Mammalian voltage-gated K\(^+\) channels are assemblies of pore-forming \(\alpha\)-subunits and modulating \(\beta\)-subunits. To operate correctly, Kv4 \(\alpha\)-subunits in the heart and central nervous system require recently identified \(\beta\)-subunits of the neuronal calcium sensing protein family called K\(^+\) channel-interacting proteins (KChIPs). Here, Kv4.2-KChIP2 channels are purified, integrity of isolated complexes confirmed, molar ratio of the subunits determined, and subunit valence established. A complex has 4 subunits of each type, a stoichiometry expected for other channels employing neuronal calcium sensing \(\beta\)-subunits.

In muscles and nerves, Kv4 (Shal family) \(\alpha\)-subunits assemble with K\(^+\) channel-interacting proteins (KChIPs)\(^3\) \(\beta\)-subunits to create mixed complexes with unique attributes and functions (1–7). Thus, Kv4 channels produce rapidly activating and inactivating currents, such as I\(_{\text{to}}\), which mediates the early repolarization phase of the cardiac action potential (8–11), and I\(_{\text{k}}\), to regulate action potential propagation and frequency in neurons (12–14). KChIP subunits 1–4 enjoy differential tissue distribution, splice variation and carry EF-hand motifs as do other neuronal calcium sensing peptides (including frequenin, recoverin, guanylyl cyclase activating protein, and the visinin-like proteins visinin-like proteins 1–3, neurexin, and hippocalin) (15); the roles of visinin-like proteins are now emerging.

KChIPs (and frequenin) (16,17) have been shown to assemble with Kv4 subunits in stable fashion leading to increased current density due to enhanced surface expression, activation at more hyperpolarized potentials, slowed inactivation, and speeded recovery from inactivation (1, 2, 16). These \(\beta\)-subunits permit kinase regulation (18), control trafficking, and alter surface half-life (6). So, the increase in I\(_{\text{to}}\) across canine cardiac surface half-life (6). So, the increase in I\(_{\text{to}}\) across canine cardiac

Current-voltage curves were evoked by depolarizing from a holding membrane voltage of +10 mV to test potentials of +80 to 70 mV with 10 mV steps lasting 500 ms every 20 s. Current-voltage curves were evoked by depolarizing from a holding potential of −100 mV to test potentials of −80 to 70 mV with 10 mV steps lasting 500 ms every 5 s. Steady state inactivation was examined from a holding potential of −100 mV with test pulses from −120 to 10 mV held for 2.5 s with a second pulse at 40 mV to measure currents that were not inactivated. Recovery from inactivation was measured by
driving channels to an inactivated state at 40 mV, hyperpolarizing to
−100 mV, and then applying a second pulse to 40 mV for various
intervals (increments of 5 and 50 ms). Inactivation was fit to a double
exponential equation. Recovery from inactivation was fit to a single
exponential. Activation $V_{1/2}$ and $V_{o}$ were calculated by fitting the con-
ductance-voltage relationship to a Boltzmann function. Similarly, inac-
tivation $V_{1/2}$ and $V_{o}$ were calculated by fitting the normalized current-
voltage relationship to a Boltzmann function.

**Expression and Purification with COS7 Cells**—Kv4.2* was co-ex-
pressed with KChIP2 in COS7 cells using LipofectAMINE 2000 (In-
vitrogen, Carlsbad, CA) and 2.5 and 5 μg cDNA/plate, respectively.
10–25 100 mm plates were harvested 2 days after transfection and
solubilized for 1 h at 4 °C in lysis buffer containing 2.5% CHAPS, 100
mm NaCl, 40 mm KCl, 1.0 mg/ml Escherichia coli lipid, 1 mM dithio-
reitol, 0.2 mM leupeptin/pepsatin, 1 mM EDTA, 20 mM HEPES-KOH,
pH 7.4, and 10% glycerol with Complete protease inhibitors (Roche
Applied Science). Soluble material (SM) was collected after centrifuga-
tion at 100,000 × g for 45 min. The extract was incubated with anti-
1D4-coated beads for 2 h at 4 °C with agitation often between New
Haven, CT, and Waltham, MA (Honda Civic 1999). The column was
washed with 50 ml of wash buffer containing 0.7% CHAPS, 300 mm
NaCl, 40 mm KCl, 0.01 mM leupeptin/pepsatin, 1 mM EDTA, 20 mm
HEPES-KOH, pH 7.4 with Complete protease inhibitors (Roche Applied
Science). The protein was then eluted (E) in wash buffer with 100 mm
NaCl and 0.2 mM/MLD peptide (Yale University Reck Facility). 1D4 mon-
oclonal antibody was purchased (NCCG, Minneapolis MN) and coupled
to beads as before (21). KChIP2 antibodies were a generous gift (J.
Trimmer, University of California, Davis).

