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Post-Transcriptional Gene Silencing of KChIP2 and $Na_v\beta1$ in Neonatal Rat Cardiac Myocytes Reveals a Functional Association between Na and Ito currents

Isabelle Deschênes[1,*], **Antonis A. Armoundas**[1,2,#], **Steven P. Jones**[1,†], and **Gordon F. Tomaselli**[1]

1*Department of Medicine, Division of Cardiology, Johns Hopkins University, 720 N Rutland Avenue, Ross 844, Baltimore, MD, USA, 21205.*

2*Department of Biomedical Engineering, Johns Hopkins University, 720 N Rutland Avenue, Ross 844, Baltimore, MD, USA, 21205.*

3*Heart & Vascular Research Center, MetroHealth Campus of Case Western Reserve University, 2500 MetroHealth Drive, Cleveland, OH, USA, 44109.*

Abstract

The Ca^{2+}-independent transient outward potassium current (I_{to}) encoded by the Kv4 family of potassium channels, is central to normal repolarization of cardiac myocytes. KChIPs are a group of Ca^{2+}-binding accessory subunits that modulate Kv4-encoded currents. However, the biophysical effects of KChIP2 on Kv4 currents raise questions about the role that KChIP2 plays in forming the native I_{to}. Previous heterologous expression studies demonstrated that the Na channel β1 subunit modulates the gating properties of Kv4.3 to closely recapitulate native I_{to} suggesting that $Na_v\beta1$ may modulate the function of Kv4-encoded channels in native cardiomyocytes. Therefore we hypothesized the existence of a structural or functional complex between subunits of I_{to} and I_{Na}. In co-immunoprecipitation of proteins from neonatal rat ventricular myocardium (NRVM), $Na_v\beta1$ was pulled-down by Kv4.x antibodies suggesting a structural association between subunits that comprise I_{to} and I_{Na}. Remarkably, post-transcriptional gene silencing of KChIP2 in NRVM, using small interfering RNAs specific to KChIP2, suppressed both cardiac I_{to} and I_{Na} consistent with a functional coupling of these channels. KChIP2 silencing suppressed Na channel α and β1 subunit mRNA levels, leaving Kv4.x mRNAs unaltered, but reducing levels of immunoreactive proteins. Post-transcriptional gene silencing of $Na_v\beta1$ reduced its protein expression. Silencing of $Na_v\beta1$ also reduced mRNA and protein levels of its α-subunit, $Na_v1.5$. Surprisingly, silencing of $Na_v\beta1$ also produced a reduction in KChIP2 mRNA and protein as well as Kv4.x proteins resulting in remarkably decreased I_{Na} and I_{to}. These data are consistent with a novel structural and functional association of I_{Na} and I_{to} in NRVMs.

Corresponding Authors: Gordon F. Tomaselli The Johns Hopkins University, 720 N Rutland Avenue, Ross 844, Baltimore, MD, USA, 21205, Phone: 410-955-2774, Fax: 410-955-7953, Email: gtomasel@jhmi.edu. Isabelle Deschênes, Case Western Reserve University, 2500 MetroHealth Drive,Rammelkamp658, Cleveland, OH, USA, 44109, Phone : 216-778-5166, Fax : 216-778-1261, Email : ideschenes@metrohealth.org.

[*]**Current Address:** Case Western Reserve University, MetroHealth Campus, Heart & Vascular Research Center 2500 MetroHealth Drive, Rammelkamp 658, Cleveland, OH 44109

[#]Harvard Medical School, Massachusetts General Hospital, Cardiovascular Research Center, 149 13th Street Charlestown, MA 02129

[†]Institute of Molecular Cardiology, University of Louisville, Baxter II - Room 404C, 580 S. Preston St. Louisville, KY 40202

Keywords

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Introduction

The Ca^{2+}-independent transient outward potassium current (I_{to}) plays a central role in the repolarization of cardiac myocytes. The pore-forming subunit that underlies I_{to} in the mammalian heart is a member of the Kv4 family of genes and is hKv4.3 in human ventricle [1]. Heterologous expression of Kv4 α-subunits generates currents similar, but not identical to native I_{to}, suggesting that accessory subunits functionally contribute to the channel phenotype. K channel interacting protein (KChIP), specifically KChIP2 is expressed in the heart and modulates expressed Kv4 currents but again, does not reproduce the native I_{to} with complete fidelity [2] [3]. These data suggest the existence of other factors in cardiac myocytes that are essential to complete functional expression of the native I_{to}.

Potentially lethal arrhythmias in rare inherited syndromes (idiopathic ventricular fibrillation) [4] and more common, acquired heart diseases (cardiac hypertrophy and failure) [5] [6] have been associated with an imbalance of depolarizing (Na current) and repolarizing (I_{to}) current early in the ventricular action potential and a down regulation of I_{to} respectively. Delineation of the molecular basis of I_{to} is therefore essential for an accurate understanding of cardiac ventricular repolarization and its derangements that are associated with lethal ventricular arrhythmias. Our previous work in an expression system (HEK 293 cells) suggests an unexpected modulation of Kv4 by the sodium channel β1 subunit [3]. This suggests the existence of an interaction between subunits of I_{to} and I_{Na}, and the possibility of a macromolecular complex between these two ion channels. This is not unprecedented as it has become clear that cardiac ion channels function as components of macromolecular complexes, comprising the α-subunits, one or more accessory subunits, and a variety of other regulatory proteins. In addition, these macromolecular channel protein complexes appear to also interact with the actin cytoskeleton and/or the extracellular matrix, suggesting important functional links between channel complexes and the cytoskeleton that influence electrical functioning [7]. Based on the functional effects of the Na channel β_1 subunit on Kv4 currents and the colocalization of the channel subunits underlying I_{to} and I_{Na}, we hypothesize the existence of a structural or functional complex between subunits of I_{to} and I_{Na}. In order to test this hypothesis, co-immunoprecipitation experiments were done to elucidate the structural link between subunits of both channels. In addition, sequence-directed RNA interference (RNAi) with small interfering RNAs (siRNA) specific to KChIP2 and $Na_v\beta1$ were used in order to delineate the molecular basis of I_{to} and to delineate the functional link between I_{to} and I_{Na}.

Material and Methods

siRNAs

siRNA corresponding to rat KChIP2 was designed as recommended [8], with 5’ phosphate, 3’ hydroxyl, and two base overhangs on each strand; it was chemically synthesized and annealed by Qiagen (Valencia, CA). The following gene-specific sequences to all known rat KChIP2 splice variants (5’-AATGCCTTTGACACCAACCAC-3’) and the rat Navβ1 subunit (5’-AATTACGAGCACAACACCAGC-3’) were used successfully. A Blast search of these sequences against the rat genome was performed and did not reveal matches with any other gene. In order to identify transfected cells, the siRNAs were labelled on the 3’-end of the sense strand with Rhodamine.

Two different negative controls were used for both KChIP2 and Navβ1 silencing. A commercially available non-silencing control siRNA labelled with fluorescein (Qiagen) was used. In addition, we used single base pair mismatch siRNAs for both KChIP2 and the Navβ1 subunit: KChIP2 (5'-AATGCCTATGACACCAACCAC-3') and rat Navβ1 subunit (5'-AATTACGGGCACAACACCAGC-3'). The mismatched base is underlined. None of these 2 sequences matched any genes in the rat genome.

Neonatal Rat ventricular Myocytes Isolation and Transfections

Neonatal rat ventricular myocytes (NRVMs) were isolated from 1- or 2-day old Sprague-Dawley rats and cultured as previously described [9]. Briefly, hearts were obtained following decapitation. The hearts were immersed in CBFHH (Calcium, Bicarbonate-Free Hank's with HEPES) and minced with a straight razor. Cells were then digested with a Trypsin 250 solution (0.2g of trypsin in CBFHH) at 37°C and then put in FBS. Cells were pelleted by centrifugation at 1000 RPM for 5 minutes and were resuspended in warm DMEM containing 5% FBS, 1% Penicillin-Streptomycin and Vitamin B_{12} (1:1000). Cells were then preplated for 30 minutes at 37°C to allow fibroblasts to adhere to the plate. Cells were pelleted again and resuspended in DMEM containing 5% FBS, 1% Penicillin-Streptomycin, Vitamin B_{12} (1:1000) and bromodeoxyuridine (1:100, to inhibit fibroblast growth). NRVMs were then plated at a concentration of about 1 million cells/35mm plate.

