4 channel protein is clearly attenuated after 15–30 minutes cells incubation with 10 μ M PP2 as compared to the control (0 min incubation with PP2).

Active Src in HEK293 cells

To become active and bind to its targets for phosphorylation, Src needs to be translocated to the membrane from the residing intracellular sites (32,35). Although Src is present in the HEK293 cells (4,20), it is unknown whether they are all in active forms. It has been shown that in other cell lines Src are primarily in inactive form (16) with only 5–10% being in active state (32). To examine the endogenous active Src kinase levels in HEK293 cells, we immunoprecipitated the cell lysate with clone 28 – an antibody that recognizes only the active form (dephosphorylated at Y529) of Src and followed by detection of Src signals with Src antibody. As shown in Fig. 5C, the active Src levels are nearly 50 times lower in HEK293 cells than in Src529 (a constitutively active form of Src tyrosine kinase (4)) transfected cells.

Reduced Src activity decreases I_f in HL-1 cells

If reduced Src tyrosine kinase activity is associated with the smaller HCN currents with slower activation kinetics, PP2 should also inhibit and slow i_f in myocytes. We tested this hypothesis using HL-1 cardiomyocytes that were originally derived from cardiac atrial cells (38). HL-1 myocytes exhibit spontaneous pacemaker activity at passages between 74–93 (30) and have been used for electrophysiological studies of pacemaker channels (30,46). Figure 6 shows that PP2 (10 μ M) can reversibly inhibit i_f and slow the activation kinetics. Averaging from 5 cells, i_f was decreased by $23.6 \pm 2.7\%$ and slowed by $60.4 \pm 4.1\%$ in the presence of PP2 at -110 mV.

PP2 reduces the mouse heart rate

To explore the potential physiological role of Src tyrosine kinases in the modulation of cardiac pacemaker activity, we wondered whether PP2 can decrease the heart rate via inhibiting HCN currents. Using a Langendorff-perfused mouse model (23), PP2 exhibited variable effects ranging from nearly 35% (one mouse), 9% (two mice), and 1–3% (three mice) inhibition on the resting heart rate. On the other hand, PP2 at 1 μ M can reproducibly reduce the isoproterenol increased heart rate by about 10% after 10 minutes perfusion ($9.6 \pm 1.9\%$, $n=4$). In comparison, ZD7288 (at 1 μ M acted as a selective i_f channel blocker (8)) reduced the heart rate close to 66% ($66 \pm 2\%$, $n=4$).

Discussion

Inhibition of HCN4 by decreased Src activity

Using a constitutively active Src (Src529) and a dominant negative Src (Src296), we have previously demonstrated that Src tyrosine kinase can phosphorylate HCN4 channel protein (3,4). Src induced phosphorylation of HCN4 channel protein can shift the voltage dependent activation to more positive potentials and speed the channel kinetics (3,4). In the present work, we used PP2 as a probe to study the tyrosine residue(s) that mediate the effects of Src tyrosine kinases on HCN4 in the same cells.

As expected, PP2 induced a negative shift of HCN4 activation and slowing of channel activation and deactivation by reducing the Src tyrosine kinase activity. The slowing of HCN4 activation is more significant near diastolic potentials, in agreement with our previous observation of acceleration of HCN4 activation by Src529 (4). Unexpectedly, PP2 also reduced the maximal whole-cell channel conductance, which was not observed in our previous studies on Src modulation of HCN4 in HEK293 cells (3,4). The difference could be due to the variable basal levels of active Src in HEK293 cells. Our results are also different from a recent report where the authors found that PP2 only slowed HCN4 kinetics without affecting the voltage-dependent activation (Zong et al., 46). The discrepancy may be caused by different methods used to study the effects of PP2 on HCN4 channel. Zong et al. recorded the currents in two separate groups of cells: one without PP2 as a control and the other incubated with PP2 for 10 minutes before patch clamp recordings; and the comparison between the two groups was performed using normalized currents (Fig. 1 in ref. 46). This approach masks the changes in the voltage dependence of channel activation and in whole-cell channel conductance (see Supplemental Figure 5).

