Modulation of the Cardiac Sodium Channel Na\textsubscript{v}1.5 by Fyn, a Src Family Tyrosine Kinase

Christopher A. Ahern, Ji-Fang Zhang, Marilyn J. Wookalis, Richard Horn

Abstract—Dynamic modulation of ion channels can produce dramatic alterations of electrical excitability in cardiac myocytes. This study addresses the effects of the Src family tyrosine kinase Fyn on Na\textsubscript{v}1.5 cardiac sodium channels. Sodium currents were acquired by whole cell recording on HEK-293 cells transiently expressing Na\textsubscript{v}1.5. Acute treatment of cells with insulin caused a depolarizing shift in steady-state inactivation, an effect eliminated by the Src-specific tyrosine kinase inhibitor PP2. Sodium channels were coexpressed with either constitutively active (Fyn\textsuperscript{CA}) or catalytically inactive (Fyn\textsuperscript{KD}) variants of Fyn. Fyn\textsuperscript{CA} caused a 10-mV depolarizing shift of steady-state inactivation compared with Fyn\textsuperscript{KD} without altering the activation conductance-voltage relationship. Comparable effects of these Fyn variants were obtained with whole-cell and perforated-patch recording. Tyrosine phosphorylation of immunoprecipitated Na\textsubscript{v}1.5 was increased in cells expressing Fyn\textsuperscript{CA} compared with Fyn\textsuperscript{KD}. We show that Fyn is present in rat cardiac myocytes, and that Na\textsubscript{v}1.5 channels from these myocytes are tyrosine-phosphorylated. In HEK-293 cells the effect of Fyn\textsuperscript{CA} on Na\textsubscript{v}1.5 inactivation is abolished by the single point mutation Y1495F, a residue located within the cytoplasmic linker between the third and fourth homologous domains of the sodium channel. We provide evidence that this linker is a substrate for Fyn in vitro, and that Y1495 is a preferred phosphorylation site. These results suggest that cardiac sodium channels are physiologically relevant targets of Src family tyrosine kinases.

Key Words: cardiac sodium channel ■ tyrosine kinase ■ Src ■ Fyn ■ phosphorylation

Voltage-gated sodium channels play a pivotal role in cardiac excitability by controlling the upstroke of the cardiac action potential. These ion channels display exquisite sensitivity to changes in membrane potential and, as a consequence, minor alterations in their biophysical properties can have dramatic effects on cardiac function. Multiple isoforms of sodium channels are expressed in the heart, including the neuronal isoforms Na\textsubscript{v}1.1, 1.3, and 1.6, which can have been shown to reside within T-tubules and participate in excitation-contraction coupling.\textsuperscript{1} However, in terms of conducting the cardiac action potential, the tetrodotoxin-resistant isoform, Na\textsubscript{v}1.5, is dominant in both its crude expression level and its contribution to the myocytes’ sodium current.\textsuperscript{1} For this reason, identification of regulatory factors that modify the behavior of Na\textsubscript{v}1.5 is crucial in understanding cardiac excitability.

Both Src family and receptor tyrosine kinases are known to be potent modulators of ion channels (see reviews\textsuperscript{2,3}) Moreover, tyrosine kinase activity is enlisted in numerous signal transduction pathways. For example, multiple ligands such as insulin result in elevated tyrosine kinase activity in the heart.\textsuperscript{4,5} Relatively little is known, however, about the modulation of voltage-gated sodium channels by tyrosine kinases. In pheochromocytoma cells, acute application of receptor tyrosine kinase agonists results in decreased sodium current, caused by a hyperpolarizing shift in the voltage dependence of steady-state inactivation.\textsuperscript{6} Moreover, sodium channels from brain microsomes form a complex with the receptor protein tyrosine phosphatase β (RPTPβ), and activation of RPTPβ shifts the voltage dependence of inactivation to depolarized potentials.\textsuperscript{7} Although different mechanisms may be at play, in each case, tyrosine phosphorylation of these sodium channels is associated with a hyperpolarizing shift in steady-state inactivation, resulting in fewer available channels for generating an action potential. Limited evidence suggests that cardiac sodium currents behave oppositely from their neuronal counterparts. Specifically, the application of tyrosine kinase inhibitors to cardiac myocytes causes a hyperpolarizing shift in the inactivation-voltage relationship, suggesting that the phosphorylated form of the cardiac channel displays enhanced excitability.\textsuperscript{8} We describe in this study the effects of the Src family kinase Fyn on the cardiac sodium channel Na\textsubscript{v}1.5, and Fyn’s potential target residue in the channel’s cytoplasmic inactivation gate.