Transfections of siRNA for endogenous gene targeting were carried out using TransMessenger transfection reagent according to the manufacturer's instructions (Qiagen) 24 to 72 hours after initial plating. Briefly, 2 μg of siRNA was mixed with the transfection reagent and incubated at 37°C with the cells in serum free DMEM for a minimum of 3 hours. Complete DMEM (with FBS and Penicillin-Streptomycin) was then added to the cells without removing the siRNA mixture. The following siRNAs were transfected into NRVMs: KChIP2 siRNA, $Na_v\beta1$ siRNA, control non-silencing siRNA, single base pair mismatch siRNA for KChIP2 (control) and single base pair mismatch siRNA for Navβ1 subunit (control). Cells were used 48 to 72 hours after transfections.

HEK 293 Cells Transfection

Transient transfections into human embryonic kidney (HEK293) cells were performed using the Polyfect transfection kit (Qiagen) according to the manufacturer's protocol for 24 hours for electrophysiological recordings and 48 hours for co-immunoprecipitations. 1μg of each cDNA was used.

Real-time PCR, Western Blotting and Co-Immunoprecipitation

Fluorescence-based kinetic real-time PCR was performed using a Perkin-Elmer Applied Biosystems Model 7900 sequence detection system as previously described; in brief, the relative mRNA abundance expressed as arbitrary units was calculated using the expression levels of all transcripts normalized to 18S rRNA. This value was then normalized to mRNA levels measured from NRVMs transfected with either the non-silencing control siRNA or the control single base pair mismatch siRNA for the appropriate transcript [2]. For Western Blotting and co-immunoprecipitation, cell lysates were prepared as previously described [10]. Briefly, cells were washed with cold PBS and then harvested in lysis buffer. Cells were briefly sonicated and then lysed for an hour at 4°C. Cells were then centrifuged for 4 minutes at 4000 rpm at 4°C. Protein concentration of the supernatant was measured. The lysis buffer contained for 10 ml: 0.05M HEPES, 0.15 M KCl, 0.5 mM CaCl2, 1% IGEPAL, 1 tablet of the proteases inhibitor cocktail Complete Mini (Roche) and 100 μl of 40 mM AEBSF. All samples were run in duplicate on Tris-HCl precast gels (Bio-Rad, Hercules, CA) in 25mmol/L Tris, 192mmol/L glycine and 0.1% (w/v) SDS running buffer as previously described [11]. Primary antibody incubations were performed overnight at 4°C using antibodies to the following:

KChIP2 (gift of Dr. James Trimmer), Kv4.2 (AB560, Chemicon), Kv4.3 (SC-11686, Santa Cruz), Kv1.4 (AB5180, Chemicon), $Na_v1.5$ (Upstate), $Na_v\beta1$ (antibody raised to the following epitope: KRRSETTAETFTEWTFR), Cav1.2 (ACC-003, Alomone) and Kir2.1 (AB5374, Chemicon). Bands on the Western blots were considered specific if they were not present when the gel was stained with pre-immune serum and if the peptide epitope reduced the band intensity. Further, antibody specificity was assessed by isolation of proteins from cells transfected with cDNAs encoding the proteins of interest. Only with satisfaction of these criteria were antibodies then used in immunopreciptation experiments. Relative band densities were quantified using ImageQuant software (Molecular Dynamics). To normalize for protein loading, we used GAPDH (from RDI-TRK #5G4-6C5) for Kv4.2, Kv4.3, Kv1.4 and $Na_v1.5$ and calsequestrin (from RDI #CALSEQabR) for KChIP2 and $Na_v\beta1$ because of their respective sizes.

Co-immunoprecipitation experiments were performed using lysates from NRVMs transfected with a control non-silencing siRNA for interaction between $Na_v\beta1$ and Kv4.2 or Kv4.3, or from transfected HEK 293 cells for interaction experiments between KChIP2 and Nav1.5 or Kv4.3. The cell lysates were pre-absorbed with Protein A Dynabeads (Dynal, Norway) for 2 hours at 4°C. The unbound extracts were then incubated overnight at 4°C with Protein A Dynabeads crosslinked to either Kv4.2, Kv4.3 or KChIP2 polyclonal antibodies. The beads were then washed four times with lysis buffer. The bound complexes were eluted from the beads by adding 2X loading buffer to the beads and heating the samples at 70°C for 10 minutes. For Co-immunprecpitation between KChIP2 and $Na_v1.5$, the elution was done at 37°C for 20 minutes. Western blots were then performed and probed with the appropriate antibodies.

Electrophysiology

Macroscopic currents from transfected NRVMs were recorded using the whole-cell configuration of the patch-clamp technique [12]. Currents were recorded using an Axopatch 200B patch-clamp amplifier (Axon Instruments, Foster City, CA) interfaced to a personal computer. Voltage commands were issued and data collected with custom-written software. Low resistance electrodes (~2MΩ when filled with internal solution) were pulled from borosilicate, and a routine series resistance compensation of the Axopatch 200B amplifier was performed to values >80% to minimize voltage-clamp errors. To ensure the quality of the voltage clamp, we measured the time constant of membrane charge capacitance. The time constant of membrane charge capacitance was measured by fitting the decay of the uncompensated capacity transient. The cell capacitance (C_m) was calculated by integrating the area under the uncompensated capacity transient elicited by a 20 mV hyperpolarizing test pulse from a holding potential of −80 mV and averaged18 pF. The uncompensated R_{series} was therefore less than 2MΩ. The internal solution contained (in mM): potassium glutamate 130, KCL 9, NaCl 10, MgCl2 0.5 and MgATP 5 and HEPES 10 (pH 7.2 with KOH). Myocytes were superfused with a physiological salt solution containing (in mM): 138 NaCl, 4 KCl, 2 CaCl2, 1 MgCl2, 0.33 NaHPO4, 10 HEPES, 10 Glucose (pH 7.4 with NaOH). The pipette-to-bath liquid junction potential was −17 mV and was corrected off-line. Membrane currents were filtered at 5 kHz and digitized with 12-bit resolution. Experiments were performed at room temperature (22–23°C). Whole-cell currents were elicited by a family of depolarizing voltage steps from a holding potential of −80 mV. A 40 ms prepulse to −40 mV was used to inactivate I_{Na}. In order to record the current-voltage (I/V) relationships for I_{to}, Ca^{2+} currents were blocked with nifedipine after action potential and all other current recordings were made.

For sodium channel family recordings both in NRVMs and HEK 293 cells the following solutions were used: the internal solution contained (in mmol/L) NaCl 10, CsF 105, EGTA 10, and Cs-HEPES 10 adjusted to pH 7.4. The bath solution contained (in mmol/L) NaCl 140, KCl 5, $MgCl_2$ 1, $CaCl_2$ 2, HEPES 10, and glucose 10 adjusted to pH 7.4.For sodium currents

recordings in control NRVM, a low external sodium concentration was used (25mM). Whole cell sodium current densities were measured by dividing the peak current obtained from stepping in 10 mV intervals from −80 to 50 mV from the holding potential of −120 mV (I/V protocol) by the cell capacitance. Time course of recovery from inactivation (τ_{rec}) was studied using a 2-pulse protocol with a 30 ms prepulse to −30 mV followed by varying rest intervals at −120 mV and a 30 ms test pulse to −30 mV. The recovery kinetics were determined by fitting the normalized peak current amplitudes to the following equation:

$$I_{test}/I_{pre-pulse}=1-\exp(-t/\tau_{rec})$$

The voltage dependence of steady state inactivation was determined by 500 ms prepulses ranging from −140 to −30 mV. The voltage dependence of inactivation was determined by fitting the normalized peak currents to a Boltzmann distribution:

$$I/I_{max}=(1+\exp[(V-V_{1/2})/k_v])^{-1}$$

Statistical analysis

Data are expressed as mean ± SEM. When indicated, a Student's t-test was performed using statistical software in Origin (Microcal ™ Software, Inc). Differences were considered to be significant at a *P* value <0.05.

