Identification of phosphorylation sites and binding pockets for modulation of Na$_V$1.5 channel by Fyn tyrosine kinase

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Cardiac sodium channel Na$_V$1.5 is the predominant form of sodium channels in cardiomyocytes, which exists as a macromolecular complex and interacts with multiple protein partners. Fyn kinase is one of the interacting proteins which colocalize, phosphorylate and modulate the Na$_V$1.5 channel. To elaborate this interaction we created expression vectors for the N-terminal, intracellular loop, and C-terminal regions of the Na$_V$1.5 channel, to express in HEK-293 cells. By co-immunoprecipitation and anti-phosphotyrosine blotting, we identified proline-rich binding sites for Fyn kinase in the N-terminal, IC-loop$_{i-ii}$ and C-terminal. After binding, Fyn kinase phosphorylates tyrosine residues present in the N- and C-terminal, which produce a depolarizing shift of 7 mV in fast inactivation. The functional relevance of these binding and phosphorylation sites was further underpinned by creating full length mutants masking these sites sequentially. An activation and inactivation curves were recorded with or without co-expressed Fyn kinase which indicates that phosphorylation of tyrosine residues at positions 68, 87, 112 in the N-terminal and at positions 1811 and 1889 in the C-terminal creates a depolarizing shift in fast inactivation of Na$_V$1.5 channel.

Introduction

Phosphorylation is a major post-translational modification carried out by approximately 518 kinases and can take place on nine amino acids under physiological conditions. The process of phosphorylation and dephosphorylation regulates most cellular functions. This simple and reversible reaction with readily available phosphoryl donor in the form of ATP is one of the most robust regulatory cellular mechanisms [1–3]. Voltage gated ion channels are integral components of excitable machinery in cells which are finely regulated by phosphorylation [4].

Cardiac sodium channel Na$_V$1.5 is the predominant form of sodium channel present in cardiomyocytes and responsible for initial upstroke during action potential generation. The channels are localized at intercalated discs, t-tubules and lateral membranes in the form of a macromolecular complex, where they interact and are modulated by a number of interacting protein partners. Phosphorylation of Na$_V$1.5 at serine and threonine residues by PKA, PKC and CaMKII is well documented, which either increase or decrease sodium current (I_{Na}) by modifying single channel conductance, alter gating or kinetics and affect trafficking of Na$_V$1.5 [5]. Besides serine and threonine, Na$_V$1.5 is also finely tuned by phosphorylation at tyrosine residues. Inhibition of tyrosine phosphorylation by tyrosine kinase

inhibitors decreased I_{Na} and produced a hyperpolarizing shift in voltage dependence of steady-state inactivation in rabbit ventricular myocytes [6]. Similarly, dephosphorylation of tyrosine residues in Na$_V$1.5 by co-expressing protein tyrosine phosphatase-1 (PTPH1) in HEK-293 cells produced a hyperpolarizing shift in voltage dependence of fast inactivation [7].

Fyn kinase is a member of the nonreceptor Src family of tyrosine kinases, synthesized by soluble ribosomes and expressed ubiquitously [8]. In cardiomyocytes Fyn kinase, along with other Src protein kinases, regulates stability of adherens junctions by tyrosine phosphorylation of catenin molecules [9]. Fyn kinase is localized at caveolae along with Na$_V$1.5 channels in ventricular myocytes [10,11] and is reported to interact and regulate multiple ion channels [12–14]. Previously, we and other researchers have described the modulation by Fyn kinase, of voltage gated sodium channels in an isoform and splice variant dependent manner [15–17]. Fyn kinase is colocalized with voltage gated neuronal sodium channel (Na$_V$1.2) in rat hippocampal neurons and also co-immunoprecipitated when heterogeneously expressed in tsA-201 cells, indicating the potential interaction. This interaction results in phosphorylation of tyrosine residues in Na$_V$1.2 channel leading to decreased I_{Na}, accelerated inactivation and a hyperpolarizing shift in fast inactivation [18]. Fyn kinase also interacts and phosphorylates Na$_V$1.5 channels, as indicated by co-immunoprecipitation studies [17]. Interestingly, Fyn kinase modulates Na$_V$1.5, by producing a depolarizing shift in steady state fast inactivation and increases rate of recovery from fast inactivation [16], oppositely to its modulation of the counterpart Na$_V$1.2 channel. Modulation of voltage gated sodium channels by Fyn kinase is a complex and multistep process, involving phosphorylation of various tyrosine residues [15–17]. The functional modulation of Na$_V$1.5 channel by Fyn kinase has been described, but information regarding binding sites for Fyn kinase and subsequent phosphorylation of tyrosine residues is still missing. So, in the present study we describe proline-rich binding motifs for Fyn kinase and phosphorylated tyrosine residues required for modulation of Na$_V$1.5 channel by using a combination of molecular biology, biochemistry and electrophysiological techniques.

Results

Fyn kinase interacts with intracellular domains of Na$_V$1.5 channel

Interaction of Fyn kinase with Na$_V$1.5 has been described previously where it phosphorylates and in-turn finely tunes voltage dependence of steady-state

