Sites and Molecular Mechanisms of Modulation of Na_v1.2 Channels by Fyn Tyrosine Kinase

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Voltage-gated sodium channels are important targets for modulation of electrical excitability by neurotransmitters and neurotrophins acting through protein phosphorylation. Fast inactivation of Na_v1.2 channels is regulated via tyrosine phosphorylation by Fyn kinase and dephosphorylation by receptor phosphoprotein tyrosine phosphatase- β , which are associated in a signaling complex. Here we have identified the amino acid residues on Na_v1.2 channels that coordinate binding of Fyn kinase and mediate inhibition of sodium currents by enhancing fast inactivation. Fyn kinase binds to a Src homology 3 (SH3)-binding motif in the second half of the intracellular loop connecting domains I and II (L_{I-II}) of Na_v1.2, and mutation of that SH3-binding motif prevents Fyn binding and Fyn enhancement of fast inactivation of sodium currents. Analysis of tyrosine phosphorylation sites by mutagenesis and functional expression revealed a multi-site regulatory mechanism. Y66 and Y1893, which are in consensus sequences appropriate for binding to the Fyn SH2 domain after phosphorylation, are both required for optimal binding and regulation by Fyn. Y730, which is located near the SH3-binding motif in L_{I-II}, and Y1497 and Y1498 in the inactivation gate in L_{III-IV}, are also required for optimal regulation. Phosphorylation of these sites likely promotes fast inactivation. Fast inactivation of the closely related Na_v1.1 channels is not modulated by Fyn, and these channels do not contain an SH3-binding motif in L_{I-II}. Subtype-selective modulation by tyrosine phosphorylation/dephosphorylation provides a mechanism for differential regulation of sodium channels by neurotrophins and tyrosine phosphorylation in unmyelinated axons and dendrites, where Na_v1.2 channels are expressed in brain neurons.

Key words: sodium channel; tyrosine; phosphorylation; modulation; Fyn; kinase

Introduction

Voltage-gated sodium channels are responsible for action potential initiation and propagation, placing them in ideal position to regulate firing and conduction in neurons. Biochemical and physiological studies have shown that sodium channels are regulated by phosphorylation of their α subunits by the serine/threonine kinases PKA and PKC, which reduces the availability of sodium channels for activation by enhancing their intrinsic slow inactivation gating process (Catterall and Catterall, 2001; Carr et al., 2003; Chen et al., 2006). Tyrosine phosphorylation and dephosphorylation have also been implicated in rapid sodium channel modulation (Hilborn et al., 1998; Ratcliffe et al., 2000), and we have recently found that a neural-specific form of Fyn kinase, a member of the Src family of cytosolic nonreceptor tyrosine kinases, binds to the rat brain sodium channel Na_v1.2, enhances fast inactivation, and mediates inhibition of sodium currents by brain-derived neurotrophic factor (BDNF), acting

through the neurotrophin receptor tyrosine receptor kinase B (TrkB) (Ahn et al., 2007). Na_v1.2 channels are preferentially expressed in unmyelinated axons and dendrites of central neurons (Westenbroek et al., 1989; Gong et al., 1999; Boiko et al., 2003), suggesting that this form of sodium channel regulation may be most important for excitability in these neuronal compartments.

Src-family kinases like Fyn have conserved Src homology (SH) domains, which regulate phosphorylation by specific interactions with substrate proteins and with lipid membranes (Brickell, 1992; Kefalas et al., 1995; Resh, 1998; Pawson, 2004). SH4 domains, located at the N terminus, contain a Met-Gly-Cys motif that specifies attachment of myristoyl and palmitoyl lipid anchors for membrane tethering of the kinases. Adjacent SH3 domains bind to proline-rich PXXP motifs in target substrates and direct phosphorylation of nearby tyrosine residues to mediate a wide variety of cellular processes. SH2 domains, situated toward the C terminus from the SH3 domain, bind to sites containing phosphorylated tyrosine residues and further localize src-family kinases for signal transduction.

To determine the molecular mechanism underlying modulation of Na_v1.2 channels by Fyn, we combined computer-assisted motif scanning and mutagenesis to identify an SH3-binding site, SH2-binding sites, and individual tyrosine phosphorylation sites that are required for Fyn regulation of Na_v1.2 channels. Our results show that interaction between the SH3 domain of Fyn and an SH3-binding motif in the intracellular loop connecting domains I and II (L_{I-II}) of Na_v1.2 channels is required for both

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binding and modulation. Bound Fyn, and possibly other Src-family kinases, phosphorylate both Y66 in the N-terminal domain and Y1893 in the C-terminal domain to form SH2 motifs that bind Fyn. Y730 in L_{I-IV}, near the SH3-binding motif, and Y1497 and Y1498 in the inactivation gate also are required for Fyn enhancement of fast inactivation. In contrast, fast inactivation of Na_v1.1 channels, which lack the SH3-binding motif in L_{I-IV}, was not modulated by Fyn kinase, consistent with the idea that this motif is essential for Fyn modulation of Na_v1.2 channels in the brain. These results point to a multisite mechanism for subtype-selective modulation of fast inactivation of Na_v1.2 channels by associated Fyn tyrosine kinase.