Results

Co-Immunoprecipitation

In heterologous expression studies, co-transfection of $Na_v\beta1$ subunits with Kv4.3 produces a current that appeared to recapitulate cardiac I_{to} with the highest fidelity compared to other known K^+ channel accessory subunits [3]. Thus we sought to determine if the subunits underlying I_{to} and I_{Na} interact in cardiac myocytes. Antibodies to either anti-Kv4.2 or Kv4.3 precipitated $Na_v\beta1$ subunits from NRVM protein extracts (n=4) (Fig. 1A).

In order to further evaluate the interactions of Na and K channel subunits, channel proteins were expressed in a cell culture system. First, in order to validate our technique, we performed a co-immunoprecipitation between KChIP2 and Kv4.3, which are known to interact. As expected, in HEK 293 cells expressing both KChIP2 and Kv4.3, the KChIP2 antibody was able to pull-down the Kv4.3 protein (Fig. 1B). Co-immunoprecipitation experiments were then performed using an anti-KChIP2 antibody, which did not reveal an association between $Na_v1.5$ and KChIP2 (n=4) (Fig. 1B, lane 1). Figure 1B (lane 1) shows the IP lane of HEK 293 cells transfected with both $Na_v1.5$ and KChIP2 which did not exhibit a band for $Na_v1.5$. $Na_v1.5$ was detected in the supernatant, consistent with the absence of direct association between $Na_v1.5$ and KChIP2.

KChIP2 modulation of $Na_v1.5$ biophysical properties

We have previously shown that $Na_v\beta1$ can modulate the biophysical properties of Kv4.3 [3] and this is consistent with the interaction of these channel subunits as assessed by co-immunoprecipitation (Fig. 1A). However, it appears that KChIP2, an accessory subunit of Kv4.3, does not reciprocally interact with $Na_v1.5$ (Fig. 1B). To further assess the potential interaction between KChIP2 and $Na_v1.5$, we co-expressed these channel subunits in HEK 293 cells and studied the biophysical properties of I_{Na} using the patch-clamp technique (Fig. 2). Co-expression of KChIP2 did not affect the gating properties of the Na current (Fig. 2). However, in this heterologous expression system, KChIP2 did increase the $Na_v1.5$ current density and this increase was greater when co-expressing both KChIP2 and $Na_v\beta1$ compared with KChIP2 alone (At −30 mV, $Na_v1.5$: 495 pA/pF ± 67 n=8, $Na_v1.5$ + KChIP2: 648 pA/pF ± 52* n=14, $Na_v1.5$ + $Na_v\beta1$: 682 pA/pF ± 71* n=12, $Na_v1.5$ + $Na_v\beta1$ + KChIP2: 795 pA/pF

± 55* n=10) (* $p<0.05$ for voltages −40 to +10 mV compared to $Na_v1.5$) (Fig. 2A). Still, KChIP2 did not modulate steady-state inactivation (Fig. 2B) and recovery from inactivation (Fig. 2C). The $V_{1/2}$ values for steady-state inactivation are: $Na_v1.5$: −88.6 mV ± 2.5 n=8, $Na_v1.5$ + KChIP2: −93.4 mV ± 2.2 n=14, $Na_v1.5$ + $Na_v\beta1$: −90.2 mV ± 1.8 n=12, $Na_v1.5$ + $Na_v\beta1$ + KChIP2: −91.4 mV ± 2.5 n=10 (Fig. 2B). The time constant of recovery from inactivation are $Na_v1.5$: 8.2 ms ± 1.7 n=8, $Na_v1.5$ + KChIP2: 10.1 ± 1.1 n=14, $Na_v1.5$ + $Na_v\beta1$: 8.9 ms ± 1.4 n=12, $Na_v1.5$ + $Na_v\beta1$ + KChIP2: 10.9 ms ± 1.9 n=10 (Fig. 2C)

Gene silencing of KChIP2

In order to further explore the association between Na channel and I_{to} channel subunits and to examine the functional consequences of this interaction, we used RNA interference (RNAi) to silence the expression of KChIP2 and examine the electrophysiological consequences in NRVMs. Small interfering RNAs (siRNAs) are potent RNAi reagents for sequence-specific, post-transcriptional gene silencing [13] [14] [15] [16]. The use of siRNA has been remarkably robust in selective gene suppression in *Caenorhabditis elegans*, *Drosophila melanogaster*, *Trypanosoma brucei* and plants[17] [14] [15]. Direct introduction of siRNA into mammalian cell lines, [18] [19] [20] cultured mammalian neurons [21] and native cardiac myocytes [22] has been effective in suppressing endogenous and heterologously expressed genes.

An siRNA was designed to a common region of rat KChIP2 to silence the expression of all splice variants in NRVMs. Ventricular myocytes were successfully transfected (>75% efficiency) with either KChIP2 specific siRNA labelled with rhodamine or a commercially available non-silencing control siRNA labelled with fluorescein (Qiagen), or as another non-silencing control, a single base pair mismatch siRNA against KChIP2. KChIP2 siRNA nearly completely eliminated the expression of KChIP2 mRNA as measured using kinetic quantitative real-time PCR with primers designed to recognize all KChIP2 splice variants (Fig. 3A). RNA isolated from non-transfected myocytes, myocytes transfected with the non-silencing control siRNA and myocytes transfected with the control single base pair mismatch siRNA for KChIP2 were not statistically different and exhibited approximately 20-fold greater levels of KChIP2 mRNA.

KChIP2 immunoreactive protein was effectively suppressed by siRNA. Proteins isolated from ventricular myocytes transfected at the same time as those used for mRNA measurements, revealed robust expression of KChIP2 protein in non-transfected controls and cells transfected with non-silencing siRNA (Fig. 3B). The KChIP2-specific siRNA essentially eliminated KChIP2 protein expression (Figs. 3B and 3C). Thus the expression of KChIP2 can effectively be knocked down in NRVMs in primary culture using siRNA.

We next examined the effect of KChIP2 gene silencing on the expression of other potassium channel subunits expressed in the ventricle. We found no significant difference in the steady-state levels of Kv4.2 or Kv4.3 mRNAs, the α subunits that underlie cardiac I_{to} in the rat[1], in the KChIP2 specific siRNA transfected cells compared with the controls (Fig. 3A). Kv1.4 is the channel subunit that underlies ventricular I_{to} early in development. Kir2.1 is the α subunit that encodes for the inward rectifier K current (I_{K1}). KVLQT1 and erg are the α subunits that encode for IKs and IKr respectively. KChIP2 siRNA did not significantly alter the steady-state mRNA levels of Kv1.4, Kir2.1, KVLQT1 or erg when compared to non-silencing siRNA-transfected controls (Fig. 3A). To further assess the possible link between I_{to} and I_{Na}, we then investigated the effect of KChIP2 silencing on the sodium channel subunits. Surprisingly, the steady-state level of $Na_v\beta1$ mRNA was as drastically reduced as KChIP2 mRNA in presence of KChIP2 siRNA compared to controls (Fig 3A). The level of $Na_v1.5$ mRNA was also significantly reduced in the presence of KChIP2 siRNA while the levels of $Ca_v1.2$ (L-type Ca^{2+} channel α1 subunit), Kir2.1, erg and KVLQT1 were unchanged (Fig. 3A).