inactivation of Na$_V$1.5 [16,17]. However, specific binding sites for Fyn kinase in the Na$_V$1.5 channel and tyrosine residues undergoing phosphorylation are still unknown. Fyn kinase binds to proline-rich regions (PxxP) in substrate protein through its SH$_3$ domain [8]. Na$_V$1.5 channel contains 11 potential proline-rich binding motifs for Fyn kinase and 16 tyrosine residues in intracellular domains. In order to identify binding motifs and potential tyrosine residues, we created expression vectors for the N-terminal, IC-loops and C-terminal. These recombinant proteins, along-with FynKa were transiently cotransfected in HEK-293 cells to confirm their expression. Figure 1A shows expression of N-terminal (lane 2), IC-loop$_{i–ii}$ (lane 3), IC-loop$_{ii–iii}$ (lane 4) and C-terminal (lane 6); we were unable to express IC-loop$_{iii–iv}$ (lane 5) despite multiple attempts. IC loop$_{iii–iv}$ was therefore excluded and expression experiments were performed with the remaining constructs. Co-expression with FynKa resulted in sufficient expression of the respective protein as shown in Fig. 1B (lane 2–6). Fyn kinase is an endogenous protein in HEK-293 cells [16] as indicated
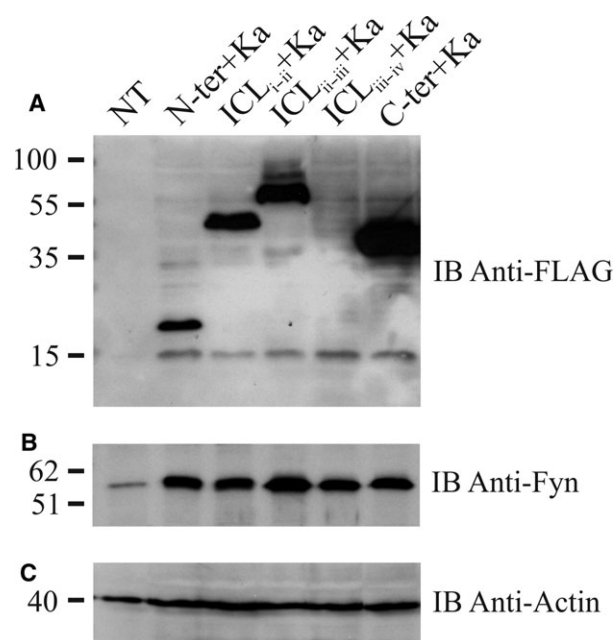
Fig. 1. Co-expression of IC-loops, N- and C-terminal along with FynKa. (A) IC-loops, N- and C-terminal of Na$_V$1.5 were co-expressed with FynKa, solubilized and separated followed by probing with anti-FLAG antibody. All the proteins were expressed except IC-loop$_{iii–iv}$ and specific bands were evident at the respective molecular weights in kDa. (B) Co-expression of FynKa was confirmed by probing with anti-Fyn antibody which indicates robust expression along with Na$_V$1.5 recombinant proteins. (C) Actin was used as a loading control.

in Fig. 1B (lane 1), however its expression levels are increased by transient transfection of FynKa (lane 2–6). FynKa is catalytically hyperactive due to deletion of regulatory amino acids from its C-terminal [19] and this catalytic hyperactivity has been confirmed previously [17]. After verifying satisfactory expression of the required proteins, we next aimed to identify binding motifs in intracellular loops by co-immunoprecipitation experiments.

For co-immunoprecipitation, Na$_V$1.5 channel recombinant proteins and FynKa were co-expressed in HEK-293 cells. Cellular lysate from transfected cells was prepared and IC-loops were pulled down by anti-FLAG M2 magnetic beads. Proteins were separated and transferred to nitrocellulose membrane followed by probing with anti-Fyn and anti-His antibodies. Figure 2A (lanes 2–5), display purified N-terminal, IC loops and C-terminal while panel B shows co-immunoprecipitated FynKa. Strong signals for FynKa appeared at 55 kDa in lanes representing IC-Loop$_{i–ii}$ and C-terminal whilst a weak signal was evident in the lane representing N-terminal, indicating that the binding motifs for Fyn kinase reside in these domains. Both the IC-Loop$_{i–ii}$ and C-terminal contain two proline rich binding regions each, i.e. $P_{465}LAP_{468}$ and $P_{637}GGP_{640}$ present in IC-Loop$_{i–ii}$, while $P_{1959}LGP_{1962}$ and $P_{2005}PSP_{2008}$ present in C-terminal. We created proline to alanine point mutations at positions 465,

637, 1959 and 2008 respectively in IC-Loop$_{i–ii}$ and C-terminal, to dismantle the proline rich binding sites and performed co-immunoprecipitation experiments by coexpressing FynKa in HEK-293 as shown in Fig. 3. Disrupting any of these sites disturb the binding of FynKa with these loops thus indicating potential role of both IC-Loop$_{i–ii}$ and C-terminal in FynKa binding with Na$_V$1.5 channel. After binding with proline-rich motifs through its SH$_3$ domain, Fyn kinase phosphorylates nearby tyrosine residues. These phosphorylated tyrosines either modulate the substrate protein or create SH$_2$ binding motif to stabilize the interaction of Fyn kinase [15].

To identify the phosphorylated tyrosines, we again co-expressed FynKa with intracellular loops in HEK-293 cells and cell lysate was prepared with urea lysis buffer for maximal solubilization and to denature the phosphatases [20]. Utmost care was taken to preserve phosphorylated tyrosines by using phosphatase inhibitors at every step. Each intracellular loop contained 9x histidine-tags, so these IC-loops were pulled down by cobalt resin and probed with anti-FLAG and anti-p-Tyr antibodies. Purified N-terminal, C-terminal and intracellular loops are shown at respective molecular weights in Fig. 4A (lanes 3–6) whilst panel B shows phosphorylated tyrosines indicated by arrow signs (lane 3 and 6). Thus, it indicates phosphorylation of tyrosine residues in both N- and C-terminal.
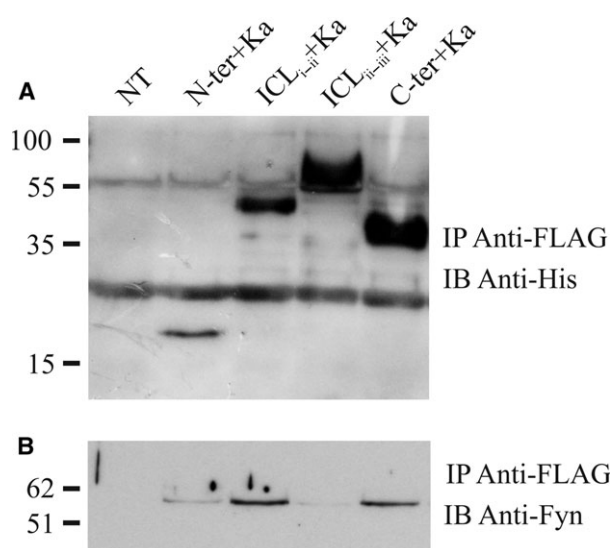
Proline-rich motifs in Na$_V$1.5 channel required for binding of Fyn kinase

Co-immunoprecipitation experiments with recombinant proteins indicated interaction of Fyn kinase with Na$_V$1.5 by binding proline rich motifs at N-terminal, IC-loop$_{i–ii}$ and C-terminal. To further investigate the physiological relevance of these proline-rich motifs, we disrupted these binding sites by creating mutants in which proline was replaced with alanine in full-length Na$_V$1.5 channel, thus masking the proline-rich binding motifs in the respective loops. One proline-rich site in IC-loop$_{iii–iv}$, was also mutated to check its possible physiological relevance. These mutants were transiently transfected in HEK-293 cells with or without FynKa and voltage dependence of activation and inactivation values were recorded by whole-cell patch clamp experiments (Table 1).

