Interaction of Syntaxin 1A with the N-Terminus of Kv4.2 Modulates Channel Surface Expression and Gating†

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ABSTRACT: Kv4.2 channels are responsible in the heart for the Ca^{2+}-independent transient outward currents and are important in regulating myocardial excitability and Ca^{2+} homeostasis. We have identified previously the expression of syntaxin 1A (STX1A) on the cardiac ventricular myocyte plasma membranes, and its modulation of cardiac ATP-sensitive K^+ channels. We speculated that STX1A interacts with other cardiac ion channels, thus we examined the interaction of STX1A with Kv4.2 channels. Co-immunoprecipitation and GST pulldown assays demonstrated a direct interaction of STX1A with the Kv4.2 N-terminus. We next investigated the functional alterations of Kv4.2 gating by STX1A in *Xenopus* oocytes. Coexpression of Kv4.2 with STX1A (1) resulted in a reduction of Kv4.2 current amplitude; (2) caused a depolarizing shift of the steady-state inactivation curve; (3) enhanced the rate of current decay; and (4) accelerated the rate of recovery from inactivation. Additional coexpression of botulinum neurotoxin C, which cleaves STX1A, reversed the effects of STX1A on Kv4.2. STX1A inhibited partially the gating changes by KChIP2, suggesting a competitive interaction of these proteins for an overlapping binding region on the N-terminus of Kv4.2. Indeed, the N-terminal truncation mutants of Kv4.2 (Kv4.2Δ2−40 and Kv4.2Δ7−11), which form part of the KChIP2 binding site, displayed reduced sensitivity to STX1A modulation. Our study suggests that STX1A directly modulates Kv4.2 current amplitude and gating through its interaction with an overlapping region of the KChIP binding motif domain on the Kv4.2 N-terminus.

The calcium-independent transient outward K^+ current (I_{to}) contributes to the early repolarizing phase of the cardiac ventricular action potential and modulates action potential duration (reviewed in refs *1*, *2*). I_{to} is generated by both Kv4.2 and Kv4.3 in rodent hearts (*3*, *4*) and Kv4.3 alone in larger mammals such as human and canine hearts (*3*). Four Kv4 α subunits are sufficient to assemble into a functional K^+ channel in heterologous expression systems, but the magnitude and gating of I_{to} are distinctive of Kv4.2 or Kv4.3 channels when expressed alone (*5*), suggesting the presence of modulatory auxiliary proteins. Kv[1] channel-interacting protein (KChIP), first identified by An and colleagues, were shown to interact specifically with the Kv4 channel family (*6*). KChIP2 is the primary isoform expressed in the heart, and knock-out of this protein in mice shows a virtual loss of I_{to} (*6*, *7*).

Kv4 α subunits are promiscuous given their ability to interact with numerous cytosolic and integral membrane proteins other than KChIP. Studies have shown that Kv4 channels are modulated by KChAP (*8*), minK (KCNE1; *9*), MiRP1 (KCNE2; *10*), dipeptidyl peptidase 6 (DPP6) and DPP10 (*11*, *12*), the β-subunits of Kv and Nav channels (*9*, *13*) and a pentraxin-like protein, PTTX (*14*). These auxiliary channel subunits increase Kv4 channel expression and/or alter its gating. Another class of proteins capable of binding Kv channels is the SNARE (soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor) proteins, and in particular, the plasma membrane associated syntaxin 1A (STX1A). It has been well-established that SNARE proteins are involved in membrane fusion during neurotransmitter exocytosis in neurons and neuroendocrine cells (*15*). Recent studies from our laboratory and others have demonstrated SNARE proteins to directly bind and modulate Kv1.1 and Kv2.1 channels (*16*−*20*). STX1A binds to the N-terminus of Kv1.1 (*19*) while it interacts with the C-terminus of Kv2.1, resulting in an inhibition of Kv2.1 surface expression and current density (*18*). Concomitant to these changes are a slowing in channel activation and hyperpolarizing shift in the voltage dependence of channel inactivation. More recently, we have reported STX1A to be expressed on the plasma membrane of rat ventricular myocytes, and influence the activity of cardiac ATP-sensitive

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[1] Abbreviations: BoNT/C1, botulinum toxin C1; GST, glutathione S-transferase; K_{ATP}, ATP-sensitive potassium; KChIP, Kv channel-interacting protein; Kv, voltage-gated potassium channel; STX1A, syntaxin 1A; SNAP-25, synaptosomal-associated protein of 25 kDa; SNARE, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor.

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K^+ channels (K_{ATP}) (*21*). This led us to speculate that STX1A may interact with other cardiac channels.

In the present study, we demonstrate that STX1A directly binds to Kv4.2 channels leading to changes in current magnitude and gating. These effects can be reversed by coexpression of the clostridium toxin, botulinum toxin C1 (BoNT/C1), which specifically cleaves STX1A leading to its proteolytic degradation. More remarkably, STX1A modified Kv4.2 channels coexpressed with KChIP2, suggesting a shared binding motif of KChIP2 and STX1A on Kv4.2. Indeed, N-terminal Kv4.2 mutants (Kv4.2Δ2−40 and Kv4.2Δ7−11) show reduced sensitivity to STX1A, suggesting that STX1A interacts within the vicinity of the KChIP2 binding domain to modulate Kv4.2 channel surface expression and activity.