In contrast to the mRNA levels, KChIP2 siRNA dramatically suppressed the levels of Kv4.2 and Kv4.3 immunoreactive proteins. Figure 3B shows Western blots employing polyclonal antibodies specific to Kv4.2, Kv4.3 and Kv1.4. The levels of protein expression normalized to GAPDH revealed at least a 10-fold reduction in the levels of Kv4.2 and Kv4.3 immunoreactive proteins in NRVMs transfected with KChIP2 siRNA when normalized to myocytes transfected with a non-silencing siRNA (Fig. 3C). There was also a significant reduction in the level of Kv1.4 protein (Fig. 3B) in cells transfected with KChIP2 siRNA when normalized to myocytes transfected with a non-silencing siRNA but this reduction was not as dramatic as that of Kv4.x proteins (Fig. 3C). Thus, silencing KChIP2 expression does not suppress Kv4.x subunit transcription but reduces the expression of the immunoreactive proteins. The possible mechanisms include suppression of translation or a failure of trafficking Kv subunits to the cell membrane with accelerated protein degradation. In contrast, both the mRNA and protein levels of $Na_v1.5$ and $Na_v\beta1$ were significantly reduced following KChIP2 silencing (Fig. 3B, C), suggesting a functional link between KChIP2 and I_{Na}. This was not a generalized effect on ion channel subunit expression as Cav1.2 and Kir2.1 protein levels were unchanged (Fig. 3B, C).

Electrophysiological effects of KChIP2 silencing

In order to study the functional consequences of KChIP2 gene suppression, we performed patch clamp recordings on the isolated NRVMs. For currents recordings (Fig. 4A–C, middle panel), cells were held at −80 mV, stepped to −40 mV (to inactivate I_{Na}) followed by voltage steps from −70 to +70 mV in 10 mV increments for 500 msec. Only current records elicited by steps to −70, 0, 30, 50 and 70 mV are shown for clarity. $I_{Ca,L}$ is observed with steps to 0 mV and I_{to} at 30, 50 and 70 mV. Not surprisingly, whole-cell current recordings revealed a complete absence of I_{to} in KChIP2 siRNA transfected cells, (Fig. 4C middle panel and 4E) consistent with the reduction seen in mRNA and protein levels (Fig. 3). In contrast, non-transfected NRVMs, and NRVMs transfected with control non-silencing siRNA consistently expressed I_{to} (density range: 8 – 11 pA/pF) (Fig 4A, B middle panel and Table 1). The absence of I_{to} would have predicted prolongation of the action potential duration; however, quite surprisingly we could not elicit action potentials in any myocytes transfected with KChIP2 siRNA (Fig. 4C left panel) despite the presence of a normal resting membrane potential (KChIP2 siRNA −79.5 ± 12.2 *vs* controls −84.5 ± 7.8, p=N.S.) (Table 1). In order to confirm that we were injecting sufficient current in these cells to generate an action potential, we determined the threshold of excitability of cells in all groups. Despite injecting current (100pA for 4ms) several times threshold for eliciting an action potential in control cells, action potentials were never elicited in the myocytes transfected with the KChIP2 siRNA. The voltage trace seen in Fig. 4C left panel, most likely corresponds to the discharge of the membrane capacity after the large current injection.

Remarkably, post-transcriptional gene silencing of KChIP2 led to complete suppression of voltage-dependent sodium current (I_{Na}) (Fig. 4C middle and right panel) thus explaining the absence of action potentials in these NRVMs. To characterize the I_{Na} in the different cell groups, families of sodium currents were recorded using the protocols shown in the inset of the right panels (Fig. 4A–C). Low external sodium (25 mM) was used for the non-transfected and control siRNA cells in order to maintain a controlled sodium current clamp. However, in presence of KChIP2 siRNA, even with physiological extracellular sodium concentration, little to no sodium current was elicited (Fig. 4C right panel, Fig. 4D). To ensure that the absence of I_{Na} was not the result of a shift in the voltage dependence of gating, we held the cells at a more negative potential (−120 mV) and delivered test pulses over a wider voltage range and were still unable to elicit significant sodium currents (Fig.4D). The absence of I_{to} and I_{Na} was not the result of an artefact or a non-specific toxic effect in the KChIP2 siRNA transfected cells since other ionic currents such as the L-type Ca^{2+} current and I_{K1} were still present and their

current densities were not different than control cells (Fig. 4C middle panel and Table 1). Further, cells transfected with control siRNA exhibited the same complement of ionic currents as the non-transfected controls and robust action potentials (Fig 4A–B and Table 1).

Gene silencing of $Na_v\beta1$

To further examine the link between $Na_v\beta1$ and I_{to} channel subunits, we used an siRNA specific for $Na_v\beta1$. Transfection of NRVMs with $Na_v\beta1$ siRNA significantly reduced both the mRNA and protein level of $Na_v\beta1$ (Fig. 5A). In addition to $Na_v\beta1$, the mRNA levels of $Na_v1.5$ and surprisingly KChIP2 were also significantly reduced (Fig. 5A) in presence of $Na_v\beta1$ siRNA compared to non-transfected and control transfected cells. Silencing $Na_v\beta1$ did not affect the steady-state levels of Kv4.x mRNA but significantly reduced the expression of the immunoreactive proteins (Fig. 5A and B). However it is unclear whether the reduction in Kv4 proteins is caused directly by $Na_v\beta1$ silencing or is the result of KChIP2 mRNA reduction following $Na_v\beta1$ silencing. Patch clamp recording of isolated ventricular myocytes transfected with $Na_v\beta1$ siRNA revealed a marked reduction in I_{Na} and I_{to} in $Na_v\beta1$ siRNA transfected cells (Fig. 5C). Current densities of I_{to} at +70 mV were 10.7 ± 2.6 pA/pF in control cells compared to 2.5 ± 1.2 pA/pF in $Na_v\beta1$ siRNA transfected cells; however, the biophysical properties of I_{to} were not different compared to control cells (Fig. 4E, 5C and Table 1). Measurement of I_{Na} in control cells were made in presence of low extracellular sodium (25mM), whereas physiological extracellular sodium concentration was used in cells transfected with the $Na_v\beta1$ siRNA. There was a dramatic reduction of I_{Na} in presence of the Navβ1 siRNA compared to control cells or NRVMs transfected with control non-silencing siRNA (Fig. 5C–D and Fig. 4D). Action potentials could not be elicited in the $Na_v\beta1$ siRNA transfected cells, likely the result of the very small I_{Na}.

Discussion

These data reveal a complex interaction between the subunits of the cardiac sodium and transient outward potassium channels in neonatal rat ventricular myocytes. Co-immunoprecipitation experiments are consistent with a physical association between Kv4.x and $Na_v\beta1$ subunits but not KChIP2 and $Na_v1.5$. Knock down experiments using specific siRNAs demonstrate that KChIP2 and $Na_v\beta1$ are required for functional expression of I_{Na} and I_{to} in NRVMs.