In N-terminal there is one potential proline rich binding motif ($P_{116}FHP_{119}$) and replacing proline at position 116 does not disturb the interaction of Fyn kinase with Na$_V$1.5 which was evident by a significant shift in voltage dependence of steady-state inactivation compared to the control. In IC-loop$_{i–ii}$ there are two

Fig. 2. Co-immunoprecipitation of Fyn kinase with Na$_V$1.5 recombinant proteins. (A) IC-loops, N- and C-terminal were pulled down by anti-FLAG M2 magnetic beads, separated and probed with anti-His antibody which shows bands at respective molecular weights in kDa. (B) Probe with anti-Fyn antibody shows co-immunoprecipitated Fyn kinase with IC-loop$_{i–ii}$, N- and C-terminal.
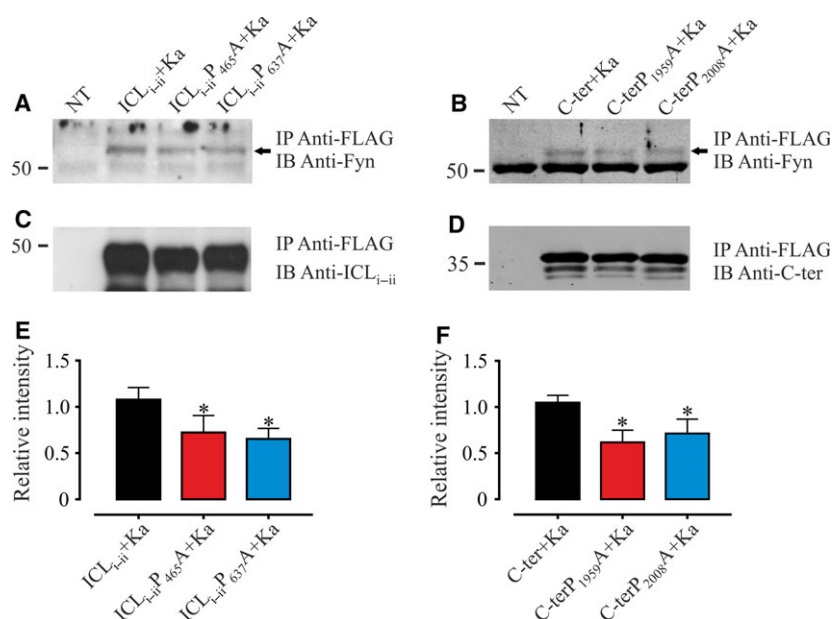
Fig. 3. Co-immunoprecipitation of Fyn kinase with IC-loop$_{i-ii}$ and C-terminal of Na$_V$1.5. IC-loop$_{i-ii}$ and C-terminal, both wild-type and mutants were co-expressed with FynKa and pulled down by anti-FLAG M2 magnetic beads. Purified proteins were separated and probed by either anti-Fyn, anti-ICL$_{i-ii}$ or anti-C-ter antibodies. A and B, represent co-immunoprecipitated Fyn kinase as indicated by arrow signs while, C and D, shows immunoprecipitated IC-loop$_{i-ii}$ and C-terminal, respectively, at mentioned molecular weights. E and F, represent densitometric analysis using IMAGEJ software where co-immunoprecipitated Fyn kinase was normalized with the respective immunoprecipitated IC-loop$_{i-ii}$ and C-terminal mutants ($n = 3$). Disruption of Fyn kinase binding in mutant loops was assessed while significance in mean difference (*$P < 0.05$) was determined by applying ANOVA and compared to the control (WT) by applying Dunnett's post-test. [Colour figure can be viewed at wileyonlinelibrary.com]
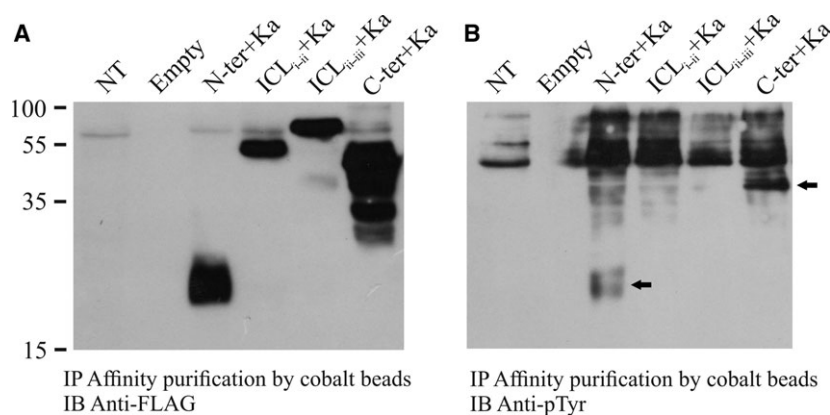

Fig. 4. Detection of phosphorylated tyrosines. IC-loops, N- and C-terminal were purified from cellular extract by using cobalt beads which recognize His-tag. Purified proteins were eluted and equal amounts were separated on two gels. A, was probed with anti-FLAG antibody to blot purified loops. B, was probed with anti-pTyr antibody, which identified phosphorylated tyrosine residues in N- and C-terminal indicated by arrows. Since Fyn kinase is auto-phosphorylated and also purified along with IC-loops, N- and C-terminal, a strong signal appeared at 55 kDa, while the two bands appearing above 55 kDa are some endogenously phosphorylated proteins.

potential proline-rich binding sites (P$_{465}$LAP$_{468}$ and P$_{637}$GGP$_{640}$) and disruption of any of these sites interfere with interaction between Fyn kinase and Na$_V$1.5. The substitution of proline at position 465 with alanine shows a depolarizing shift of 1.7 mV in steady

state inactivation, but it was statistically not significant; however when proline at position 637 was replaced with alanine this completely removed the depolarizing shift in voltage dependence of steady-state inactivation curve indicating the interaction of Fyn

Table 1. Voltage dependence of activation and steady-state inactivation values for Na$_V$1.5 wild-type and mutants representing masked proline-rich regions in which proline was changed to alanine at the respective positions. Significance of Fyn kinase co-expression was determined independently for activation and inactivation, in each mutant by applying two-way ANOVA followed by Bonferroni's post-test where ***$P < 0.001$.