Materials and Methods

DNA Clones, Transfection, and Cell Culture

Na\textsubscript{v}1.5 and the Na\textsubscript{v}1.5 Y1494F, Na\textsubscript{v}1.5 Y1495F point mutants were generated as described previously.\textsuperscript{9} A C-terminal 14-amino-acid V5 tag was introduced using standard cloning procedures.\textsuperscript{10} V5 had no

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effect on the fast inactivation of the sodium currents (data not shown). The pHOOK, pCS2-c-Fyn(A), and c-FynKD clones were gifts from Dr T. Holmes and Alberto Llamas (New York University, New York, NY). Calcium phosphate was used to transiently transfected HEK-293 cells. For electrophysiology, cells cotransfected with pHOOK (Invitrogen) were identified by their binding to pHOX (4-ethoxymethylene-2-phenyl-2-oxazolin-5-one)–coated 2.0 to 2.5 μm magnetic beads (Spherotech), which were synthesized using previously described methods.12

Tissue Lysates, Immunoprecipitation, and Western Blots

HEK-293 cells were lysed ∼48 hours after transfection in ice-cold lysis buffer containing (in mmol/L) 25 TRIS, 150 NaCl, 100 CsF, 100 NaF, 5 EDTA, 1 Na3VO4, and 1% Triton X-100 (pH 7.5), supplemented with a protease inhibitor cocktail (Roche), and lysates clarified by centrifugation. For antiphosphotyrosine assays, cells were supplemented with 100 μmol/L peroxide-treated Na3VO4, 5 minutes before lysis. Minced adult rat hearts were homogenized 3×10 seconds at 10K with a Polytron homogenizer in lysis buffer without Triton X-100 and centrifuged to remove nuclei, then membranes pelleted by centrifugation at 50 000g. The membrane pellet was resuspended in lysis buffer containing 1% Triton X-100, then clarified by centrifugation. The resulting supernatant was assayed for protein using the Bradford assay.

Transfected sodium channels were immunoprecipitated with anti-V5 antibody (Invitrogen), whereas tyrosine-phosphorylated protein from rat heart was immunoprecipitated with the 4G10 antibody (Upstate) from 500 μg lysate with protein G agarose (Sigma Chemical). Immunoprecipitated proteins were separated by SDS-PAGE and transferred to PVDF membranes (Millipore). Blots were probed with antibodies against V5, Fyn (Transduction Laboratories), or the nonconserved I-II loop of NaV1.5 (Chemicon), and detection was by enhanced chemiluminescence.

The top panel of Figure 1A shows a representative V5 Western blot of HEK-293 lysates from cells nontransfected (NT), expressing NaV1.5 alone, or coexpressed with either FynCA (Constitutively Active) or FynKD (Kinase Dead). NaV1.5 is robustly expressed in HEK-293 cells and cotransfection with NaV1.5-NT or transfected with NaV1.5 alone or in combination with either FynCA or FynKD. Bottom, Fyn blot of HEK-293 cells either nontransfected or expressing either FynCA or FynKD, as well as 20 ng of an isolated cardiac membrane preparation. Each blot was representative of 3 separate experiments. B, Fyn mutants are functional. Five micrograms of whole-cell lysates (Bradford-assayed) from indicated cell types were exposed to the anti-phosphotyrosine 4G10 antibody.

