KChIP3 Rescues the Functional Expression of Shal Channel Tetrimerization Mutants*

KChIP proteins regulate Shal, Kv4.x, channel expression by binding to a conserved sequence at the N terminus of the subunit. The binding of KChIP facilitates a redistribution of Kv4 protein to the cell surface, producing a large increase in current along with significant changes in channel gating kinetics. Recently we have shown that mutants of Kv4.2 lacking the ability to bind an intersubunit Zn$^{2+}$ between their T1 domains fail to form functional channels because they are unable to assemble to tetramers and remain trapped in the endoplasmic reticulum. Here we find that KChIPs are capable of rescuing the function of Zn$^{2+}$ site mutants by driving the mutant subunits to assemble to tetramers. Thus, in addition to known trafficking effects, KChIPs play a direct role in subunit assembly by binding to monomeric subunits within the endoplasmic reticulum and promoting tetrameric channel assembly. Zn$^{2+}$-less Kv4.2 channels expressed with KChIP3 demonstrate several distinct kinetic changes in channel gating, including a reduced time to peak and faster entry into the inactivated state as well as extending the time to recover from inactivation by 3–4 fold.

The formation of voltage-gated potassium (Kv)$^{3}$ channels is a multistep process with many different interactions and folding events required to form the completed channel (1). The common functional core of all Kv channels assembles as a tetramer of pore-forming $\alpha$-subunits. This tetramer is the core of the functional ion channel signal transduction complex, but additional folding steps as well as interactions with auxiliary proteins occur before the final functional channel complex at the cell surface is formed. Many auxiliary subunit proteins that bind to Kv $\alpha$-subunits have been identified, but precisely when these interactions occur during channel complex formation and what role these interactions play in helping the channels to assemble, traffic, and function are topics of great interest (1–4). Through the use of heterologous expression systems and mutagenesis studies, we can expose many of these important interactions and folding events, and reveal the processes by which Kv channel complexes form. A comparison of channel expression and functional properties with and without specific auxiliary proteins reveals how these different processes contribute to the formation and function of ion channel complexes.

An early step in Kv channel formation involves the tetramerization of the $\alpha$-subunit T1 domains at the cytoplasmic N terminus of the protein (5–7). For Kv4.2 channels, a critical component of the T1 domain interaction involves the coordination of an intersubunit Zn$^{2+}$ ion found on non-Shaker type Kv channel T1 domains (8–10). Although Zn$^{2+}$-binding sites are common in proteins, intersubunit Zn$^{2+}$-binding sites, as found in the T1 domain, are relatively rare. To determine what functions might be regulated by the T1 intersubunit Zn$^{2+}$ site, we generated a series of mutations to the Zn$^{2+}$-coordination residues and tested them for cell surface expression (8). We found that mutation to any of the Zn$^{2+}$ binding site amino acids caused a block of functional channel expression by disrupting subunit tetramerization. This disruption traps the protein within the endoplasmic reticulum and produces a shift in the migration of solubilized subunits on size exclusion chromatography to monomeric molecular weights (8). Therefore, we can clearly say that one important function of the Zn$^{2+}$ site is to stabilize channel assembly. Because functional channels could not be formed without the intact Zn$^{2+}$ site, we sought to determine if it was possible to rescue these mutant channels by co-expression with KChIP auxiliary subunit proteins that are known to enhance Shal channel expression by 10–50 times (3).

Kv4.2 subunits form channels in the absence of KChIP auxiliary proteins; however, co-expression with KChIP enhances current expression by altering the trafficking of Kv4 channels (11). KChIP auxiliary subunits interact with Kv4.2 channels by binding to the first 14 residues of the cytoplasmic N terminus and possibly through direct binding interactions with the T1 domain (3, 12–14) containing the zinc site. Also, the KChIP subunit itself is capable of multimeric interactions following binding to the Kv4 N terminus, providing an alternative and non-zinc-dependent pathway to channel complex multimerization (12). The KChIP enhancement of expression has been attributed to a trafficking action of KChIPs, causing channel proteins to redistribute to the cell surface (3, 11). The mechanism responsible for this redistribution is not known. KChIPs could be acting to enhance channel assembly, enhance folding of the assembled channels, block the action of a retention sequence on Kv4.2 N terminus, providing a stronger surface expression motif to the channel complex, or a combination of effects (1). In addition to increasing expression, KChIP binding to the Kv4.2 N terminus modulates the functional gating properties of the channels. The most dramatic effects are on the inactivation properties of the channels, where KChIP drastically slows inactivation and speeds the recovery from inactivation. Inactivation is an important feedback mechanism that

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* The abbreviations used are: Kv, voltage-gated potassium; EGFP, enhanced green fluorescent protein; CHO, Chinese hamster ovary; SEC, size exclusion chromatography; FPLC, fast protein liquid chromatography; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]-1-propanesulfonic acid; ANOVA, analysis of variance.
limits Shal channel function at depolarized potentials and gives these channels their characteristic “A” current waveform. The N terminus of Kv4.2 is clearly involved in inactivation gating, since deletion of this region produces slowed inactivation. Moreover, experiments on the functional properties of the N terminus show that it can act as a pore-binding peptide (15–17). Therefore, the gating changes produced by KChIPs might depend on the binding and sequestering of the Kv4 N terminus, causing an alteration to a complex set of allosteric interactions that involve other structural elements of the channel (18). Here we have tested whether KChIP3 binding promotes assembly of the Zn²⁺ site mutant Kv4.2 subunit proteins by testing for the rescue of channel tetramerization and cell surface function by KChIP3. We then characterized the resultant complexes as to their assembly and biophysical properties in comparison to wild-type Kv4.2 co-expressed with KChIP3.