MATERIALS AND METHODS

DNA Constructs. Kv4.2, Kv4.2Δ2−40, and Kv4.2Δ7−11 were kindly provided to us by Dr. Peter Backx (University of Toronto) (*5*), Dr. Robert Bähring (Universität Hamburg, Hamburg, Germany) (*22*), and Dr. KeWei Wang (Wyeth Research, Princeton, NJ) (*23*), respectively. Channel constructs were subcloned into pcDNA3 (Invitrogen, Burlington, ON). STX1A in pcDNA3 was generously provided by Dr. Richard Scheller (Stanford University, Palo Alto, CA) (*24*). KChIP2 (a generous gift from Dr. David McKinnon, SUNY, Stony Brook, NY) (*25*) was subcloned into pcDNA3. BoNT/C1 plasmid was provided by Dr. Heiner Niemann (Hanover, Germany) (*26*) and subcloned into pcDNA3. The N-terminal (aa 1−182) and C-terminal (aa 409−630) domains of Kv4.2 were amplified by PCR and inserted into pcDNA3/myc-His vector.

Cell Culture and Transfections. HEK 293 cells were grown at 37 °C in 5% CO_2 in DMEM supplemented with 10% FBS and penicillin-streptomycin (100 U/mL, 100 μg/mL). (Invitrogen). Cells were transiently transfected with Kv4.2 in the absence or presence of STX1A and/or KChIP2 using Lipofectamine 2000 (Invitrogen).

Recombinant GST Fusion Proteins and In Vitro Binding Studies. STX1A in pGEX-4T-1 was generously provided by Dr. W. Trimble (Hospital for Sick Children, Toronto, ON). Generation of the GST fusion proteins STX1A and GST pulldown assays were performed as previously described (*18*, *27*). Eluted proteins were analyzed by SDS−PAGE. Protein was transferred to PVDF membranes, blotted with primary Kv4.2 antibody (Alomone Laboratory, Jerusalem, Israel) and secondary goat anti-rabbit antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and detected using ECL Plus (GE HealthCare).

Co-immunoprecipitation Assay. HEK 293 cells were plated on 10 cm culture dishes and transfected with Kv4.2 and STX1A cDNA using Lipofectamine 2000. Thirty hours after transfection, cells were washed twice with ice-cold phosphate-buffered saline and lysed with 0.3 mL of immunoprecipitation buffer (150 mM NaCl, 5 mM $MgSO_4$, 1 mM EDTA, 1 mM EGTA, 20 mM Tris-HCl pH 7.4, protease inhibitors) supplemented with 1% Triton X-100. Rat cardiac lysate was prepared as described previously (*21*). Fifty microliters of either HEK 293 or cardiac lysate was saved as the non-immunoprecipitated sample (input control), whereas 10 μL of a 50% slurry of Protein A Sepharose CL-4B resin (GE HealthCare) and 0.2 μL of antibody was added to the remainder and mixed gently for 2 h at 4 °C. The mixture was centrifuged for 10 s at 15000*g*, and the pellet was washed twice to capture the immunoprecipitated proteins, which were then eluted with 3 × 35 μL of SDS buffer (50 mM Tris-HCl, pH 6.8, 5% glycerol, 1.67% SDS, 83 mM DTT, and 0.0002% bromophenol blue). The samples were run on SDS−PAGE to separate proteins, which were identified by immunoblotting analysis.

Oocyte Isolation and Recording. Isolation of *Xenopus laevis* oocytes was performed as previously described (*5*). Oocytes were nuclearly injected with cDNA for expression of Kv4.2 channels alone (0.3 μg/μL cDNA), or coexpressed with STX1A, BoNT/C1, and/or KChIP2 (0.15 μg/μL cDNA) (total volume, 50 nL). Current recordings and analysis were performed as described previously (*5*). Comparisons were made from oocytes obtained from at least three frogs.

Data Analysis. Results are represented as the mean ± SEM. Statistical analysis was determined using an unpaired Student's *t* test or one-way ANOVA followed by a Student−Newman−Keuls test. A P value <0.05 was used to denote statistical significance.

RESULTS

We have recently demonstrated the expression of STX1A in isolated rat ventricular myocytes, and suppression of cardiac ATP-sensitive K^+ currents through its binding to the nucleotide binding domains (*21*). We speculated that STX1A interacts with other cardiac ion channels. In particular, we focused on Kv4.2 channels given its importance in cardiac excitability and its ability to interact with numerous auxiliary proteins.

Kv4.2 Binds to Syntaxin 1A. We investigated initially the ability of STX1A to interact directly with Kv4.2 by performing co-immunoprecipitation and GST pulldown assays with native and recombinant protein. First, we immunoprecipitated rat cardiac STX1A and ran the eluted protein sample on SDS−PAGE, followed by immunoblotting for Kv4.2. Kv4.2 came down with STX1A as shown in Figure 1A (left panel). We performed the converse experiments, and observed Kv4.2 co-immunoprecipitated both STX1A and STX1B isoforms (Figure 1A, right panel). In the absence of primary antibody, no immunoprecipitation of either Kv4.2 or STX1A was observed (Figure 1A). Similar results were obtained when Kv4.2 was coexpressed with STX1A in HEK 293 cells (Figure 1B). Previous work revealed that STX1A interacts with the N-terminus of Kv1.1 (*19*) and the C-terminus of Kv2.1 (*18*).