Fusion Proteins and Autoradiogram Procedures

The sodium channel III-IV loop amino acids Gly1481 to Asp1523 were cloned into the pQE-30 Xa vector (Qiagen) resulting in a fusion protein containing a N-terminal 6-histidine repeat followed by the sequence: GSQSGQIEGRPFTGSLGQDFIPTEEQKYYNA-MKKLGSKKPQYKPRPLNKQFGIDFKLN. The Tyr-to-Phe mutations at Y1494 and Y1495 were produced sequentially with Quickchange site-directed mutagenesis (Stratagene). All clones contained an additional mutation at Y1517F. Fusion proteins (2 μg), isolated on Ni-NTA columns (Qiagen), were incubated with 5 ng recombinant Fyn (Invitrogen) in a buffer containing (in mmol/L) 25 Tris-HCl, 30 MgCl2, 5 MnCl2, 0.5 EGTA, 0.05 Na3VO4, 0.5 DT, pH 7.2, and 2.5 μCi 32P-ATP. Reactions were incubated for 1 hour at 30°C, separated on 16.5% Tris-Tricine gels (BioRad), transferred to PVDF membranes and quantified with a phosphorimager.

Electrophysiology

In most experiments, standard whole-cell recording methods13 were used to record ionic currents. The patch pipette contained (in mmol/L) 105 CsF, 35 NaCl, 10 EGTA, and 10 Hepes (pH 7.4). The bath contained (in mmol/L) 150 NaCl, 2 KCl, 1.5 CaCl2, 1 MgCl2, and 10 Hepes (pH 7.4). Liquid junction potentials between the bath and the pipette solution were corrected. Electrode resistance was in the range of 1 to 1.5 MΩ. Data were collected 10 to 15 minutes after the establishment of the whole cell configuration. In one set of experiments (Figure 5), perforated patch recording was used with the antibiotic amphotericin B, according to previously described methods.14 The pipette solution contained (in mmol/L) 75 CsSO4, 35 NaCl, 20 CsCl, 8 MgCl2, and 10 Hepes (pH 7.4) (junction potential = −7.9 mV). The access resistance at the time of recording ranged from 2.0 to 8.7 MΩ. The voltage errors because of series resistance in both standard whole cell and perforated patch recordings were always <3 mV after compensation. All experiments were performed at room temperature.

Data Analysis

Data were analyzed using pCLAMP (Axon Instruments) and ORIGIN 7.0 (OriginLab). Throughout the article, error bars represent the standard error of the mean. G-V relations were fitted to the Boltzmann equation:

$$G(V) = \frac{1}{1 + \exp\left(-g(V - V_{mid})/RT\right)}$$

where $RT/F = 25$ mV at room temperature.

Results

Acute Modulation of NaV1.5 by Insulin and Reversal of This Effect by PP2

We investigated whether tyrosine kinases modulate the cardiac sodium channel by transiently expressing the NaV1.5...
isoform in HEK-293 cells and assaying the expressed channels’ response to 1 μg/mL insulin, a concentration ~2-fold higher than the effective dose in vascular endothelium. The insulin receptor endogenous to HEK-293 cells is a receptor tyrosine kinase that has been shown to activate Src family tyrosine kinases. Bisindolylmaleimide (100 nmol/L) was present in both pretreatment and bath solutions to ablate any competing effects of conventional protein kinase C, whose activation has been implicated in the insulin response pathway of HEK-293 cells. The effects of insulin incubation on expressed cardiac sodium channels were ascertained by recording sodium currents in the whole-cell patch clamp configuration.

Figure 2A shows the effects of insulin and of the specific Src family kinase inhibitor PP2 on families of sodium currents resulting from depolarizing steps from a holding potential of −140 mV. Steady-state inactivation curves are shown in Figure 2B. Insulin pretreatment caused a small but significant depolarizing shift of the inactivation midpoint (V_{mid}) from −95.0 ± 2.4 mV to −89.2 ± 1.9 mV for 7 control and 10 insulin-treated cells, respectively (P = 0.039). This result is consistent with hyperpolarizing shifts caused by specific inhibitors of Src family kinases on cardiac myocytes. It is possible that this modulation is because of the tyrosine kinase activity of the insulin receptor itself or one of its downstream effectors. If the modulation is because of the activation of an endogenous Src family tyrosine kinase, the effect should be blocked by PP2. Consistent with this, 1 μmol/L PP2 completely reversed the insulin-induced depolarizing shift back to control levels, V_{mid} = −96.2 ± 1.6 mV (n = 6), even though insulin was present in pretreatment and bath solution (P < 0.01 for PP2 + insulin versus insulin alone; Figure 2A and 2B). Figure 2C displays the peak activation conductance-voltage (G-V) relationships for the 3 classes of cell treatments. In contrast to the modulation of sodium channel inactivation properties, no evidence of either the insulin or PP2 treatment was seen in the G-V curves. The rightward shift of inactivation alone by insulin should produce an enhanced window current, ie, an increased overlap of steady-state activation and inactivation curves, which would result in enhanced excitability in the region of the resting potential. These results suggest that the Na_{a1.5} sodium channel is a target of a Src family tyrosine kinase.