Concerning the tolerance of cells and the current “rundown” (current amplitude decreases over repeated recordings) over prolonged whole-cell patch recordings (42), we first recorded currents in the presence of PP2 (10 minutes incubation), and then washed out PP2 for 15–20 minutes before recording the currents as a control. This method avoids the cell-to-cell variations and overestimate of the effects of PP2 due to current rundown, since current rundown can induce a hyperpolarizing shift of i_f voltage dependent activation (42).

The notion that the inhibition of HCN4 channel activity by PP2 is mediated by reduced Src phosphorylation on HCN4 tyrosines is further supported by the direct biochemical evidence in which the phosphorylation levels are diminished with PP2 incubation (Fig. 5B).

Mechanism of Src phosphorylation of HCN4

To pinpoint the tyrosine residues that mediate the modulation by Src tyrosine kinase of HCN4 channels, we studied the actions of PP2 on mutants with tyrosine substituted by phenylalanine at sites that can be potentially phosphorylated. If the tyrosine residue(s) mediates the Src effects on HCN4, the mutant should be insensitive to PP2. Our data have shown that Src induced effects on the gating of HCN4 channels can be largely abolished by Y531F mutant. This is different from a recent report (46) showing that Tyr⁵⁵⁴ is *the only functional site* that is phosphorylated by Src since the Y554F mutant *completely* abolished the sensitivity to PP2. By contrast, we found that PP2 can also affect the gating of Y554F channel. PP2 caused a similar hyperpolarizing shift for the voltage dependent activation of HCN4 and Y554F. It is noticed, however, that the percentage of PP2 inhibition is smaller in Y554F than in WT (Supplemental Figure 2). This is in agreement with the smaller effect in the reduction of channel conductance. Therefore, Tyr⁵⁵⁴ also contributes to the reduced channel conductance, in addition to the slowing of channel kinetics, in response to the reduced Src tyrosine kinase activity.

Both Y531F and Y554F mutants are still phosphorylated by tyrosine kinases, as being supported by the mass spectroscopy data (46) and the protein chemistry data presented in this work. There are other tyrosine residues on HCN4 channel proteins that can be potentially phosphorylated by tyrosine kinases (NetPhos database, <http://www.cbs.dtu.dk/services/NetPhos/>). The functions of those residues on the regulation of HCN4 channel properties are unclear.

Src modulation of i_f

We showed in this work that in HEK293 cells transfected with Src529 the active Src levels are about 50 times higher compared to non-transfected cells. This result may explain why in our previous studies we observed more significant effects with Src529 than with Src296 on the voltage dependent activation of HCN4 expressed in HEK293 cells (3,4). To test whether results obtained from HEK293 cells can be applied to myocytes, we employed HL-1 cardiomyocytes in which all four HCN isoforms are present (30). As expected, i_f is not only slowed, but also reversibly inhibited by PP2.

In addition, reduced heart rate in an isolated mouse model associated with the decreased Src tyrosine kinase activity provides further pharmacological evidence supporting the role for Src regulation of HCN channels. In comparison to ZD7288, PP2 exhibited less reduction of heart rate. It makes sense since Src has also been suggested in the modulation of other cardiac ion channels that can contribute to the decreased heart rate (20,9,10,35), whereas the inhibitory effect of ZD7288 reflects mostly the i_f contribution to the increased heart rate. The time PP2 takes to elicit its maximal effect on heart rate is comparable to that on HCN channels in HEK293 cells and i_f in HL-1 myocytes. The effect of PP2 on heart rate is reversible, which is also in agreement with PP2's actions on I_{HCN} and i_f . It is unclear why PP2 exhibited large variation on inhibiting resting heart rate (from 1–35%) in the mouse model. It is worth emphasizing that PP2's near zero effect on resting heart rate in three mice does not necessarily mean that PP2 has no effect on HCN channels *in vivo* since Src tyrosine kinase also modulates other ion channels. Src phosphorylation of one group of ion channels (including HCN channels) that contribute to the increased heart rate could counterbalance to that of another group of ion channels that contributes to the decreased heart rate.