The absence of I_{to} after transfection with KChIP2 siRNA is concordant with the absence of this current in KChIP2(−/−) mice [23]. However the absence of I_{Na} was surprising, although in our previous work, coexpression of Kv4.3 with Navβ1 appeared to most closely recapitulate cardiac I_{to} when compared to a number of other K channel accessory subunits [3]. The germline knock-out of KChIP2 in mice produces a phenotype which includes the absence of I_{to}, action potential prolongation and susceptibility to ventricular arrhythmias [23]. Additionally, a recent paper reported the effects of a germ-line knockout of $Na_v\beta1$ [24]. Quite similar to Kuo et al [23], the authors find action potential prolongation, but no change in excitability. The prolongation in QT observed in this model may be due to effects on sodium currents, however, effects on potassium currents have not been excluded and could be explained by effects of $Na_v\beta1$ on the density and biophysics of Kv4.3-based currents [3]. Post-natal reduction of the expression of KChIP2 and $Na_v\beta1$ in our work produces a distinct phenotype with absent or dramatically reduced I_{to} and I_{Na} and myocyte inexcitability. There are several possibilities for this discrepancy between our gene silencing model and the knock out animal models. With respect to the knock out animals, clearly our experimental design was different; the reduction in KChIP2 and $Na_v\beta1$ was neither complete nor present from the earliest stages of development and was performed in a cell culture system. Elimination of the protein in the germline of a knockout animal may allow for the recruitment of compensatory mechanisms for the

development of cardiac excitability to compensate for some functions of KChIP2 (e.g. expression of Na channel subunits) otherwise this would have been an embryonic lethal transgenic. This type of developmental plasticity may not exist in a terminally differentiated cell such as a cardiac myocyte. In contrast to the KChIP2-silenced NRVMs, knockout mouse cells exhibit apparently normal excitability and presumably Na^+ currents although not measured specifically [23]. Our data suggest that the effect of KChIP2 on Na channel subunits involves alteration of transcription or mRNA stability compared with the effects on Kv4 channel expression which is clearly post-transcriptional. Another possibility is that experimental conditions, specifically isolated myocytes in culture, reveal an effect of KChIP2 and $Na_v\beta1$ not normally present in a developing animal. Finally but less likely, there could be a fundamentally different relationship between I_{to} and I_{Na} in mice compared to rats. The absence of an effect of control non-silencing siRNA on the molecular biology and electrophysiology of the myocyte and the complementary effects of the KChIP2 and the $Na_v\beta1$ siRNAs argue strongly against a non-specific effect of the siRNA knockdown in this cell system.

It is remarkable that post-transcriptional silencing of KChIP2 suppressed Kv4 channel translation and/or accelerated protein degradation but dramatically and selectively reduced mRNA levels of Navβ1 and to a lesser extent $Na_v1.5$. It is not clear whether the suppression of $Na_v1.5$ mRNA is a direct effect of KChIP2 gene silencing or is secondary to the dramatic reduction in Navβ1 expression. Another possibility could be that the changes in I_{to} could affect sodium current as a consequence of potassium ion levels. In either case, gene suppression of KChIP2 has revealed and suggested a novel role for this K channel ancillary subunit in cardiac myocytes. In NRVMs in culture, KChIP2 appears to act as an “excitability switch”, inducing the expression of depolarizing I_{Na} and repolarizing I_{to}, making a polarized myocyte excitable. Similarly post-transcriptional silencing of $Na_v\beta1$ suppressed both I_{Na} and I_{to}. Our data do not allow us to determine if this is due to a reduction in $Na_v\beta1$ or KChIP2 expression. In either case, $Na_v\beta1$ silencing complements the KChIP2 siRNA data and reinforces this hypothesis that there is a link between I_{to} and I_{Na} possibly through their accessory subunits.

The association between subunits of the I_{Na} and I_{to} appears to be both structural and functional in nature and all of the effects do not necessarily require being part of a macromolecular complex. Indeed the transcriptional effects of KChIP2 suppression on $Na_v1.5$ and $Na_v\beta1$ mRNA are likely to be due to a direct effect on the transcriptional machinery or RNA stability. We have previously shown [2] that KChIP2 protein is expressed in the nucleus of cardiac myocytes consistent with, but certainly not proving such a mechanism. The effect of suppression of $Na_v\beta1$ expression, may be working primarily through its effects on KChIP2. It is fascinating to speculate, but with no evidence at this point that a surface complex of ion channel subunits might regulate its own transcription, translation and/or trafficking. On the other hand, the co-immunoprecipitation of $Na_v\beta1$ by anti-Kv4.2 and anti-Kv4.3 antibodies from lysates of NRVMs is consistent with an association of these subunits in heart cells. The precise nature of this complex required the co-immunoprecipation of other putative members with the appropriate antibodies (e.g. immunoprecipitation Nav1.5 with anti-KChIP2). However, we did not find an interaction between $Na_v1.5$ and KChIP2. This further suggests a transcriptional effect of KChIP2 on $Na_v1.5$ and $Na_v\beta1$ rather than a physical association in the membrane.

The molecular mechanism of the reciprocal interactions between these subunits of I_{to} and I_{Na} that occur at the protein and post-transcriptional levels remains uncertain. We can hypothesize that the molecular phenotypes of the KChIP2 and $Na_v\beta1$ knockdown in NRVMs are similar but distinct in detail. KChIP2 knockdown produces a more complete inhibition of I_{to} and suppression of Kv4 protein expression. If KChIP2 is the pivotal mRNA/protein acting as a nodal point, suppression of this subunit could reduce steady state mRNA levels of $Na_v1.5$

and $Na_v\beta1$ and suppression of $Na_v\beta1$ through a reduction in KChIP2 could exhibit a similar phenotype. Any one of a number of mechanisms may be operative including: 1. RNA regulation by co-transcription; 2. transcriptional enhancement by KChIP2 in NRVMs, as has been described in some neuronal preparations; 3. Alterations of mRNA transcription, splicing and/or stability; 4. Induction of RNA binding proteins; 5. KChIP2 induced alterations in local calcium; 6. Alterations in cellular excitability producing downstream, specific effects on the maintenance of expression of certain ion channels. The reduction of Kv channel protein by KChIP2 siRNA appears to be post transcriptional. As for the effect seen on Kv1.4, even though there is no apparent association of the mature Kv1.4 channel with KChIP2, this does not exclude a direct or indirect role in Kv1.4 biogenesis, trafficking or membrane insertion.

In conclusion, previous studies have reported that siRNA could be used to specifically suppress endogenous and exogenous gene expression in mammalian cell lines [18] and also recently in native cardiac myocytes [22]. The use of RNAi in NRVMs has provided us with novel insights into the molecular basis of native I_{to}. We have confirmed the known important interaction of KChIP2 with Kv4.3 and Kv4.2, but more importantly we have demonstrated a previously unknown functional link between cardiac I_{to} and I_{Na}. Coordinate regulation of expression of subunits of I_{to} and I_{Na} is demonstrated by this study. Our previous work [3] had raised the possibility that subunits underlying these two channels may form a macromolecular complex that is required to reproduce the phenotypic characteristics of I_{to} in heart cells. Co-immunoprecipitation and gene silencing now suggest the existence of a complex involving subunits from both channels in NRVMs. Further, expression of KChIP2 is required for the development of normal excitability of rat ventricular myocytes in culture. These data provide new insights into the functional association of ionic currents that are essential to normal depolarization and repolarization in the heart. The structural and developmental link between I_{to} and I_{Na} suggests the possibility of new candidate genes (i.e. KChIP2 and Kv4.3) for heritable "Na^+ channel–related" arrhythmia syndromes such as idiopathic ventricular fibrillation.