Materials and Methods

Expression in mammalian cells

TsA-201 cells were grown in DMEM/F-12 medium (Invitrogen, Rockville, MD), supplemented with 10% fetal bovine serum (Invitrogen) and 100 U/ml penicillin and streptomycin, and incubated at 37°C in 10% CO₂. Neither Fyn nor Na_v1.2 channels are endogenously expressed in tsA-201 cells as assessed by immunoblotting (data not shown), so coexpression experiments can be used to analyze the interactions and regulation of these two exogenous proteins without interference from their endogenous counterparts. The tsA-201 cells were grown to ~70% confluence, suspended with trypsin-EDTA, and plated onto 150 mm culture dishes at 40% confluence 24 h before transfection. Before transfection, the medium was replaced with fresh medium, and the cells were transiently transfected with cDNAs using the calcium phosphate technique (Chen and Okayama, 1988; Qu et al., 1994). Cells were incubated overnight at 37°C in 3% CO₂. After 12–16 h, the medium was replaced, and the cells were allowed to recover for 9–24 h before experiments.

cDNA constructs

Fyn constructs. pCMV5-huFyn was a kind gift from Dr. M. Resh (Memorial Sloan Kettering Cancer Center, New York, NY) (Alland et al., 1994). To create ΔSH3Fyn, pCMV5-huFyn was cut with *Bsi*WI and *Bst*EII, thus deleting amino acids L86 to D142, and religated. ΔSH3,4Fyn was constructed by PCR amplification of amino acids S143 to L537 of pCMV5-huFyn, resulting in *Hind*III and *Bam*HI sites in the N-terminal end and C-terminal end, respectively. This fragment was subcloned into pcDNA3.1myc/his vector (Invitrogen) such that a myc-epitope tag was appended to the C terminus of the construct. The myristoylation and palmitoylation sequence (MGALCC) of A kinase-anchoring protein 15 (AKAP15) (Hulme et al., 2002) was added to the N terminus of ΔSH3,4Fyn by PCR amplification of amino acids S143 to L537 of pCMV5-huFyn to create lipΔSH3,4Fyn. The fragment was subcloned into pcDNA3.1myc/his vector.

Na_v1.2 constructs. All Na_v1.2 channel constructs were based on the rat brain type IIA sodium channel cDNA (Auld et al., 1990). Full-length Na_v1.2 channels were expressed in the mammalian expression vector pCDM8 (Linford et al., 1998). Na_vβ1 subunits (Isom et al., 1992) in the pCDM8 vector were coexpressed, where indicated. Short loops, truncations, and single amino acid mutations were constructed using PCR between unique restriction sites, and fragments containing the mutant sequence or deletion were subcloned back into the host vector with a Fast-Link ligation kit (Epicenter Biotechnologies, Madison, WI). Mutant fragments were sequenced with PCR across both junctions to confirm the presence of mutant nucleotides and the absence of spontaneous mutations during PCR.

cDNAs encoding the intracellular domains of Na_v1.2 channels were constructed in the vector pcDNA3.1myc/his with the Kozak sequence and lipid anchor motif of AKAP15 (see above) at the N terminus and a Myc tag at the C terminus. These fusion proteins contained the following amino acid residues from Na_v1.2: N terminus, amino acids 1–124; L_{I-II}, amino acids 428–753; L_{II-III}, amino acids 984–1203; L_{III-IV}, amino acids 1464–1526; C terminus, amino acids 1776–2005; L_{I-IIa}, amino acids 428–519; and L_{I-IIb}, amino acids 590–681. Fusion protein constructs were expressed in tsA201 cells by the calcium phosphate transfection method (see above).

Na_v1.2/G638, Na_v1.2/P636A, and Na_v1.2/Y730F were made by inserting a glycine at position 638 to produce the sequence PTGLP or by PCR point mutations to the cDNA fragment encoding S632 to M900 of Na_v1.2. This cDNA fragment was amplified with the flanking restriction enzyme sites, and the resulting PCR product was cut with those restriction enzymes and subcloned back into Na_v1.2 in the pCDM8 vector that had been similarly cut. Na_v1.2/Y66F was made by PCR point mutation of the cDNA fragment from the *Spe*I site to the *Sph*I site of the host vector subcloned in Bluescript, and the resulting PCR product was subcloned back into the host vector cut with the same restriction enzymes. Na_v1.2/Y1497F and Na_v1.2/Y1498F were made by PCR point mutations to the cDNA fragment between L1220 and L1776 of Na_v1.2 by PCR amplification with flanking *B*spI and *X*hoI sites. Na_v1.2/Y1893F and Na_v1.2/Y1975F were made by PCR point mutations to the cDNA fragment extending from a silent *X*hoI site inserted at the position of L1776 to a *Cl*aI site in the host vector subcloned in Bluescript, followed by subcloning back into the full-length cDNA cut with the same restriction enzymes.

Membrane preparation from transfected cells

Transfected cells were treated with pervanadate for 5 min at room temperature to inhibit phosphotyrosine phosphatases. The cells were harvested and lysed in (in mM) 25 Tris-HCl, 150 NaCl, 1 EDTA, 5 pervanadate, and protease inhibitors. The supernatant was collected after centrifugation at 3000 rpm for 1 min, and pellets of the membrane fraction were collected after centrifugation at 26,000 rpm for 30 min. Collected pellets were resuspended, and the protein concentration was measured by the BCA assay (Pierce Biotechnology, Rockford, IL).

Immunoprecipitation and immunoblot analysis

Membrane lysates were precleared with protein-A agarose beads (Sigma-Aldrich, St. Louis, MO) for 1 h, and 500 μg of lysate protein were incubated with 5–25 μg of the appropriate antibody. After 3 h of incubation, protein-A agarose beads were added and incubated overnight. The next day, beads were washed with washing buffer (25 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 5 mM sodium phosphate, 1 mM pervanadate, 0.01% Na₃S, and 1% Triton X-100) and eluted by boiling for 5 min. For detection of phosphotyrosine, 2 mM EGTA, 30 mM NaF, and 30 mM β-glycerophosphate were included in the lysis and immunoprecipitation buffer. Immunoprecipitated proteins were separated by SDS-PAGE and transferred to polyvinylidene difluoride membrane. Blots were probed with anti-Fyn (Millipore), anti-Fyn3G, anti-Myc (Santa Cruz Biotechnology, Santa Cruz, CA), anti-phosphotyrosine 4G10 (Millipore), anti-SP19 (Gordon et al., 1988), or anti-SP20 (Westenbroek et al., 1989) recognizing the sodium channel α subunit, and detected by chemiluminescence (GE Healthcare, Piscataway, NJ).