EXPERIMENTAL PROCEDURES

DNA Subcloning—Rat Kv4.2 cDNA was obtained as a gift from the L. Jan laboratory (pBS-Kv4.2). The Kv4.2 coding region was transferred to a bacteriophage vector (pBluescript, Stratagene, Inc.), and also used to generate a vector for bacterial expression of the T1 domain, as described previously (8). KChIP3 was obtained as an EST clone, 2403205, and inserted into the same vector using available XhoI + NotI restriction sites.

In addition, as previously described (8), zinc coordinate site mutants were made using the QuikChange™ strategy and reagents (Stratagene, Inc.), with oligonucleotides that were 36–48 bases in length. All mutations were confirmed by DNA sequence analysis through the entire coding region.

Cell Culture—CHO-K1 cells were obtained from ATCC and maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 40 μg/ml l-proline, 100 units/ml penicillin, and 100 units/ml streptomycin.

Transfection—CHO cells were transiently transfected with cDNAs using Lipofectamine, as recommended by the manufacturer (Invitrogen Life Technologies, Inc.). Enhanced GFP (EGFP, Clontech, Inc.) was coexpressed with the channel to assess transfection efficiency and to identify expressing cells for voltage clamp experiments (26). One day prior to transfection, cells were plated onto cover slips. At 18–24 h, confluency of the cells was about 50–60% and considered optimal for transfection. We used a mixture of 0.5 μg of cDNA (Kv4.2 or zinc site mutants), 0.5 μg of GFP, and 3 μl of Lipofectamine in 0.5 ml of serum-free Dulbecco’s modified Eagle’s medium for about 2 h after which regular medium was restored. All the recordings were done 18–20 h after transfection.

For the Kv4.2 and KChIP3 ratio study, total cDNA used per transfection was 4.5 μg. The Kv4.2 cDNA level was fixed at 0.8 μg and EGFP cDNA at 0.5 μg in the transfection mix. Different amounts of KChIP3 were used to generate the different expression ratios. Red fluorescent protein cDNA was used as a filler to keep the cDNA total amount fixed. Transfection was carried out as described above. Recordings were done at 18–20 h after transfection. For the comparative studies on the effects of different KChIP3 ratios, the experiment was performed identically with the zinc triple mutant (Kv4.2-“ZnB3”) cDNA level also fixed to 0.8 μg. Because of the limited cDNA capacity of our transfection reagent, this experimental design limited us to a maximal Kv4.2 to KChIP3 cDNA ratio of 4:1. Therefore we could not test whether the mutant expression had completely plateaued. However, a Student’s t test between wild type and mutant at this cDNA ratio revealed no significant difference in the expression level.

Electrophysiology—Ionic currents were recorded with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA) using whole cell voltage clamp configuration (27). Current stimulus protocols and data collection were controlled by pClamp 6.05 software (Axon Instruments, Foster City, CA). The pipette contained (in mM): 140 KCl, 1 MgCl₂, 1 EGTA, 10 HEPES (pH 7.4), and 0.133 CaCl₂. The calculated free Ca⁺⁺ concentration was 1.5 nM at pH 7.4. The bath solution was (in mM): 2 KCl, 138 NaCl, 1.8 CaCl₂, 1 MgCl₂, 10 HEPES (pH 7.4). Data were collected at 10 kHz and filtered at 5 kHz using an Axopatch 200B amplifier. Data recorded in a cell containing KCl were identified by fluorescence microscopy using an inverted Diaphot microscope (Nikon, Inc.) equipped for fluorescence detection. Pipettes were pulled on a microprocessor-controlled, multiple stage puller (Sutter Instruments, Novato, CA) from borosilicate tubing with resistance of 0.9–1.2 MΩ.

Whole cell currents were sampled at 5 kHz and filtered at 2 kHz (~3 dB, four pole Bessel filter). Series resistance was compensated for 90–95%. Data were leak-subtracted online using P/8 protocol. The holding (Vh) potential was −80 mV. Records on SDS-PAGE gels were digitized (Axon Instruments) and stored directly on a hard disk using pClamp version 6.0.5. All recordings were done at room temperature (25 ± 2 °C).

Size Exclusion Chromatography—Transiently transfected cells were solubilized in CHAPS (2% final concentration) and run on a Superose 6 column using an Amersham Biosciences FPLC system as described previously (8). Fractions were run on polyacrylamide gels and Western blotted to determine the fractions containing the proteins of interest. Molecular weight standards were run and the standard curve corrected to the appropriate fraction by the dead time between the detector and collector. Kv4.2 protein often runs as a doublet on SDS-PAGE gel, with a fraction of the protein running at a high molecular size. There was no difference in the FPLC profiles for these two bands, and the higher molecular size band appeared to be protein that either remained tetrameric or aggregated in the running of the gel. The Western blots in Fig. 3 only show the profile for the immunoreactive material at the correct size for Kv4.2, ~70 kDa.

