Modulation of the Cardiac Sodium Channel NaV1.5 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 NaV1.5 cardiac sodium channels. Sodium currents were acquired by whole cell recording on HEK-293 cells transiently expressing NaV1.5. 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 (FynCA) or catalytically inactive (FynKD) variants of Fyn. FynCA caused a 10-mV depolarizing shift of steady-state inactivation compared with FynKD 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 NaV1.5 was increased in cells expressing FynCA compared with FynKD. We show that Fyn is present in rat cardiac myocytes, and that NaV1.5 channels from these myocytes are tyrosine-phosphorylated. In HEK-293 cells the effect of FynCA on NaV1.5 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. (Circ Res. 2005;96:991-998.)

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 NaV1.1, 1.3, and 1.6, which can have been shown to reside within T-tubules and participate in excitation-contraction coupling. However, in terms of conducting the cardiac action potential, the tetrodotoxin-resistant isoform, NaV1.5, is dominant in both its crude expression level and its contribution to the myocytes’ sodium current. For this reason, identification of regulatory factors that modify the behavior of NaV1.5 is crucial in understanding cardiac excitability.

Both Src family and receptor tyrosine kinases are known to be potent modulators of ion channels (see reviews2,3). 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.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.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.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.8 We describe in this study the effects of the Src family kinase Fyn on the cardiac sodium channel NaV1.5, and Fyn’s potential target residue in the channel’s cytoplasmic inactivation gate.

Materials and Methods

DNA Clones, Transfection, and Cell Culture

NaV1.5 and the NaV1.5 Y1494F, NaV1.5 Y1495F point mutants were generated as described previously.9 A C-terminal 14-amino-acid V5 tag was introduced using standard cloning procedures.10 V5 had no
effect on the fast inactivation of the sodium currents (data not shown). The pHOOK, pCS2-c-Fyn<sup>CA</sup>, and c-Fyn<sup>KD</sup> clones were gifts from Dr T. Holmes and Alberto Llamas (New York University, New York, NY).<sup>11</sup> Calcium phosphate was used to transiently transfec

HEK-293 cells. For electrophysiology, cells cotransfected with pHOOK (Invitrogen) were identified by their binding to pHOX (4-ethylmethylen-2-phenyl-2-oxazolidin-5-one)-coated 2.0 to 2.5 μm magnetic beads (Spherotech), which were synthesized using previously described methods.<sup>12</sup>

**Tissue Lysis, Immunoprecipitation, and Western Blots**

HEK-293 cells were lysed ~48 hours after transfection in ice-cold lysis bufer containing (in mmol/L) 25 TRIS, 150 NaCl, 100 CsF, 100 NaF, 5 EDTA, 1 Na<sub>2</sub>VO<sub>4</sub>, 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 Na<sub>2</sub>VO<sub>4</sub>, 5 minutes before lysis. Mined adult rat hearts were homogenized 3×10 seconds at 10K with a Polytron homogenizer in lysis bufer without Triton X-100 and centrifuged to remove nuclei, then membranes pelleted by centrifugation at 50 000g. The membrane pellet was resuspended in lysis bufer 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 protease (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 Na<sub>1</sub>,1.5 (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 Na<sub>1</sub>,1.5 alone, or coexpressed with either Fyn<sup>CA</sup> (Constitutively Active) or Fyn<sup>KD</sup> (Kinase Dead). Na<sub>1</sub>,1.5 is robustly expressed in HEK-293 cells and cotransfection with either Fyn<sup>CA</sup> or Fyn<sup>KD</sup> is comparably expressed, ruling out differences in effect on this expression. In the bottom panel of Figure 1 is a Fyn HEK-293 cells and cotransfection with either Fyn mutant has no effect on these cell types were assayed by WB: V5 and Fyn blots verify expression of Na<sub>1</sub>,1.5-V5 and Fyn. Top, Anti-V5 blot of HEK-293 cells nontransfected (NT) or transfected with Na<sub>1</sub>,1.5 alone or in combination with either Fyn<sup>CA</sup> or Fyn<sup>KD</sup>. Bottom, Fyn blot of HEK-293 cells either nontransfected or expressing either Fyn<sup>CA</sup> or Fyn<sup>KD</sup>, as well as 20 μg 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.
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.\(^\text{15}\) The insulin receptor endogenous to HEK-293 cells is a receptor tyrosine kinase that has been shown to activate Src family tyrosine kinases.\(^\text{16}\) Bisindolylmaleimide (100 nm/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.\(^\text{17}\) 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 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.\(^\text{8}\) 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\(_{\alpha 1.5}\) sodium channel is a target of a Src family tyrosine kinase.