**Modulation of Na_{a1.5} by Fyn Is Manifested in Inactivation Properties**

To test whether Na_{a1.5} can be modulated by a Src family kinase, we coexpressed Na_{a1.5} in HEK-293 cells with either of 2 mutants of the kinase Fyn. The constitutively active mutant, FynCA, lacks its inhibitory carboxyl terminus, whereas the “kinase-dead” mutant, FynKD, has a single point mutation at K299M rendering it catalytically inactive. Figure 3A shows families of sodium currents from cells expressing Na_{a1.5} alone or with either of the 2 Fyn mutants. Consistent with our experiments with insulin, channel coexpression with FynCA causes an 11-mV depolarizing shift in the steady-state inactivation curve (Figure 3B) from an average V_{mid} of −100.4 ± 2.0 mV for 9 FynKD cells to −89.4 ± 0.9 mV for 12 FynCA cells (P < 0.001). The steady-state inactivation curve for Na_{a1.5} alone (9 cells) is shown as a line without symbols for clarity. This line is a Boltzmann fit of the Na_{a1.5} data and is bounded on the right and left by the mutant kinases, suggesting that a baseline tyrosine phosphorylation of the channel exists when it is expressed alone in HEK-293 cells. This result also suggests that FynKD is capable of displacing endogenous Fyn from binding sites in the vicinity of its phosphorylation targets in the sodium channel. There were no significant differences in the G-V relationships for the 3 cell types shown (Figure 3C).

Although coexpression of FynCA clearly shifts the steady-state inactivation curve, it has a smaller effect on the rate of fast inactivation. Figure 3D shows that FynCA causes a slight slowing of fast inactivation. This difference is statistically insignificant (P > 0.05). Consistent with the depolarizing shift of steady-state inactivation, however, the rate of recovery from inactivation was increased in Na_{a1.5} channels coexpressed with FynCA (Figure 4). Moreover, channels in the presence of FynCA had a decreased entry rate into a slow-inactivated state (Figure 3E). Here, significance (P < 0.01) is shown as an asterisk at the relevant time point. These results, like those with insulin, provide evidence for modulation of Na_{a1.5} by a tyrosine kinase.

Our whole-cell recordings use a pipette solution containing 105 mmol/L CsF. To test whether our conclusions might be confounded by fluoride, a known inhibitor of serine-threonine phosphatases, we examined the effects of these 2 Fyn variants on Na_{a1.5} currents in the absence of fluoride using perforated patch recording, a technique that causes less perturbation of the intracellular milieu than whole-cell recording. Figure 5 shows that, in agreement with our standard whole-cell recording, FynCA produces a 10-mV depolarizing shift of steady-
state inactivation without affecting the activation $G-V$ relationships.

Tyrosine kinase modulation of an ion channel may be mediated either by phosphorylation of a tyrosine residue or by the allosteric consequences of the kinase binding to the channel.\textsuperscript{18} In our case, the latter of these 2 possibilities is unlikely, given that both of our kinase mutants contain unperturbed SH3 binding domains and differ only in their ability to phosphorylate tyrosine residues.\textsuperscript{11} Furthermore, biophysical consequences of the expressed \textsc{Fyn$^{CA}$} on sodium channel inactivation were similar to those observed for an acute insulin treatment, suggesting that these effects are because of direct tyrosine phosphorylation of the channel rather than alterations of the channel protein during biogenesis and trafficking.