Potential physiological implications of Src modulation of HCN channels

The hierarchy of cardiac pacemaker activity begins at sinoatrial node which paces at the highest rate (60–80 beat/min). The electrical conducting system Purkinje fibers also have spontaneous pacemaker activity which pace at slower rate (40–60 beat/min). The working ventricle paces at slowest rate (20–40 beat/min) under pathophysiological conditions. Associated with this tissue-specific pacemaker activity, the cardiac pacemaker current (i_f , which is generated by HCN channels) is activated around –40/–50mV in the sinoatrial node, about –70/–80mV in Purkinje fiber and at potentials more negative than –100mV in ventricular myocytes in physiological conditions (6,39,41–43). Thus, there exists –60mV to –70mV negative shift in i_f voltage-dependent activation between the sinoatrial node and ventricle. In addition, i_f activates around –70mV in neonatal rat ventricle and around –100mV/–110mV in adult rat ventricles, shifting its activation –30mV to –40mV during the development (27). The cellular and molecular factors that control this mobility of i_f activation are still unclear.

Previous studies have revealed some mechanistic insights into the regulation of HCN channels that may contribute to the voltage-dependent activation of i_f . Gating of HCN channels can be regulated by interacting with cAMP (36), other HCN isoforms (2), its β -subunit (MiRP1) (13,40), and by post-translational modulation (5,24). Most recently, new evidence has shown that the voltage dependent activation of HCN channels are also regulated by p38 (26), PIP2 (25,45), and 4 β -phorbol-12-myristate-13-acetate (14). The evidence presented in this work

provides a novel mechanism that Src tyrosine kinase might contribute to the positive shift of i_f voltage dependent activation in cardiac myocytes, since a shift in HCN channel activation in a depolarizing direction by enhanced Src tyrosine kinase activity provides more inward current at diastolic potentials, which can increase the phase-4 depolarization leading to the increased pacemaker activity (1).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The human HCN4 cDNA plasmid was originally provided as a gift by Dr. UB Kaupp (Institut für Biologische Informationsverarbeitung, Germany). This work was supported by grants from National Heart, Lung, and Blood Institute to HGY (HL-075023) and SJM (HL-027339).