To determine the putative cytoplasmic domain of Kv4.2 that binds to STX1A, we coexpressed myc-tagged N- (aa 1−182) or C-terminal (aa 409−630) domains of Kv4.2 in HEK 293 cells and performed pulldown assays using GST fusion proteins (Figure 2A). HEK 293 cell lysates were eluted on glutathione agarose beads bound with either GST-STX1A or GST alone. GST-STX1A bound to only the N- but not C-terminal domain of Kv4.2 (Figure 2A). Neither Kv4.2 cytoplasmic domain bound to GST. Overall, these results demonstrate the direct physical interaction of STX1A with N-terminus of Kv4.2 channels.

STX1A exists in a "closed" configuration in which the N-terminal H_{ABC} domain folds over to inhibit the C-terminal

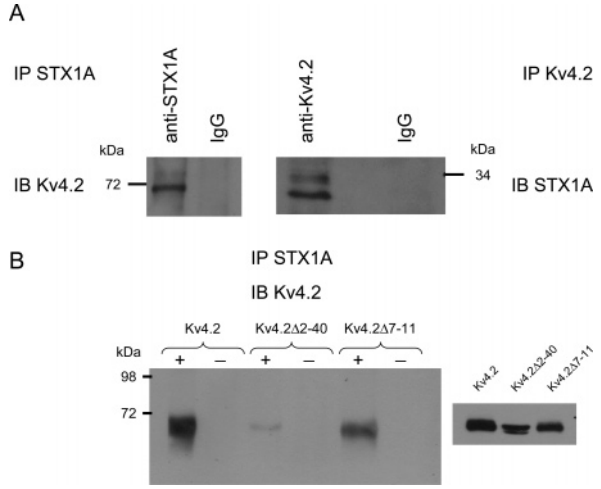

FIGURE 1: Kv4.2 binds STX1A. (A) Co-immunoprecipitation of native Kv4.2 and STX1A. Rat cardiac lysate was used to pull down STX1A (left panel) and Kv4.2 (right panel). Immunoprecipitated proteins were run on SDS−PAGE and immunoblotted with either Kv4.2 (left panel) or STX1 (right panel) polyclonal antibody. The STX1 antibody recognizes both STX1A and STX1B. IgG lanes represent the immunoprecipitation assay performed in the absence of the primary antibody. (B) Co-immunoprecipitation of recombinant Kv4.2 with STX1A. Kv4.2 wild-type or mutant channels were coexpressed with STX1A in HEK 293 cells, and immunoprecipitation with anti-STX1A was performed. Samples were run on SDS−PAGE and then immunoblotted with anti-Kv4.2. Co-immunoprecipitation assays were performed in the presence (+) or absence (−) of anti-STX1A antibody. Inset shows expression levels of the Kv4.2 constructs as assessed by Western blot analysis of whole cell lysate. Full-length Kv4.2 and Kv4.2Δ7−11 co-immunoprecipitated with STX1A, while Kv4.2Δ2−40 interacted much more weakly with STX1A.

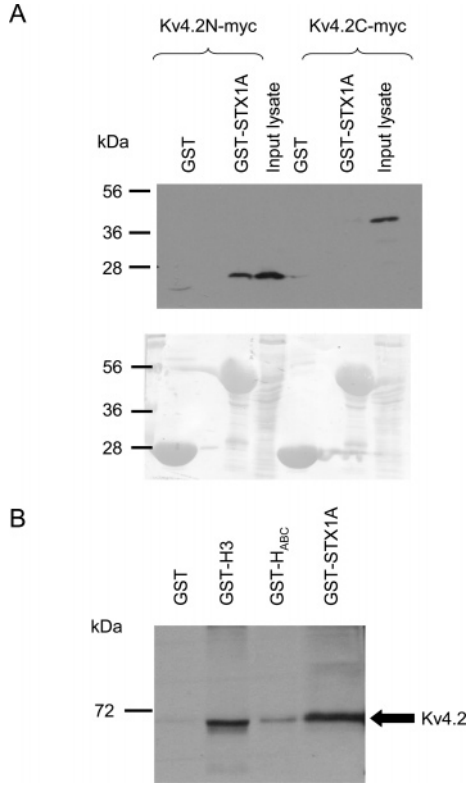

FIGURE 2: Regions on STX1A and Kv4.2 important for interaction. (A) GST and GST-STX1A-WT fusion proteins (all bound to glutathione agarose beads, 550 pmol protein each) were used to pull down the N- (left panel) or C-terminal (right panel) myc-tagged domains of Kv4.2 expressed in HEK293 cells. Only the N-terminus of Kv4.2 bound to GST-STX1A. The corresponding Ponceau S stained membrane is shown below, which demonstrates that similar quantities of GST fusion proteins were used for the pull-down assay. (B) Kv4.2 binds preferentially to the H3 domain of STX1A. GST, GST-H3, GST-HABC, and GST-STX1A fusion proteins were bound to glutathione agarose as described above and were used to pull down Kv4.2.

H3 domain from assembling with its cognate SNARE proteins. Activation of STX1A to the "open" configuration is required to release the inhibitory action of the H_{ABC} domain on the H3 domain (*15*). Therefore, we examined which domain of STX (H_{ABC} or H3) interacts with Kv4.2. Pulldown experiments with GST fusion proteins of H_{ABC} and H3 demonstrated that H3 domain physically bound greater to Kv4.2 than the H_{ABC} domain (Figure 2B).

