Src tyrosine kinase alters gating of hyperpolarization-activated HCN4 pacemaker channel through Tyr<sup>531</sup>

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Li C-H, Zhang Q, Teng B, Mustafa SJ, Huang J-Y, Yu H-G. Src tyrosine kinase alters gating of hyperpolarization-activated HCN4 pacemaker channel through Tyr<sup>531</sup>. Am J Physiol Cell Physiol 294: C355–C362, 2008.—We recently discovered that the constitutively active Src tyrosine kinase can enhance hyperpolarization-activated, cyclic nucleotide-gated (HCN) 4 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, 4-amino-5-(4-chlorophenyl)-7-(4-butyl)pyrazolo[3,4-d]pyrimidine (PP2), on HCN4 and its mutant channels expressed in HEK 293 cells by using a 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; Tyr<sup>531</sup> and Tyr<sup>554</sup>. Substituting HCN4-Tyr<sup>531</sup> with phenylalanine largely abolished the effects of PP2 on HCN4 channels. Replacing HCN4-Tyr<sup>554</sup> with 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’s actions on HCN4 channels is Tyr<sup>531</sup>.

hyperpolarization-activated, cyclic nucleotide-gated channels; 4-amino-5-(4-chlorophenyl)-7-(4-butyl)pyrazolo[3,4-d]pyrimidine

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<sub>AP</sub>) is a major contributor to the autonomous regulation of cardiac pacemaker activity in heart (1, 6). We have previously reported that the reduced tyrosine kinase activity inhibits I<sub>AP</sub> in sinoatrial node and ventricular myocytes as well as in HCN channels expressed in Xenopus oocytes (39, 44). Recently, we (3, 4) 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, whereas a negative dominant Src slows the HCN4 activation without apparent effect on the voltage dependence of HCN4 activation.

The effects of Src on HCN4 channel properties are mediated by phosphorylation via a possible binding to the HCN4 channel protein. A recent study (46) reported that inhibition of Src by 4-amino-5-(4-chlorophenyl)-7-(4-butyl)pyrazolo[3,4-d]pyrimidine (PP2) only slows HCN4 channel activation kinetics without affecting the voltage dependence of channel activation. Moreover, the phosphotyrosines that mediate acceleration of activation kinetics by Src were tracked to HCN4-Tyr<sup>531</sup> (46).

In this report, we searched a 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 the Src tyrosine kinase family, to study its effects on these mutant channels.

MATERIALS AND METHODS

Cell culture and transfection. Human embryonic kidney (HEK) 293 cells were grown in DMEM (Invitrogen), supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, and 100 μg/ml streptomycin. Cells with 90–95% confluence in a six-well plate were used for plasmid transfection with Lipofectamine 2000 (Invitrogen), and the plasmid phrGFP (Stratagene) was cotransfected 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, LA). Cells were maintained in “Claycomb medium” (38), supplemented with 10% fetal bovine serum (SAFC Biosciences), 2 mM l-glutamine, 100 μM norepinephrine, and 100 μM linopirdine-streptomyacin. The medium was changed every 24 h. 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% CO<sub>2</sub> and 95% air at a relative humidity of ~95%.

Plasmid preparation and site-directed mutagenesis. Human HCN cDNA was originally provided as a gift by Dr. U. B. Kaupp (Institut für Biologische Informationsverarbeitung, Jülich, Germany). Two single HCN4 mutants, designated Y531F and Y554F, were generated by replacing tyrosine residues at the positions 531 and 554, respectively, with phenylalanine by using Stratagene QuikChange II XL site-directed mutagenesis kits (Stratagene). Two pairs of oligonucleotide primers were used for mutagenesis: HCN4-Y531F, F1: 5'-CTGCTCACCTGTCAGACCTTTCTGTGATCAAGCCG-3'; R1: 5'-CGCAGTACCAGAAAAATTTCAAGCGGTGAGCG-3'; and HCN4-Y554F, F1: 5'-GGAGGCGATCCGAGCTTACCATGCGA-3'; R1: 5'-GGATACGGCGTCTGATGAAAAGGTCTC-3'. All mutations were verified by DNA sequencing analysis.

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Protein extraction, immunoprecipitation, and Western blot analysis. Total protein extracts were prepared from transfected cells after 18–48 h incubation with CytoBuster protein extraction reagent (Novagen). The protein concentration of the lysate was determined by using the Bradford method. Equal amounts of total protein (1 g) 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 extensively with cold PBS buffer and were resuspended in 2× Laemmli sample buffer. The immune complexes were separated by SDS-PAGE and were 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 by using a densitometer (Molecular Devices) and signals were analyzed by using ImageQuant software. All protein experiments were repeated at least three times.