Data Analysis—Data analysis was performed using Clampfit version 6.0.5 (Axon Instruments) and Origin 6.1 software (Microcal Software, Inc.). Pooled data are expressed as mean ± S.E. Statistical comparisons (t test for paired or unpaired data or 1 way ANOVA using Origin and Analyze-it for Excel (Analyze-it Software). Tests of significance were performed based on standard hypothesis testing approaches. The analysis method of choice depended upon the null hypothesis to be tested: comparison of two variables or sets of variables. Student’s t tests were performed to determine if a single measured variable was significantly different between mutant and wild type. ANOVA comparisons were performed to test if a variable range was significantly different between mutant and wild type; for example, if mutants produce overall changes in the time constants of inactivation. Similarly ANOVA comparisons were used to test whether a channel property, such as inactivation time constant, showed significant changes with voltage for mutant or wild type. Values of p < 0.05 were considered statistically significant.

Reversal Potential Measurement—Cells were stepped to ±50 mV for 15 ms and then to different potentials from −140 mV to +50 mV at 10 mV steps. The holding potential was at −80 mV. An I-V curve was plotted with the peak tail current as y-axis and command potentials as x-axis. The potential where the peak tail current intersected the x-axis was recorded as the reversal potential. Under typical recording conditions, reversal potential was measured as approximately −90 mV.

RESULTS

KChIP3 Rescues Function of Zn²⁺ Site Mutant—The interaction of KChIP auxiliary subunits with Kv4.2 subunits is an important step in the formation of functional A-type Kv channels (3, 11). We were interested in testing if the intersubunit interactions of KChIPs might also have a direct role in channel assembly beyond their established role in redistributing channels onto the cell surface. In order to test this hypothesis, we sought to determine if KChIP co-expression could rescue the loss of A-current produced by Zn²⁺ site assembly mutants of Kv4.2. For rat Kv4.2, the residues involved in coordination of Zn²⁺ are His105, Cys111, Cys132, and Cys133 (8). Previous studies have shown that mutations to these sites eliminate functional channel expression by disruption of subunit assembly and trapping of monomeric subunits on intracellular membranes (8). For these studies, we have adopted the following nomenclature to refer to these mutations: single mutants are indicated by the mutation in parentheses after the construct name; multiple mutants are called Kv4.2-“ZnB” for “minus Zn²⁺ binding,” with a specific number to indicate the sites mutated. Thus, Kv4.2-“ZnB2” has the mutations C132A and C133A, Kv4.2-“ZnB3” has the additional mutation C111A, and Kv4.2-“ZnB4” has the additional mutation H105A. In general, we have focused our studies on the Kv4.2-“ZnB3” mutant, lacking all 3 coordinating Cys residues, but have performed complementary expression, assembly, and biophysical studies.
are seen with higher levels of KChIP3 cDNA. The 10-fold lower requirement for KChIP3 cDNA is likely caused by more efficient synthesis of this soluble protein compared with the synthesis of the polytransmembrane channel protein.

With Kv4.2(−ZnB3), the dose response relationship is significantly different, with additional KChIP cDNA required for maximal current expression. No expression of channels was observed with a 1:10 ratio of KChIP3 to Kv4.2(−ZnB3) (see Fig. 2B; ratio = 0.1). Expression was first evident at a ratio of 1:4 (Fig. 2B; ratio = 0.25), with increasing current at increasing KChIP3 to Kv4.2(−ZnB3) ratios until functional expression levels comparable to wild-type channels were achieved at a KChIP3 to Kv4.2 ratio of 4:1 (Fig. 2B; ratio = 4.0). Although, the cDNA ratios obviously do not accurately reflect the amount of protein being expressed, the 40-fold right shift in the curve with Kv4.2(−ZnB3) suggests that a mass action effect is occurring, where KChIP3 binding to Zn2+ site mutant channel subunits is driving functional expression of these channels. These results strongly support our previous hypothesis that the loss of function produced by Zn2+ site mutations is a specific problem in the assembly of the channel rather than a complete nonspecific disruption of the subunit protein, and suggest that the primary action of KChIP3 is to overcome this assembly defect (8, 10). To further confirm this conclusion, we sought to determine whether the assembly state of the channel had in deed changed following KChIP3 co-expression.

**Tetramerization of Zn2+ Site Mutants**—Our previous studies have shown that the Kv4.2(−ZnB) subunit protein runs as a monomer on SEC-FPLC compared with the normal tetrameric position of the wild-type channel (8). In order to test the hypothesis that KChIP3 was rescuing the function of Kv4.2(−ZnB) by driving tetramer assembly, cells were transfected with KChIP3 and Kv4.2, either with or without Zn2+ site mutations, and then the proteins were solubilized and the channel assembly state characterized by SEC. In the absence of KChIPs, wild-type Kv4.2 runs in fractions consistent with its self-assembly into tetramers, whereas Kv4.2(−ZnB3) is found in lower molecular size fractions consistent with monomers (see Fig. 3A and Ref. 8).