**Whole-cell CTX Binding**—[3H]-CTX binding to cells was performed as
described previously (22). Briefly, cell samples were divided into control
and test groups. Control assessed nonspecific binding of [3H]-CTX (36 nm)
that took place in the presence of 1 μM non-radioactive CTX. Test
groups measured total [3H]-CTX binding and specific binding was deter-
mined by subtracting the nonspecific from the total binding. All steps
were performed at 4 °C. Cells were exposed to [3H]-CTX in binding buffer
(20 mM KCl, 10 mM NaPi, 100 mM sucrose, and 1 mg/ml human serum
albumin) for 30 min, collected by centrifugation for 1 min at 3,000
rpm, and the supernatant was removed. The cell pellet was washed in 100
μl binding buffer, centrifuged, and after removal of supernatant, suspen-
sed in 100 μl of buffer, transferred to a scintillation vial with 10 ml of
scintillation fluid (SafeScint, American Bioanalytical, Natick, MA)
for liquid scintillation counting.

**CTX Binding to Purified Complexes**—A 50 μl elution of purified
material was exposed to 36 nm [3H]-CTX with or without 1 μM unlabelled
CTX. As above, the two samples were used to assess nonspecific and
total binding of [3H]-CTX. Binding was performed at 4 °C for 30 min,
8 h, 16 h, or 24 h, after which unbound toxin was removed with an
Amicon Centricron 100 (Millipore, Billerica, MA) by centrifugation
for 10 s at 9,000 rpm and a wash with 20 μl wash buffer with only 100 mm
NaCl by centrifugation. The filter was then placed in a liquid scintilla-
tion vial and studied in a scintillation counter as above. The dose-
titration assay was performed in a similar manner with 25 μl aliquots
of purified channel complex, 0.45, 1.33, 4, 12, or 36 nm [3H]-CTX was
applied to a control and test sample for 30 min at 4 °C and processed as
above.

**Glycerol Gradient Analysis**—Kv4.2*KChIP2 protein complex (0.5
ml, ~2 μg, E2–4) was layered on a 12 ml 10–40% (v/v) linear glycerol
gradient containing 0.7% CHAPS, 100 mM NaCl, 40 mM KCl, 1 mM
EDTA, 20 mM HEPES-KOH, pH 7.4, and Complete protease inhibitors
(Roche Applied Science). A parallel gradient was layered with 20
ml aliquots of each of the following molecular mass markers (Amersham Biosciences);
chymotrypsinogen (25 kDa), albumin (67 kDa), aldolase (158 kDa),
catalase (232 kDa), and ferritin (440 kDa). Gradients were centrifuged
at 100,000 × g, E2–4. Gradients were centrifuged using an SW41 rotor
(Beckman, Fullerton, CA); 1 ml fractions were collected by upward
displacement. Proteins were precipitated with 5 volumes acetone at −20 °C
for analysis.