References

1. Accili EA, Proenza C, Baruscotti M, DiFrancesco D. From funny current to HCN channels: 20 years of excitation. *News Physiol Sci* 2002;17:32–37. [PubMed: 11821534]
2. Altomare C, Terragni B, Brioschi C, Milanese R, Pagliuca C, Viscomi C, Moroni A, Baruscotti M, DiFrancesco D. Heteromeric HCN1-HCN4 channels: a comparison with native pacemaker channels from the rabbit sinoatrial node. *J Physiol* 2003;549:347–359. [PubMed: 12702747]
3. Arinsburg S, Pastor D, Torres G, Hallas B, El-Maghrabi MR, Cohen IS, Yu HG. Src changes gating of HCN4 channels through direct binding to the channel proteins. *Biophysical J* 2005;88:465A.
4. Arinsburg SS, Cohen IS, Yu HG. Constitutively active Src tyrosine kinase changes gating of HCN4 channels through direct binding to the channel proteins. *J Cardiovasc Pharmacol* 2006;47:578–586. [PubMed: 16680072]
5. Barbuti A, Gravante B, Riolfo M, Milanese R, Terragni B, DiFrancesco D. Localization of pacemaker channels in lipid rafts regulates channel kinetics. *Circ Res* 2004;94:1325–1331. [PubMed: 15073040]
6. Baruscotti M, DiFrancesco D. Pacemaker channels. *Ann N Y Acad Sci* 2004;1015:111–121. [PubMed: 15201153]
7. Bain J, McLauchlan H, Elliott M, Cohen P. The specificities of protein kinase inhibitors: an update. *Biochem J* 2003;371:199–204. [PubMed: 12534346]
8. BoSmith RE, Briggs I, Sturgess NC. Inhibitory actions of ZENECA ZD7288 on whole-cell hyperpolarization activated inward current (I_f) in guinea-pig dissociated sinoatrial node cells. *Br J Pharmacol* 1993;110:343–349. [PubMed: 7693281]
9. Bowlby MR, Fadool DA, Holmes TC, Levitan IB. Modulation of the Kv1.3 potassium channel by receptor tyrosine kinases. *J Gen Physiol* 1997;110:601–610. [PubMed: 9348331]
10. Browe DM, Baumgarten CM. Stretch of beta 1 integrin activates an outwardly rectifying chloride current via FAK and Src in rabbit ventricular myocytes. *J Gen Physiol* 2003;122:689–702. [PubMed: 14610020]
11. Chen S, Wang J, Siegelbaum SA. Properties of hyperpolarization-activated pacemaker current defined by coassembly of HCN1 and HCN2 subunits and basal modulation by cyclic nucleotide. *J Gen Physiol* 2001;117:491–504. [PubMed: 11331358]
12. Davis MJ, Wu X, Nurkiewicz TR, Kawasaki J, Gui P, Hill MA, Wilson E. Regulation of ion channels by protein tyrosine phosphorylation. *Am J Physiol Heart Circ Physiol* 2001;281:H1835–1862. [PubMed: 11668044]
13. Decher N, Bundis F, Vajna R, Steinmeyer K. KCNE2 modulates current amplitudes and activation kinetics of HCN4: influence of KCNE family members on HCN4 currents. *Pflügers Arch* 2003;446:633–640. [PubMed: 12856183]
14. Fogle KJ, Lyashchenko AK, Turbendian HK, Tibbs GR. HCN pacemaker channel activation is controlled by acidic lipids downstream of diacylglycerol kinase and phospholipase A2. *J Neurosci* 2007;27:2802–2814. [PubMed: 17360902]