Following co-expression of wild-type Kv4.2 with KChIP3, the channel remains in the tetramer fractions (Fig. 3B). KChIP3 is found to co-migrate with the channel in addition to the normal low molecular mass position seen for unassembled KChIP3, indicating a stable incorporation into the channel complex (see Fig. 3C). Although KChIP addition is expected to increase the channel protein mass by ~25%, there was no large change in the position of the channel protein in the fractionation, suggesting that KChIP3 is tightly integrating into the structure and not significantly increasing the hydrodynamic radius. Support for this conclusion comes from single particle reconstruction studies on Kv4.2 channels co-expressed with KChIP2, where the KChIP2 density is found to be tightly integrated with the T1 domain and the cytoplasmic C terminus (19).

For Kv4.2(−ZnB3), the results of forming a channel complex with KChIP3 are much more dramatic. Expressed alone, Kv4.2(−ZnB3) is found exclusively in the monomeric fractions (see Fig. 3A). Co-expression with KChIP3 at a 1:1 cDNA ratio (Fig. 3B) produces a partial redistribution of Kv4.2(−ZnB3), where both tetrameric and monomeric channel protein is seen on SEC-FPLC. If the KChIP cDNA level is further boosted to 4× the level of Kv4.2(−ZnB3) cDNA, then the shift to tetramer is complete with all protein found in the tetrameric fractions (4× in Fig. 3B). These results on assembly state correlate well with the expression level dependence for KChIP3 rescue of Kv4.2(−ZnB3) function. Co-expressed KChIP3 is stably incorporated into the

**Kv4.2 (+ZnB3) mutant channel by KChIP3.** Currents in response to depolarizing voltage steps in 10-mV increments to +50 mV were recorded by whole cell voltage clamp from CHO-K1 cells. A, Kv4.2 expressed alone produces low levels of A-type K-current. B, transfection with the same amount of cDNA for the Kv4.2(−ZnB3) mutant fails to generate any functional A-current. C, co-transfection of equal amounts of Kv4.2 and KChIP3 cDNA dramatically enhances the level of A-current. D, co-transfection of equal amounts of Kv4.2(−ZnB3) and KChIP3 cDNA rescues mutant function, resulting in significant levels of A-current, ~25% less than seen with wild-type channels. E, elevation of the level of KChIP3 cDNA to 4× higher than Kv4.2 cDNA does not further enhance current levels. F, elevation of KChIP3 cDNA to 4× higher than Kv4.2(−ZnB3) cDNA results in complete rescue of current to wild-type levels.

on other −ZnB mutants and EGFP-tagged versions of these channels, and found that they behave similarly.

As expected from our previous results, wild-type Kv4.2 expression produces A-currents in CHO cells (Fig. 1A); however, the Kv4.2(−ZnB3) mutant showed no functional expression without KChIP co-expression (Fig. 1B and Ref. 8). With co-transfection of KChIP cDNA at a 1:1 cDNA ratio, wild-type Kv4.2 shows a large increase in functional A-current (see Fig. 1C). Interestingly, co-expression with KChIP3 rescues Kv4.2(−ZnB3) mutant subunits, producing functional A-current; however, the level of current seen is only ~25% of that seen with wild-type Kv4.2 channels (Fig. 1D). We confirmed this basic finding with several other Zn2+ site mutants, and the summary results are presented in Fig. 2A. Although none of the Zn2+ site mutants functioned without KChIP3, they were all rescued to a similar degree, ~25% of wild-type, when co-expressed with KChIP3 at a 1:1 cDNA ratio.

To determine whether this reduced expression level could be further enhanced with higher levels of KChIP3 expression, we boosted the KChIP3 cDNA levels to 4× greater than Kv4.2 and retested for A-current levels. Under these conditions, there was no change in wild-type Kv4.2 expression (Fig. 1E); however, the Kv4.2(−ZnB3) current is now 4× larger, reaching the same level of functional expression seen with wild-type channels (Fig. 1F).

**Higher KChIP3 Levels Are Required to See Zn2+ Mutant Expression**—The previous results suggest that there is a distinct dose response relationship for KChIP3 enhancement of Kv4.2 channels versus Kv4.2(−ZnB3). To further explore this effect, we kept the channel cDNA level constant and varied the amount of KChIP3 cDNA, keeping the total amount of cDNA constant. Fig. 2B shows the results of a dose response experiment using different ratios of wild-type Kv4.2 and KChIP3 cDNA. With wild-type Kv4.2, significant increases in current are seen with a 1:30 ratio of KChIP3 to Kv4.2 (Fig. 2B; ratio = 0.033). At a 1:10 ratio (Fig. 2B; ratio = 0.1), the enhancement of expression is maximal, and no further increases in current...
channel complex since it co-migrates with this tetrameric Kv4.2 (ZnB3); however, the majority of the KChIP3 protein is found in the free KChIP fractions, as expected for our mass action hypothesis (Fig. 3C). Similar results were obtained with EGFP-tagged versions of the ZnB3 mutant, (+EGFP)Kv4.2 (ZnB3) (Fig. 3, A–C). We also confirmed with several other Zn2+ site mutant constructs that co-expression of KChIP3 with mutant channels at a 4:1 ratio produces shifts in channel protein migration from monomer to tetramer fractions (data not shown). These results strongly support our hypothesis that the action of KChIP3 in the rescue of Kv4.2 (ZnB) mutants overcomes an assembly defect and drives the subunits to tetramer even in the absence of the Zn2+ binding interactions that would normally stabilize the T1 tetrameric interaction.