Syntaxin 1A Regulates Kv4.2 Expression and Gating. To probe the functional effects of STX1A on Kv4.2 channel activity, we coexpressed both proteins in *Xenopus* oocytes. Kv4.2 expressed alone displayed robust currents in oocytes (Figure 3A), with maximal peak outward current of 3.6 ± 0.4 μA (n = 26) measured at +60 mV (Figure 3B). Coexpression of STX1A (Kv4.2:STX1A; 2:1 wt:wt cDNA) markedly reduced Kv4.2 currents to 1.7 ± 0.2 μA (n = 23; P < 0.05), but had no effect on the voltage dependence of channel activation (data not shown). Concomitant to the reduction in current magnitude, there was a modest acceleration in current decay. Current decay at +60 mV was fit to a biexponential function: $I = A_1 \exp(-x/\tau_1) + A_2 \exp(-x/\tau_2) + c$, where I is the current amplitude, A_1 and A_2 are the amplitudes of the time constants, τ_1 and τ_2, respectively, and c is the non-inactivating component. STX1A increased the rate of current decay of the faster time constant, τ_1, from 26.4 ± 0.9 ms to 21.1 ± 1.6 ms (P < 0.05; n = 8), without affecting the slow time constant, τ_2 (Table 1).

To further examine the effects of STX1A on Kv4.2 gating, we measured steady-state inactivation and recovery from inactivation. Steady-state inactivation was recorded using a two-pulse protocol with 500 ms conditioning depolarizing pulses from −120 to −20 mV followed by a test pulse to +60 mV (Figure 3C). STX1A elicited a significant depolarizing shift in the steady-state inactivation curve from −61.8 ± 1.2 mV (n = 17) to −56.6 ± 0.9 mV (n = 17) (P < 0.05), with no significant difference in the slope factor between control (9.7 ± 0.7 mV) and STX1A group (10.0 ± 0.3 mV). Recovery from inactivation was examined using a two-pulse protocol using a 500 ms depolarizing pulse to +60 mV followed by repolarization to −100 mV for varying recovery intervals prior to a second depolarizing test pulse to +60 mV (Figure 3D). Kv4.2 currents recovered from inactivation with a single time constant of 274 ± 28 ms (n = 17). There was a significant acceleration of the recovery (182 ± 13 ms, n = 17; P < 0.05) when STX1A was coexpressed with the channel.

Botulinum Toxin C1 Abolishes the Effects of Syntaxin 1A. The clostridium neurotoxin, botulinum toxin C1 (BoNT/C1), specifically cleaves STX1A leading to the degradation of the SNARE protein (*28*). To demonstrate that the effects of STX1A on Kv4.2 were due to the specific protein−protein interactions, and not the result of overexpressing an additional protein in the oocytes, we coexpressed BoNT/C1 with Kv4.2 and STX1A. BoNT/C1 abrogated the effects of STX1A on channel magnitude and gating. Kv4.2 channel magnitude (3.2