15. Gauss R, Seifert R, Kaupp UB. Molecular identification of a hyperpolarization-activated channel in sea urchin sperm. *Nature* 1998;393:583–587. [PubMed: 9634235]
16. Goodsell DS. The molecular perspective: the src oncogene. *Oncologist* 2001;6:474–476. [PubMed: 11675527]
17. Hanke JH, Gardner JP, Dow RL, Changelian PS, Brissette WH, Weringer EJ, Pollok BA, Connelly PA. Discovery of a novel, potent, and Src family-selective tyrosine kinase inhibitor. Study of Lck- and FynT-dependent T cell activation. *J Biol Chem* 1996;271:695–701. [PubMed: 8557675]
18. Headrick JP, Hack B, Ashton KJ. Acute adenosinergic cardioprotection in ischemic-reperfused hearts. *Am J Physiol Heart Circ Physiol* 2003;285:H1797–1818. [PubMed: 14561676]
19. Headrick JP, Peart J, Hack B, Flood A, Matherne GP. Functional properties and responses to ischaemia-reperfusion in Langendorff perfused mouse heart. *Exp Physiol* 2001;86:703–716. [PubMed: 11698964]
20. Holmes TC, Fadool DA, Ren R, Levitan IB. Association of Src tyrosine kinase with a human potassium channel mediated by SH3 domain. *Science* 1996;274:2089–2091. [PubMed: 8953041]
21. Ludwig A, Zong X, Jeglitsch M, Hofmann F, Biel M. A family of hyperpolarization-activated mammalian cation channels. *Nature* 1998;393:587–591. [PubMed: 9634236]
22. Ma YC, Huang J, Ali S, Lowry W, Huang XY. Src tyrosine kinase is a novel direct effector of G proteins. *Cell* 2000;102:635–646. [PubMed: 11007482]
23. Morrison RR, Teng B, Oldenburg PJ, Katwa LC, Schnermann JB, Mustafa SJ. Effects of targeted deletion of A1 adenosine receptors on postischemic cardiac function and expression of adenosine receptor subtypes. *Am J Physiol Heart Circ Physiol* 2006;291:H1875–1882. [PubMed: 16679400]
24. Much B, Wahl-Schott C, Zong X, Schneider A, Baumann L, Moosmang S, Ludwig A, Biel M. Role of subunit heteromerization and N-linked glycosylation in the formation of functional hyperpolarization-activated cyclic nucleotide-gated channels. *J Biol Chem* 2003;278:43781–43786. [PubMed: 12928435]
25. Pian P, Bucci A, Robinson RB, Siegelbaum SA. Regulation of gating and rundown of HCN hyperpolarization-activated channels by exogenous and endogenous PIP₂. *J Gen Physiol* 2006;128:593–604. [PubMed: 17074978]
26. Poolos NP, Bullis JB, Roth MK. Modulation of h-channels in hippocampal pyramidal neurons by p38 mitogen-activated protein kinase. *J Neurosci* 2006;26:7995–8003. [PubMed: 16870744]
27. Robinson RB, Yu H, Chang F, Cohen IS. Developmental change in the voltage-dependence of the pacemaker current, *I_f*, in rat ventricle cells. *Pflugers Arch* 1997;433:533–535. [PubMed: 9000433]
28. Sanna PP, Berton F, Cammalleri M, Tallent MK, Siggins GR, Bloom FE, Francesconi W. A role for Src kinase in spontaneous epileptiform activity in the CA3 region of the hippocampus. *Proc Natl Acad Sci U S A* 2000;97:8653–8657. [PubMed: 10890901]
29. Santoro B, Liu DT, Yao H, Bartsch D, Kandel ER, Siegelbaum SA, Tibbs GR. Identification of a gene encoding a hyperpolarization-activated pacemaker channel of brain. *Cell* 1998;93:717–729. [PubMed: 9630217]
30. Sartiani L, Bochet P, Cerbai E, Mugelli A, Fischmeister R. Functional expression of the hyperpolarization-activated, non-selective cation current *I_f* in immortalized HL-1 cardiomyocytes. *J Physiol* 2002;545:81–92. [PubMed: 12433951]
31. Sato K, Fukami Y, Stith BJ. Signal transduction pathways leading to Ca²⁺ release in a vertebrate model system: lessons from *Xenopus* eggs. *Semin Cell Dev Biol* 2006;17:285–292. [PubMed: 16584903]
32. Shenoy S, Chackalaparampil I, Bagrodia S, Lin PH, Shalloway D. Role of p34cdc2-mediated phosphorylations in two-step activation of pp60c-src during mitosis. *Proc Natl Acad Sci U S A* 1992;89:7237–7241. [PubMed: 1379736]
33. Shi W, Wymore R, Yu H, Wu J, Wymore RT, Pan Z, Robinson RB, Dixon JE, McKinnon D, Cohen IS. Distribution and prevalence of hyperpolarization-activated cation channel (HCN) mRNA expression in cardiac tissues. *Circ Res* 1999;85:e1–6. [PubMed: 10400919]
34. Timpson P, Jones GE, Frame MC, Brunton VG. Coordination of cell polarization and migration by the Rho family GTPases requires Src tyrosine kinase activity. *Curr Biol* 2001;11:1836–1846. [PubMed: 11728306]

