Differential Modulation of Fast Inactivation in Cardiac Sodium Channel Splice Variants by Fyn Tyrosine Kinase

Shahid M. Iqbal a Gowri S. B. Andavan a,b Rosa Lemmens-Gruber a

a Department of Pharmacology and Toxicology, University of Vienna, Vienna, Austria; b Department of Biotechnology, Bannari Amman Institute of Technology, Tamil Nadu, India

Key Words
Na v 1.5 channel splice variants • Fyn kinase • Phosphorylation

Abstract

Background/Aims: Post-translational modifications such as phosphorylation and dephosphorylation can finely tune the function of ion channels. Na v 1.5 is the main sodium channel in human hearts and alternative splicing of the transcript generates two major splice variants, characterized by the presence (Q-pre) or absence (Q-del) of glutamine at position 1077. In the heart, both the Na v 1.5 channel and Fyn tyrosine kinase are colocalized at adherens junctions. This study aimed to investigate the modulation of the aforementioned splice variants by Fyn tyrosine kinase.

Methods and Results: Q-del and Q-pre were transiently expressed alone, with catalytically active Fyn kinase (FynKa) or with a catalytically dead Fyn kinase (FynKd). Co-expression of Na v 1.5 channel splice variants and Fyn kinase was confirmed by Western blotting and their Interaction was established by co-immunoprecipitation experiments. The enzymatic activity of Fyn kinase and phosphorylation of Na v 1.5 channel were ascertained by immunoprecipitation and anti-phosphotyrosine immunoblotting. Whole-cell ionic currents were recorded in patch clamp experiments to examine the modulation of Na v 1.5 channel variants by Fyn kinase, which indicated a hyperpolarizing shift of 9.68 mV in fast inactivation of Q-del. In contrast, a depolarizing shift of 8.77 mV in fast inactivation was observed in the case of Q-pre, while activation curves remained unaltered for both splice variants. This differential modulation in fast inactivation was further assessed by mutating tyrosine 1495 to phenylalanine in the inactivation loop, which completely removed the modulatory effect of Fyn kinase in Q-pre splice variant, while in Q-del variant hyperpolarizing shift in fast inactivation was reduced to 4.74 mV. Finally, the modulatory effect of Fyn kinase was compensated at a mid-value of 94.63 ± 0.34, when both splice variants were co-expressed at a normal physiological ratio. Conclusion: Q-del and Q-pre were differentially modulated by Fyn kinase, and this fine modification resulted in smooth electrical activity in the heart.
Introduction

The human heart contains multiple ion channels, among which the cardiac sodium channel, encoded by SCN5A gene, is the major ion channel responsible for large sodium influxes resulting in cardiac excitability. Makielski and coworkers [1], first described that alternative splicing of the Na\textsubscript{v}1.5 channel transcript generates multiple variants, of which two major ones are distinguished by the presence (Q-pre) or absence (Q-del) of glutamine at position 1077. Q-del and Q-pre account for 65 and 35% of the total Na\textsubscript{v}1.5 channel population in the heart, respectively, with a transcript ratio of 2:1. This ratio remains consistent in all of the cardiac chambers, which renders Q-del, consisting of 2015 amino acids instead of 2016 [1], the most abundant Na\textsubscript{v}1.5 splice variant in the human heart. The Na\textsubscript{v}1.5 channel is strictly regulated by various mechanisms, and minor variation in its biophysical characteristics can cause life-threatening disorders [2, 3]. Regulation of the Na\textsubscript{v}1.5 channel by phosphorylation of tyrosine residues has been reported, but is still not well understood [4-7].

Phosphorylation plays a role in cardiac pathophysiology, and the biophysical kinetics of the Na\textsubscript{v}1.5 channel are finely tuned by a complex interplay of protein kinase A (PKA), protein kinase C (PKC), calcium/calmodulin-dependent protein kinase II (CaMKII) and Fyn tyrosine kinase [8]. PKA activation phosphorylates the Na\textsubscript{v}1.5 channel and produces a negative shift in activation as well as in the inactivation curve [9], which in the ischemic heart may generate re-entrant arrhythmias on adrenergic stimulation [10]. Similarly PKC upregulation is observed in heart failure due to ischemia [11], and activation of PKC decreases \(I_{Na}\) by phosphorylating the Na\textsubscript{v}1.5 channel, which produces a negative shift in steady-state inactivation [12]. CaMKII also phosphorylates the Na\textsubscript{v}1.5 channel, and produces a negative shift in inactivation causing both, gain and loss of function effects. These effects include late inward current, enhanced intermediate inactivation, slowed fast inactivation and recovery from inactivation [10]. It is reported that Na\textsubscript{v}1.5 channels and Src family tyrosine kinases are colocalized at adherens junctions in myocytes [13-15]. Caveolae located on the membranes of ventricular myocytes serve as sodium channel reservoirs [16]. Fyn is a member of the non-receptor Src family of tyrosine kinases, and is also present in caveolae, indicating possible interaction with Na\textsubscript{v}1.5 channels [17]. Interaction of Fyn kinase with the hH1 variant of Na\textsubscript{v}1.5 channel was found to cause a depolarizing shift in the inactivation curve, while activation remained unaffected [7].