Electrophysiology

Sodium channels were transiently expressed for electrophysiological analysis by transfecting 35 mm dishes of tsA-201 cells with 1.2 μg of cDNA encoding the Na_v1.2 α subunit with or without 0.1 μg of Fyn cDNA using the calcium phosphate method (Chen and Okayama, 1988; Qu et al., 1994). Transfected cells were identified with anti-CD8-coated beads (Dyna, Oslo, Norway) (Jurman et al., 1994). Currents were recorded in the whole-cell configuration using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Recording solutions were identical to those described previously (Ratcliffe et al., 2000) (in mM): 189 N-methyl-D-glucamine, 1 NaCl, 4 MgCl₂, 0.1 BAPTA, 25 Tris-phosphocreatine, 2 Na-ATP, 0.2 Na-GTP, and 40 HEPES, adjusted to pH 7.2 with H₂SO₄ in the recording pipette, and 140 NaCl, 5.4 CsCl, 1.8 CaCl₂, 1 MgCl₂, and 10 HEPES, adjusted to pH 7.4 with NaOH in the bath. Recording pipettes were pulled from borosilicate glass transfer tubes and fire polished to resistances of 2–4 MΩ on the day of recording. Data were acquired using Pulse software (Heka, Lambrecht, Germany) controlling an ITC 18 DA/AD interface (Instrutech, Great Neck, NY). All analyses were performed using Igor Pro (Wavemetrics, Lake Oswego, OR). Standard P/4 subtraction of linear leak and capacitance was used. Series resistance was typically 6–8 MΩ, of which >80% was compensated electronically.

The voltage dependence of activation was measured from a holding potential of −110 mV. Cells were depolarized to potentials from −110 to

Figure 1. Effect of deletion of the SH3 domain of Fyn on association with Na_v1.2 channels. **A**, Na_v1.2 and wild-type Fyn with a C-terminal Myc epitope tag were coexpressed in tsA-201 cells. **B**, Na_v1.2 and ΔSH3,4Fyn with a C-terminal Myc tag were coexpressed in tsA-201 cells. **C**, Na_v1.2 and lipΔSH3,4Fyn with a C-terminal Myc tag were coexpressed in tsA-201 cells. **D**, Na_v1.2 and ΔSH3Fyn with a C-terminal Myc tag were coexpressed in tsA-201 cells. **A–D**, Anti-rabbit IgG was used as a negative control (lane 1), and anti-SP19, an antibody which recognizes the Na_v1.2 α subunit, was used to immunoprecipitate sodium channel complexes (lane 2). Immunoprecipitation (IP) samples were probed with either 4G10 (middle) or anti-Myc (**B**, **C**) or anti-Fyn (**A**, **D**) (bottom). Then the blot was stripped and reprobed with SP20 recognizing the Na_v1.2 α subunit (top). Similar results were obtained in at least three experiments like those illustrated in **A–D** and in additional experiments with similar, but not identical, experimental design.

+20 mV in 10 mV increments, and peak inward currents were measured. Conductance (G)–voltage relationships were determined from peak current (I) versus voltage relationships as $G = I/(V - V_{\text{Rev}})$, where V was the test potential, and V_{Rev} was the extrapolated reversal potential. The voltage dependence of inactivation was measured from a holding potential of −110 mV. Cells were depolarized for 100 ms to potentials from −110 to −15 mV in 5 mV increments followed by test pulses to −10 mV. Peak sodium currents were normalized and plotted versus prepulse potential. Statistical significance between the means of two samples were estimated by Student's t test. For comparison of means of multiple samples, ANOVA followed by Tukey's posttest was used.

Results

Role of the SH3 domain in Fyn binding and tyrosine phosphorylation of Na_v1.2 channels

Fyn kinase often associates with the plasma membrane through its SH4 motif and with its physiological substrates through its SH2 and SH3 domains (Dunant et al., 1997; Klein et al., 2002; Ulmer et al., 2002; Qiu et al., 2005). To determine the importance of the SH3 and SH4 domains of Fyn kinase in binding to Na_v1.2 channels, we tested the interaction of a Fyn truncation mutant lacking the SH3 and SH4 domain (Fig. 1). As we reported in the accompanying study (Ahn et al., 2007), wild-type Fyn binds to Na_v1.2 channels and can be coimmunoprecipitated with specific antibodies (Fig. 1A). In contrast, we did not detect binding of ΔSH3,4Fyn to Na_v1.2 channels in transfected tsA-201 cells (Fig. 1B). Moreover, measurement of phosphotyrosine content of the immunoprecipitated Na_v1.2 channels with an anti-phosphotyrosine antibody that recognizes all phosphorylated tyrosine residues showed that tyrosine phosphorylation of these channels was much reduced when coexpressed with ΔSH3,4Fyn compared with wild-type Fyn (Fig. 1B). These results implicate the SH3 domains of Fyn in binding and tyrosine phosphorylation of Na_v1.2 channels.