**Amino Acid Analysis**—After SDS-PAGE and staining with Coomasie
Blue, Kv4.2 and Kv4.2* channels with and without KChIP2

| Parameter | Kv4.2 | Kv4.2*KChIP2 | Kv4.2* | Kv4.2*+KChIP2 |
|-----------|-------|-------------|-------|--------------|
| Activation $V_{1/2}$ (mV) | −24 ± 0.6 | −28.8 ± 0.9 | −28.9 ± 1.0 | −30.2 ± 0.9 |
| Activation $V_{1/2}$ (mV) | 13.7 ± 0.3 | 10.3 ± 0.2 | 12.0 ± 0.3 | 8.5 ± 0.1 |
| $V_{peak}$ at 40 mV (μA) | 1.5 ± 0.1 | 4.9 ± 0.4 | 2.2 ± 0.3 | 4.9 ± 0.6 |
| Inactivation $V_{1/2}$ (mV) | −62.2 ± 0.8 | −50.3 ± 1.0 | −58.6 ± 0.6 | −52.5 ± 0.4 |
| Inactivation $\tau_{inact}$ (ms) | 6.5 ± 0.63 | 6.6 ± 0.32 | 3.3 ± 0.02 | 3.3 ± 0.02 |
| Inactivation $\tau_{inact}$ (ms) | 204 ± 20 | 197 ± 15 | 140 ± 21 | 172 ± 9 |
| $A_{low}/A_{low}$ | 0.17 | 0.48 | 0.20 | 0.48 |
| $A_{low}/A_{low}$ + $A_{tetrad}$ | 0.83 | 0.52 | 0.80 | 0.52 |
| Recovery $\tau_{rec}$ (ms) | 192 ± 8 | 19.3 ± 0.6 | 221 ± 8 | 24.5 ± 1.5 |
| n (no. of oocytes) | 5 | 5–8 | 5–6 | 5–11 |
RESULTS

First, a variant of human Kv4.2 α-subunits (Kv4.2*) was produced that maintained the functional attributes of wild-type but had two modifications, a pore binding site for the peptide toxin CTX and an 8 residue C-terminal epitope tag (1D4, ETSQVADA). The rationale for these embellishments was as follows. A single CTX molecule binds in the ion conductive pore of toxin-sensitive K⁺ channels to occlude the permeation pathway only when 4 permissive α-subunits are assembled in correct fashion; as a result, small changes in pore (or toxin) structure are registered with great sensitivity as altered blockade (23–25). Thus, CTX and related toxins were used to locate the external aspect of the conduction pore (26), assess its dimensions (27, 28), count channels (22), and, as here, assess the integrity of purified channel protein (19). The 1D4 epitope allows purification of membrane proteins under mild conditions via monoclonal antibody binding and peptide elution (21).

The CTX-sensitive variant of Kv4.2 was produced by introduction of 3 mutations in the pore loop (Fig. 1a) chosen in an iterative fashion based on residues found to be important for high-affinity toxin binding in other K⁺ channels (29, 30). Although wild-type Kv4.2 channels are insensitive to application of CTX, those with Kv4.2* α-subunits show half-maximal block (Kᵢ) at 0.35 ± 0.04 nM (Fig. 1, b and c). Similarly, wild-type Kv4.2-KChIP2 channels are insensitive to toxin whereas those with Kv4.2* α-subunits showed Kᵢ = 0.76 ± 0.03 nM (Fig. 1, b and c). As CTX is external and KChIP2 subunits are intracellular, the increase in Kᵢ observed on KChIP2 incorporation was unexpected. Relaxation to equilibrium blockade upon acute toxin application revealed KChIP2 to have little effect on the rate of recovery of Kv4.2. But to destabilize bound toxin, increasing its off-rate −3-fold (Kᵢ ′ = 8.0 ± 0.6 × 10⁻³ s⁻¹ versus Kᵢ = 22.9 ± 2.6 × 10⁻³ s⁻¹). This indicates small KChIP2-induced changes in the external pore of the closed channel (the predominant state in this protocol).

Despite modifications to confer CTX sensitivity and append
the epitope tag, channels with Kv4.2* behaved almost like those with wild-type subunits (Table I and Fig. 2). Kv4.2* channels are like wild-type in their voltage-dependence for half-maximal activation and half-maximal steady state inactivation. Although small differences in inactivation kinetics were observed for slow ($\tau_{\text{slow}}$) and fast ($\tau_{\text{fast}}$) time constants, these were without associated changes in their relative ratio, or rates of recovery from inactivation. Moreover, the archetypal effects of KChIP2 were unaltered by the $\alpha$-subunit mutations. Thus, KChIP2 produced similar increases in current density and rates of recovery from inactivation with both wild type Kv4.2 and Kv4.2*, and similar decreases in rates of inactivation (due to increased contribution of the slow component) with expected small shifts in the voltage required to achieve half-maximal activation and half-maximal steady state inactivation.