Drugs. Small-molecule 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-phenylpyrazolo[3,4-d]pyrimidine (PP3), was 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) by using a murine model that has been fully characterized (19). Animals were anesthetized with intraperitoneal pentobarbital sodium (50 mg/kg). The heart was superfused with a modified Krebs-Henseleit buffer (in mM: 120 NaCl, 25 NaHCO3, 4.7 KH2PO4, 2.5 CaCl2, 1.2 MgSO4, 15 glucose, and 0.05 EDTA). 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 multichannel data-acquisition system and was processed by using PowerLab Chart 5 (ADInstruments).

Whole cell patch-clamp recordings. After 24–96 h transfection, cells with green fluorescence were selected for patch-clamp experiments. The HEK 293 cells were placed in a Lucite bath in which the temperature was maintained at 20 ± 1°C by a temperature controller (Cell MicroControls, Norfolk, VA). IHCN4 currents were recorded by 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 (in mM: 6 NaCl, 130 K-aspartate, 2 MgCl2, 5 CaCl2, 11 EGTA, and 10 HEPES; pH adjusted to 7.2 by KOH). The external solution contained (in mM) 120 NaCl, 1 MgCl2, 5 HEPES, 30 KCl, and 1.8 CaCl2, with pH adjusted to 7.4 with NaOH. 4-amino-pyrididine (2 mM), was added to the external solution to inhibit the endogenous transient outward potassium current (Ito), which can overlap with and obscure I1 tail currents recorded at +20 mV. Data were acquired by Clampex and analyzed by Clampfit (pCLAMP 8, Axon). The current traces are presented after leak subtraction.

Because of 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., −75 to −95 mV) did not reach steady states under the 15-s pulse conditions we used for current recordings of HCN4 channels expressed in HEK 293 cells. We thus either applied 20- to 60-s pulses (exemplified in Supplemental Fig. 1, available online at the American Journal of Physiology-Cell Physiology website) or fit the current traces to the steady states. The results were 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-s current amplitude and obtained a ratio (always >1). We then multiplied this ratio by the corresponding tail currents, which were then used for construction of the activation curves, for calculation of the deactivation kinetics, and for calculation of the channel conductance. Dashed lines in patch-clamp recordings indicate zero currents.

Only effects of PP2 after at least 10 min perfusion/incubation and 15–20 min washout from the same cell were considered. Data are shown as means ± SE. The data comparison was considered statistically significant if P < 0.05 (by Student’s t-test).

RESULTS

Inhibition of Src decreases HCN4 channel activity. Figure 1 provides a representative set of recordings on HCN4 expressed in a HEK 293 cell in the presence (incubation; Fig. 1A) and absence (washout; Fig. 1B) of 10 μM PP2 in the same cell. Held at −10 mV, the membrane was hyperpolarized for 15 s to potentials ranging from −65 to −135 mV in 10-mV increments and was stepped back to +20 mV for 2 s to record tail currents for channel deactivation. After PP2 was washed out, HCN4 currents were significantly incremented. 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 min incubation, and after washout of PP2 (Supplemental Fig. 2A). The inhibitory effect of PP2 on HCN4 was due to the reduced phosphorylation, not due to the direct action on the channel, because 10 μM PP3 (an inactive
isoform of PP2) did not affect HCN4 current after at least 10 min incubation (Supplemental Fig. 2B).

Figure 1C shows the activation curves constructed from the data presented in Fig. 1, A and B. The Boltzmann’s fit yielded a midpoint (V_{1/2}) of −97 and −80 mV in the presence and absence of PP2, respectively. Averaging over eight cells, the mean V_{1/2} was −100 ± 2 mV with PP2 and −88 ± 2 mV without PP2. The 12-mV difference is statistically significant (P = 0.0025). On the other hand, no significant difference in the slope of activation curves was observed (slope = 9.6 ± 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. The time-dependent inward current began to activate at −65 mV without PP2 (Fig. 1B) and at −75 mV with PP2 (Fig. 1A). This negative shift of voltage threshold activation is in agreement with the negative shift in the midpoint of activation without changing the slope of the activation curve.