As previously mentioned the most abundant splice variant of the Na\textsubscript{v}1.5 channel in the human heart is Q-del, so the present work aimed to investigate the effect of Fyn kinase on Q-del and compare it with the Q-pre splice variant. Plasmid borne Fyn kinase and Na\textsubscript{v}1.5 channel variants were transfected into mammalian cells and their expression was verified by Western blotting. The interaction of Fyn kinase with Na\textsubscript{v}1.5 channel variants was established by co-immunoprecipitation, followed by immunoprecipitation and immunoblotting to assess the phosphorylation of tyrosine residues. Functional modulation of Q-del and Q-pre by Fyn kinase was examined by patch clamp experiments.

Material and Methods

Cell Culture, DNA Clones and Transfection

FLAG tagged and untagged Q\textsubscript{1077}-deleted and Q\textsubscript{1077}-present both in pcDNA3-N were kindly provided by Prof. Jonathan C Makielski, University of Wisconsin, USA, while inactive Fyn tyrosine kinase on pCS2-c-Fyn\textsuperscript{KD} and active kinase on pCS2-c-Fyn\textsuperscript{CA} were a donation by Dr. Richard Horn, Jefferson Medical College, USA. HEK-293 cells were grown in DMEM growth medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. Cells were transiently transfected with cDNA by using X-tremeGene HP DNA transfection reagent (Roche), while mutations were introduced into the cDNA by using Quikchange lightning site-directed mutagenesis kit (Agilent Technologies) according to the manufacturer’s protocol and the intended mutations were confirmed by sequencing. According to experimental setting transfected cells were treated by src kinase blocker PP2 (1 µM) when required.
Western Blotting Immunoprecipitation and Co-immunoprecipitation

After 48 hours of transfection, cells were lysed by using RIPA lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% NP40, 0.1% sodium deoxycholate, 0.1% SDS), freshly supplemented with 1 mM sodium orthovanadate, 2 mM sodium pyrophosphate, 2 mM β-glycerophosphate, 2 mM phenylmethylsulfonyl fluoride, 20 µg/ml aprotinin and 10 µg/ml leupeptin. For anti-phosphotyrosine assay cells were treated with 0.1 mM sodium orthovanadate for 2 hours before lysis. Lysate was cleared by centrifugation and protein concentrations were determined by Bradford’s assay. For immunoprecipitation and co-immunoprecipitation experiments about 500 µg of lysate was incubated with 3-5 times pre-washed anti-FLAG M2 magnetic beads (Sigma-Aldrich), overnight with shaking at 4 °C. Next day beads were washed 3-5 times with washing buffer (150 mM NaCl, 10 mM Tris-Cl, 5 mM EDTA, 5 mM EGTA), and eluted by heating at 60°C for 5 min with Laemmli buffer. The eluted proteins were separated on 7% SDS-PAGE followed by transfer onto the nitrocellulose membrane through semidry method and probed by incubating overnight at 4°C with anti-Na\(\text{v}\)1.5 (diluted 1:400, Alomone labs), anti-Fyn, anti-phosphotyrosine and anti-β actin (diluted 1:500, Santa Cruz), antibodies. Next day membranes were washed and incubated with HRP-conjugated anti-rabbit (diluted 1:5000, Cell Signaling), or anti-mouse (diluted 1:5000, Sigma-Aldrich), antibodies for 90 min at room temperature. Binding of antibodies were detected by ECL reagent (GE Healthcare Life Sciences).

Electrophysiology

For electrophysiological experiments HEK-293 cells were transiently transfected and whole-cell ionic currents were measured by using Axopatch 200B. Borosilicate glass electrodes were pulled by using DMZ universal puller with a resistance less than 5 MΩ. The bath solution contained 140 mM NaCl, 4 mM KCl, 0.75 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 5 mM HEPES, while the composition of pipette solution was 120 mM CsF, 20 mM CsCl, 5 mM HEPES, 5 mM EGTA, and pH of both solutions was adjusted to 7.4. Before starting the experiment liquid junction potential was corrected in online mode by using Clampex. After obtaining whole-cell configuration, series resistance and whole-cell capacitance were also compensated. Data was digitized at 100 kHz and low pass filtered at 5 kHz. Pulse protocol for activation consisted of depolarizations to various levels between -100 to +70 mV for 24 ms, with holding potential of -140 mV. 10 s were given between each sweep. The pulse protocol for fast inactivation included current recordings from a holding potential of -140 mV to 1000 ms prepulses at -150 to -30 mV in 5 or 10 mV increments, followed by a 24 ms test pulse to 0 mV.

Data Analysis

For activation and inactivation curve fitting, cells having peak current amplitude less than 3 nA were used. Statistical analysis was performed by using Origin Pro8 statistical software (OriginLab Corporation) and pCLAMP (Axon Instruments). Steady state activation (\(m\_\infty\)) curves were created by fitting Boltzmann function: \(m\_\infty = \{1 + \exp \left[ -(V - V_m)/s \right] \}^{-1}\) to the normalized conductance-voltage values, While steady state inactivation (\(h\_\infty\)) was determined by fitting normalized peak currents to Boltzmann equation: \(h\_\infty = \{1 + \exp \left[ -(V - V_h)/s \right] \}^{-1}\). Whereas \(V_m\) and \(V_h\) are mid activation and inactivation potentials respectively, \(V\) is test potential and \(s\) is slope factor. Data was presented as mean ± SEM, while significance in difference (\(P<0.05\)) was calculated by Student’s t-test.