The Fyn SH4 domain specifies association with the membrane by an N-terminal myristoyl and palmitoyl anchor (van't Hof and

Figure 2. Binding of Fyn by L_{1-II} of the Na_v1.2 α subunit. **A**, L_{1-II}, L_{1-III}, L_{1-IV}, and C-terminal (C-term) domains of Na_v1.2 with N-terminal lipid anchors and C-terminal Myc epitope tags were expressed in tsA-201 cells, solubilized, and immunoblotted with anti-Myc as described in Materials and Methods. **B**, Coimmunoprecipitation of Na_v1.2 intracellular domains with Fyn. The indicated Na_v1.2 domain constructs and Fyn were coexpressed in tsA-201 cells and solubilized. Lysate samples for each construct were resolved by SDS-PAGE and immunoblotted with anti-Fyn (lane 1). The indicated Na_v1.2 domain constructs were immunoprecipitated with nonimmune rabbit IgG (lane 2) or anti-Myc (lane 3) and analyzed by SDS-PAGE and immunoblotting with anti-Fyn. **C**, Immunoprecipitation of L_{1-IIa} and L_{1-IIb} with Fyn. L_{1-IIa} and L_{1-IIb} with N-terminal lipid anchor and C-terminal Myc epitope tag were analyzed as in **B**. Similar results were obtained in at least three experiments like those illustrated in **A–C** and in additional experiments with similar, but not identical, experimental design. IP, Immunoprecipitation; WT, wild-type.

Resh, 1997; Liang et al., 2004). To test the significance of lipid anchoring of Fyn, we added a lipid anchor motif from AKAP15 (Hulme et al., 2002), which specifies a similar myristoyl and palmitoyl anchor, to the N terminus of FynΔSH3,4 to yield lipΔSH3,4Fyn. This lipid-anchored mutant also was not coimmunoprecipitated with Na_v1.2 channels, and the tyrosine phosphorylation of Na_v1.2 channels was substantially reduced (Fig. 1C). These results indicate that binding to the SH3 domain of Fyn is required for association with Na_v1.2 channels, even when Fyn is anchored in the cell membrane.

To further define the role of the SH3 domain, we deleted it specifically from the Fyn kinase, retaining the SH4 domain and its lipid anchor. Deletion of only the SH3 domain, ΔSH3Fyn, was sufficient to nearly completely prevent binding of Fyn to Na_v1.2 channels and to substantially reduce tyrosine phosphorylation of Na_v1.2 channels (Fig. 1D). These results indicate that the SH3 domain of Fyn is required for normal binding to Na_v1.2 channels and for their tyrosine phosphorylation.

Interaction of Fyn kinase with intracellular domains of Na_v1.2 channels

To identify the segments of Na_v1.2 channels that bind Fyn kinase, we constructed a series of expression plasmids encoding five intracellular domains (N-terminal, L_{1-II}, L_{1-III}, L_{1-IV}, and C-terminal), which all have potential SH3-binding motifs, SH2-binding motifs, or potential sites of tyrosine phosphorylation by

Figure 3. Effect of mutation of the SH3-binding motif and phosphorylated tyrosine residues in Nav1.2 channels. **A**, Nav1.2/638G and Fyn were coexpressed in tsA-201 cells and solubilized. Lysate samples (lane 1) were analyzed by SDS-PAGE and immunoblotting with anti-SP20 (top) or anti-Fyn (bottom). Additional samples were immunoprecipitated with nonimmune rabbit IgG (lane 2) or anti-SP19 (lane 3) and analyzed by SDS-PAGE and immunoblotting with anti-SP20 (top) or anti-Fyn (bottom). **B**, Nav1.2/638G, $\beta 1$ subunit, and Fyn were coexpressed in tsA-201 cells and analyzed as in **A**. **C**, Left, Nav1.2 or Nav1.2/638G mutant channels were coexpressed with Fyn in tsA-201 cells, solubilized, immunoprecipitated with nonimmune rabbit IgG (lane 1) or anti-SP19 (lane 2), analyzed by SDS-PAGE, and immunoblotted with anti-Fyn (bottom). **C**, Right, Nav1.2/Y66F or Nav1.2/Y730F mutant channels were coexpressed with Fyn in tsA-201 cells, solubilized, immunoprecipitated with nonimmune rabbit IgG (lane 1) or anti-SP19 (lane 2), analyzed by SDS-PAGE, and immunoblotted with anti-SP20 (Nav1.2; top) or anti-Fyn (bottom). **D**, Nav1.2, Nav1.2/Y1497F/Y1498F (YYFF), or Nav1.2/Y1893F channels were coexpressed with Fyn in tsA-201 cells, solubilized, immunoprecipitated with nonimmune rabbit IgG (lane 1) or anti-SP19 (lane 2), analyzed by SDS-PAGE, and immunoblotted with anti-Fyn. Similar results were obtained in at least three experiments like those illustrated in **A–D** and in additional experiments with similar, but not identical, experimental design.

Src-family kinases, as identified by Scansite (<http://scansite.mit.edu>). The L_{I-II}, L_{II-III}, L_{III-IV}, and C-terminal constructs were successfully coexpressed with recombinant Fyn kinase in tsA-201 cells (Fig. 2*A*), but we were unable to express a similar construct encoding the N-terminal domain at sufficient level for analysis. The most robust interaction of Fyn kinase was observed with L_{I-II} (Fig. 2*B*). No interaction was observed with the L_{II-III} or L_{III-IV} segments, and a much lower level of coimmunoprecipitation was observed for the C-terminal domain (Fig. 2*B*). To further localize the site of interaction, each half of the L_{I-II} segment was expressed separately, and coimmunoprecipitation of Fyn was measured. Binding activity for Fyn was retained by the L_{I-IIb} construct, which contains the second half of this loop, but no interaction was observed with L_{I-IIa} containing the first half of the loop (Fig. 2*C*). These results identify the second half of L_{I-II} as the interaction site in Nav1.2 channels with highest affinity for Fyn.

