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

ROLES OF Src, Fyn, AND Yes TYROSINE KINASES

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 lowest 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 Ca²⁺-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 \( I_p \) 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 \( I_H \) 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 \( I_H \) by binding to the cyclic nucleotide-binding domain of the HCN channels, respectively (7).

Other important mechanisms for the modulation of \( I_H \) include long QT (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 \( I_H \) 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 \( \alpha \) (RPTPα) 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, 16). 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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stituting aspartic acid with asparagine using PCR. HCN4-D553N-DsRed was made by subcloning HCN4-D553N into the DsRed vector. RPTPα 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, FynY531, and YesY537 in the text for interchangeable use with the constitutively active form of each kinase: Src529F, 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 CellLytic 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× 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 μg/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 MΩ 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_{in} 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 ± 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×/0.75 objective or a Plan-Apochromat 63×/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 RPTPα**—We have recently demonstrated that RPTPα 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 RPTPα may also inhibit the surface expression of HCN4. Fig. 1 shows a typical current
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 RPTPα 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, RPTPα almost eliminated the current expression of HCN4 (Fig. 1C). The effect of RPTPα on HCN4 current expression is similar to that on HCN2 current expression (17). As a control, the empty vector, pRK5 (used to subclone RPTPα), 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 RPTPα**—Wondering whether HCN4 current inhibition of RPTPα 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 RPTPα (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 RPTPα (left lane). These results suggested that the altered membrane expression of HCN4 is possibly caused by the increased or decreased tyrosine phosphorylation of the channel protein by Src tyrosine kinase and RPTPα 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.
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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 RPTPα, 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 points (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 expression 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 ~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 Src kinases on D553N gating, we examined the biophysical properties of D553N co-expressed with Src/Fyn/Yes (Fig. 4I) in comparison.
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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 RPTPα (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
significant on D553N surface expression, as evidenced by a significant expression of D553N. However, Fyn has been much less effective in rescuing HCN4 from loss of surface expression at the plasma membrane by Src/Fyn/Yes kinases.

The cellular evidence for the functional rescue of D553N current expression was 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.

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 RPTPα (17). The inhibited HCN2 current expression was due to the reduced surface expression of HCN2 channels via association between RPTPα 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.

**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, \( I_{\text{f}} \), 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 \( I_{\text{f}} \).

**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.
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 nearby tyrosine residues from being phosphorylated. Asso-
ciation 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 Asp475 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 inter-residue 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 Tyr544) 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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