** Trafficking of Zn2+ Mutants out of the Endoplasmic Reticulum by KChIP3—**Our results have shown that KChIP3 co-expression rescues the function of Zn2+ binding site mutants by driving the assembly of these subunits to tetramer. We next confirmed that in the presence of KChIP3 the Zn2+ site mutant channel protein was released from the endoplasmic reticulum to allow trafficking to the cell surface. For these studies we have used COS-7. COS-7 cells are large flat cells that provide a beautiful view of the internal membrane systems of the cell. In Fig. 4A, we show the distribution of EGFP-tagged Kv4.2 protein, (+EGFP)Kv4.2, in COS-7 cells in the absence of KChIP3. Without KChIP3, the protein is strongly retained in endoplasmic reticulum and Golgi membranes, as can be seen by strong fluorescence within the internal membrane systems of the cell (8, 11). Addition of low or high levels of KChIP3 cDNA to the transfection does not noticeably change the protein distribution; however, with a 4× higher level of KChIP3 cDNA, the protein is clearly released from the internal membrane and produces a diffuse fluorescence pattern, similar to wild-type channels (Fig. 4D).

**Functional Properties of Zn2+ -less Kv4 Channels—**The ability to rescue Kv4.2 (ZnB) function by co-expression with KChIP3 provides us with an opportunity to characterize the changes in channel functional properties without Zn2+ bound to the Kv4.2 T1 domain. Because we were unable to express Kv4.2 (ZnB) without KChIP co-expression we focused our studies on answering the question of how the A-channels produced by co-expression of KChIP3 and Kv4.2 (ZnB) constructs are different from channels produced by co-expression of KChIP3 and Kv4.2. In Fig. 5A, we have plotted voltage-clamped current traces for Kv4.2 and Kv4.2 (ZnB3) co-expressed with KChIP3 in response to voltage steps from −30 mV to +50 mV. The currents are normalized to the same peak current, and the traces overlaid to allow a general comparison of the waveforms for normal and mutant channels. The first effect that is quite obvious is that the A-currents decay more rapidly for the Kv4.2 (ZnB3) mutant channels than wild type. We have examined other Zn2+ site mutants and find this to be a general property of these channels; loss of Zn2+ binding accelerates the inactivation of channels during sustained depolarizations. A second effect is seen by examination of the currents in response to the smallest shown depolarization to −30 mV, which shows a higher level of current for wild-type channels than for Kv4.2 (ZnB3) channels (Fig. 5A). Finally, examination of the traces at higher sweep speeds shows that the Kv4.2 (ZnB3) currents rise, reach their peak, and begin decaying before wild-type Kv4.2 currents (see Fig. 5A, inset).

We next characterized the steady state inactivation and peak activation properties for normal and Zn2+ site mutant channels. Steady state inactivation curves were measured for both
KChIP Promotes Shal Channel Assembly

Channel Kinetic Properties—To quantify the kinetic differences in the current waveforms that underlie these differences in function between Kv4.2 channels and Kv4.2(−ZnB3) channels expressed with KChIP3, we measured the time to peak activation and characterized the inactivation kinetics. For the time required to peak (see Fig. 6A), the Kv4.2(−ZnB3) channel was consistently faster than wild type, particularly at more depolarized potentials. At +50 mV, the time to peak for Kv4.2(−ZnB3) channels was 4.3 ± 0.3 ms compared with 7.7 ± 0.8 ms for wild-type channels.

We next examined the changes in inactivation kinetics that produce the more rapid current decay in Zn2+ site mutant channels. Kv4 channel inactivation has been dissected in numerous biophysical studies into three separate processes: open state, closed state, and slow inactivation (15, 20, 21). Open state inactivation utilizes a pore block produced by the Kv4 cytoplasmic N terminus and is lost by deletion of the Kv4 N terminus or binding of KChIP proteins to the N terminus (16, 22). Closed state inactivation is a separate process from open state inactivation. In contrast to open state inactivation, closed state inactivation is not lost by removal of the Kv4 N-terminal inactivation peptide (21). Although the precise mechanism responsible for this inactivation is not known, it is sensitive to mutations within the channel vestibule and results in an apparent uncoupling between S4 movements and channel pore opening (20–23). A third slower inactivation process also occurs with Kv4 channels that is evident with longer depolarizations and is thought to be a distinct process from C-type inactivation seen in Shaker channels; however, the mechanism for this slow inactivation is poorly understood (21). In the presence of KChIPs, the decay of the current for both normal and Zn2+ site mutants can be as well fit as the sum of 2 exponentials (see Fig. 6B). The faster exponential process is proposed to be dominated by the entry of flickering Kv4.2 channels into closed state inactivation, and the slower process is dominated by movement into the slow inactivated state (16, 22). From these fits, we see that loss of the Zn2+ site slightly accelerates the kinetics of both inactivation components as well as shifting the relative weight of the two components toward the faster kinetic (Fig. 6, C and D). These effects all combine to accelerate the decay of A-current for the mutant channels.