Requirement for an SH3-binding motif in L_{I-II} for binding and modulation of Nav1.2 channels

SH3 domains bind to target proteins at canonical sequences containing Arg-X-X-Pro-X-Leu-Pro motifs (Li et al., 1992; Songyang and Cantley, 1995; Pawson, 2004). Using Scansite, we identified a putative SH3-binding motif in the second half of L_{I-II} of Nav1.2. To test the role of this motif in binding and functional modulation of Nav1.2 channels by Fyn, we introduced a glycine residue into the putative SH3-binding motif to change the spacing of the

Figure 4. Effects of mutations in the SH3 domain of Nav1.2 on modulation by Fyn. **A**, Examples of effects of Fyn coexpression on the time course of sodium currents during depolarizations to -15 mV from a holding potential of -110 mV. Normalized currents recorded in the absence [slower trace] and presence (faster trace) of coexpressed Fyn from WT channels (top) or Nav1.2/638G (bottom) are superimposed. **B**, Mean time constants for inactivation of currents conducted by Nav1.2/638G channels during depolarizations to the indicated potentials in the absence (filled circles) or presence (open squares) of coexpressed Fyn. The dotted and dashed curves represent the Nav1.2 WT data reported by Ahn et al. (2007) (accompanying study) for the channel expressed alone and in the presence of Fyn, respectively. The effect of Fyn on the time constants of fast inactivation of Nav1.2/638G is significantly less than wild type at all potentials ($p < 0.05$). **C**, Normalized mean conductance–voltage relationships for cells expressing Nav1.2/638G in the absence (filled circles) and presence (open squares) of coexpressed Fyn derived from depolarizations to the indicated potentials. **D**, Mean normalized inactivation curves of Nav1.2/638G channels in the absence (filled circles) and presence (open squares) of coexpressed Fyn. The voltage dependence of inactivation of WT channels as determined by Ahn et al. (2007) (accompanying study) in the absence (dotted line) and presence (dashed line) of coexpressed Fyn are superimposed. Cells were depolarized for 100 ms with prepulses to the indicated potentials (-110 to -15 mV in 5 mV steps) followed by a 5 ms test pulse to 0 mV. Mean normalized peak test pulse current is plotted as a function of prepulse potential. **E**, Mean normalized inactivation curves of Nav1.2/P636A channels in the absence (filled circles) and presence (open squares) of coexpressed Fyn. The protocol was identical to that of **D**. **F**, Mean time constants for inactivation of Nav1.2/P636A channels during depolarizations to the indicated potentials in the absence (filled circles) or presence (open squares) of coexpressed Fyn. Time constants were derived from monoexponential fits to the decaying phase of the sodium current recorded in response to depolarizations to the indicated potentials. Error bars represent SEM.

two proline residues from P T L P to P T G L P. This mutant (Nav1.2/638G) was expressed and immunoprecipitated by anti-SP19 against Nav1.2 but was unable to coimmunoprecipitate Fyn kinase from cell lysates (Fig. 3*A,C*), compared with wild type (Fig. 1*A*). Coexpression of the Nav1 $\beta 1$ subunit did not restore

Table 1. Voltage dependence of inactivation for control and mutant channels

	Control		+ Fyn		<i>p</i> (control vs Fyn)
	<i>V_h</i> (mV)	<i>n</i>	<i>V_h</i> (mV)	<i>n</i>	
WT	−64.2 ± 0.42	40	−70.1 ± 0.32	34	<i>p</i> < 0.01
638G	−59.4 ± 0.51	11	−59.9 ± 1.21	4	<i>p</i> > 0.05
P636A	−63.4 ± 1.39	12	−65.5 ± 0.41	9	<i>p</i> > 0.05
Y730F	−66.8 ± 0.85	11	−66.8 ± 1.53	8	<i>p</i> > 0.05
Y1497F	−67.5 ± 1.44	10	−70.5 ± 0.89	11	<i>p</i> > 0.05
Y1498F	−64.9 ± 1.03	17	−62.2 ± 2.44	10	<i>p</i> > 0.05
Y1893F	−62.5 ± 0.72	4	−65.7 ± 0.36	3	<i>p</i> > 0.05*
Y66F	−60.7 ± 1.12	10	−62.8 ± 0.84	4	<i>p</i> > 0.05
Y1795F	−60.6 ± 1.28	5	−71.6 ± 0.75	3	<i>p</i> < 0.01

Half inactivation (*V_h*) values derived from fits of a Boltzmann function to normalized inactivation curves determined from individual cells as described in Materials and Methods. Differences between groups were determined by ANOVA followed by Tukey's posttest. **p* < 0.05 for this mutant by Student's *t* test.

coimmunoprecipitation of Fyn by Na_v1.2/638G (Fig. 3*B*). These results implicate the SH3-binding motif in L_{I-II} in binding of Fyn.

The kinetics of activation and fast inactivation of mutant Na_v1.2/638G were similar to wild-type channels, but coexpression of Fyn had a much-reduced effect on the rate of fast inactivation (Fig. 4*A*) over a wide range of membrane potentials (Fig. 4*B*). As for wild-type channels, Fyn had no effect on the voltage dependence of activation of Na_v1.2/638G (Fig. 4*C*). On the other hand, we found no effect of Fyn on the voltage dependence of fast inactivation of Na_v1.2/638G channels, in contrast to the negative shift observed with wild type (Fig. 4*D*, Table 1). We also mutated the first proline residue in the SH3-binding motif to alanine, generating the mutant Na_v1.2/P636A. This mutant also failed to coimmunoprecipitate with Fyn kinase (data not shown) and was less sensitive to Fyn modulation when expressed in tsA-201 cells (Fig. 4*E,F*, Table 1). Together, these results show that Fyn kinase interacts with this SH3-binding motif in L_{I-II} of Na_v1.2 and that this interaction is required for modulation of fast inactivation of Na_v1.2 channels by Fyn kinase.