Results

Functional expression of Fyn kinase with Na\(\text{v}\)1.5

To determine whether Fyn kinase and Na\(\text{v}\)1.5 variants would be co-expressed by host cells, we transfected HEK-293 cells with plasmids carrying one of the two types of Fyn kinase, and each of the two variants of Na\(\text{v}\)1.5 channel. Transfected cells were grown for 48 hours and then soluble protein extracts were prepared in RIPA lysis buffer. Whole cell lysates from non-transfected cells were used as a control. Both control and transfected cells were separated on SDS-PAGE and immunoblotted using anti-Na\(\text{v}\)1.5 and anti-Fyn antibodies.

The results of immunoblotting (Fig. 1A) showed that transfected cells (lanes 2-7) yielded detectable signals around 255 KDa indicating expression of Q-del and Q-pre, whereas no corresponding band was seen in the non-transfected control (lane 1). In addition, at about
Both active and inactive Fyn kinase interact with Na\textsubscript{\(v\)}\textsubscript{1.5} variants

Co-immunoprecipitation experiments were performed to detect any potential interaction between Fyn kinase and Na\textsubscript{\(v\)}\textsubscript{1.5} variants. Anti-FLAG M2 magnetic beads were used to pull down FLAG-tagged Na\textsubscript{\(v\)}\textsubscript{1.5} variants from whole cell lysate of HEK-293 cells, transiently transfected with Q-del or Q-pre and Fyn kinase active or inactive variants. Co-immunoprecipitated proteins were separated on SDS-PAGE and probed with anti-Na\textsubscript{\(v\)}\textsubscript{1.5} and anti-Fyn antibodies. Whole cell lysates of non-transfected cells were used as a control.

The results showed bands at 59 KDa and 255 KDa indicating interaction of Fyn kinase with Na\textsubscript{\(v\)}\textsubscript{1.5} channel variants. The results were consistent with previous reports \cite{7}. Since all four proteins were detectable in host cells, our aim was to detect physical interaction between Fyn kinase and Na\textsubscript{\(v\)}\textsubscript{1.5} variants.

Tyrosine phosphorylation of Na\textsubscript{\(v\)}\textsubscript{1.5} splice variants by Fyn kinase

Since our working hypothesis was that the Na\textsubscript{\(v\)}\textsubscript{1.5} splice variants were modulated through phosphorylation by Fyn kinase, we expressed FynK\textsubscript{a} or FynK\textsubscript{d} with either Q-del or Q-pre and the soluble protein lysate was separated on SDS-PAGE followed by transfer to nitrocellulose membrane. The membrane was probed with anti-phosphotyrosine antibody to check phosphorylation and to confirm the enzymatic activity of Fyn kinase variants. Protein extract from non-transfected cells was used as a negative control (lane 1), while Q-del or Q-pre alone were used to represent the basal phosphorylation levels (lane 2 and 5). When Q-del or Q-pre was expressed with FynK\textsubscript{a}, phosphorylation was increased.
compared to Q-del or Q-pre alone (Fig. 2A). On the other hand there was evident reduction in phosphorylation when both variants were expressed with FynKd. These experiments provided preliminary evidence of phosphorylation and confirmed enzymatic activity of Fyn kinase. Next we examined direct phosphorylation of Na\textsubscript{v1.5} channel splice variants by immunoprecipitating FLAG-tagged Q-del and Q-pre either transfected alone or with FynKa and FynKd. Immunoprecipitated proteins were probed with anti-phosphotyrosine antibody which indicated both Q-del and Q-pre were phosphorylated when expressed with FynKa (Fig. 2B, lane 2 and 5). Weak signals for phosphorylation were also detected when both splice variants were expressed either alone (lane 1 and 4) or with FynKd (lane 3 and 6), which conforms to our previous observation that Fyn kinase was also expressed endogenously in HEK-293 cells. Anti-phosphotyrosine antibodies were then stripped and membranes were re-probed by anti-Na\textsubscript{v1.5} antibody to ensure equal loading of immunoprecipitated Na\textsubscript{v1.5} channel variants.

The results from Western blotting experiments illustrated that both Fyn kinase and Na\textsubscript{v1.5} splice variants were expressed in HEK-293 cells at detectable levels and their interaction was demonstrated by co-immunoprecipitation. The interaction involved phosphorylation of Na\textsubscript{v1.5} channel variants by Fyn kinase; analysis of the effect of phosphorylation on biophysical kinetics of Q-del and Q-pre was thus subsequently performed.