Radioactive CTX ($^{3}$H-CTX) was synthesized as previously reported (22) and used first to monitor and optimize surface expression of channels on COS7 cells (see “Materials and Methods”). As expected based on measurement of channel currents, KChIP2 increased the absolute number of channels on the cells 2.6-fold, from 1.6 ± 0.2 pmol/plate ($n = 3$) to 4.0 ± 0.2 pmol/plate ($n = 11$). Purification of Kv4.2*-KChIP2 complexes was achieved via the 1D4 epitope on Kv4.2* with anti-1D4 antibodies covalently bound to beads (Fig. 3). Thus, COS7 cells induced to transiently express Kv4.2* and KChIP2 at high levels were solubilized with the detergent CHAPS (2.5%), and complexes isolated by binding, washing with buffer, and elution with 1D4 peptide (see “Materials and Methods”). This led to co-purification of Kv4.2* and KChIP2 at high levels to be rich in the two channel subunits (Fig. 3b). Staining with Coomassie Blue confirmed the E2 fraction to be rich in the two channel subunits (Fig. 3b).

Purified material was subjected to centrifugation through a glycerol gradient and studied by Western blot analysis after SDS-PAGE to estimate the size of Kv4.2*-KChIP2 complexes. The complex had a molecular mass between 232–440 kDa in trials with four separate preparations (Fig. 3c). Because Kv4.2* subunits carry no carbohydrate, each 642 residue protein has a predicted mass of 72 kDa and each tetramer a mass of 288 kDa (an underestimate due to detergent binding (21)). Because KChIP2 has 252 residues and a predicted mass of 28 kDa this result argues that complexes contain between 1 and 6 KChIP2 subunits ($\sim$316–456 kDa). The predicted mass for an octameric complex with 4 Kv4.2* subunits and 4 KChIP2 subunits is 400 kDa.

To verify the stability and integrity of purified Kv4.2*-KChIP2 complexes over time, binding of $^{3}$H-CTX to isolated complexes was assessed. After 24 h at 4 °C, ~90% of the binding activity was retained (Fig. 4a). Indeed, despite residence in detergent rather than a lipid membrane, purified complexes bound $^{3}$H-CTX with high affinity. Binding studies with four separate preparations Kv4.2*-KChIP2 were well fit to a single-site function reveals $K_{d}$ ~4.8 nM ($n = 4$), as in “Materials and Methods.”

![Figure 3](image3.png)

**FIG. 3.** Purification of Kv4.2*-KChIP2 complexes and estimation of mass by glycerol gradient centrifugation. a, purified Kv4.2*-KChIP2 channels separated by 10% SDS-PAGE, transferred to nitrocellulose, and probed with anti-1D4 and anti-KChIP2 antibodies. $\alpha$- and $\beta$-subunit mutants were excised and subjected to amino acid analysis (Table II). The band with a star is a minor contaminant (~1%) of 1D4 purifications (21). b, E2 fraction applied to a 10% glycerol gradient subjected to centrifugation and examined by 10% SDS-PAGE and stained with Coomassie Brilliant Blue. The bands corresponding to each subunit were excised and subjected to amino acid analysis (Table II). The band with a star is a minor contaminant (~1%) of 1D4 purifications (21). c, E2 fraction applied to a 10–40% glycerol gradient subjected to centrifugation and examined by 10% SDS-PAGE and Western blot analysis with anti-1D4 and anti-KChIP2 antibodies. Peak fractions for Kv4.2*-KChIP2 complexes are between markers at 232 and 440 kDa (catalase and ferritin, respectively).