The maximal whole cell channel conductance also is decreased in the presence of PP2 (Fig. 1, A vs. B). By dividing the tail currents by the driving force (the reversal potential was measured in each cell), we calculated the maximal whole 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 and +20 mV were fit with a 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 were 1.59 ± 0.42 s (n = 8) without PP2 and 2.39 ± 0.31 s (n = 8) with PP2, and the mean deactivation time constants at +20 mV were 0.25 ± 0.07 s (n = 8) without PP2 and 0.41 ± 0.09 s (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 an average slowing of 53.4 ± 20% (n = 8) on current activation at −135 mV and 53.5 ± 15% (n = 8) on deactivation at +20 mV (see Supplemental Fig. 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 and −85 mV, P = 0.04 and 0.002, respectively) but are less significant at the rest of voltages tested, although the slowing is apparent for each cell studied.

Ty^{531}, not Ty^{554}, 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 cyclic nucleotide binding domain) region that are likely to be phosphorylated (see the schematic diagram in Supplemental Fig. 4). We substituted Ty^{531} with phenylalanine to obtain the Y531F mutant. Figure 2 shows in a HEK 293 cell the Y531F currents in the presence (Fig. 2A) and absence (washout; Fig. 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} was decreased from 3.1 nS (Fig. 2B) to 2.5 nS by PP2 (Fig. 2A). However, the V_{1/2} and the slope of activation were unchanged (PP2: V_{1/2} = −96 mV, slope = 8.7; washout PP2: V_{1/2} = −98 mV, slope = 8.6). Averaging over six cells, the mean G_{max} was 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} was 11.0 ± 2.6%. The averaged V_{1/2} and slope were −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 (τ_{act}) and deactivation (τ_{deact}) kinetics were also unchanged by PP2: τ_{act}(−135 mV) = 2.24 ± 0.24 s in control; τ_{act}(−135 mV) = 2.10 ± 0.18 s in the presence of PP2 (P = 0.66); τ_{deact}(+20 mV) = 0.324 ± 0.054 s (n = 6) in control; τ_{deact}(+20 mV) = 0.321 ± 0.052 s (n = 6) in the presence of PP2 (P = 0.97).

The percent changes in time constants by PP2 are summarized in Supplemental Fig. 3C. These data support the role of Ty^{531} in mediating the increased channel activity of HCN4 by Src tyrosine kinase.

It has been recently reported (46) that the Src tyrosine kinase can speed HCN4 channel activation and that the effect is mediated by Ty^{554}. 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 HEK 293
cell. The currents were elicited by 15-s hyperpolarizing pulses from −65 to −135 mV (Fig. 3B) or −65 to −145 mV (Fig. 3A). The activation curves were plotted for control and PP2 and were normalized to $G_{\text{max}}$ (Fig. 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 ($Y554F = 14$; $PP2 = 17$). The mean values of $V_{1/2}$ and slope were $-88 \pm 3$ mV and $12 \pm 1$ for control and $-101 \pm 1$ mV and $14 \pm 1$ for PP2 ($n = 5$). The 13-mV negative shift in $V_{1/2}$ induced by PP2 was 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 Supplemental Fig. 2, $A$ and $B$, in which PP2 has little effect on Y531F, whereas it reversibly inhibits Y554F.

We next examined the effects of PP2 on Y554F maximal whole cell channel conductance and kinetics. Averaged $G_{\text{max}}$ 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 $G_{\text{max}}$ by PP2 is $31 \pm 2\%$ ($n = 6$; Supplemental Fig. 3A). The mean activation time constants at −135 mV were $1.70 \pm 0.20$ s for control and $1.96 \pm 0.30$ s for PP2 ($n = 6$, $P = 0.48$). The mean deactivation time constants at +20 mV were $268 \pm 23$ ms for control and $260 \pm 33$ ms for PP2 ($n = 6$, $P = 0.84$). Although statistically insignificant by comparing the means, the slowing of kinetics in each cell induced by PP2 was consistent for all cells tested. The percent slowing was $16 \pm 6\%$ ($n = 6$) for activation and $12 \pm 4\%$ for deactivation ($n = 6$), respectively (Supplemental Fig. 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 the voltages tested. On the other hand, no significant slowing of kinetics by PP2 was observed on Y531F within the entire voltage range in all cells tested. Figure 4D shows the average time constants of wild-type, Y531F, and Y554F activation. The slowing effect of channel activation was more significant for Y531F than Y554F compared with the wild type.