Identification of tyrosine phosphorylation sites required for modulation by Fyn

As a first step toward identification of potential tyrosine residues for phosphorylation by Fyn, we analyzed the amino acid sequence of L_{I-II} using the Scansite algorithm, and we also conducted a further visual scan to identify tyrosine residues near the SH3-binding site that might be substrates, even without a classical consensus sequence for Fyn phosphorylation. Scansite did not identify any of the tyrosine residues in L_{I-II} as potential Fyn phosphorylation sites in its least stringent mode, which identifies the top 5% of tyrosine residues predicted to be phosphorylated by Fyn. On the other hand, Y730 is located just downstream of the SH3-binding site in the sequence --PPCWYKFAN--. We constructed the mutant Y730F and expressed it in tsA-201 cells with-out and with Fyn. Fyn could be immunoprecipitated normally with these mutant channels from cotransfected cells (Fig. 3*C*). Analysis of the functional properties of Na_v1.2/Y730F channels by whole-cell voltage clamp showed that this mutation did not alter the kinetics or voltage dependence of activation (Fig. 5*A*, Table 1). However, it eliminated the effect of Fyn on the voltage dependence of inactivation (Fig. 5*B*, Table 1) and substantially reduced its effect on the rate of inactivation (Fig. 5*C*). These results suggest that phosphorylation of Y730 is required for the functional effects of Fyn.

A second region of interest in the Na_v1.2 channel is the inactivation gate, which is formed by the linker between domains III and IV (Vassilev et al., 1988; Stühmer et al., 1989; Rohl et al., 1999). Phosphorylation of a serine residue (S1506) in the inacti-

Figure 5. Effect of mutation Na_v1.2/Y730F on modulation by Fyn. *A*, Mean voltage dependence of activation of Na_v1.2/Y730F channels in the absence (filled circles) and presence (open squares) of coexpressed Fyn kinase. Protocol was as described in Figure 4*C*. *B*, Mean normalized inactivation curves of Na_v1.2/Y730F channels in the absence (filled circles) and presence (open squares) of coexpressed Fyn, determined as described in Figure 4*D*. *C*, Mean time constants for inactivation of Na_v1.2/Y730F channels during depolarizations to the indicated potentials in the absence (filled circles) or presence (open squares) of coexpressed Fyn, determined as described in Figure 4*B*. Error bars represent SEM.

vation gate by protein kinase C slows fast inactivation (West et al., 1991; Qu et al., 1996), and two tyrosine residues located nearby (Y1497 and Y1498) are possible substrates for Fyn and might contribute to its modulation of fast inactivation. These two resi-

Figure 6. Effect of mutations Na_v1.2/Y1497F and Na_v1.2/Y1498F on modulation by Fyn. **A, C**, Mean normalized inactivation curves from cells expressing Na_v1.2/Y1497F (**A**) and Na_v1.2/Y1498F (**C**) channels in the absence (filled circles) and presence (open squares) of coexpressed Fyn determined as described in Figure 4*D*. **B, D**, Mean time constants for inactivation of Na_v1.2/Y1497F (**B**) and Na_v1.2/Y1498F (**D**) channels during depolarizations to the indicated potentials in the absence (filled circles) or presence (open squares) of coexpressed Fyn, determined as described in Figure 4*B*. Error bars represent SEM.

dues have been shown to be phosphorylated by Fyn *in vitro* and are implicated in modulation of cardiac Na_v1.5 channels by Fyn (Ahern et al., 2005). To test the possible role of these two tyrosine residues in modulation of Na_v1.2 channels by Fyn, we constructed two mutants, Na_v1.2/Y1497F and Na_v1.2/Y1498F, and expressed them without and with Fyn. Neither mutation prevented Fyn interaction with Na_v1.2 channels (Fig. 3*D*) (data not shown). Analysis of their functional properties by whole-cell voltage clamp showed that each of these mutations reduced the effects of Fyn expression on the voltage dependence of inactivation and the rate of inactivation substantially (Fig. 6, Table 1). The Y1497F mutation negatively shifted the voltage dependence of inactivation in the absence of Fyn by −3.4 mV (Fig. 6*A*, Table 1), which may have contributed to its reduced level of Fyn regulation. Nevertheless, the most straightforward interpretation of these results is that both Y1497 and Y1498 play a significant role in Fyn modulation.

Finally, we used the Scansite algorithm to identify SH3-binding sites, potential sites of phosphorylation by Fyn, and potential sites at which phosphorylation would create an SH2-binding site for Fyn in the other intracellular domains of Na_v1.2 channels. Scansite did not recognize any additional SH3-binding sites in Na_v1.2 channels, even at low stringency (<5 percentile). However, phosphorylation of Y66 in the N-terminal domain and both Y1893 and Y1975 in the C-terminal domain would form SH2-binding sites that are predicted to interact with Fyn. To test their possible roles in Fyn regulation of Na_v1.2 channels, we constructed the corresponding mutants Na_v1.2/Y66F, Na_v1.2/Y1893F, and Na_v1.2/Y1975F, expressed them without and with Fyn, and analyzed their functional properties by whole-cell voltage clamp. Mutation of Y66 had a small but significant ($p < 0.05$ by ANOVA followed by Tukey's posttest) effect on the voltage dependence of fast inactivation in the absence of Fyn, but this