DNA	Activation (mV)		Inactivation (mV)	
	− FynKa	+ FynKa	− FynKa	+ FynKa
WT	-32.3 ± 0.15 ($n = 5$)	-31.0 ± 0.46 ($n = 5$)	-92.2 ± 0.59 ($n = 11$)	-85.6 ± 0.80*** ($n = 13$)
P$_{116}$A	-29.8 ± 0.31 ($n = 6$)	-29.2 ± 0.55 ($n = 6$)	-91.9 ± 0.68 ($n = 13$)	-87.2 ± 0.41*** ($n = 10$)
P$_{465}$A	-28.7 ± 0.27 ($n = 6$)	-28.8 ± 0.32 ($n = 3$)	-89.4 ± 0.29 ($n = 10$)	-87.7 ± 0.43 ($n = 12$)
P$_{637}$A	-31.0 ± 0.36 ($n = 12$)	-30.4 ± 0.40 ($n = 12$)	-88.2 ± 0.22 ($n = 10$)	-88.7 ± 0.40 ($n = 10$)
P$_{1509}$A	-32.3 ± 0.60 ($n = 4$)	-30.8 ± 0.21 ($n = 5$)	-89.8 ± 0.35 ($n = 10$)	-85.9 ± 0.60*** ($n = 10$)
P$_{1959}$A	-28.4 ± 0.35 ($n = 4$)	-29.4 ± 0.51 ($n = 6$)	-87.2 ± 0.34 ($n = 10$)	-88.3 ± 0.38 ($n = 14$)
P$_{2008}$A	-28.9 ± 0.23 ($n = 4$)	-27.2 ± 0.34 ($n = 4$)	-85.4 ± 0.27 ($n = 13$)	-85.9 ± 0.35 ($n = 12$)

kinase with Na$_V$1.5 is lost. The proline-rich binding site in IC-loop$_{iii-iv}$ (P$_{1506}$QKP$_{1509}$) does not interfere in interaction of Fyn kinase with Na$_V$1.5 because depolarizing shift of 4 mV was present compared to the control. In C-terminal there are again two potential proline-rich binding sites (P$_{1959}$LGP$_{1961}$ and P$_{2005}$PSP$_{2008}$). The substitution of proline residues with alanine at position 1959 and 2008 completely removed the depolarizing shift in voltage dependence of steady-state inactivation indicating the disruption of Fyn Kinase interaction with Na$_V$1.5. Hence, this mutational analysis elucidates that proline-rich binding sites in IC-loop$_{i-ii}$ (P$_{637}$GGP$_{640}$) and C-terminal (P$_{1959}$LGP$_{1962}$ and P$_{2005}$PSP$_{2008}$) are required for interaction of Fyn kinase with Na$_V$1.5, while proline-rich motif (P$_{465}$LAP$_{468}$) may play a secondary role since disruption of this site was unable to completely remove the depolarizing shift in steady state inactivation.

Fyn kinase-mediated phosphorylation of tyrosine residues in Na$_V$1.5 channel

Immunoprecipitation of IC-loops to identify tyrosine phosphorylation indicated that Fyn kinase phosphorylates tyrosine residues present in both N- and C-terminal (Fig. 4B). There are three tyrosine residues in N-terminal and six in C-terminal. To pinpoint potential tyrosine residues which are phosphorylated by Fyn kinase, we mutated single tyrosine residues into phenylalanine in N- and C-terminal. These mutants were transiently expressed in HEK-293 cells with or without FynKa and voltage dependence of activation and inactivation curves were recorded (Table 2). Figure 5B shows half maximal inactivation curves of Na$_V$1.5 tyrosine mutants in N-terminal. Replacing any tyrosine in N-terminal (at position 68, 87 and 112) completely removed depolarizing shift in inactivation curves,

which indicate their significance in modulation of Na$_V$1.5 by Fyn kinase.

In C-terminal, replacing tyrosines at position 1811 and 1889 abolished the depolarizing shift in voltage dependence of steady-state inactivation curve indicating phosphorylation of these tyrosine residues, however, there was significant depolarizing shift in mutants Y$_{1795}$F, Y$_{1950}$F, Y$_{1977}$F and Y$_{1995}$F compared to control which indicates that these tyrosines do not play a role in modulation of Na$_V$1.5 by Fyn kinase as shown in Fig. 6.

Discussion

The present work elaborates functional interaction of Fyn kinase with Na$_V$1.5 channel which in-turn modulates fast inactivation. By using a combination of biochemical and electrophysiological techniques, we found that Fyn kinase binds to proline-rich regions in intracellular connecting loop$_{i-ii}$ and C-terminal. After binding it phosphorylates adjacent tyrosine residues in N-terminal and C-terminal which produces a depolarizing shift in voltage dependence of steady-state inactivation of Na$_V$1.5 channel.

The Na$_V$1.5 channel exists as a macromolecular complex with multiple interacting protein partners which aid in trafficking, regulating the biophysical activity and/or degradation of the ion channel [5,21,22]. Fyn kinase is one of these interacting partners, which interacts and phosphorylates tyrosine residues in Na$_V$1.5 channel to produce a depolarizing shift in voltage dependence of steady-state inactivation [16]. The Src tyrosine kinases and Na$_V$1.5 are colocalized at adherens junctions to influence electrical coupling and propagation of action potential [16]. Since phosphorylation of connexins by Src tyrosine kinases reduce junctional coupling [9,23], the increased excitability of Na$_V$1.5 by tyrosine phosphorylation can play a

Table 2. Voltage dependence of activation and steady-state inactivation values for Na$_V$1.5 mutants representing masked tyrosine sites in N- and C-terminal. Significance of Fyn kinase expression was determined independently for activation and inactivation, in each mutant by applying two-way ANOVA followed by Bonferroni's post-test where $*P < 0.05$ and $***P < 0.001$.