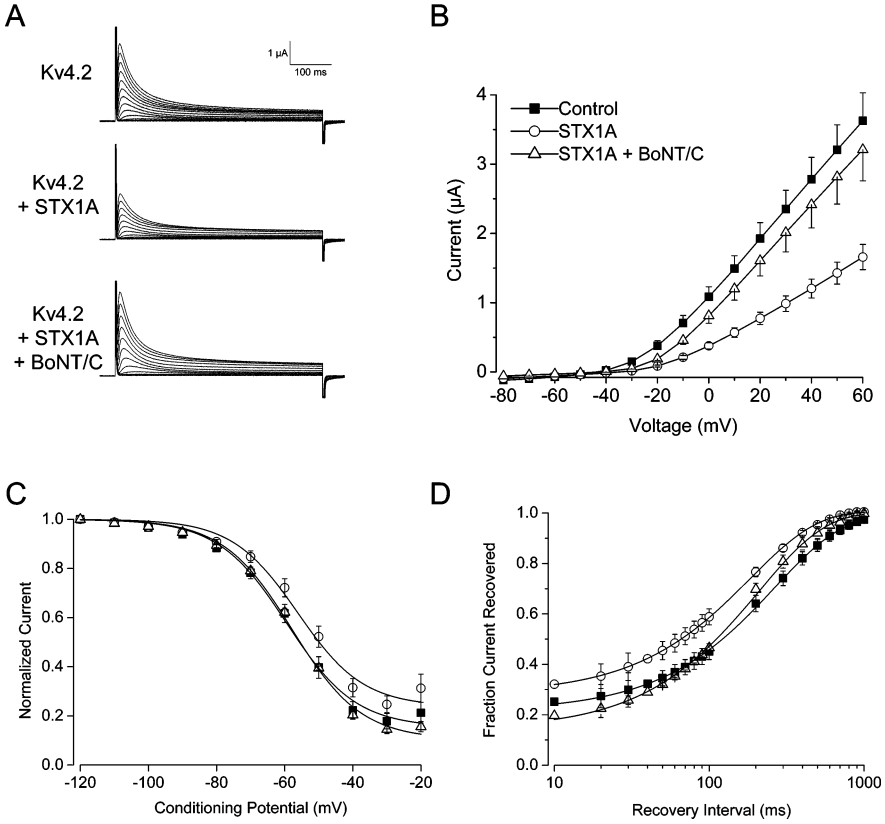

FIGURE 3: STX1A modulated Kv4.2 amplitude and gating. (A) Kv4.2 currents expressed alone in *Xenopus* oocytes or in the presence of STX1A or STX1A/BoNT/C. The amount of Kv4.2 cDNA was constant (7.5 ng) in all groups. Currents were elicited from 500 ms step depolarizations from −80 to +60 mV from a holding potential of −80 mV. (B) Current−voltage ($I-V$) relationship of peak outward current plotted as a function of the test potential. $I-V$ relationships of Kv4.2 expressed alone ($n = 26$), or coexpressed with STX1A ($n = 23$) and STX1A + BoNT/C ($n = 11$). (C) Steady-state inactivation of Kv4.2. Peak currents measured during the test pulse were normalized to the current at −120 mV and plotted as a function of the conditioning potential. Data were fitted with a Boltzmann function $I/I_{max} = 1/\{1 + \exp[(V - V_{1/2})/k]\}$, where $V_{1/2}$ is the half-maximal inactivation potential and k is the slope factor. The fitted values for control Kv4.2 ($n = 17$), Kv4.2 + STX1A ($n = 17$), and Kv4.2 + STX1A + BoNT/C ($n = 10$) are given in the text. (D) Summarized recovery from inactivation data for Kv4.2 expressed alone or in the presence of STX1A with or without BoNT/C. Peak outward currents measured during the second test pulse were normalized to the first peak outward current and plotted as a function of the recovery interval. Data were fitted with a monoexponential function $I/I_{max} = A \exp^{-t/\tau}$, where A is the amplitude, t is time, and τ is the time constant for recovery. The fitted values for control Kv4.2 ($n = 17$), Kv4.2 + STX1A ($n = 17$), and Kv4.2 + STX1A + BoNT/C ($n = 10$) are given in the text.

± 0.5 μA, $n = 11$) (Figure 3A) and current decay were not significantly different from control (27.5 ± 1.1 ms, $n = 11$). The midpoint of the steady-state inactivation curve was shifted leftward to −58.9 ± 0.9 mV ($n = 10$; Figure 3C), and there was a partial restoration of the recovery from inactivation (216 ± 27 ms, $n = 10$) (Figure 3D). BoNT/C1 expressed with Kv4.2 channel alone had no significant effect on current magnitude or channel gating (Table 1).

Surface Protein Biotinylation. The decrease in Kv4.2 currents by STX1A may be attributable to impaired channel surface expression and/or the inability of the channel to open. We have shown previously that STX1A reduces Kv2.1 trafficking to plasma membrane, in addition to having a direct inhibitory action on the channel (*18*). To determine whether these SNARE proteins affect Kv4.2 surface expression, we measured the levels of surface protein expression by biotinylation. Figure 4 shows Western blot of biotinylated membrane Kv4.2 protein. STX1A reduced the surface expression of Kv4.2 to 45 ± 11% of control ($n = 4$). These results suggest that the reduction current magnitude is in part due to the decrease in channel protein levels at the surface membrane, possibly due to impaired channel trafficking to the membrane. However, we cannot rule out the possibility that STX1A reduces surface membrane stability of the Kv4.2 leading to enhanced channel endocytosis. Moreover, the effects on open channel probability and single channel conductance may also be contributing factors to the reduced currents observed.

STX1A Attenuates KChIP2 Modulation of Kv4.2. KChIP2 interacts with Kv4.2 to form a macromolecular complex, which constitutes I_{to} in the heart (*6*, *7*). Therefore, we determined whether the modulation of KChIP2 on Kv4.2 was influenced by the presence of STX1A. Coexpression of KChIP2 alone resulted in larger Kv4.2 currents (6.5 ± 0.7 μA, $n = 25$) compared to control (3.6 ± 0.4 μA, $n = 26$) ($P < 0.05$) (Figure 5A,B). KChIP2 also shifted the steady-state inactivation curve rightwards ($V_{1/2}$ −48.6 ± 0.5 mV, $k = 4.6 \pm 0.1$ mV, $n = 25$; Figure 5C) and accelerated the rate of recovery from inactivation (21.1 ± 1.4 ms, $n = 20$; Figure 5D), as has been reported (*6*). When Kv4.2/KChIP2 was coexpressed with STX1A (Kv4.2:KChIP2:STX1A 2:1:1 wt ratio), STX1A reduced channel amplitude to 4.2 ± 0.4 μA ($n = 20$, $P < 0.05$, Figure 5A,B) and slowed the fast time constant of current decay from 45.1 ± 4.8 ms to 64.2 ± 5.5 ms ($P < 0.05$). STX1A produced no significant shift in the steady-state inactivation (−50.4 ± 0.4 mV, $n = 20$, Figure 4C) or change in the slope factor (4.2 ± 0.1 mV), but significantly slowed the rate of recovery from inactivation

Table 1: Effects of STX1A on Kv4.2 N-Terminal Deletion Mutants[a]