Figure 7. Effects of mutations in predicted SH2-binding domains on modulation by Fyn. **A, C, D**, Mean normalized inactivation curves from cells expressing Na_v1.2/Y66F (**A**), Na_v1.2/Y1893F (**C**), and Na_v1.2/Y1975F (**D**) channels in the absence (filled circles) and presence (open squares) of coexpressed Fyn, determined as described in Figure 4*D*. **B**, Mean time constants for inactivation of Na_v1.2/Y66F channels during depolarizations to the indicated potentials in the absence (filled circles) or presence (open squares) of coexpressed Fyn determined as described in Figure 4*B*. The dotted and dashed curves represent the Na_v1.2 wild-type (WT) data reported by Ahn et al. (2007) for the channel expressed alone and in the presence of Fyn, respectively. Inactivation of Y66F channels in control was significantly slower than WT channels at potentials between −40 and −15 mV ($p < 0.05$ by ANOVA with Tukey's posttest). Error bars represent SEM.

mutation completely prevented the effect of Fyn on the voltage dependence of inactivation (Fig. 7*A*, Table 1). Mutation Y66F also slowed the rate of inactivation in the absence of Fyn (Fig. 7*B*). Mutation of Y1893 impaired Fyn regulation of the voltage dependence of inactivation (Fig. 7*C*, Table 1). In contrast, mutation of Y1975 did not reduce the effect of Fyn on the functional properties of Na_v1.2 channels (Fig. 7*D*, Table 1). Consistent with these functional effects, Na_v1.2/Y66F lost its ability to associate with Fyn (Fig. 3*D*), whereas Na_v1.2/Y1893F retained its interaction with Fyn, but with substantially reduced coimmunoprecipitation under our assay conditions, consistent with the impaired regulation (Fig. 3*D*). These results suggest a complex mechanism of Fyn binding and modulation involving multiple sites of interaction and phosphorylation. A molecular model that includes these multiple sites of Fyn binding, phosphorylation, and regulation is presented in the Discussion.

Modulation of Na_v1.1 channels by Fyn

The Na_v1.1 channel is the closest relative to Na_v1.2 in amino acid sequence, with 87% identity over the entire protein (Numa and Noda, 1986). Inspection of the amino acid sequence of L_{I-II} revealed that Na_v1.1 has the equivalent of Y730 but does not have a conserved SH3-binding motif in L_{I-II}. We expressed Na_v1.1 channels in tsA-201 cells without or with Fyn kinase and analyzed their functional properties by whole-cell voltage clamp (Fig. 8). Fyn kinase had no effect on the voltage dependence of inactivation (Fig. 8*A*) and little effect on the kinetics of fast inactivation (Fig. 8*B*). The lack of response of Na_v1.1 channels to Fyn and the lack of the SH3-binding motif in L_{I-II} support the conclusions that regulation of sodium channel

Figure 8. Effect of Fyn coexpression on the inactivation properties of Na_v1.1 channels. **A**, Mean normalized inactivation curves from cells expressing Na_v1.1 channels in the absence (filled circles) and presence (open squares) of coexpressed Fyn, determined as described in Figure 4D. **B**, Mean time constants for inactivation of Na_v1.1 channels during depolarizations to the indicated potentials in the absence (filled circles) or presence (open squares) of coexpressed Fyn, determined as described in Figure 4B. Error bars represent SEM.

fast inactivation by tyrosine phosphorylation is subtype-specific and that one of the molecular elements that defines specificity is the SH3-binding motif in L_{I-II}.

Discussion

Binding of Fyn via an SH3 interaction is required for modulation of Na_v1.2 channels

Our results indicate that Fyn must bind to Na_v1.2 channels to regulate them. Deletion of the Fyn SH3 domain prevents binding to the sodium channels, and mutation of the SH3-binding motif in L_{I-II} of Na_v1.2 blocks binding and regulation by Fyn. The interaction of Fyn with the SH3-binding motif in L_{I-II} brings the bound kinase within ~90 amino acid residues of Y730, one of its putative targets of phosphorylation. This nearby binding interaction would be crucial for phosphorylation of Y730 because that tyrosine residue is in an atypical sequence for Fyn phosphorylation. Thus, tethering of Fyn in L_{I-II} may enhance both the rate and specificity of phosphorylation of Y730 and thereby allow regulation of sodium channel function.

Two consensus SH2 interaction sites are involved in modulation by Fyn

Y66 and Y1893 are in consensus sequences (YGDI and YEPI) that can potentially bind the SH2 domain of Fyn after phosphorylation. Mutation of each of these Y residues to F impairs modulation by Fyn. The mutation of Y to F causes little perturbation of the protein environment, because the aromatic character of the amino acid residue is retained. Consistent with this, we did not find substantial changes in baseline function of Na_v1.2 channels caused by these mutations. On the other hand, phosphorylation of Y causes a substantial local change in hydrophilicity, which is sufficient to form an active SH2-binding site for Src-family kinases. Our results indicate that both of these putative SH2-binding sites are important for optimal Fyn binding and regula-

tion. It is a surprise that two SH2-binding sites and an SH3-binding site are all involved in Fyn regulation. We speculate that these SH2 and SH3-binding sites all participate in regulation by maintaining a high local concentration of Fyn, dependent on binding of Fyn to the SH3 domain and additional phosphorylation of Y66 and Y1893 to form active SH2-binding motifs. This high local concentration may be required to overcome continuous dephosphorylation by bound receptor protein tyrosine phosphatase β (RPTP β), which we have demonstrated in previous work (Ratcliffe et al., 2000). In this model, the dynamic equilibrium between phosphorylation of the SH2 sites by Fyn and/or other tyrosine kinases and dephosphorylation by bound RPTP β would set the local concentration of Fyn and thereby determine the extent of regulation of Na_v1.2 channels.