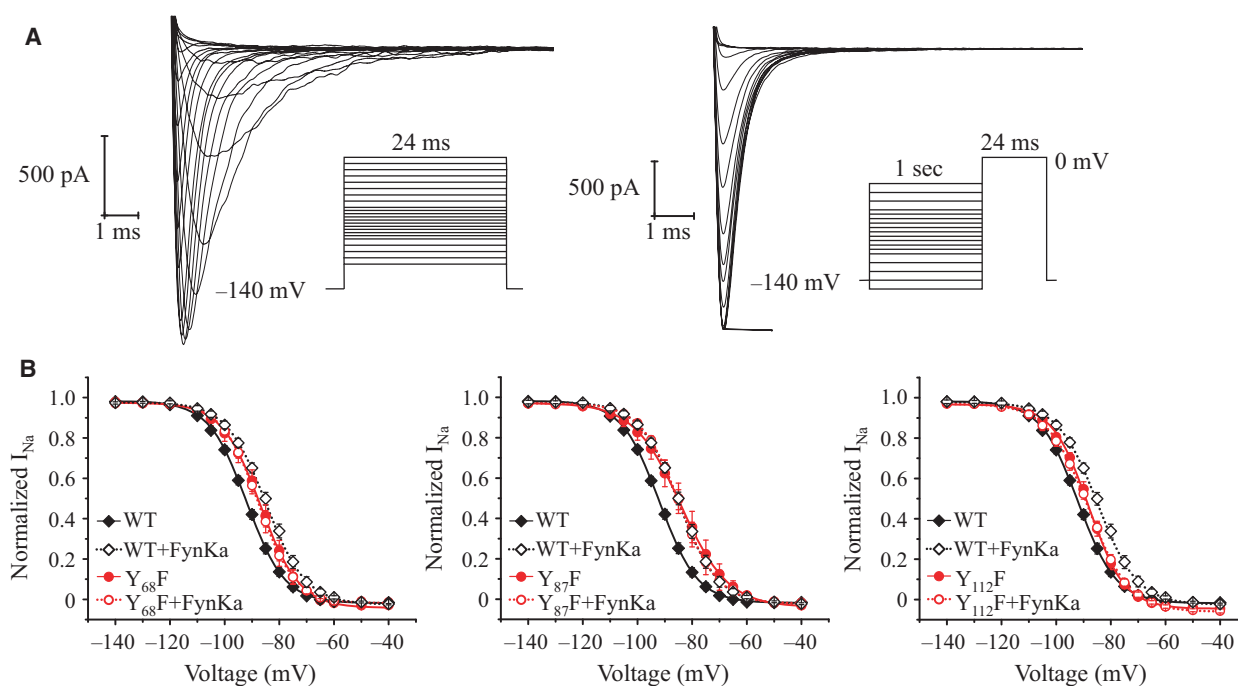| Mutant	Activation (mV)		Inactivation (mV)	
	− FynKa	+ FynKa	− FynKa	+ FynKa
Y$_{68}$F	−29.4 ± 0.75 ($n = 4$)	−31.1 ± 0.30 ($n = 5$)	−87.0 ± 0.32 ($n = 10$)	−87.7 ± 0.20 ($n = 10$)
Y$_{87}$F	−30.1 ± 0.55 ($n = 4$)	−31.8 ± 0.29 ($n = 4$)	−84.1 ± 0.34 ($n = 10$)	−84.6 ± 0.16 ($n = 12$)
Y$_{112}$F	−30.6 ± 0.44 ($n = 4$)	−29.5 ± 0.70 ($n = 3$)	−88.2 ± 0.43 ($n = 11$)	−88.2 ± 0.48 ($n = 10$)
Y$_{1795}$F	−33.1 ± 0.22 ($n = 3$)	−32.7 ± 0.49 ($n = 5$)	−92.4 ± 0.36 ($n = 11$)	−85.4 ± 0.30*** ($n = 12$)
Y$_{1811}$F	−33.0 ± 0.15 ($n = 4$)	−31.1 ± 0.59 ($n = 5$)	−90.7 ± 0.42 ($n = 10$)	−89.6 ± 0.30 ($n = 11$)
Y$_{1889}$F	−31.7 ± 1.51 ($n = 3$)	−32.7 ± 0.30 ($n = 3$)	−89.0 ± 0.42 ($n = 13$)	−89.8 ± 0.32 ($n = 12$)
Y$_{1950}$F	−31.2 ± 0.33 ($n = 6$)	−30.4 ± 0.34 ($n = 4$)	−93.1 ± 0.41 ($n = 11$)	−87.0 ± 0.32*** ($n = 11$)
Y$_{1977}$F	−34.3 ± 0.51 ($n = 3$)	−32.4 ± 0.31 ($n = 3$)	−92.5 ± 0.35 ($n = 11$)	−87.7 ± 0.31*** ($n = 11$)
Y$_{1995}$F	−31.8 ± 0.29 ($n = 4$)	−30.8 ± 0.29 ($n = 4$)	−92.4 ± 0.47 ($n = 9$)	−90.6 ± 0.48* ($n = 10$)

Fig. 5. Current tracings and half maximal inactivation curves for Na$_V$1.5 WT channel or mutants. (A) Representative current recordings showing activation (left) and inactivation (right) of Na$_V$1.5 wild-type channel. (B) Boltzmann fit for half maximal inactivation curves of Na$_V$1.5 WT and N-terminal mutants, where tyrosine was replaced with phenylalanine to mask the effect of phosphorylation by Fyn kinase. The statistical analysis for each mutant is shown in Table 2.

compensatory role. Tyrosine kinase activity can be increased by physiological (stimulation by adrenergic receptors, insulin, angiotensin II and epidermal growth factor) or pathological (post infarct left ventricular remodeling, cardiac ischemia and reperfusion injury) mechanisms [16]. Recently it has been shown in humans that Fyn kinase is down-regulated in end-stage failing hearts [24]. In the heart, Fyn kinase negatively regulates reactive oxygen species production