**Differential regulation of fast Inactivation in Na\textsubscript{v1.5} splice variants**

To examine the effect of Fyn kinase on Na\textsubscript{v1.5} variants we expressed either Q-del or Q-pre in HEK-293 cells with or without FynKa, and ionic currents were recorded by pulse protocol as described in Methods. Particular attention was paid to recording fast inactivation because it has been previously reported that Fyn kinase altered fast inactivation in Na\textsubscript{v1.2} [18, 19] and in the hH1 variant of Na\textsubscript{v1.5} [7], but activation remained unaffected. The activation and inactivation currents from both Na\textsubscript{v1.5} variants were recorded (Fig. 3A and B) and graphs were plotted by fitting the Boltzmann function onto these recordings to calculate the sodium channel kinetics. The values of Q-del or Q-pre alone were taken as baseline values. The half maximal activation and inactivation values of Q-del were -37.06 ± 0.23 mV (n = 6) and -88.92 ± 0.34 mV (n = 6) respectively, while the half maximal activation and inactivation values of Q-pre were -38.69 ± 0.30 mV (n = 7) and -97.41 ± 0.57 mV (n = 7), respectively (Fig 3C and D). Both splice variants were then expressed with FynKa to check its possible modulatory
effect. With Q-pre co-expression of FynKa produced a depolarizing shift of 8.77 mV of half maximal inactivation value (Fig. 4), which was statistically significant \( (P<0.05, n = 7) \), while the activation remained unaffected. On the other hand when Q-del was co-expressed with FynKa, a hyperpolarizing shift of 9.68 mV of half maximal inactivation was observed (Fig. 4), which was also statistically significant \( (P<0.05, n = 11) \), and the activation values of both splice variants were almost identical.

Our results with Q-pre appeared to be consistent with those with the hH1 variant of Na\(_{1.5}\) channel which exhibited a 5.5 mV depolarizing shift of half maximal inactivation \[7\]. In contrast, Q-del variant behaved oppositely to its Q-pre counterpart, but was consistent with neuronal sodium channel (Na\(_{V}\)1.2), which showed 5.9 mV hyperpolarizing shift of half maximal inactivation \[18\]. This raises the questions, a) why splice variants exerted opposite effects when co-expressed with FynKa, and whether these effects were due to the physical presence of Fyn kinase, or due to its phosphorylating activity and b) what role does splice variant background play in modulation by Fyn kinase, according to reports that different Na\(_{1.5}\) splice variants may differ in biophysical characteristics under certain conditions \[3, 20\].
**Inactive FynKd does not influence Q-pre or Q-del**

To exclude the possibility that previous modulation of Q-del and Q-pre was due simply to the binding effect of Fyn kinase, we repeated the same experiments with enzymatically inactive Fyn, FynKd. Co-expression of FynKd either with Q-del or Q-pre failed to cause any significant alteration in the half maximal inactivation of both Q-del and Q-pre variants, which remained near the base line values (Fig. 4). Thus the shift in half maximal inactivation, seen in previous experiments, was modulated by FynKa. The enzymatic activity of FynKa can be hindered by the specific Src family kinase inhibitor, PP2. So blockade of FynKa with PP2 would serve to pinpoint the phosphorylation activity of Fyn kinase as the source of modulation in Na\(_{\text{v}1.5}\) variants.

**FynKa blockade restores steady state inactivation of Q-del and Q-pre to their basal values**

To ascertain the differential modulation of Q-del and Q-pre as a consequence of phosphorylation by FynKa, we expressed FynKa either with Q-del or Q-pre, and determined fast inactivation in the presence of 1 µM PP2. FynKa in the presence of the Src kinase blocker PP2 did not alter the half maximal inactivation values for Q-del or Q-pre and the values remained near baseline. This established that the shift in half maximal inactivation was due to modulation by FynKa through phosphorylation (Fig. 4). The half maximal activation and inactivation values for both splice variants with or without FynKa, FynKd and FynKa+PP2 are summarized in Table 1. Using this strategy it became clear that Fyn kinase differentially regulated both Q-del and Q-pre variants by phosphorylation, but the question of differential modulation still remained to be answered.

**Probing differential modulation of Na\(_{\text{v}1.5}\) splice variants**

To explore the underlying reason for this differential modulation of Na\(_{\text{v}1.5}\) variants by Fyn kinase, we then transiently co-transfected both Q-pre and Q-del splice variants at a normal human heart ratio [1], by using 350 ng of Q-pre cDNA (35%) and 650 ng of Q-del cDNA (65%), in HEK-293 cells. The half maximal activation and inactivation values of co-transfected Q-pre\(_{\text{(35%)}}\) and Q-del\(_{\text{(65%)}}\) were -35.58 ± 0.31 (n = 6) and -94.12 ± 0.28 (n = 22), respectively. Co-expression of the FynKa with Q-pre\(_{\text{(35%)}}\) + Q-del\(_{\text{(65%)}}\) did not produce any hyperpolarizing or depolarizing shift, with half maximal activation and inactivation values of -36.06 ± 0.22 (n = 5) and -94.63 ± 0.34 (n = 20), respectively (Fig. 5). Hence it was revealed that Fyn kinase harmonized the half maximal inactivation values of Na\(_{\text{v}1.5}\) variants, if both Q-del and Q-pre co-existed.