	Kv4.2 ($n = 8-26$)	Kv4.2Δ2−40 ($n = 8-10$)	Kv4.2Δ7−11 ($n = 10-15$)	Kv4.2 + BoNT/C1 ($n = 8$)
current amplitude at +60 mV (μA)				
control	3.6 ± 0.4	4.2 ± 0.6	6.1 ± 0.8	3.1 ± 0.6
STX1A	1.7 ± 0.2[b]	3.2 ± 0.5	3.6 ± 0.6[b]	
current decay at +60 mV (ms)				
control				
τ_1	26.4 ± 0.9	122 ± 26	56.3 ± 5.2	24.8 ± 2.0
τ_2	147 ± 6	294 ± 28	241 ± 65	149 ± 12
STX1A				
τ_1	21.1 ± 1.6[b]	146 ± 23	73.9 ± 3.7[b]	
τ_2	75 ± 29	265 ± 10	291 ± 47	
steady-state inactivation (mV)				
control				
$V_{1/2}$	−61.8 ± 1.2	−61.7 ± 2.3	−60.4 ± 2.3	−60.9 ± 0.6
k	9.7 ± 0.7	6.8 ± 0.4	6.5 ± 0.8	10.3 ± 0.6
STX1A				
$V_{1/2}$	−56.6 ± 0.9[b]	−60.8 ± 1.7	−61.3 ± 1.6	
k	10.0 ± 0.3	6.7 ± 0.3	6.1 ± 0.2	
recovery from inactivation (ms)				
control	274 ± 28	243 ± 19	193 ± 15	267 ± 26
STX1A	182 ± 13[b]	226 ± 21	209 ± 10	

[a] Recordings and measurements were made as described in the Materials and Methods section. The effects of BoNT/C1 alone on Kv4.2 channel gating is shown. Data represent the mean ± SEM. [b] $P < 0.05$ from corresponding control values in the absence of STX1A.

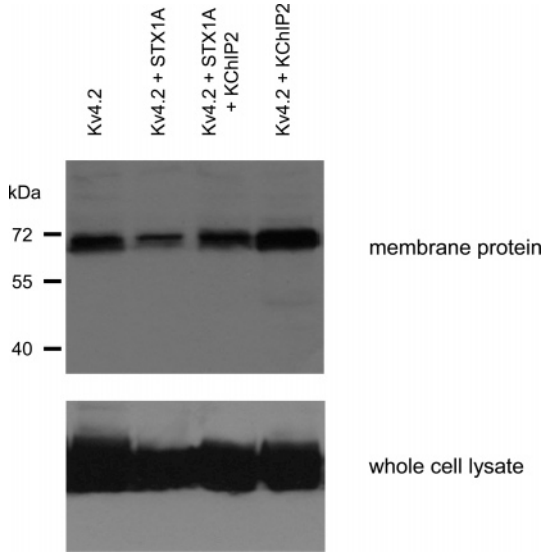

FIGURE 4: STX1A decreases the surface expression of Kv4.2. Transfected HEK293 cells were biotinylated and solubilized as described under "Materials and Methods". Biotinylated proteins (plasma membrane fraction) were isolated using the streptavidin resin. The proteins eluted from the resin (upper panel), and whole cell lysates obtained prior to the streptavidin precipitation (lower panel) were then separated by PAGE, and the Kv4.2 protein was identified by a specific antibody by Western blotting.

(33.7 ± 1.9 ms, $n = 20$; $P < 0.05$) (Figure 5D). The surface level of Kv4.2 in the presence of both KChiP2 and STX1A was 64 ± 7% ($n = 4$) of Kv4.2 expressed alone (Figure 4). These results suggest that STX1A interacts with an overlapping binding motif on Kv4.2 as KChIP2, and that KChIP2 can compete with STX1A to partially block STX1A inhibition of Kv4.2 surfacing to the membrane surface.

KChIP binds to the N-terminal domain of Kv4.2/Kv4.3 (*6*). Early work by Bähring et al. demonstrated that deletion of 39 amino acids in the proximal portion of the N-terminus of Kv4.2 (Kv4.2Δ2−40) prevented the functional association of KChIP2 with the channel and observed a lack of channel gating modifications (*22*). We have also demonstrated that STX1A binds to the N-terminus of Kv4.2. Therefore, we examined whether STX1A affected Kv4.2Δ2−40 channel expression and gating. The effects of STX1A were attenuated on Kv4.2Δ2−40 channels compared to the full-length wild-type Kv4.2, as reflected by the lack of effect on both channel amplitude and gating (Table 1). This was further supported by the negligible binding of Kv4.2Δ2−40 channels to STX1A, as shown by the co-immunoprecipitation assay (Figure 2A).

More recently, the interacting domains on the N-terminal of Kv4.2 for KChIP were determined by X-ray crystallography (*23*). Two regions on Kv4.2, residues 7−11 and 71−90, were found to be critical for KChiP binding and modulation of Kv4.2. We therefore examined whether STX1A interacted with the similar region of Kv4.2 as KChIP. We coexpressed Kv4.2Δ7−11 with STX1A (2:1 wt:wt cDNA), given that this region overlaps with Kv4.2Δ2−40. As shown in Table 1, the effects of STX1A on Kv4.2Δ7−11 channels were markedly attenuated but not completely eliminated. Here, STX1A was able to significantly reduce peak current amplitude and slow the rate of current decay (Table 1). Furthermore, a comparable level of STX1A binding to Kv4.2Δ7−11 channels as wild-type channels is observed (Figure 1B). These results suggest that STX1A shares an overlapping binding site on the N-terminus of Kv4.2 as KChIP2.