after pressure overload, thus playing a protective role during oxidative stress and preventing pathological cardiac hypertrophy [24]. Fyn kinase consists of four domains (SH$_1$–SH$_4$). The SH$_1$ is a tyrosine kinase domain, located at the C-terminal. The SH$_2$ domain recognizes phosphorylated tyrosines whilst the SH$_3$ domain is noncatalytic and consists of 50–70 amino acids [8]. The SH$_3$ domain is found in a diverse range of intracellular signaling proteins which recognize
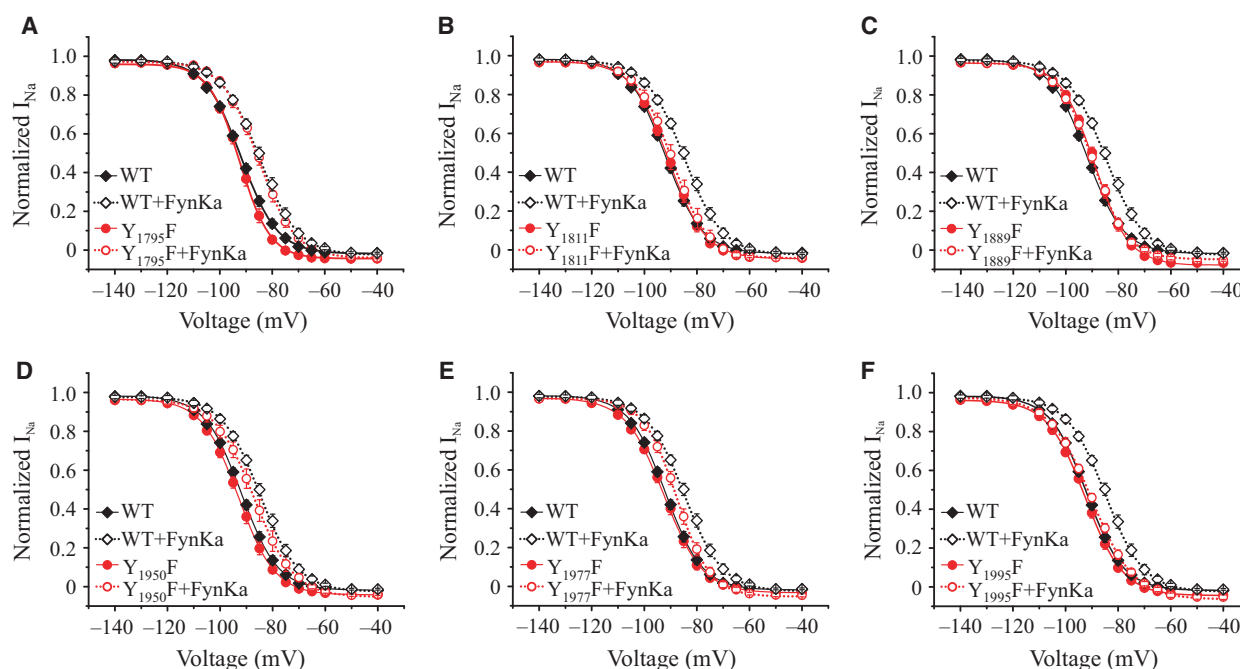
Fig. 6. Boltzmann fit for half maximal inactivation of Na$_V$1.5 WT and C-terminal mutants, where tyrosine was replaced with phenylalanine to mask the effect of tyrosine phosphorylation by Fyn kinase. B and C, shows tyrosine mutants at position 1811 and 1889 which are sites for phosphorylation by Fyn kinase while A, D, E, and F, represent mutants for unphosphorylated tyrosine sites. The statistical analysis for each mutant is shown in Table 2.

proline-rich (PxxP) regions and there are two widely accepted consensus motifs for this domain: (R/K) xxPxxPx and PxxPx(R/K) named as class I or class II, respectively [25,26]. The final SH4 domain consists of myristoylation and palmitoylation motifs for lipid anchoring [8]. We and other researchers have previously shown that, co-immunoprecipitation of Fyn kinase with Na$_V$1.5 and phosphorylation of tyrosine residues at position 1494 and 1495 in the inactivation gate produced a depolarizing shift in fast inactivation, whilst activation remained unaffected [16]. However, no binding site for Fyn kinase SH$_2$ or SH$_3$ domains have been described and the probability for involvement of other tyrosine residues as a potential candidate for phosphorylation by Fyn kinase is also highly pertinent.

The Na$_V$1.5 channel consists of four homologous domains with each domain comprising six transmembrane subunits. These domains are connected by intracellular loops which also contain several consensus motifs for interaction with other protein partners [27]. To pinpoint the potential proline-rich binding sites for Fyn kinase SH$_3$ domain in Na$_V$1.5 channel, we co-expressed intracellular loops along with N- and C-terminal as recombinant proteins with Fyn kinase in HEK-293 cells (Fig. 1). After successful co-expression

we performed co-immunoprecipitation experiments which established the proline-rich binding sites for SH$_3$ domain of Fyn kinase reside in N-terminal, IC-loop$_{i-ii}$ and C-terminal (Fig. 2). There is one proline-rich site in N-terminal and two each in IC-loop$_{i-ii}$ and C-terminal. Surprisingly, there are five proline-rich regions in IC-loop$_{ii-iii}$ but Fyn kinase did not co-immunoprecipitate with this loop. We were unable to express IC-loop$_{iii-iv}$ separately, but this loop contains one proline-rich region, so we considered this site in our electrophysiological experiments with full length Na$_V$1.5 protein. To further specify the Fyn kinase binding site each in IC-loop$_{i-ii}$ and C-terminal we created mutants P465A, P637A, P1959A and P2008A masking the proline-rich regions and performed co-immunoprecipitation experiments. Disrupting any of these sites disturbed the binding of Fyn kinase, thus indicating both sites in IC-loop$_{i-ii}$ and C-terminal are involved in Fyn kinase binding (Fig. 3).

The physiological importance for these sites was further underpinned by creating full length Na$_V$1.5 mutants where proline-rich regions were masked sequentially in respective loops. Recording of half maximal inactivation curves in the absence and presence of catalytically active Fyn kinase specified proline-rich regions in IC-loop$_{i-ii}$ and C-terminal serve as

the binding sites for SH_3 domain. Disrupting the proline-rich sites in IC-loop$_{i–ii}$ ($P_{637}GGP_{640}$) and C-terminal ($P_{1959}LGP_{1962}$ and $P_{2005}PSP_{2008}$) completely neutralized the effect of Fyn kinase which indicates interruption of Fyn kinase binding with $Na_V1.5$. When the other proline-rich site in IC-loop$_{i–ii}$ ($P_{465}LAP_{468}$) was masked, it still displayed the tendency of depolarizing shift in fast inactivation but it was statistically not significant which suggests it might play a secondary role in Fyn kinase binding. In the $Na_V1.5$ C-terminal, proline-rich motif ($P_{2005}PSP_{2008}$) conforms to the class II consensus motif, so it may be a preferred site for Fyn kinase binding. After binding to $Na_V1.5$ through SH_3 domain, Fyn kinase phosphorylates nearby tyrosine residues to modulate the fast inactivation.