![Fig. 5. Steady-state fast inactivation of both Q-del and Q-pre splice variants co-transfected at a ratio of 65% and 35% respectively with or without FynKa. Co-expression of FynKa did not lead to any change in fast inactivation.](image1)

**Fig. 6.** A linear relationship ($R^2 = 0.98$) observed between hydrophobicity of the substituted amino acids, where glutamine at position 1077 is replaced with lysine (K), proline (P), tyrosine (T), alanine (A), and the hyperpolarizing shift in half maximal inactivation of different point mutants of Na\(_{\text{v}1.5}\) channel.
Glutamine is a key player at position 1077

To emphasize the presence of glutamine amino acid at position 1077, we generated four different mutants, where glutamine at position 1077 was replaced with lysine (Q\textsubscript{1077}K), proline (Q\textsubscript{1077}P), tyrosine (Q\textsubscript{1077}Y) and alanine (Q\textsubscript{1077}A). The half maximal inactivation values for these mutants with and without FynKa or FynKd were recorded and presented in Table 2. All the four mutants exhibited hyperpolarizing shift when expressed with FynKa, and these values remained near baseline when expressed with FynKd. We then established a correlation between different properties of amino acids like hydrophobicity, average mass, isoelectric point, van der Waals volume, molar mass, dissociation constant (\(\alpha\)-COOH), dissociation constant (\(\alpha\)-NH\textsubscript{3}) and the shift in fast inactivation curves. A linear relationship (\(R^2 = 0.98\)) was observed only in correlation between hydrophobicity [21], and shift in fast inactivation of the four mutants (Fig. 6), while there was a weak correlation (data not shown), with molar mass (\(R^2 = 0.76\)) and with the van der Waals volume (\(R^2 = 0.67\)). Thus it became clear that glutamine at position 1077 plays part in differential modulation of Na\textsubscript{v}1.5 variants by Fyn kinase.

Inactivation loop contains tyrosine involved in phosphorylation

To check out the possible tyrosine residue involved in phosphorylation by Fyn kinase, we created a point mutation (Y\textsubscript{1495}F), where tyrosine at position 1495 was replaced by phenylalanine in both Q-pre and Q-del variants. The involvement of this tyrosine has been reported in the hH1 variant of Na\textsubscript{v}1.5 channel [7]. Q-del (Y\textsubscript{1495}F) or Q-pre (Y\textsubscript{1495}F) mutants were either expressed alone or with FynKa, FynKd, FynKa+PP2 and their half maximal activation and fast inactivation values were recorded which are shown in Table 3. This Y\textsubscript{1495}F point mutation completely abolished the depolarizing shift by FynKa in Q-pre splice variant. Contrarily in Q-del splice variant the point mutation Y\textsubscript{1495}F did not completely remove the hyperpolarizing shift in fast inactivation (Fig. 7), instead it was reduced to 4.74 mV but still

### Table 2. Half maximal inactivation values of Na\textsubscript{v}1.5 channel point mutants either expressed alone or with FynKa or with FynKd. Data presented as mean ± standard error of mean. n: number of experiments. *: P<0.05 compared to mutant alone (Student’s t-test)

| Mutant | +FynKa (mV) | +FynKd (mV) |
|--------|-------------|-------------|
| Q\textsubscript{1077}Y | -90.43 ± 0.35 | -99.79 ± 0.18* | -90.45 ± 0.32 |
| (n = 4) | (n = 3) | (n = 3) |
| Q\textsubscript{1077}P | -88.21 ± 0.47 | -97.71 ± 0.38* | -88.85 ± 0.89 |
| (n = 6) | (n = 4) | (n = 4) |
| Q\textsubscript{1077}K | -97.47 ± 0.23 | -102.95 ± 0.43* | -97.63 ± 0.98 |
| (n = 6) | (n = 3) | (n = 3) |
| Q\textsubscript{1077}A | -101.61 ± 0.30 | -118.02 ± 0.36* | -105.33 ± 0.51 |
| (n = 6) | (n = 3) | (n = 5) |

### Table 3. Half maximal activation and inactivation values of Q-delY\textsubscript{1495}F and Q-preY\textsubscript{1495}F mutants either expressed alone or with FynKa or with FynKd or with FynKa+PP2. Data presented as mean ± standard error of mean. n: number of experiments. *: P<0.05 compared to Q-delY\textsubscript{1495}F alone (Student’s t-test)

| Mutant | Activation (mV) | Inactivation (mV) |
|--------|-----------------|-------------------|
| Q-delY\textsubscript{1495}F | -40.90 ± 0.20 | -94.10 ± 0.42 |
| (n = 5) | (n = 9) |
| Q-delY\textsubscript{1495}F+FynKa | -40.23 ± 0.19 | -98.84 ± 0.42* |
| (n = 8) | (n = 11) |
| Q-delY\textsubscript{1495}F+FynKd | -39.14 ± 0.29 | -95.02 ± 0.32 |
| (n = 4) | (n = 6) |
| Q-delY\textsubscript{1495}F+FynKa+PP2 | -37.04 ± 0.20 | -91.84 ± 0.39 |
| (n = 4) | (n = 8) |
| Q-preY\textsubscript{1495}F | -32.69 ± 0.25 | -92.32 ± 0.52 |
| (n = 3) | (n = 10) |
| Q-preY\textsubscript{1495}F+FynKa | -32.31 ± 0.34 | -93.43 ± 0.53 |
| (n = 7) | (n = 9) |
Fig. 7. Steady-state fast inactivation curves of \( Y_{1495}F \) mutant for both Q-del and Q-pre splice variants. Q-del\( Y_{1495}F \) was either expressed alone or with FynKa, with FynKd or with FynKa+PP2. Curves with FynKd and FynKa+PP2 are shown in dotted lines for clarity and remained near the base line value. Q-pre\( Y_{1495}F \) was either expressed alone or with FynKa and co-expression with FynKa did not produce any shift in fast inactivation.