Low Voltage Inactivation—To explain the shifts in steady state inactivation produced by Zn2+ site mutations, we need to examine the time course for entry into the low voltage inactivated state as well as the time course for the recovery from inactivation. For Kv4 channels, the inactivation at negative potentials, near the steady state V1/2, is dominated by entry into the closed state inactivation process, because it occurs at potentials where channel opening rarely occurs (20, 21, 23). In Fig. 7, we examined the kinetics for inactivation for wild type as well as Zn2+ mutant channels expressed with KChIP3 at potentials near the V1/2 for steady state inactivation. In Fig. 7A, we show the closed state inactivation of wild-type Kv4.2 expressed with KChIP3, with an interpulse potential of −55 mV. Fig. 7B, shows the closed state inactivation of Kv4.2(−ZnB3) channels with KChIP3 in response to the same inactivation protocol. As expected from the shifts in steady state inactivation, the Kv4.2(−ZnB3) channels inactivate more completely than wild-type Kv4.2 channels when stepped to the same potential. Average responses are compared in Fig. 7C for inactivation to the two potentials that bracket the V1/2 for inactivation of these channels. Two differences between wild-type and Kv4.2(−ZnB3) channels is evident. The first is that Zn2+ mutant channels reach the same level of inactivation at more negative potentials than wild type, as expected from the shift in steady state inactivation. The second is that the kinetics for entry into the closed inactivated state are different between wild-type and Zn2+ mutant channels, inactivating to the same level. At potentials near the V1/2, Zn2+ site, mutant channels normal and Zn2+ mutant channels in the presence of KChIP3, see Fig. 5B. For wild-type Kv4.2, the midpoints for the inactivation curve was −51.1 ± 1.2 mV in the presence of KChIP3. For Kv4.2(−ZnB3) half-inactivation occurred at −58.6 ± 0.7 mV indicating an enhanced tendency to inactivate for these mutant channels. Peak activation curves were measured for both normal and Zn2+ mutant Kv4.2 channels expressed in the presence of KChIP3 (Fig. 5B). The activation midpoints of Kv4.2 wild-type channels is −27.8 ± 0.4 mV compared with −25.1 ± 0.2 mV for the mutant Kv4.2(−ZnB3). The activation of Kv4.2(−ZnB3) channels also showed a slightly steeper slope of 6.0 ± 0.2 compared with 7.0 ± 0.3 for wild-type Kv4.2 channels expressed with KChIP3. Whereas these effects on activation are small there is a significant difference in the overlap between activation and inactivation curves for mutants versus wild type. The decreased overlap for Kv4.2(−ZnB) channels is expected to produce a much smaller “window” of sustained current between −60 mV and −30 mV.

Fig. 3. KChIP3 co-expression drives the Kv4.2(−ZnB3) protein into a stable tetrameric state. SEC-FPLC profiles for channels expressed with and without KChIP3 are shown. Superose 6 column fractions from 22 to 38 were run on SDS-PAGE gels and Western-blotted with either anti-Kv4.2 or anti-KChIP3 antisera to identify the fractions containing the protein of interest. Each panel shows the results of a set of FPLC runs for the different Kv4.2 channel constructs indicated on the left, grouped according to whether the protein was co-expressed with KChIP3. Lower number fractions, eluting sooner from the column, contain proteins with a larger hydrodynamic radius. For different conditions, the set of fractions that are expected to contain tetrameric channels are boxed in black, fractions that are expected to contain monomeric subunits are boxed in gray, and fractions that are expected to contain free KChIP3 are boxed in dashes. A, channels expressed without KChIP Western-blotted to identify fractions containing Kv4.2 subunits. Wild-type Kv4.2 channels run in the tetrameric fractions even without KChIP3; however, Kv4.2(−ZnB3) channels run as monomers either with or without an EGFP epitope tag. B, channels expressed with KChIP3 Western-blotted for Kv4.2 protein. Level of KChIP3 cDNA added is indicated to the right of the fractionation. As expected, Kv4.2 channels are still localized in the tetrameric fractions. Kv4.2(−ZnB3) channels show a progressive shift to tetrameric fractions with increasing KChIP3 cDNA. An EGFP-tagged version of this mutant shows a similar shift to tetramer with expression of KChIP3 at levels 4X greater than channel cDNA. C, channels co-expressed with KChIP3 Western-blotted for Kv4.2 protein. Level of KChIP3 cDNA added is indicated to the right of the fractionation. As expected, Kv4.2 channels are still localized in the tetrameric fractions.
FIG. 4. KChIP3 changes the subcellular distribution of wild-type and Zn²⁺ site mutant channels. EGFP-tagged wild-type and −ZnB3 mutant channels were co-expressed with 4× level of KChIP3 cDNA and then visualized by confocal microscopy 36 h later. A, (+EGFP)Kv4.2 expressed without KChIP3 is localized to internal membranes and concentrated in perinuclear compartments, likely the Golgi. B, (+EGFP)Kv4.2(−ZnB3) mutant channels expressed without KChIP3 are localized in diffuse intracellular membrane network as expected for endoplasmic reticulum-retained proteins. C, co-expression of 1× or 4× KChIP3 with (+EGFP)Kv4.2 traffics the channel onto the cell surface and reduces the concentration of protein in the Golgi. Only 4× cells are shown. D, expression of 4× KChIP3 with (+EGFP)Kv4.2(−ZnB3) protein traffics the channels onto the cell surface. Fluorescent protein is seen to coat the cell surface.