Tyrosine phosphorylation can modulate protein function by changing protein conformation or serving as a point for cross talk with the other post translational modifications [28]. Addition of a phosphate group can impart large negative charge which can also modify local hydrophilic interaction of the amino acid [15,29]. Phosphorylated tyrosines in $Na_V1.5$ were identified by extracting intracellular loops from the cell lysate and then by probing with anti-pTyr antibody which revealed tyrosine phosphorylation in N- and C-terminal (Fig. 4B). There are three tyrosine residues in the N-terminal and six in the C-terminal, so we individually mutated them with phenylalanine and recorded half maximal inactivation with or without co-expressed FynKa to distinguish the phosphorylated tyrosines. Figure 5B shows that the depolarizing shift in fast inactivation was neutralized when any of the three tyrosine residues in N-terminal were replaced which underpin their role in Fyn kinase mediated modulation of $Na_V1.5$. In the C-terminal, replacing tyrosine at positions 1811 and 1889, abolished the Fyn kinase mediated depolarizing shift in fast inactivation compared to the control which indicate phosphorylation of tyrosine residues at these positions, whilst in other mutants 1795, 1950, 1977 and 1995 modulation by Fyn kinase was not affected and they behaved like a WT channel (Fig. 6). Phosphorylated tyrosine at position 1889 (pYEPI) can also serve as a binding site for Fyn kinase SH_2 domain, since it closely resembles to the consensus site (pYEEI) recognized by SH_2 domain [15,30]. This multisite phosphorylation of tyrosine residues in $Na_V1.5$ was anticipated previously [16,17] and evidenced in $Na_V1.2$ channel [15]. There are three binding sites in $Na_V1.2$; one proline-rich site for SH_3 ($P_{636}ILP_{639}$) and two phosphorylated tyrosines for SH_2 (66 and 1893) domain. After binding, Fyn kinase can phosphorylate tyrosine at positions 730,

1497 and/or 1498 to modulate the fast inactivation in $Na_V1.2$ channel [15]. Mass spectrometric-based proteomic studies have revealed that on average a protein can be phosphorylated at five different sites which can modulate protein in conjunction [28]. The C-terminal of $Na_V1.5$ has been previously described to play a role in channel inactivation [31]. Binding of Fyn kinase at the C-terminal of $Na_V1.5$ and successive phosphorylation of tyrosine residues modulated inactivation, as evidenced by a depolarizing shift.

To summarize, we described interaction and modulation of $Na_V1.5$ channel fast inactivation by Fyn tyrosine kinase. This interaction is mediated by proline-rich regions in intracellular connecting loop$_{i–ii}$ and the C-terminal of $Na_V1.5$ channel, which create a binding site for the SH_3 domain of Fyn kinase. After binding, Fyn kinase phosphorylates surrounding tyrosine residues in the C-terminal, which may create additional binding site for Fyn kinase through the SH_2 domain. Binding of the SH_2 domain further stabilizes the interaction, whilst phosphorylation of other tyrosine residues modulates fast inactivation of $Na_V1.5$ channel.

Limitations of study

We would like to mention that *in vitro* heterologous expression system is a widely used technique to characterize ion channels, as it closely resembles *in vivo* system but still it does not truly reflect cardiomyocytes. Fyn kinase is endogenously expressed in HEK293 cells (at low levels) as observed by us and other researchers in previous studies [16,17]. We transiently expressed catalytically active Fyn kinase to continuously phosphorylate tyrosine residues in $Na_V1.5$ channel because tyrosine phosphorylation is a rare event and it represents around 0.5% of total phosphorylation events in human, with majority of phosphorylation occurring on serine (90%) and/or threonine (10%) residues [32]. Moreover, the presence of tyrosine phosphatases dephosphorylates the tyrosine residues thus making it is difficult to detect tyrosine phosphorylation and be able to detect changes due to phosphorylation of tyrosine residues. Secondly, in expression experiments we were unable to express short IC-loop$_{iii–iv}$ and thus we were not able to perform co-immunoprecipitation experiments for this loop to detect Fyn kinase binding. However, by using full length $Na_V1.5$ channel and electrophysiological experiments we reported this loop does not play a role in Fyn kinase binding. Finally, the auto-phosphorylated form of Fyn kinase purify along with $Na_V1.5$ intracellular loops which appear in the form of a strong signal around 55 kDa (Fig. 4B). We used multiple approaches like cobalt beads, protein

G-HRP to minimize this strong signal but we could not overcome this problem. There are two tyrosine residues each in IC-loop$_{i–ii}$ and IC-loop$_{ii–iii}$ which are located near to the intracellular domains. The phosphorylation of these tyrosine residues is less likely to occur, but still their phosphorylation and in turn involvement in modulation of Na$_V$1.5 channel cannot be completely ruled out.

Materials and Methods

DNA constructs and transfection

Plasmids, pcDNA3 encoding Na$_V$1.5 (Genbank accession no. NM_198056) and pCS2-c-Fyn^{CA} encoding catalytically active Fyn kinase (Genbank accession no. AK056699) were kindly provided by Jonathan C Makielski, University of Wisconsin, and Richard Horn, Jefferson Medical College, USA, respectively. Na$_V$1.5 channel N-terminal, intracellular loops (IC-loops) and C-terminal were PCR amplified, and flanked by recognition sequences specific for KpnI and XhoI for later cutting and then ligation in pcDNA3.1/His A plasmid. The plasmid also contained FLAG tag in frame to N-terminal and additionally palmitoylation and myristoylation sequence (MGALCC) at C-terminal for lipid anchoring as described previously [15]. Mutations were introduced in Na$_V$1.5 cDNA by using Quickchange lightning site-directed mutagenesis kit (Agilent Technologies, Inc., Cedar Creek, TX, USA). All the constructs were sequenced and sub cloned for subsequent use by using commercially available kits according to manufacturer′s protocol. The plasmid carrying either full length Na$_V$1.5 channel or recombinant proteins and/or catalytically active Fyn kinase (FynKa) were transfected in HEK-293 cells by using X-tremeGene HP DNA transfection reagent (Roche, Mannheim, Germany). HEK-293 cells were maintained in DMEM growth medium supplemented with 10% fetal bovine serum, 2 mM L-glutamine and 1% penicillin/streptomycin.