Discussion

Here we demonstrate for the first time that Fyn kinase, a member of the non-receptor src family of tyrosine kinases, differentially modulates Na\(_{1.5}\) channel splice variants. Fyn kinase phosphorylated Na\(_{1.5}\) variants Q-del and Q-pre, which resulted in a hyperpolarizing shift in fast inactivation of Q-del and a depolarizing shift in fast inactivation of Q-pre. When both splice variants co-existed, this differential modulation by Fyn kinase was abolished.

Na\(_{1.5}\) channel has two major splice variants classified by the presence or absence of glutamine at position 1077 [1]. Splice variant background plays an important role in ion channel pathophysiology [22], as it has been previously reported that two common polymorphisms, \( S_{24}Y \) and \( H_{558}R \), cause an increased expression defect in Q-pre splice variant but not in Q-del [23]. Similarly a loss of function missense mutation, \( G_{1406}R \), was exacerbated in a Q-pre background [24]. Inactivation kinetics of sodium channel exhibit diversity, depending on the location of sodium channels in the heart. The availability curve of the sodium channel remained 9.6 mV more hyperpolarized in atrial compared to epicardial ventricular myocytes of guinea pigs [25]. Also subcellular differences in sodium current have been described in adult rat ventricular myocytes, showing that half maximal inactivation value of sodium channel in lateral membranes is 12.9 mV more hyperpolarized when compared to sodium channels present at the intercalated disc region. It was speculated that most of the cardiac sodium channels present at the lateral membranes remain inactivated during resting membrane potential [26]. This raised the question, how cardiac sodium channels present at the lateral membranes play their part during action potential propagation [27]. In our experiments, half maximal inactivation value of Q-pre was 8.24 mV more hyperpolarized when compared to Q-del variant. This could indicate the possibility that Q-pre splice variant is more likely to be localized on lateral membranes of the myocytes, while Q-del variant might be present at intercalated disc regions.

More recently it has been proposed that in cardiac-myocytes, a multiple pool of sodium channels exists and they are differentially regulated [27]. In our experiments Fyn kinase interacted differently with both Q-del and Q-pre, as Fyn kinase produced about 8 mV
depolarizing shift in fast inactivation of Q-pre splice variant and contrarily a hyperpolarizing shift of about 9 mV was produced in Q-del variant. The shift in fast inactivation remained near the baseline value in both splice variants when they were expressed either with FynKd or treated with 1 µM PP2. Co-transfection of both Q-pre and Q-del splice variants at a normal ratio of human heart i.e. 35% and 65% [1] respectively, resulted in a fast inactivation value of -94.12 ± 0.28 (n = 22), which was more hyperpolarized from Q-del alone and more depolarized from Q-pre alone. When FynKa was co-expressed with Q-pre (35%) + Q-del (65%), surprisingly it did not produce any shift in fast inactivation suggesting that Fyn kinase synchronized both splice variants to a midpoint for smooth electrical activity of cardiac myocytes. The absence of a modulating shift in fast inactivation of co-transfected Q-del and Q-pre can be attributed to the possibility of differential phosphorylation levels of both splice variants by FynKa. Since wild type Fyn kinase is also endogenously expressed in HEK-293 cells, the possibility of competitive binding on Na,1.5 cannot be excluded. Secondly tyrosine phosphatases also play part in maintaining a balance between phosphorylated and de-phosphorylated states, as the interaction of protein tyrosine phosphatase (PTPH1) with the hH1 variant of Na,1.5 has been described [6]. The type of heterologous expression system might also play a role; HEK-293 cells may lack certain components of the cellular machinery of cardiac myocytes. All these factors may play a contributory role, since the expected shift in fast inactivation depending on transfected ratios of Q-del and Q-pre was not observed.

The fact that tyrosine phosphorylation of Na,1.5 channel has received little attention until now, may be in part due to low levels of tyrosine phosphorylation compared to that of serine and threonine residues. To date eight phosphorylated serines and threonines have been identified by in silico/in vitro studies and 15 sites identified by native proteomic studies, while only one phosphotyrosine has been identified by in vitro methods [28]. To our knowledge not a single study has reported the direct effect of tyrosine phosphorylation on human cardiac myocytes, although a few studies have reported the effect of tyrosine kinase inhibitors on rabbit and guinea pig ventricular myocytes. Genistein, AG957, PP2 and ST638 have been shown to decrease the sodium current in rabbit ventricular myocytes, while the first two have also shifted fast inactivation towards more negative potentials [4]. Contrarily, epidermal growth factor (EGF) increased sodium current in guinea pig ventricular myocytes by tyrosine phosphorylation with accelerated activation and inactivation of ionic currents [5]. In neuronal sodium channel, it has been reported that Fyn kinase first binds to proline rich motif in the intracellular loop connecting domain I and II, followed by phosphorylation of tyrosines at position 66 and 1893 to create a binding site for the SH2 domain of Fyn kinase. Fyn that binds via its SH3 domain phosphorylates tyrosine at position 730, while if it is bound via SH2 domain it phosphorylates tyrosines present at position 1497 or 1498 [18]. In the case of the hH1 variant of the cardiac sodium channel, tyrosine at position 1495 plays a critical role in modulation through phosphorylation with Fyn kinase; when this tyrosine was replaced with phenylalanine, the depolarizing shift in inactivation was eliminated. Since immunoprecipitated Y1495F or Y1498F point mutants of Na,1.5 channels have roughly equal phosphotyrosine levels, the possibility of multiple targets for Fyn kinase in the cardiac sodium channel cannot be ruled out [7]. We created the point mutation Y149F in both Q-pre and Q-del variants to try to elucidate this differential modulation of both splice variants by Fyn kinase. Our results illustrated that in Q-pre splice variant this mutation completely removed the depolarizing shift in inactivation by FynKa, which was in accordance with the hH1 variant of Na,1.5. On the other hand in Q-del splice variant a hyperpolarizing shift was not completely removed, but rather reduced from 9.68 mV to 4.74 mV, which suggested the possible involvement of other tyrosine residues in interaction with Fyn kinase.