**Biochemical Evidence for Tyrosine Phosphorylation of Na$_{v}$.1.5**

Figure 6A shows the results of immunoprecipitation of V5-tagged Na$_{v}$.1.5 channels from cells also expressing \textsc{Fyn$^{CA}$} or \textsc{Fyn$^{KD}$}, an experiment designed to test whether kinase

\begin{figure}[h]
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\includegraphics[width=\textwidth]{figure3}
\caption{Coexpression of Fyn alters the inactivation properties of Na$_{v}$.1.5. A, Representative families of sodium currents from the indicated cell types elicited with depolarizing steps in 10-mV increments from $-100$ to $+60$ mV from a holding potential of $-140$ mV. Scale bar represents 5 ms and 2 nA. B, Steady-state inactivation. Solid line in B corresponds to a Boltzmann fit, $V_{\text{mid}}=-94.9\pm2.4$ mV, of Na$_{v}$.1.5 cells expressed alone with the symbols removed for clarity. Symbols for \textsc{Fyn$^{CA}$} and \textsc{Fyn$^{KD}$} expressing cells correspond to the indicated cell types in A. C, Conductance-voltage curves for the same cell types with no significant differences in either $V_{\text{mid}}$ or slope. D, Time course of fast inactivation from currents produced by a depolarization from $-140$ mV to the indicated voltage was fit with a single exponential. Although currents originating from cells expressing Na$_{v}$.1.5 and \textsc{Fyn$^{CA}$} tended to display slower inactivation, these differences were insignificant ($P>0.05$) at all voltages. E, Entry into the slow inactivated state was assayed using a standard two-pulse protocol (inset) with the duration of the conditioning pulse to 0 mV indicated on the abscissa and the current during the test pulse to 0 mV for 20 ms normalized on the ordinate. Holding potential, $-120$ mV. Reset at $-120$ mV, 50 ms. Na$_{v}$.1.5 currents from cells coexpressing \textsc{Fyn$^{CA}$} when compared with \textsc{Fyn$^{KD}$} were reluctant to enter the slow inactivated state as evidenced by larger test currents after prepulses of 1, 10, 15, and 20 seconds in duration ($P<0.01$).

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\caption{Fyn speeds recovery from inactivation. Normalized time course of recovery from inactivation at $-120$ mV for WT channels either coexpressed with \textsc{Fyn$^{CA}$} (\textbullet, $n=8$ cells) or in the presence of tyrosine kinase inhibitors (1 \textmu mol/L PP2+100 \textmu mol/L Tyrophostin AG957; \textbullet, $n=6$ cells). Degree of inactivation during the 8-ms prepulse to 0 mV (more than 96% complete) was the same in both classes of cells examined. Data fit to a single exponential with time constants of 12.9 ms and 29.1 ms for \textsc{Fyn$^{CA}$} and kinase inhibited cells, respectively.

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\caption{Fyn produces similar biophysical effects during perforated patch recording. All experiments were done in the presence of bisindolylmaleimide (100 nmol/L). Cells coexpressing \textsc{Fyn$^{KD}$} were additionally exposed to the specific tyrosine kinase inhibitors PP2 (1 \textmu mol/L) and Tyrophostin AG957 (100 \textmu mol/L; Calbiochem). A, Families of sodium currents elicited with depolarizing steps in 5-mV increments from $-90$ mV to $+30$ mV from a holding potential of $-120$ mV. Scale bars represent 4 ms and 2 nA (left), and 4 ms and 4 nA (right). B, Steady-state inactivation curves. $V_{\text{mid}}$'s for \textsc{Fyn$^{CA}$} ($-77.0\pm2.5$ mV, $n=4$) and \textsc{Fyn$^{KD}$} ($-86.9\pm0.9$ mV, $n=3$) are significantly different ($P<0.03$). C, Peak activation $G$-$V$'s for \textsc{Fyn$^{CA}$} ($-40.2\pm1.8$ mV, $n=3$) and \textsc{Fyn$^{KD}$} ($-45.9\pm1.8$ mV, $n=3$) are not significantly different.
affects the levels of tyrosine phosphorylation of the coexpressed sodium channel. The V5 Western blots show that sodium channels were efficiently isolated in equal proportions from both cell types. Antiphosphotyrosine blots of the same samples demonstrate that the channels’ phosphotyrosine levels are markedly increased in cells coexpressing FynCA compared with those coexpressing FynKD.