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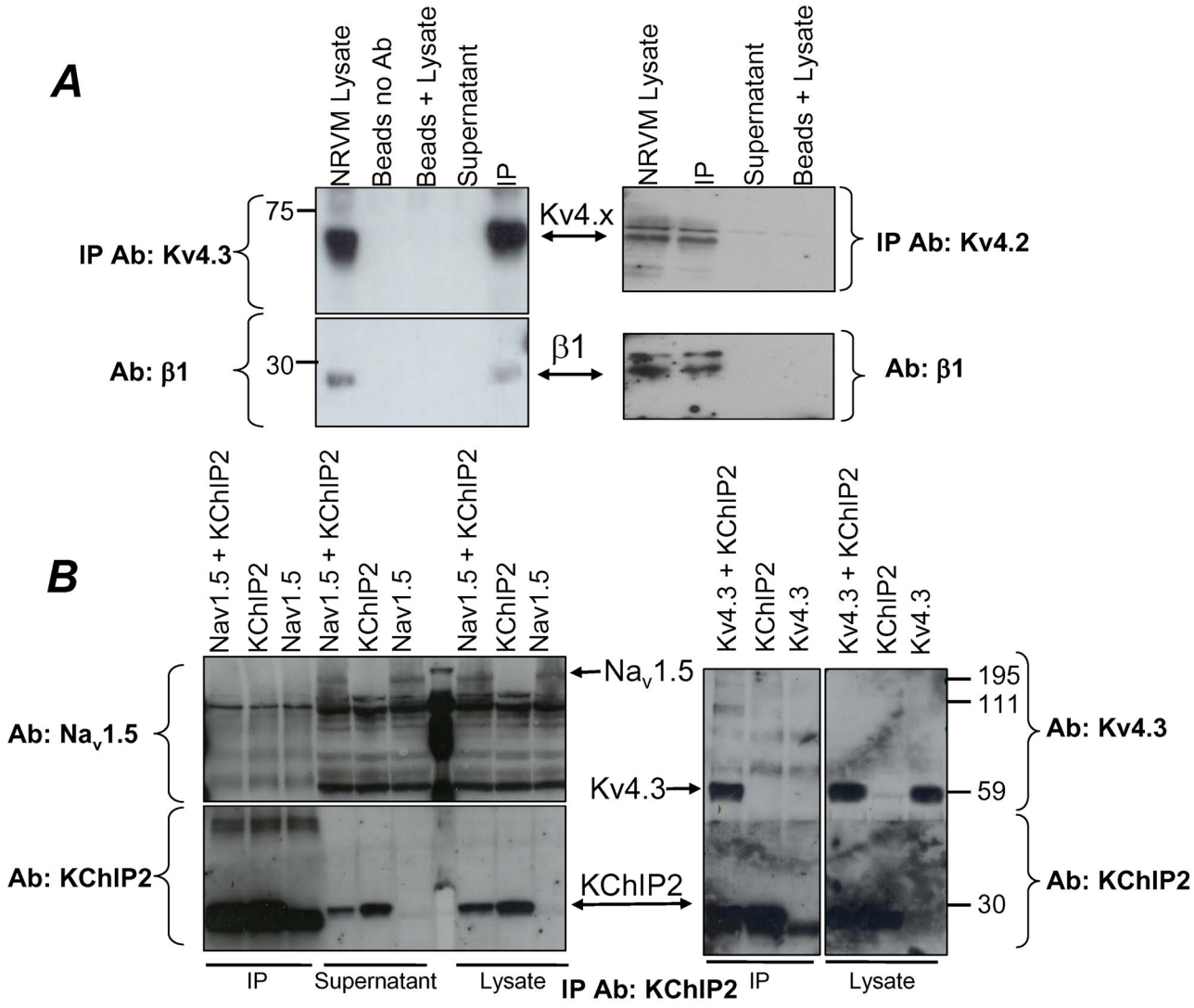

Figure 1.

Co-Immunoprecipitation of potassium and sodium channel subunits (A) Co-Immunoprecipitation of the $Na_v\beta1$ subunit by Kv4.2 and Kv4.3 antibodies. Lysates from NRVMs transfected with a control non-silencing siRNA were immunoprecipitated with Kv4.x antibodies (IP antibodies). The blot was then hybridized with an anti-$Na_v\beta1$ antibody and the Kv4.x IP antibodies. The NRVM lysate lanes represent total cell lysates from control cells transfected with a control non-silencing siRNA. The supernatant lanes are the non-bound lysate after incubation with the beads crosslinked with anti-Kv4.2 or Kv4.3 IP antibodies. The IP lanes correspond to the elution from beads crosslinked with either antibodies against Kv4.2 or Kv4.3 respectively and incubated with the protein lysate. The beads + Lysate lanes correspond to non-specific binding of the lysates on beads with no antibody and the Beads no Ab lane represents beads with neither antibody nor lysate. The results demonstrate an interaction between $Na_v\beta1$ and Kv4.x proteins in NRVM. These are representative blots from a total of five identical experiments. (B) Co-immunoprecipitation in transfected HEK 293 cells with KChIP2 antibody to assess interaction of KChIP2 with $Na_v1.5$ and Kv4.3. Each lane corresponds to a different transfection, the bottom gels are probed with the IP antibody, KChIP2, and the top gels with either Nav1.5 or Kv4.3 antibodies. The IP lanes correspond to

the elution from beads crosslinked with KChIP2 antibody and incubated with the protein lysates from HEK 293 cells for the specific transfection indicated in each lane. Lysate lanes correspond to the transfected HEK 293 cell lysates before IP. The supernatant lanes are the non-bound lysate after incubation with the beads crosslinked with anti-KChIP2 antibody. The data demonstrate that Kv4.3, as previously reported, associates with KChIP2, while $Na_v1.5$ does not

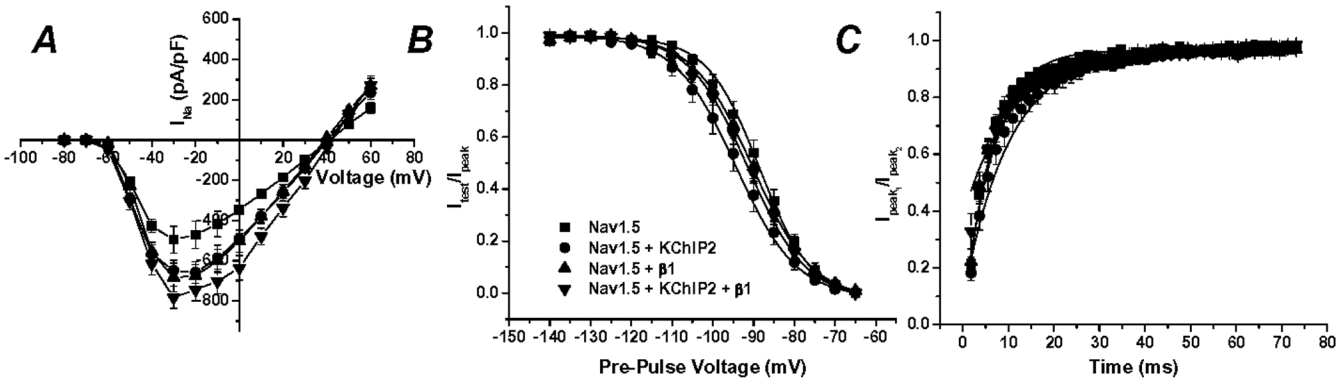

Figure 2.

Effect of KChIP2 on $Na_v1.5$ biophysical properties. HEK 293 cells were transfected with either Nav1.5 (n=8), $Na_v1.5$ + KChIP2 (n=14), $Na_v1.5$ + $Na_v\beta1$ (n=12) or $Na_v1.5$ + KChIP2 + $Na_v\beta1$ (n=10). (A) I/V relationship revealed a significant increased in peak current densities (at voltages −40 to +10 mV) when Nav1.5 was co-expressed with either KChIP2 or $Na_v\beta1$ and both ($p<0.05$). Neither steady-state inactivation (B) nor recovery from inactivation (C) were modified.