FIG. 5. Functional differences in Kv4.2(−ZnB3) channels compared with wild-type channels expressed with KChIP3. A, activation curves in response to step depolarization from −30 mV to +50 mV in 10-mV increments. Currents for Kv4.2(−ZnB3), shown in red, and Kv4.2, shown in black, were normalized to the peak current at +50 mV and plotted on the same time base. The Zn²⁺ site mutant channels activate slightly less at the lower potentials and inactivate more rapidly at all potentials. An expanded time base inset compares the activation of the two channels at +50 mV. As can be seen, the Zn²⁺ site mutant channels appear to activate more rapidly, reaching a peak sooner than wild-type channels. B, steady state inactivation and peak activation properties of normal and Zn²⁺ site mutant channels. Kv4.2(−ZnB3) channels show a significant leftward shift in inactivation and a rightward shift in activation compared with wild-type channels. Statistical analyses were performed on the set of fits to individual oocytes using Student’s 𝑡 tests. Significant differences were found between mutant and wild type in the half-activation voltages, half-inactivation voltages, and activation slopes, but not inactivation slopes.
take longer to reach a steady state for inactivation. Fig. 7D compares the kinetics for entry into the closed inactivated state for wild-type and Zn²⁺ site mutant channels. We have plotted the average and standard deviations for the best single exponential fits to individual experiments at different potentials. At more negative potentials, the inactivation of Kv4.2(ZnB3) channels is significantly slower than wild-type Kv4.2 channels. Inactivation of the Kv4.2(ZnB3) channels however shows a much stronger voltage dependence than wild-type Kv4.2, and the traces cross over each other at approximately −52 mV. Above this potential, inactivation of Kv4.2(ZnB3) channels is more rapid, in agreement with our results on depolarized potentials shown in Fig. 6.

Recall that the rate of recovery from the inactivated state is controlled by the ratio of the rate for entry into the inactivated state to the rate of recovery from the inactivated state. We therefore were interested in examining if changes in the recovery from inactivation, indicative of a more stable inactivated state, are significantly altered with the loss of the Zn²⁺ site. Recovery was characterized using a typical two-pulse protocol where the time spent at the interpulse potential was varied to characterize the recovery time course. Fig. 8, A and B shows the recovery profiles for wild-type Kv4.2 and Kv4.2(ZnB3) channels. The amplitude of the second peak was measured and plotted versus the time spent at the interpulse potential for normal and ZnB channels in Fig. 8C. It is clear from these plots that the −ZnB mutants recover more slowly from inactivation. For wild-type Kv4.2 expressed with KChIP3, the time constant to recover from inactivation at −80 mV is 81.7 ± 4.1 ms; however, for Kv4.2(ZnB3) the
recovery is dramatically slowed to 254.3 ± 8.2 ms. In fact, this value is almost as slow as Kv4.2 expressed without KChIP. Therefore, loss of the Zn$^{2+}$ site eliminates much of the effect that KChIP3 produces to accelerate the recovery of Kv4.2 from inactivation.

**DISCUSSION**

In these studies, we have found that the KChIP3 auxiliary subunit protein has a dramatic ability to rescue the function of an assembly mutation of Kv4.2 subunit proteins. Kv4.2 subunits with the zinc site mutated are completely unable to form functional channels because of a loss of subunit tetramerization. Our results strongly suggest that this loss of function in these mutants is a specific effect related to the loss of Zn$^{2+}$ binding, rather than a nonspecific effect of these mutants: 1) All mutants to Zn$^{2+}$ coordination residues tested (single, double, triple, and quadruple) have very similar effects on channel assembly and have similar functional changes when rescued by KChIP3. 2) The four coordination residues mutated are located in 3 different secondary structural elements. 3) The coordination residue Cys111 is found on the opposite surface and only joins the other three residues following folding and interaction of the assembly interface (9). The only clear relationship between these mutants is that they all disrupt Zn$^{2+}$ binding to this site.