**Modulation of Na\(_{\alpha 1.5}\) by Fyn Is Manifested in Inactivation Properties**

To test whether Na\(_{\alpha 1.5}\) can be modulated by a Src family kinase, we coexpressed Na\(_{\alpha 1.5}\) in HEK-293 cells with either of 2 mutants of the kinase Fyn. The constitutively active mutant, Fyn\(_{\text{CA}}\), lacks its inhibitory carboxyl terminus, whereas the “kinase-dead” mutant, Fyn\(_{\text{KD}}\), has a single point mutation at K299M rendering it catalytically inactive.\(^\text{11}\) Figure 3A shows families of sodium currents from cells expressing Na\(_{\alpha 1.5}\) alone or with either of the 2 Fyn mutants. Consistent with our experiments with insulin, channel coexpression with Fyn\(_{\text{CA}}\) 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 Fyn\(_{\text{KD}}\) cells to −89.4±0.9 mV for 12 Fyn\(_{\text{CA}}\) cells (P<0.001). The steady-state inactivation curve for Na\(_{\alpha 1.5}\) alone (9 cells) is shown as a line without symbols for clarity. This line is a Boltzmann fit of the Na\(_{\alpha 1.5}\) 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 Fyn\(_{\text{KD}}\) 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 Fyn\(_{\text{CA}}\) clearly shifts the steady-state inactivation curve, it has a smaller effect on the rate of fast inactivation. Figure 3D shows that Fyn\(_{\text{CA}}\) 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\(_{\alpha 1.5}\) channels coexpressed with Fyn\(_{\text{CA}}\) (Figure 4). Moreover, channels in the presence of Fyn\(_{\text{CA}}\) 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\(_{\alpha 1.5}\) 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\(_{\alpha 1.5}\) currents in the absence of fluoride using perforated patch recording, a technique that causes less perturbation of the intracellular milieu than whole-cell recording.\(^\text{14}\) Figure 5 shows that, in agreement with our standard whole-cell recording, Fyn\(_{\text{CA}}\) 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. 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. Furthermore, biophysical consequences of the expressed 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\(_\text{V}1.5\)

Figure 4 shows the results of immunoprecipitation of V5-tagged Na\(_\text{V}1.5\) channels from cells also expressing Fyn\(^{CA}\) or Fyn\(^{KD}\), an experiment designed to test whether kinase
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. The structure of this sequence has been solved using solution nuclear magnetic resonance (NMR) and is shown in Figure 7B with the relevant residues marked for clarity. 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 through 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, \( V_{\text{mide}} \) 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 \( V_{\text{mide}} \) 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 \( V_{\text{mide}} \) 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 \( V_{\text{mide}} \) or slope.
we observed, each was mutated individually to phenylalanine, and the single point-mutant sodium channels were coexpressed with FynCA or FynKD. These single point mutations themselves have little effect on channel function, consistent with previous results.9