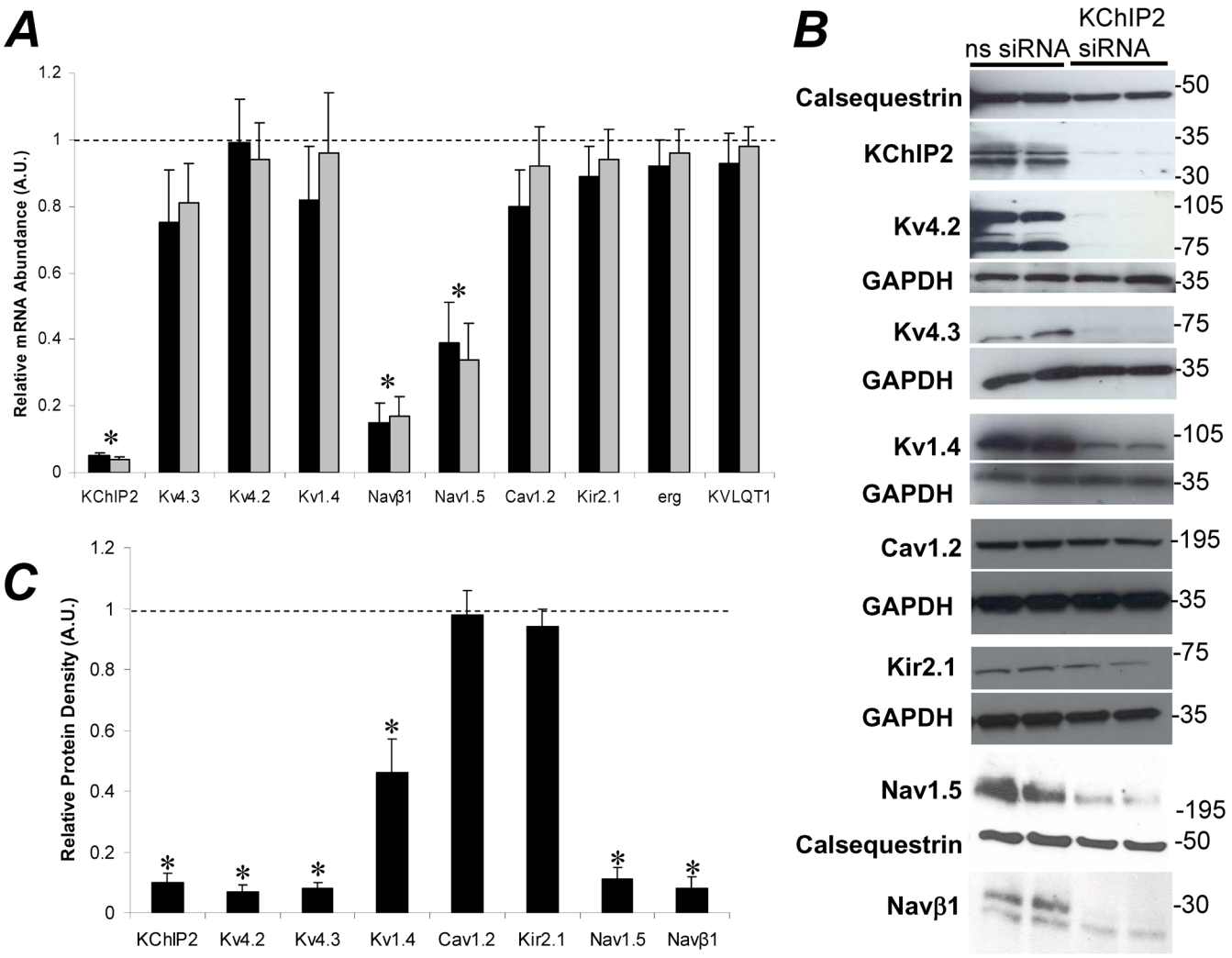

Figure 3.

Effect of KChIP2 siRNA on mRNA and protein levels. (A) Real-time PCR quantification shown in arbitrary units (A.U.) of mRNA transcripts extracted from NRVMs transfected with KChIP2 siRNA normalized to 18S rRNA and then normalized to NRVMs transfected with either a non-silencing control siRNA (black bar) or a single base pair mismatch siRNAs for KChIP2 (gray bar). (B) Representative Western blots of ion channel subunits and of housekeeping proteins for normalization. (C) A bar graph, in arbitrary units (A.U.), of the relative protein density demonstrates a dramatic reduction in KChIP2, Kv4.2, Kv4.3, $Na_v1.5$ and $Na_v\beta1$ proteins and a more modest reduction in Kv1.4 when normalized to either GAPDH or calsequestrin and then normalized to the quantity of the immunoreactive protein present in NRVMs transfected with the non-silencing control siRNA. RNA and proteins were isolated from at least three different transfections for each condition. * indicate the genes for which the mRNA or protein level was significantly reduced compared to the two control siRNAs ($p < 0.05$).

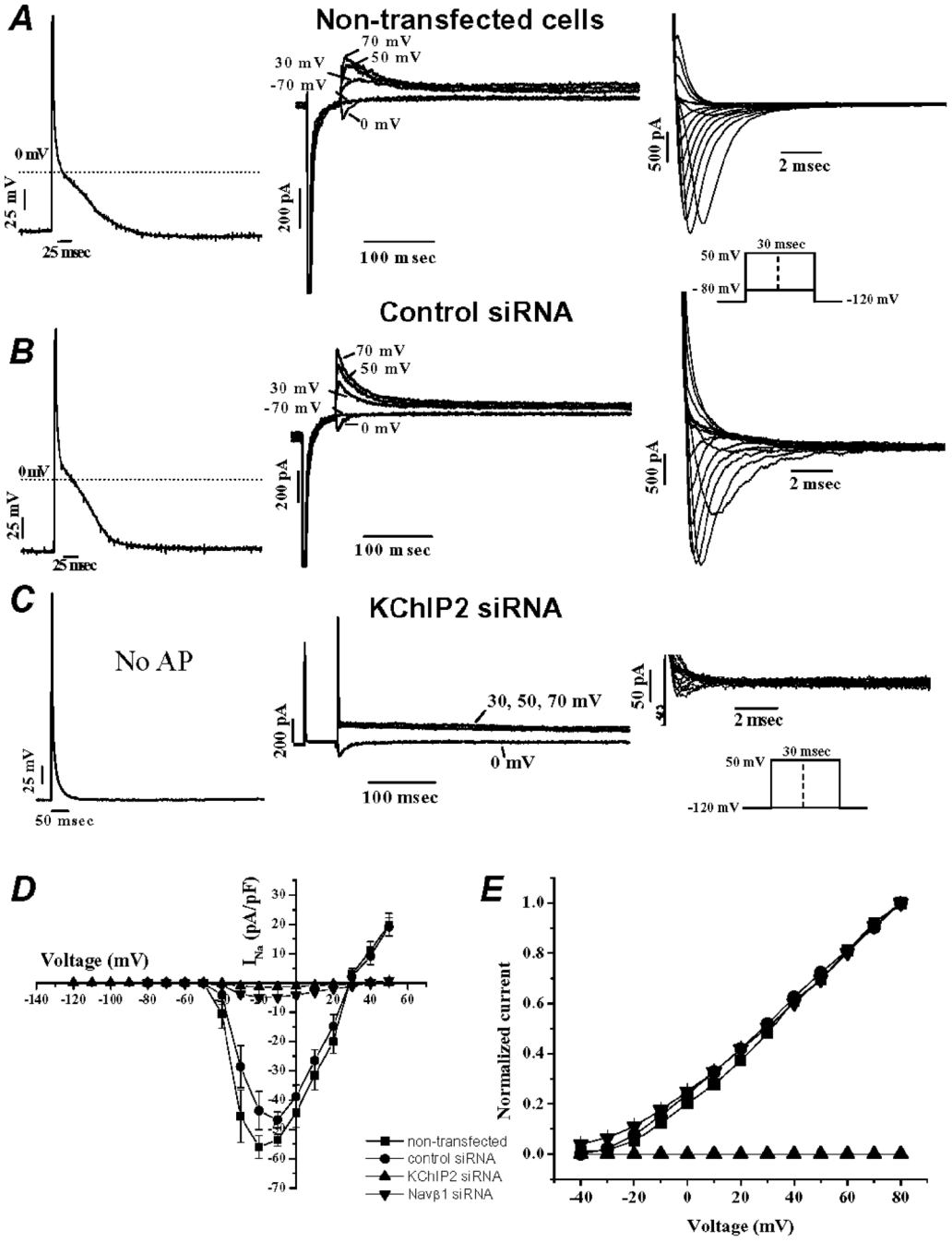

Figure 4.