DISCUSSION

In secretory and nonsecretory cells, STX1A have been shown to regulate the plasma membrane trafficking and gating of numerous ion channels, such as voltage-gated Ca^{2+} and K^+ channels, CFTR, and ENaC (*18*−*20*, *29*−*31*). Our more recent work demonstrated that STX1A is expressed in cardiac ventricular myocytes and decreases the activity of cardiac K_{ATP} channels (*21*, *32*). We speculated that STX1A regulates the trafficking and gating of other cardiac ion channels. We first examined the interaction of STX1A with Kv4.2, given that the expression and activity of this Kv α subunit is highly regulated by numerous cytosolic and membrane-associated auxiliary subunits. In the present study, we demonstrate that STX1A impairs the surface expression and modifies gating of Kv4.2. STX1A interacts directly with Kv4.2 as co-immunoprecipitation experiments of native and exogenously expressed Kv4.2 protein were able to pull down STX1A. This latter effect was mediated in part through an interaction with the proximal N-terminal domain on the channel. These effects are attenuated by proteolytic cleavage of STX1A by BoNT/C and are competed by coexpressing KChIP2. Last, STX1A binds in the vicinity as KChIP2 on the N-terminal cytoplasmic domain of Kv4.2.

In the heart, Kv4.2 is functionally associated with KChIP2 forming a multimeric complex responsible for cardiac I_{to}. Using the N-terminus of Kv4.2 and Kv4.3 (residues 1−180) as bait in a yeast two-hybrid screen, An et al. identified a novel class of proteins that selectively bound Kv4 channels