Na,1.5 channels consist of four transmembrane domains, each comprising six subunits, one intracellular N-terminal, one intracellular C-terminal and three intracellular loops connecting the four transmembrane domains (Fig. 8). There are in total 11 proline rich (P-X-X-P) binding motifs and 22 tyrosine residues in the intracellular regions of the Na,1.5 channel. Glutamine 1077 is present in the intracellular loop connecting domain-II with domain-III and contains five potential proline rich binding motifs for Fyn kinase and two...
possible tyrosine residues for phosphorylation. So, the presence or absence of glutamine at position 1077 might make different proline rich binding sites or different tyrosines more accessible to Fyn kinase for subsequent phosphorylation and in turn differential modulation in both splice variants. We evaluated the importance of the glutamine residue at position 1077 by mutating it to lysine, proline, tyrosine or alanine (Fig. 6). All the mutants at position 1077 exhibited a hyperpolarizing shift in fast inactivation indicating that the presence of glutamine accounts for differential regulation by Fyn kinase.

In conclusion, Fyn kinase differentially modulates Na\textsubscript{v}1.5 splice variants which results in synchronized steady-state fast inactivation kinetics for smooth electrical activity of the heart. Modulation by Fyn kinase is a complex, multistep process, involving several tyrosine residues for interaction, which we are currently exploring. The results of our present study will likely improve our understanding of the post translational modifications of cardiac sodium channel and their role in the cardiac electrical activity.

We would like to mention that the physiological implication of alternative splicing in Na\textsubscript{v}1.5 channel is not fully understood [22]. Makielski and coworkers described expression levels of both Q-del and Q-pre splice variants by PCR techniques; these results have not yet been corroborated by specific protein detection methods [3]. A common polymorphism (H\textsubscript{558}R) in the Na\textsubscript{v}1.5 channel is also reported to occur in the population at a frequency of 19-24% [29]. Although this point mutation has been reported not to alter the half maximal inactivation of Na\textsubscript{v}1.5 channel, a complex allosteric effect due to phosphorylation by Fyn kinase cannot be excluded. Finally \textit{in vitro} techniques are designed in close similarity with \textit{in vivo} conditions, but heterologous expression system cannot truly reflect cardiac myocytes. Despite this fact, heterologous expression system is the most widely used technique for analysis and characterization of ion channels so it is proposed that our results might be extrapolated accordingly.
Acknowledgements

We are deeply thankful to Dr. Waheed Shabbir and Dr. Muhammad Aufy for their helpful discussion and guidance on experimental work.

Disclosure Statement

The authors disclose no conflict of interest. This work was supported by Higher Education Commission of Pakistan in collaboration with OeAD-GmBH, and by Open Access Publishing Fund of the University of Vienna.