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 Fig. 5B and 5C), whereas the slowing effect of PP2 remains apparent (Supplemental Fig. 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 because phosphorylation on different sites can induce different or no functional effects (46), as evidenced by PP2’s actions on Y531F and Y554F. With the use of a phosphotyrosine-specific antibody (4G10) to immunoprecipitate the samples followed by detection with a HCN4 antibody, Fig. 4A shows that tyrosine residues of Y531F and Y554F mutant channels expressed in HEK 293 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 (Fig. 5A). HCN4 Western blot analysis often shows two bands, nonglycosylated (near 130 kDa) and glycosylated (near 150 kDa) forms, using coimmunoprecipitation 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 Fig. 5B that in HEK 293 cells expressing HCN4 channels the tyrosine phosphorylation of HCN4 channel protein is clearly attenuated after 15–30-min incubation with 10 μM PP2 compared with the control (0 min incubation with PP2).

Active Src in HEK 293 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 HEK 293 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 the active state (32). To examine the endogenous active Src kinase levels in HEK 293 cells, we immunoprecipitated the cell lysate with clone 28, an antibody that recognizes only the active form (dephosphorylated at Tyr529) 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 HEK 293 cells than in Src529 [a constitutively active form of Src tyrosine kinase (4)]-transfected cells.

Reduced Src activity decreases If 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 If in myocytes. We tested this hypothesis by using HL-1 cardiomyocytes that were originally derived from cardiac atrial cells (38). HL-1 myocytes exhibit spontaneous pacemaker activity at passages 74–93 (30) and have been used for electrophysiological studies of pacemaker channels (30, 46). Figure 6 shows that PP2 (10 μM) can reversibly inhibit If and slow the activation kinetics. Averaging from five cells, If was decreased by 23.6 ± 2.7% and slowed by 60.4 ± 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) to 9% (two mice) and 1–3% (three mice) inhibition of the resting heart rate. On the other hand, PP2 at 1 μM can reproducibly reduce the isoproterenol-increased heart rate by ∼10% after 10 min perfusion (9.6 ± 1.9%, n = 4). In comparison, ZD-7288 (1 μM) [which at 1 μM acted as a selective If channel blocker (8)] reduced the heart rate close to 66% (66 ± 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 can 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 HEK 293 cells (3, 4). The difference could be due to the variable basal levels of active Src in HEK 293 cells. Our results are also different from a recent report [Zong et al. (46)] where the authors found that PP2 only slowed HCN4 kinetics without affecting the voltage-dependent activation. The discrepancy may be caused by different methods used to study the effects of PP2 on HCN4 channel kinetics, 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 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 mediate the effects of Src tyrosine kinases on HCN4 in the same cells.

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 min incubation) and then washed out PP2 for 15–20 min 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, because current rundown can induce a hyperpolarizing shift of \( I _ { H } \) 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 because 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. We noticed, however, that the percentage of PP2 inhibition is smaller in Y554F than in wild type (Supplemental Fig. 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 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 (see the NetPhos database at http://www.cbs.dtu.dk/services/NetPhos/). The functions of those residues on the regulation of HCN4 channel properties are unclear.

Src modulation of If. We showed in this work that in HEK 293 cells transfected with Src529, the active Src levels are ~50 times higher compared with nontransfected 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 HEK 293 cells (3, 4). To test whether results obtained from HEK 293 cells can be applied to myocytes, we employed HL-1 cardiomyocytes in which all four HCN isoforms are present (30). As expected, If 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. Compared with ZD-7288, PP2 exhibited less reduction of heart rate. It makes sense because Src has also been suggested in the modulation of other cardiac ion channels that can contribute to the decreased heart rate (9, 10, 20, 35), whereas the inhibitory effect of ZD-7288 reflects mostly the If contribution to the increased heart rate. The time PP2 takes to elicit its maximal effect on heart rate is comparable with that on HCN channels in HEK 293 cells and If in HL-1 myocytes. The effect of PP2 on heart rate is reversible, which is also in agreement with PP2’s actions on HCN and If. It is unclear why PP2 exhibited large variation on inhibiting resting heart rate (from 1 to 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 because 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 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 the sinoatrial node, which faces at the highest rate (60–80 bpm). The electrical conducting system Purkinje fibers also have spontaneous pacemaker activity, which faces at a slower rate (40–60 bpm). The working ventricle paces at the slowest rate (20–40 bpm). Associated with this tissue-specific pacemaker activity, If contribution to the increased heart rate could contribute to the decreased heart rate.

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