We have demonstrated that Fyn alters the biophysical properties of NaV1.5 and that this modulation may involve the phosphorylation of 1 or more tyrosine residues on the channel. We next asked whether cardiac sodium channels are tyrosine phosphorylated in vivo. To this end, we used a commercially available antibody specific for NaV1.5 to identify these sodium channels in a rat cardiac membrane preparation. Lane 1 of Figure 6B shows a Western blot of this membrane preparation and identifies the expected endogenous sodium channel. To determine whether these sodium channels were tyrosine phosphorylated, we immunoprecipitated tyrosine-phosphorylated protein from this cardiac preparation with a phosphotyrosine antibody, 4G10, and probed with the NaV1.5 antibody. The results of this immunoprecipitation are in the adjacent lanes in Figure 6B and demonstrate that NaV1.5 is indeed tyrosine phosphorylated in vivo. These biochemical results lead us to suggest that the modulation of the inactivation properties of cardiac sodium channels is because of direct phosphorylation of tyrosine residues on the channel.

**Target Tyrosine Residue for Fyn Is in the Cytosolic III-IV Loop**

The NaV1.5 isoform contains 22 cytosolic tyrosines. Given that FynCA modulates the inactivation properties of the channel, we narrowed our attention to tyrosine residues in the cytoplasmic linker between domains III and IV, the putative inactivation gate. Figure 7A shows a schematic rendering of the NaV1.5 sodium channel highlighting the 4 homologous domains and the cytosolic linkers that connect them. The polypeptide sequence of the III-IV linker is partially expanded and 2 potential target residues at positions 1494 and 1495 are shown in bold. Also shown in bold is the IFM (Ile, Phe, Met) inactivation “ball” that is directly upstream from these 2 tyrosines. Previous work from our laboratory showed that the double Y1494Y1495/QQ mutation has pronounced effects on the rates and voltage dependence of inactivation. To test the importance of these twin residues in the modulatory effects

**Figure 6.** NaV1.5 channels are tyrosine phosphorylated when transfected into HEK-293 cells and in vivo. A, Immunoprecipitated (IP) NaV1.5-V5 channels from cells coexpressing the channel with FynCA or FynKD were exposed to either V5 or 4G10 antibodies. B, Rat heart sodium channels are tyrosine phosphorylated in vivo. Ten micrograms of a cardiac membrane preparation or immunoprecipitates from this membrane preparation with either no primary antibody or the 4G10 antibody. Cardiac sodium channels were identified by a specific NaV1.5 antibody.

**Figure 7.** Y1495F point mutation in the inactivation domain abolishes Fyn modulation. A, Topological model of NaV1.5 showing domains I though IV, the location of the V5 tag, the S4 voltage sensing segment in red, and a partial sequence of the III-IV linker. B, NMR structure of the sequence in A. Relevant residues are noted. C, V_{mid} for the steady-state inactivation curves of the Y1494F mutant is right-shifted in the presence of FynCA (values in Results). Student t test comparison for V_{mid} between 7 FynCA- and 7 FynKD-expressing cells (P<0.001). Conductance-voltage curves for the same cell types with no significant differences in either V_{mid} or slope. D, Steady-state inactivation and G-V curves for the Y1495F mutant for 8 FynCA or 7 FynKD cells with no significant differences in V_{mid} or slope.
Sodium Channel III-IV Loop Is a Substrate for Fyn

To ascertain whether the Y1495 residue is a bona fide target of Fyn, we generated a series of 6xHis-tagged fusion proteins that contained the III-IV loop of Na\textsubscript{v}1.5 for use as in vitro substrates for Fyn. We tested 4 fragments altogether: a wild-type fragment, 2 single tyrosine to phenylalanine mutants, and a double mutant with both 1494 and 1495 sites changed to phenylalanine. Figure 8A shows a representative Coomassie blue–stained gel containing these fragments. Each fragment shows the same pattern with a prominent band at roughly the predicted size of 8.5 kDa for the full-length fusion protein and 2 smaller bands, both more weakly expressed. To verify the authenticity of the fusion protein, we used an antibody directed against the N-terminal 6xHis tag, which showed reactivity exclusively with the highest molecular weight band (data not shown). Before loading the fragments in the gel shown in Figure 8A, each fragment was used as a substrate for Fyn-catalyzed \textsuperscript{32}P incorporation. An autoradiogram of the