References

1. Makielski JC, Ye B, Valdivia CR, Pagel MD, Pu JL, Tester DJ, Ackerman MJ: A ubiquitous splice variant and a common polymorphism affect heterologous expression of recombinant human SCN5A heart sodium channels. Circ Res 2003;93:821-828.
2. Abriel H: Roles and regulation of the cardiac sodium channel Na\textsubscript{v}1.5: Recent insights from experimental studies. Cardiovasc Res 2007;76:381-389.
3. Walzik S, Schroeter A, Benndorf K, Zimmer T: Alternative splicing of the cardiac sodium channel creates multiple variants of mutant T1620K channels. PloS one 2011;6:e19188.
4. Wang Y, Wagner M, Kumar R, Cheng J, Joyner R: Inhibition of fast sodium current in rabbit ventricular myocytes by protein tyrosine kinase inhibitors. Pflugers Arch - Eur J Physiol 2003;446:485-491.
5. Liu H, Sun HY, Lau CP, Li GR: Regulation of voltage-gated cardiac sodium current by epidermal growth factor receptor kinase in guinea pig ventricular myocytes. J Mol Cell Cardiol 2007;42:760-768.
6. Jespersen T, Gavillet B, van Bemmelen MX, Gordonier S, Thomas MA, Staub O, Abriel H: Cardiac sodium channel Na\textsubscript{v}(1.5) interacts with and is regulated by the protein tyrosine phosphatase FTPH1. Biochem Biophys Res Commun 2006;348:1455-1462.
7. Ahern CA, Zhang JF, Wookalis MJ, Horn R: Modulation of the cardiac sodium channel Na\textsubscript{v}1.5 by Fyn, a Src family tyrosine kinase. Circ Res 2005;96:991-998.
8. Rook MB, Evers MM, Vos MA, Bierhuizen MF: Biology of cardiac sodium channel Na\textsubscript{v}1.5 expression. Cardiovasc Res 2012;93:12-23.
9. Zhou J, Yi J, Hu N, George AL, Jr, Murray KT: Activation of protein kinase A modulates trafficking of the human cardiac sodium channel in Xenopus oocytes. Circ Res 2000;87:33-38.
10. Herren AW, Bers DM, Grandi E: Post-translational modifications of the cardiac Na channel: contribution of CaMKII-dependent phosphorylation to acquired arrhythmias. Am J Physiol Heart Circ Physiol 2013;305:H431-445.
11. Wang J, Liu X, Sentex E, Takeda N, Dhalla NS: Increased expression of protein kinase C isoforms in heart failure due to myocardial infarction. Am J Physiol Heart Circ Physiol 2003;284:H2277-2287.
12. Qu Y, Rogers JC, Tanada TN, Catterall WA, Scheuer T: Phosphorylation of S1505 in the cardiac Na\textsuperscript{+} channel inactivation gate is required for modulation by protein kinase C. J Gen Physiol 1996;108:375-379.
13. Maier SK, Westenbroek RE, Schenkman KA, Feigl EO, Scheuer T, Catterall WA: An unexpected role for brain-type sodium channels in coupling of cell surface depolarization to contraction in the heart. Proc Natl Acad Sci USA 2002;99:407-4078.
14. Tsukita S, Oishi K, Akiyama T, Yamanashi Y, Yamamoto T, Tsukita S: Specific proto-oncogenic tyrosine kinases of src family are enriched in cell-to-cell adherens junctions where the level of tyrosine phosphorylation is elevated. J Cell Biol 1991;113:867-879.
15. Hsu KL, Fan HJ, Chen YC, Huang YS, Chen CH, Wu JC, Wang SM: Protein kinase C-Fyn kinase cascade mediates the oleic acid-induced disassembly of neonatal rat cardiomyocyte adherens junctions. Int J Biochem Cell Biol 2009;41:1536-1546.
16. Yarbrough TL, Lu T, Lee HC, Shibata EF: Localization of cardiac sodium channels in caveolin-rich membrane domains: regulation of sodium current amplitude. Circ Res 2002;90:443-449.
Iqbal et al.: Fyn Kinase Synchronizes Na\textsubscript{v}1.5 Channel Splice Variants

Razani B, Woodman SE, Lisanti MP: Caveolae: from cell biology to animal physiology. Pharmacol Rev 2002;54:431-467.

Beacham D, Ahn M, Catterall WA, Scheuer T: Sites and molecular mechanisms of modulation of Na\textsubscript{v}1.2 channels by Fyn tyrosine kinase. J Neurosci 2007;27:11543-11551.

Ahn M, Beacham D, Westenbroek RE, Scheuer T, Catterall WA: Regulation of Na\textsubscript{v}1.2 channels by brain-derived neurotrophic factor, TrkB, and associated Fyn kinase. J Neurosci 2007;27:11533-11542.

Camacho JA, Hensellek S, Rougier JS, Blechschmidt S, Abriel H, Benndorf K, Zimmer T: Modulation of Na\textsubscript{v}1.5 channel function by an alternatively spliced sequence in the DII/DIII linker region. J Biol Chem 2006;281:9498-9506.

Kate J, Doolittle RF: A simple method for displaying the hydrophobic character of a protein. J Mol Biol 1982;157:105-132.

Schroeter A, Walzik S, Blechschmidt S, Haufe V, Benndorf K, Zimmer T: Structure and function of splice variants of the cardiac voltage-gated sodium channel Na\textsubscript{v}1.5. J Mol Cell Cardiol 2010;49:16-24.

Tan BH, Valdivia CR, Rok BA, Ye B, Ruwaldt KM, Tester DJ, Ackerman MJ, Makielski JC: Common human SCN5A polymorphisms have altered electrophysiology when expressed in Q1077 splice variants. Heart Rhythm 2005;2:741-747.

Tan BH, Valdivia CR, Song C, Makielski JC: Partial expression defect for the SCN5A missense mutation G1406R depends on splice variant background Q1077 and rescue by mexiletine. Am J Physiol Heart Circ Physiol 2006;291:H1822-H1828.

Li GR, Lau CP, Shrier A: Heterogeneity of sodium current in atrial vs epicardial ventricular myocytes of adult guinea pig hearts. J Mol Cell Cardiol 2002;34:1185-1194.

Lin X, Liu N, Lu J, Zhang J, Anunomwe JM, Isom LL, Fishman GI, Delmar M: Subcellular heterogeneity of sodium current properties in adult cardiac ventricular myocytes. Heart Rhythm 2011;8:1923-1930.

Shy D, Gillet L, Abriel H: Targeting the Sodium Channel Na\textsubscript{v}1.5 to Specific Membrane Compartments of Cardiac Cells: Not a Simple Task! Circ Res 2014;115:901-903.

Marionneau C, Abriel H: Regulation of the cardiac Na\textsuperscript{+} channel Na\textsubscript{v}1.5 by post-translational modifications. J Mol Cell Cardiol 2015;82:36-47.

Yang P, Kanki H, Drolet B, Yang T, Wei J, Viswanathan PC, Hohnloser SH, Shimizu W, Schwartz PJ, Stanton M, Murray KT, Norris K, George AL, Jr., Roden DM: Allelic variants in long-QT disease genes in patients with drug-associated torsades de pointes. Circulation 2002;105:1943-1948.