KChIPs are revealed here to be active participants in the assembly of subunits, capable of binding to unassembled subunits and then driving them together to form functional channels. These results clearly provide important new insights into KChIP function. The mechanism by which KChIPs are able to drive channel assembly is not clear; however, previous results suggest some possible mechanisms. First, KChIP proteins have been shown to bind to the very N-terminal region of Kv4.2 subunits (12). So, one possible mechanism is that the floppy N-terminal chain folds back onto the T1 domain and allows the KChIP protein to interact...
with and rescue the assembly interface lost with the Zn$^{2+}$ site mutants. Such a possibility is suggested by yeast 2-hybrid experiments, which have shown that KChIP proteins can interact directly with specific regions of the T1 domain, providing a direct mechanism to modulate T1-T1 interactions (13). Indeed, single particle reconstruction experiments have suggested a binding of KChIPs along the T1 interaction interface (19). These models agree with our observations in SEC-FPLC studies that show a minimal impact on KChIP binding on the hydrodynamic radius of the channel, suggesting the KChIP protein is tightly integrated into the channel structure. The crystal structure of the KChIP- Kv4.2 interaction suggests another mechanism for driving assembly by the dimeric interactions between KChIPs bound to the Kv4.2 N terminus (12). This mechanism would not directly rescue the lost interactions within the T1 domain, but rather replace the energy lost with a complementary set of interactions that occur separately from the T1 domain. Future mutagenesis studies that can separate KChIP dimerization interactions from direct KChIP interactions with the T1 domain may allow us to distinguish between these different possibilities.

Another important question remaining to be answered is the relative importance of KChIPs driving subunit assembly compared with their trafficking properties in the formation of native A-currents. Although it could be argued that there does not seem to be any problems in Kv4.2 subunit assembly without KChIPs, in native cells, where Kv4.2 expression is not being driven by a cytomegalovirus promoter, the situation could be very different. There, efficient assembly of Kv4 channels into a tetrameric channel may require auxiliary subunits to help the forming channel hold together long enough to find the required subunits out of the sea of thousands of other membrane proteins being synthesized at the same time.

Indeed, we may be creating a somewhat artificial distinction when we classify trafficking and assembly into two discrete processes. Examination of Fig. 4 shows that the subcellular distributions of wild-type and Zn$^{2+}$ site mutant channels are similar in the absence of KChIPs. Thus, cellular processes that trap the unassembled Zn$^{2+}$ site mutant channel proteins within the endoplasmic reticulum are similarly recognizing the wild-type protein as being improperly assembled until an interaction with KChIP completes the assembly and frees the protein to reach the cell surface. Biophysical studies on native neuronal A-currents and cardiac I$_{to}$-currents have suggested that formation of functional currents in these native cells likely requires interactions with auxiliary subunits such as KChIP3 (24–26). Of particular importance in determining if these processes represent distinct functional mechanisms will be to de-

![Image](image_url)

**Fig. 8.** Comparison of the rate of recovery from inactivation for Kv4.2 compared with Kv4.2(-ZnB3) both expressed with KChIP3. Channels were fully inactivated with a 1-s depolarization to +50 mV, and then the channels were allowed to recover for varying lengths of time at different potentials. A, wild-type channel recovery at −80 mV, shows a rapid recovery of channel function. B, Kv4.2(-ZnB3) mutant recovery at −80 mV is prolonged compared with wild type. C, average recovery from inactivation for normal and Kv4.2(-ZnB3) mutant channels. The recovery curves were well fit with single exponentials. D, summary data for exponential fits to individual experiments at different recovery potentials. Recovery was ~3x longer for Kv4.2(-ZnB3) mutants compared with wild type at all potentials tested. Values at all potentials were significantly different between wild type and mutant.
termine whether mutations to KChIPs can be identified, which differentially affect their ability to rescue Zn\(^{2+}\) site mutant Kv4 channel assembly without affecting wild-type channel trafficking functions, and \textit{visa versa}.

Our results further show that an intact Zn\(^{2+}\) binding site of Kv4.2 subunits is not absolutely required for channel function. For Kv4.2 channels, the most drastic effect of Zn\(^{2+}\) site mutations is a slower recovery from inactivation by 3–4-fold. At the negative potentials where this time constant is measured, its value is likely dominated by the rate constant for recovery from inactivation, and is thus a good indicator that the stability of the inactivated state of the channel has increased by Zn\(^{2+}\) site mutation. In addition, at depolarized potentials, we find that the Zn\(^{2+}\) site mutant channels peak sooner and inactivate more rapidly. Modeling studies have suggested that inactivation in Kv4 channels is caused by the channels accumulating in the closed inactivated state at both low and high voltages (21). The stabilization of the inactivated state by Zn\(^{2+}\) site mutagenesis is only expected to accelerate the rate of inactivation at positive potentials if Kv4.2 channels significantly reopen from the closed inactivated state. An alternative explanation for the reduced time to peak and faster inactivation kinetics is that Zn\(^{2+}\) site mutations also accelerate the entry into the inactivated state. The combination of these two effects would act together to left-shift the steady state inactivation curve for Zn\(^{2+}\) site mutant channels.

Interestingly, Zn\(^{2+}\) site mutants could accelerate inactivation by two different mechanisms, either accelerating the rate to enter the closed inactivated state, or increasing the fraction of activated channels that remain closed. Previous studies on Kv2.1 channels showed that chemical modification of N-terminal Cys residues prolonged the latency to first channel opening (27). Although the exact Cys residues responsible were not identified, the region implicated included the cysteine residues (27). Although the exact Cys residues responsible were not identified, the region implicated included the cysteine residues (27).

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KChIP3 Rescues the Functional Expression of Shal Channel Tetramerization Mutants
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