Figure 8. In vitro phosphorylation of the Na\textsubscript{v}1.5 inactivation domain by Fyn. A, Coomassie stain of in vitro phosphorylation reactions containing 5 ng of recombinant Fyn and 2 μg of the indicated 6xHis-tagged III-IV loop fusion protein. WT indicates the wild-type fusion protein. Arrow indicates the full-length fusion protein. All 4 constructs express equally. B, Autoradiogram of the same gel from A, top band corresponds to the autophosphorylation of Fyn. C, Average \textsuperscript{32}P incorporation normalized to the WT fusion protein phosphorylation levels.

same gel from Figure 8A is shown in Figure 8B. The band at \(\approx 55\) kDa corresponds to the autophosphorylated form of the kinase and demonstrates that there was robust and similar levels of kinase activity for each reaction. The fusion protein near the bottom of the gel gives a signal that is most intense for the wild-type fragment and diminishes with each point mutation. Using a phosphorimager, we measured the signal from the 8.5 kDa band and quantified the results from 9 \textsuperscript{32}P incorporation experiments with the averages from this analysis shown in Figure 8C. The single Y1494F and Y1495F mutants showed phosphorylation levels that were significantly different (\(P<0.001\)) from both wild-type and double-mutant substrates. It is worth noting that their phosphorylation levels were significantly different from each other as well (\(P<0.01\)). Given that the signal of the Y1494F fusion protein arises from phosphorylation at the Y1495 site, our data suggest that Y1495 is the preferred phosphorylation target for Fyn. Therefore, our results support the idea that the cytoplasmic III-IV linker is a substrate for the Src family tyrosine kinase Fyn and that Y1495 is preferred to its neighboring tyrosine at position 1494.

Discussion

We show in this study that the voltage-gated cardiac sodium channel Na\textsubscript{v}1.5 is modulated by the Src family tyrosine kinase Fyn and that Y1495 is preferred to its neighboring tyrosine at position 1494.

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Discussion

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kinase Fyn. The effect of the kinase on the channel is manifested in altered steady-state inactivation. Fyn most likely achieves this effect by increasing the rates of recovery from fast inactivated states. We further provide evidence that Na\textsubscript{v}1.5, either cotransfected with Fyn\textsuperscript{CA} or isolated from cardiac myocytes, is tyrosine phosphorylated. Lastly, the biophysical effects of Fyn\textsuperscript{CA} on Na\textsubscript{v}1.5 can be abolished with a single point mutation, Y1495F, in the cytosolic linker between domains III and IV.

Previous work on tyrosine kinase effects on voltage-gated sodium channels showed that modulation takes the form of altered inactivation.\textsuperscript{6-8} The modulation of the steady-state inactivation of Na\textsubscript{v}1.5 by Fyn\textsuperscript{CA} is in agreement with these previous observations. However, although the modulation we see is similar in magnitude, it is opposite in direction from that observed for other channel isoforms. Specifically, when neuronal sodium channel isoforms are modulated by tyrosine phosphorylation, whether through growth factors\textsuperscript{6} or via an association with protein tyrosine phosphatase \( \beta \),\textsuperscript{7} the phosphorylated channel displays a more hyperpolarized steady-state inactivation relationship. However, the depolarizing phosphorylation,\textsuperscript{32} in both physiological and pathological cases, tyrosine kinase activities are coupled to other signaling pathways, including downstream and upstream kinases. Moreover, cardiac myocytes contain numerous other isoforms of ion channels subject to modulation by kinases and phosphatases, resulting in an inherently complex system. We give evidence here that Src family tyrosine kinases provide the heart with a potent tool for fine tuning cardiac electrical excitability, demonstrating yet another adaptive pathway for regulation of heart function.

Acknowledgments
This work was supported by NIH grant AR41691 (to R.H.). We thank Todd Holmes for the Fyn\textsuperscript{CA} and Fyn\textsuperscript{KD} constructs and for generous and voluminous advice on the many pitfalls of studying tyrosine phosphorylation, and Rocky Kass for his insightful comments on the manuscript.

References
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