Whole cell electrophysiological recordings. Representative action potentials at a 1 Hz stimulation frequency (left panel), representative currents (middle panel) and Na channel family (right panel) obtained from non-transfected NRVMs (A), cells transfected with control non-silencing siRNA (B) and cells transfected with KChIP2 siRNA (C). No action potentials were elicited from NRVMs transfected with KChIP2 siRNA (C, left panel). For currents recording (middle panel), cells were held at −80 mV, stepped to −40 mV (to inactivate I_{Na}) followed by voltage steps from −70 to +70 mV in 10 mV increments for 500 msec. Only current records elicited by steps to −70, 0, 30, 50 and 70 mV are shown for clarity. $I_{Ca,L}$ is observed with steps to 0 mV in all cell types and I_{to} at 30, 50 and 70 mV only in the non-transfected

cells and cells transfected with control non-silencing siRNA (A and B middle panel). Neither I_{to} nor I_{Na} was detected in NRVMs transfected with KChIP2 siRNA (C, middle panel). The right panel represents a family of sodium currents recorded using the protocols shown in the inset. Low external sodium (25mM) was used for the non-transfected and control siRNA cells in order to ensure voltage clamp control. In presence of the KChIP2 siRNA, physiological external sodium concentrations were used and even when stepping to very negative voltages (−120mV) no sodium currents were elicited. Averaged current-voltage relationships for I_{Na} measured with reduced extracellular sodium (except for cells transfected with KChIP2 siRNA) (D) and I_{to} (E).

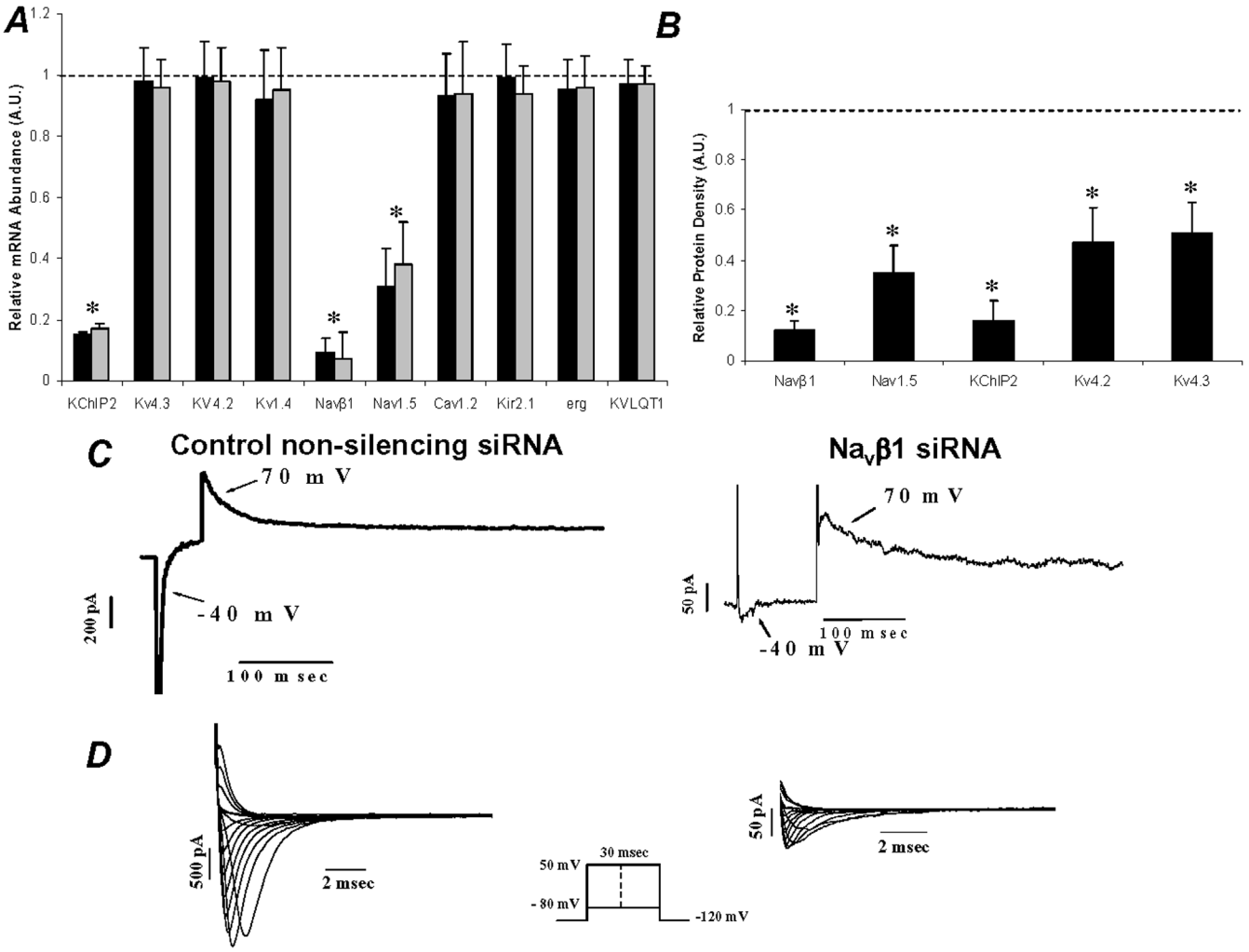

Figure 5.

Na$_v$β1 silencing (A) Real-time PCR quantification shown in arbitrary units (A.U.) of mRNA transcripts extracted from NRVMs transfected with Na$_v$β1 siRNA normalized to 18S rRNA and then normalized to NRVMs transfected with either a non-silencing control siRNA (black bar) or a single base pair mismatch siRNAs for Na$_v$β1 (grey bar). (B) A bar graph of the relative density of immunoreactive proteins in arbitrary units quantified from Western blots. The relative band intensity was normalized to either GAPDH or calsequestrin for loading and then normalized to the quantity of the immunoreactive proteins present in cells transfected with the non-silencing siRNA control. RNA and Proteins were isolated from at least three different transfections for each condition. * indicate the genes for which the mRNA or protein level was significantly reduced compared to the two control siRNAs ($p < 0.05$). (C) A two-pulse protocol is used to elicit currents from NRVM transfected with control non-silencing siRNA or Na$_v$β1 siRNA. Cells were held at −80 mV, stepped to −40 mV (to inactivate I_{Na}) followed by voltage steps from −70 to +70 mV in 10 mV increments for 500 msec. Only the sodium current present in the inactivating pulse (−40 mV) and the current elicited by the step to +70 mV (I_{to}) are shown for clarity. These cells had normal I_{K1} and $I_{Ca,L}$. (D) Sodium current family recorded using the protocol in the inset. In control cells, currents were recorded in presence of low extracellular sodium (25mM), whereas physiological sodium concentration was used in cells transfected

with the $Na_v\beta1$ siRNA. The data confirm a dramatic reduction in sodium currents in presence of the $Na_v\beta1$ siRNA.

Table 1

Electrophysiological parameters

	Resting Membrane Potential (mV)	APD_{50} (msec)	APD_{90} (msec)	Current Density (pA/pF)			I_{to} properties		n
				I_{to} (70 mV)	I_{K1} (−140 mV)	I_{CaL} (0 mV)	Steady-State Inactivation ($V_{1/2}$ in mV)	Recovery from Inactivation (τ_{rec} in msec)	
Non-transfected	−82.1 ± 10.1	10 ± 4	101 ± 10	8.4 ± 1.8	9.5 ± 3.1	4.2 ± 1.1	−37.5 ± 5.2	111 ± 19	7
Non-silencing siRNA	−84.5 ± 7.8	11 ± 3	107 ± 16	10.7 ± 2.6	10.7 ± 2.7	4.8 ± 1.1	−41.1 ± 4.2	95 ± 17	12
KChIP2 siRNA	−79.5 ± 12.2	na	na	na	10.5 ± 1.6	3.6 ± 1.3	na	na	6
β1 siRNA	−81.2 ± 8.5	na	na	2.5 ± 1.2	9.8 ± 2.1	3.5 ± 1.8	−42.7 ± 4.8	96 ± 12	8

na: not applicable