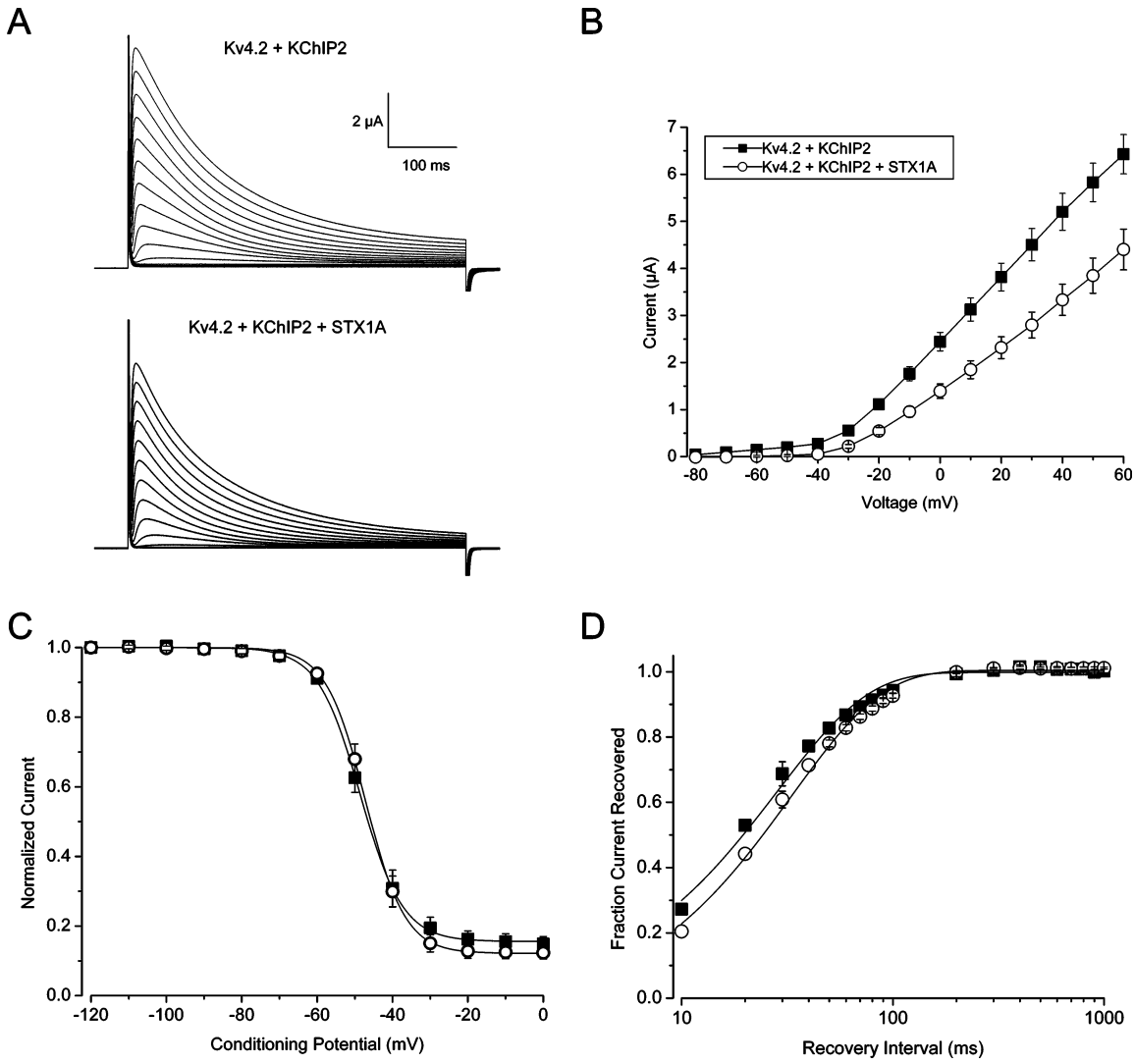

FIGURE 5: STX1A reduces the effects of KChIP2 on Kv4.2. (A) Kv4.2 channels coexpressed with KChIP2 with or without STX1A in *Xenopus* oocytes. Currents were elicited as described in Figure 2. (B) Current−voltage ($I-V$) relationship of peak outward current of Kv4.2/KChiP2 alone ($n = 25$) or in the presence of STX1A ($n = 20$). C) STX1A had no effect of the steady-state inactivation of Kv4.2/KChIP2. (D) Summarized recovery from inactivation data for Kv4.2/KChIP2 expressed alone or in the presence of STX1A. Data were fitted as described in Figure 2. STX1A ($n = 20$) slowed the rate of recovery from inactivation of Kv4.2/KChiP2 channels ($n = 20$).

(*6*). KChIPs enhance the surface expression, slow current decay kinetics, and enhance the recovery from inactivation (*6*). Heterologous expression studies show that KChIP can form an octomeric complex with Kv4.2, with four KChIPs assembling with four Kv4.2 α subunits (*33*). Bähring et al. demonstrated that the proximal N-terminal residues 2−40 of Kv4.2 (*22*), and more recently, residues between 11 and 23, as well as the T1 domain, were critical for binding and modulation of KChIP2 (*34*). Similarly, X-ray crystallography studies have revealed two regions on the N-terminus of Kv4.2 where KChIP1 specifically interacts with the channel, amino acids 7−11 and 71−90 (T1 domain) (*23*). The more recent Kv4.3-KChIP1 crystal structure showed that a single KChiP monomer laterally binds to two neighboring Kv4.3 N-terminal domains: a proximal segment and the T1 (tetramerization) domain on the adjacent Kv4.3 subunit (*35*). Pioletti and colleagues demonstrated further that the highly conserved residues on the proximal N-terminus of Kv4.3 (site 1) influence channel trafficking, while the site 2 T1 domain affects channel gating (*36*). The attenuated effects of STX1A on Kv4.2Δ2−40 channel binding and expression, and the abrogration of STX1A effects on Kv4.2 by KChiP2 as shown in the present study, are in good agreement with these recent findings detailing the importance of the Kv4.2 N-terminus on channel expression and gating. Other auxiliary subunits, such as frequenin (*37*) and PTTX (*14*), have been shown to interact with the N-teriminus of Kv4.2. Our data further reveals that the N-terminus of the Kv4.2 channel is critical for regulating channel surface expression and gating.

STX1A binds to a similar N-terminal region on Kv4.2 as KChiP2 is substantiated by our experiments showing that STX1A had limited effects on Kv4.2Δ2−40, and had less effect on Kv4.2Δ7−11, indicating that STX1A binds a smaller portion of this domain. However, we cannot exclude the possibility that STX1A may interact with a larger or more distal region of the N-terminus.

The first 20 amino acids of the N-terminus of Kv4.2 form an α-helix (*38*). Full-length STX1A also adopts a helical structure, and forms the SNARE core complex with SNAP-25 and synaptobrevin through parallel four-helix bundles (*39*). We speculate that a helical−helical interaction is involved between STX1A and the proximal N-terminal

domain of Kv4.2. The H3 domain or SNARE motif of STX1A is the region, which coordinates with SNAP-25 and synaptobrevin, and is the region which binds to Kv4.2. The H3 domain is also involved in the functional interaction with N-type Ca^{2+} channels (*29*), CFTR (*30*), and ENaC channels (*31*). We have recently demonstrated the importance of the H3 domain in modulating Kv2.1 (*27*) and K_{ATP} channel activity (*21*, *32*), and found this region to be involved in Kv4.2 binding. Therefore, it appears that the H3 domain is the active region of STX1A required for exocytosis as well as channel regulation.

A transmural expression profile of Kv4.2/Kv4.3 and KChIP has been shown in the heart. In rodent hearts, Kv4.2/Kv4.3 is highly expressed on the epicardial surface and at lower levels in the endocardium (*40*). No difference in KChIP levels across the myocardial wall is found in rodent hearts (*25*). In contrast, KChIP expression shows a transmural gradient in human and canine hearts with highest levels in the epicardium, while Kv4.3 levels are constant (*25*). This raises the possibility that some Kv4.2/Kv4.3 subunits may not be functionally assembled with KChIP in the heart. Indeed, 13% of both endocardial and epicardial rat ventricular myocytes do not express KChIP, while Kv4.2 message was detected in all myocytes (*41*). Favorable conditions may allow STX1A to functionally interact with Kv4.2 in situations where KChIP levels are low or absent. Moreover, the effects of STX1A on Kv4.2 may not be restricted to the heart, as these proteins are abundantly expressed in neurons and neuroendocrine cells. In the brain, Kv4 channels constitute the A-type Kv currents (*42*). In cerebellar granule cells, Kv4.2 is highly expressed in synaptic glomerular regions, which are enriched with SXT1A and other SNARE proteins required for exocytosis (*43*).

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