The effect of FynCA on the first mutant, Y1494F, is shown in Figure 7C. Y1494F is indistinguishable from the wild-type channel and responds to the kinase with a 12-mV rightward shift in the steady-state inactivation (Vmid from −97.3±2.1 to −85.4±1.6 mV for 7 FynCA and 7 FynKD cells, respectively). This result suggests that phosphorylation at tyrosine 1494 is unlikely to mediate the effect of the tyrosine kinase. In striking contrast, the effects of both FynCA and FynKD are abolished in the channel carrying the Y1495F mutation (Figure 7D). Y1495F channels had average Vmid values for steady-state inactivation of −93.9±1.2 mV for 8 FynCA and −93.3±1.8 mV for 7 FynKD expressing cells, respectively, both of which are indistinguishable from the Y1495F mutant alone, Vmid=−92.1±1.2. As in the case with the wild-type channel, Fyn modulation primarily affects the properties of steady-state inactivation, leaving the activation G-V relationships of both mutants unchanged (Figure 7C and 7D). Although these data strongly suggest Y1495 to be the site of phosphorylation, we cannot rule out the possibility that, in actuality, both residues (as well as other tyrosine residues in the channel) are phosphorylated yet only 1 site, Y1495, governs the kinase effect on inactivation. In support of multiple targets of Fyn, immunoprecipitation of either Y1494F or Y1495F channels from cells coexpressing FynCA had roughly equal phosphorylation levels (data not shown), consistent with work showing multiple targets of Src family kinases on other ion channels.21,22 Nonetheless, the ability of the single Y1495F mutation to abolish Fyn modulation strongly implicates this site in mediating the functional effects of the kinase. This result can be rationalized by the proximity of Y1495, but not Y1494, to M1487, a critical residue involved in inactivation.23 as shown in the NMR structure (Figure 7B) of this segment of the III-IV linker.20

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 NaV1.5 for use as in vitro substrates for Fyn. We tested 4 fragments altogether: a wild-type fragment, 2 single tyrosine to phenylalanine mutants at the 1494 and 1495 positions, and a double mutant with both 1494 and 1495 sites changed to phenylalanine. Figure 8A shows a representative Coommassie 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 32P incorporation. An autoradiogram of the same gel from Figure 8A is shown in Figure 8B. The band at 85 kDa corresponds to the autophosphorylated form of the kinase and demonstrates that there was robust and similar 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 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 32P incorporation normalized to the WT fusion protein phosphorylation levels.

Figure 8. In vitro phosphorylation of the NaV1.5 inactivation domain by Fyn. A, Coommassie 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 32P incorporation normalized to the WT fusion protein phosphorylation levels.

Discussion

We show in this study that the voltage-gated cardiac sodium channel NaV1.5 is modulated by the Src family tyrosine kinase Fyn and that Y1495 is preferred phosphorylation site.
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 NaV1.5, either cotransfected with FynCA or isolated from cardiac myocytes, is tyrosine phosphorylated. Lastly, the biophysical effects of FynCA on NaV1.5 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. The modulation of the steady-state inactivation of NaV1.5 by FynCA 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 or via an association with protein tyrosine phosphatase, the phosphorylated channel displays a more hyperpolarized steady-state inactivation relationship. However, the depolarizing phosphorylated channel is accompanied by dramatic alterations in insulin signal transduction. Moreover, activation of Src family kinases reduces the biophysical effects of FynCA on NaV1.5 can be abolished with a single point mutation, Y1495F, in the cytosolic linker between domains III and IV.

An increase in excitability because of tyrosine phosphorylation may play a compensatory role in physiological or pathological situations in which cardiac excitability has been compromised. Tyrosine kinase activity in the heart can be elevated during decedently nonpathological stimulation by adrenergic ligands, angiotensin II, epidermal growth factor, or insulin. Tyrosine kinases may be activated also through the pathological states associated with cardiac ischemia and reperfusion injury, and postinfarct left ventricular remodeling. Furthermore, end-stage dilated cardiomyopathy is accompanied by dramatic alterations in insulin signaling and tyrosine phosphorylation. In both physiological and pathological cases, tyrosine kinase activity is 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.

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