Western blotting, immunoprecipitation and co-immunoprecipitation

For the preparation of cell lysate plasmid DNA carrying either full length Na$_V$1.5 wild-type, Na$_V$1.5 mutants and recombinant proteins with or without FynKa were transiently transfected in HEK-293 cells. After 48 h incubation, cells were washed two times gently with ice cold PBS buffer and kept continuously on ice. Cells were scraped from the dishes by the addition of 500 μL RIPA buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP-40, 0.1% Na-deoxycholate, 0.1% SDS supplemented with 10 mM NaF, 5 mM Na-orthovandate, 10 mM Na-pyrophosphate, 10 mM β-glycerophosphate and 5 μL protease inhibitor cocktail) and solubilized by frequent vortexing. After

30 min the cellular mixture was centrifuged for 15 min at 16 000 *g* and supernatant was collected in fresh tube. Protein concentration was determined by Bradford assay and then lysate was stored at −20 °C for later use. For the detection of phosphorylated tyrosines in recombinant proteins, transfected cells were treated with 0.1 mM Na-orthovandate for 2 h before lysate preparation and urea lysis buffer (6 M urea, 50 mM tris-HCL, 1% triton X100, 0.1% SDS supplemented with 10 mM NaF, 5 mM Na-orthovandate, 10 mM Na-pyrophosphate, 10 mM β-glycerophosphate and 5 μL protease inhibitor cocktail) was used for protein solubilization with the same protocol as described before. Co-immunoprecipitation was performed by incubating 500 μg of protein extract with 3 times prewashed anti FLAG M2 magnetic beads (Sigma-Aldrich Co. LLC., St. Louis, MO, USA) overnight with continuous end over end rotation at 4 °C. After incubation beads were washed 5 times with wash buffer (150 mM NaCl, 10 mM tris-HCl, 5 mM EGTA, 5 mM EDTA, 0.1% Triton X100) and proteins were eluted by heating for 5 min at 60 °C with Roti protein sample buffer (8% SDS, 40% glycerol, 20% 2-mercaptoethanol, 0.015% bromophenol blue in PBS). For immunoprecipitation of recombinant proteins to detect phosphorylated tyrosines we used cobalt resin (Thermo Fisher Scientific, Rockford, IL, USA). Cobalt beads were washed 3 times with wash buffer (300 mM NaCl, 10 mM imidazole, 50 mM NaH$_2$PO$_4$, 6 M urea supplemented with 10 mM NaF, 5 mM Na-orthovandate, 10 mM Na-pyrophosphate, 10 mM β-glycerophosphate and protease inhibitor cocktail) and incubated overnight with 1000 μg of protein extract with end over end rotation at 4 °C. After incubation beads were washed 5 times with wash buffer and directly eluted in Roti sample buffer by heating at 60 °C for 5 min. Cell lysate and purified proteins were separated on 14% polyacrylamide gel and transferred onto the nitrocellulose membrane by semidry method. Membranes were incubated with respective primary antibodies in 1 : 500 dilutions [anti-Fyn, anti-pTyr, anti-βactin; SantaCruz Biotechnology, Dallas, TX, USA, anti-His; Thermo Fisher Scientific, anti-FLAG, anti-IC loop$_{i–ii}$ Na$_V$1.5 (detecting IC loop$_{i–ii}$); Sigma-Aldrich, anti-C-terminal Na$_V$1.5 (detecting C-terminal); Alomone Labs, Jerusalem, Israel], overnight at 4 °C and probed by incubating for 90 min at room temperature with relevant secondary antibodies at 1 : 10 000 dilutions (anti-mouse; Sigma-Aldrich, anti-rabbit; Cell Signalling Technology, ProteinG-HRP; ThermoFischer Scientific). Protein bands were visualized by chemiluminescent method on Hyperfilm with ECL reagent (GE Healthcare Life Sciences, Little Chalfont, Buckinghamshire, UK).

Electrophysiology

For electrophysiological experiments either wild-type Na$_V$1.5 channel or mutant channel with or without FynKa (1.5 : 0.5 ng) were transiently expressed in HEK-293 cells.

After transient transfection cells were incubated for 40 h at 37 °C, 5% CO_2 and before experiments cells were gently washed once and perfused with extracellular solution (140 mM NaCl, 4 mM KCl, 1.8 mM $CaCl_2$, 0.75 mM $MgCl_2$, 5 mM HEPES; pH 7.4). Experiments were performed as described previously [33]. Briefly, borosilicate glass electrodes were pulled from DMZ universal puller with resistance less than 5 MΩ and filled with intracellular solution (120 mM CsF, 20 mM CsCl, 5 mM EGTA, 5 mM HEPES; pH 7.4). After attaining whole-cell configuration, series resistance and whole-cell capacitance was corrected by using Axopatch 200B. Data was digitized at 100 kHz and low pass filtered at 5 kHz by using Digidata 1440A. Activation curves were recorded at a holding potential of −140 mV with 5 or 10 mV depolarizing pulses ranging from −80 to −10 mV. Similarly inactivation curves were recorded at holding potential of −140 mV with 1 s conditioning pulses ranging from −150 to 0 mV in 5 or 10 mV increments followed by a 24 ms test pulse at 0 mV.

Statistical analysis

For data analysis, cells with peak current amplitude less than 3 nA were selected. Current traces were analyzed by pCLAMP (Molecular Devices, LLC., San Jose, CA, USA), while statistical analysis and graphs were drawn with the help of GRAPHPAD PRISM 7 (GraphPad Software, La Jolla, CA, USA) and ORIGIN PRO8.5 software (OriginLab Corporation, Northampton, MA, USA), respectively. Steady state activation and inactivation curves were created by fitting Bolltzmann function:

$$G_{Na}/G_{max} \text{ or } I_{Na}/I_{max} = \{1 + \exp[(V_{1/2} - V)/V_c]\}^{-1},$$

where G_{Na}/G_{max} is normalized conductance to its maximum value, I_{Na}/I_{max} is the normalized current amplitude, V is membrane potential, V_c is a constant and $V_{1/2}$ is the voltage where current amplitude is half maximum [34]. Data was presented as mean ± SEM and statistical significance among means of different groups (+Fyn vs −Fyn) was determined by applying ANOVA followed by either Dunnett's or Bonferroni's post-test where suitable and mentioned accordingly.

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Conflict of interest

The authors declare no conflict of interest.

Authors contributions

SMI gave substantial contribution to the design of the work, performed experiments, analyzed and interpreted data, and drafted the work. MA and WS helped and performed molecular biology experiments. RLG gave substantial contribution to the conception and design of the work, interpretation of data, drafted and critically revised the work. All authors approved the version to be published and agree to be accountable for the content of the work. The experimental work was performed in the laboratory of RLG.

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