Role of phosphorylation of the inactivation gate in Fyn modulation

Fast inactivation of sodium channels is mediated by the intracellular loop connecting domains III and IV, which is thought serve as an inactivation gate by folding into the intracellular mouth of the pore and blocking it within 1–2 ms after channel opening (Vassilev et al., 1988; Stühmer et al., 1989; West et al., 1992; Kellenberger et al., 1996; Kellenberger et al., 1997a,b). A prominent feature of the structure of the inactivation gate is an α helix that includes Y1497 and Y1498, which participate in hydrophobic interactions that stabilize the folded structure of the inactivation gate in solution (Rohl et al., 1999). Our results show that mutation of these Y residues to F impairs modulation by Fyn. These mutations retain the hydrophobic character of these amino acid residues and do not disrupt sodium channel function themselves. On the other hand, phosphorylation of these residues would disrupt hydrophobic interactions in the inactivation gate and would be likely to alter its function. Phosphorylation of these residues would not form an active SH2 domain because the surrounding sequence is not appropriate for an SH2-binding site for Fyn. Therefore, it is likely that phosphorylation of Y1497 and Y1498 contributes directly to modulation of the rate and voltage dependence of inactivation by Fyn by modifying inactivation gate function rather than by enhancing Fyn binding. Consistent with this conclusion, our results show that mutation of these amino acid residues does not alter Fyn binding to Na_v1.2 channels, and previous work has shown that these two tyrosine residues can be phosphorylated *in vitro* by Fyn kinase in fusion proteins derived from the Na_v1.5 channel, whose sequence is identical in the region of phosphorylation to the Na_v1.2 channel (Ahern et al., 2005).

A multisite model for Fyn binding and regulation

Our results support a multistep model for Fyn regulation. First, it is likely that Fyn binds constitutively to the SH3 domain in L_{I-II}, which is active in Fyn binding without phosphorylation. Fyn localized to sodium channels by this protein–protein interaction, plus free Fyn and/or other Src-family kinases, can also phosphorylate Y66 in the N-terminal domain and Y1893 in the C-terminal domain to produce two SH2-binding sites and further concentrate Fyn near the sodium channel. Fyn may bind in a bidentate manner to the SH3-binding site and to one of these two SH2-binding sites to form a highly stable complex, as has been observed for another Src-family kinase (Qiu et al., 2005). Our data indicate that both Y66 and the SH3 domain make critical contributions to Fyn binding because disruption of either of them reduces affinity enough to prevent coimmunoprecipitation of Fyn. Fyn bound in a bidentate complex with these two motifs may

form a complex that is very stable during isolation by coimmunoprecipitation. Conversely, Y1893 may mediate a lower-affinity but physiologically important SH2 interaction, as the mutation Y1893F partially reduces Fyn binding and impairs its functional effect. Fyn bound via its SH3 domain is well positioned to phosphorylate the nearby Y730, and Fyn bound via its SH2 and/or SH3 domains may phosphorylate Y1497 and/or Y1498 in the inactivation gate. The extent of regulation by this multisite mechanism would be determined by the rate of phosphorylation by Fyn versus the rate of dephosphorylation by bound RPTP β . Regulation of these events by BDNF activation of Fyn and ligand regulation of RPTP β is predicted to control electrical excitability of developing axons and neurons, as discussed in the accompanying study (Ahn et al., 2007).

This multisite model for Fyn regulation resembles the mechanism of regulation of sodium channels by PKA and PKC. PKA binds to L_{I-II} of Na_v1.2 channels via interactions of L_{I-II} with AKAP15, and this interaction is required for regulation of channel function (Catterall et al., 1997, 2002). Both PKA and PKC phosphorylate a family of sites in L_{I-II} that synergistically enhance the slow inactivation of Na_v1.2 channels (Numann et al., 1991; Li et al., 1992, 1993; Murphy et al., 1993; Cantrell et al., 1997, 2002; Smith and Goldin, 1997; Carr et al., 2003; Chen et al., 2006). In addition, PKC phosphorylates a site in the inactivation gate at S1506, just downstream of the Y residues that are required for Fyn modulation (West et al., 1991). The similarities of kinase anchoring, phosphorylation, and regulation between these serine-threonine kinases and Fyn suggest that the molecular mechanisms through which they alter channel gating may overlap as well.

Subtype specificity of Fyn modulation of sodium channels

Our results show that fast inactivation of Na_v1.1 channels is not modulated by Fyn, and the SH3-binding site in L_{I-II} is not present in these channels, although they have the equivalent of Y730 in L_{I-II} as well as Y1497 and Y1498 in the inactivation gate. These results are consistent with our model of Fyn regulation, which depends on Fyn bound to the SH3 domain to initiate the regulatory process. Evidently, Fyn can selectively regulate Na_v1.2 channels, which are localized primarily in unmyelinated axons, premyelinated axons, and dendrites (Westenbroek et al., 1989, 1992), compared with Na_v1.1 channels, which are localized primarily in cell bodies (Westenbroek et al., 1989). Differential regulation by neurotrophins acting through TrkB and Fyn may contribute to the different excitability of these regions of brain neurons.

Although there is no equivalent SH3 domain in Na_v1.5 channels, which are expressed primarily in the heart, these sodium channels are modulated by Src-family kinases. Surprisingly, Fyn causes a positive shift of inactivation of Na_v1.5 channels, opposite to the effect of Fyn reported here (Ahern et al., 2005). The effect of Fyn on Na_v1.5 channels requires phosphorylation of Y1497 and/or Y1498, but the SH2 and SH3 sites that may localize Fyn for this form of regulation are not known. It will be interesting to determine whether a multisite model of Fyn binding and regulation is also found for these sodium channels in cardiac myocytes as well as other sodium channel subtypes.

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