35. Tong Y, Brandt GS, Li M, Shapovalov G, Slimko E, Karschin A, Dougherty DA, Lester HA. Tyrosine decaging leads to substantial membrane trafficking during modulation of an inward rectifier potassium channel. *J Gen Physiol* 2001;117:103–118. [PubMed: 11158164]
36. Wainger BJ, DeGennaro M, Santoro B, Siegelbaum SA, Tibbs GR. Molecular mechanism of cAMP modulation of HCN pacemaker channels. *Nature* 2001;411:805–810. [PubMed: 11459060]
37. Weernink PA, Rijksen G. Activation and translocation of c-Src to the cytoskeleton by both platelet-derived growth factor and epidermal growth factor. *J Biol Chem* 1995;270:2264–2267. [PubMed: 7530720]
38. White SM, Constantin PE, Claycomb WC. Cardiac physiology at the cellular level: use of cultured HL-1 cardiomyocytes for studies of cardiac muscle cell structure and function. *Am J Physiol Heart Circ Physiol* 2004;286:H823–829. [PubMed: 14766671]
39. Wu J-Y, Yu H-G, Cohen IS. Epidermal growth factor increases if in rabbit SA node cells by activating a tyrosine kinase. 2000
40. Yu H, Wu J, Potapova I, Wymore RT, Holmes B, Zuckerman J, Pan Z, Wang H, Shi W, Robinson RB, El-Maghrabi MR, Benjamin W, Dixon J, McKinnon D, Cohen IS, Wymore R. MinK-related peptide 1: A beta subunit for the HCN ion channel subunit family enhances expression and speeds activation. *Circ Res* 2001;88:E84–87. [PubMed: 11420311]
41. Yu HG, Chang F, Cohen IS. Pacemaker current exists in ventricular myocytes. *Circ Res* 1993;72:232–236. [PubMed: 7678078]
42. Yu HG, Chang F, Cohen IS. Pacemaker current I_f in adult canine cardiac ventricular myocytes. 1995
43. Yu HG, Chang F, Cohen IS. Phosphatase inhibition by calyculin A increases if in canine Purkinje fibers and myocytes. *Pflugers Arch* 1993;422:614–616. [PubMed: 8385773]
44. Yu HG, Lu Z, Pan Z, Cohen IS. Tyrosine kinase inhibition differentially regulates heterologously expressed HCN channels. *Pflugers Arch* 2004;447:392–400. [PubMed: 14634823]
45. Zolles G, Klocker N, Wenzel D, Weisser-Thomas J, Fleischmann BK, Roeper J, Fakler B. Pacemaking by HCN channels requires interaction with phosphoinositides. *Neuron* 2006;52:1027–1036. [PubMed: 17178405]
46. Zong X, Eckert C, Yuan H, Wahl-Schott C, Abicht H, Fang L, Li R, Mistrik P, Gerstner A, Much B, Baumann L, Michalakakis S, Zeng R, Chen Z, Biel M. A novel mechanism of modulation of hyperpolarization-activated cyclic nucleotide-gated channels by Src kinase. *J Biol Chem* 2005;280:34224–34232. [PubMed: 16079136]

Figure 1. PP2 (10 μ M) on HCN4 currents in a HEK293 cell. A). HCN4 currents in the presence of PP2. B). PP2 was washed out. C). activation curves in the presence and absence of PP2.

Figure 2. Y531F currents in the presence (A) and absence (B) of PP2. Activation curves are shown in (C).

Figure 3. Y554F currents in the presence (A) and absence (B) of PP2. Activation curves are shown in (C).

Figure 4.

PP2 on activation kinetics of HCN4, Y531F, and Y554F. Average time constants of channel activation are plotted against test voltages for HCN4 (A), Y531F (B), and Y554F (C) in the absence and presence of PP2. D: Comparison of time constants for HCN4, Y531F, and Y554F channel activation.

Figure 5.

Tyrosine phosphorylation of HCN4 and mutants expressed in HEK293 cells. (A) Tyrosine phosphorylation of HCN4, Y531F, and Y554F: samples were immunoprecipitated using a 4G10 antibody followed by Western analysis by a HCN4 antibody. Cells with no transfection (NT) and the direct immunoprecipitation of HCN4 with the same HCN4 antibody serve a negative and a positive control, respectively. (B) PP2 inhibition of phosphorylation of HCN tyrosines: cells were treated for a time period for 0, 15min, 30min before being immunoprecipitated using a HCN4 antibody followed by Western detection by a 4G10 antibody. (C) Active Src levels in non-transfected (HEK) and Src529-transfected HEK293 cells detected by immunoprecipitation using Clone 28 followed by Western blotting using a Src antibody.

Figure 6.

PP2 on the hyperpolarization-activated current (i_f) in HL-1 cells. I_f was elicited by a step to -110 mV for 2 seconds from a holding potential of -50 mV. Control, PP2, and washout (Rec) are shown.