![Figure 4](image4.png)

**FIG. 4.** $^{3}$H-CTX binding reveals stability and integrity of purified Kv4.2*-KChIP2 complexes. a, $^{3}$H-CTX binding to Kv4.2*-KChIP2 complexes at various times after isolation: 30 min, 8 h, 16 h, and 24 h ($n = 3$), as in “Materials and Methods.” b, $^{3}$H-CTX binding to Kv4.2*-KChIP2 complexes with increasing $^{3}$H-CTX (0, 0.45, 1.33, 4, 12, or 36 nM) and fit to a single-site function reveals $K_{d}$ ~4.8 nM ($n = 4$), as in “Materials and Methods.”
Kv4.2-KChIP2 Channels Have 4 Subunits of Each Type

TABLE II
Moles of Kv4.2* and KChIP2 subunits in channel complexes

| Study | Kv4.2* | KChIP2 | Ratio |
|-------|--------|--------|-------|
|       | pmol   | pmol   |       |
| 1     | 3.00 ± 0.1 | 3.47 ± 0.1 | 1.15  |
| 2     | 10.3 ± 0.2 | 11.0 ± 0.2 | 1.06  |
| 3     | 11.3 ± 0.3 | 10.3 ± 0.2 | 0.91  |

Blue dye, and the bands excised from the gel for amino acid analysis by hydrolysis, ion-exchange chromatography, and ninhydrin detection (Table II). Because the amino acid sequences of Kv4.2* and KChIP2 were known, the expected and observed amino acid content of the bands could be compared and the moles of each subunit determined independently from 9–12 residues in each of 3 independent trials. The molar ratio of Kv4.2*-KChIP2 in purified complexes was 1:1 (Table II); similar results are noted in a companion report for channels with a KChIP2 variant and others with a Kv4.2* mutant. Purified channels are shown to have 4 α-subunits because CTX binds with high affinity (Fig. 4). A 1:1 molar ratio therefore indicates that Kv4.2*-KChIP2 complexes contain 4 KChIP2 subunits.

**DISCUSSION**

Accessory β-subunits are a fundamental feature of K+ channels that determine channel location, abundance, sensitivity to stimulation, and pharmacology in vivo (6, 20, 32). Here we demonstrate that regulatory KChIP2 subunits assemble with pore-forming Kv4.2 subunits in 4:4 complexes when over-expressed in tissue culture cells to produce voltage-gated K+ channels like those in native cells (such as cardiac I_k1, (3, 4)). A 4:4 subunit arrangement appears to be the natural and stable valence for Kv4.2*-KChIP2 channels first because it was found with four separate large-scale preparations and second because KChIPs could not be separated from α-subunits without complete dissociation of the complexes to monomer form (not shown). KChIP2 increases trafficking to the surface, channel half-life, and detergent solubility of mixed complexes formed with Kv4.2 (6). This suggests why we could purify significant amounts of Kv4.2*-KChIP2 channels but did not succeed when Kv4.2* α-subunits were expressed alone (not shown). We expect KChIPs and related neuronal calcium sensing proteins (such as frequenin (16)) to assemble with other K+ channel α-subunits with 4:4 stoichiometry to yield similar effects on stability, structure, and function.

A 4:4 subunit stoichiometry is also seen with the soluble intracellular regulator Kvβ2 in assemblies with the N-terminal segments of Kv1 α-subunits (33). Similarly, KCNMBI-encoded β-subunits (two span transmembrane proteins) and voltage- and calcium-gated BK α-subunits assemble with 4:4 valence (34, 35). The same subunit ratio is found with SUR β-subunits (bearing 17 transmembrane segments) and Kir6.2 α-subunits (36–38). In contrast, just 2 KCNE1-encoded Mink β-subunits (peptides with a single transmembrane span) assemble with 4 KCNQ1 α-subunits to form cardiac I_k1 channels (39, 40); a 2:4 ratio is therefore expected for other channels with Mink-related peptides (MIRP1–4) (20). How many DPPX monomers (another single span β-subunit) assemble with Kv4.2 α-subunits (41) is yet to be determined.

In a companion study, Kv4.2*-KChIP2 complexes purified by the strategy described here were found amenable to visualization by negative stain electron microscopy; demonstration here that isolated complexes maintain structural integrity and have 4:4 subunit valence allowed three-dimensional images of the channels to be reconstructed with a resolution of 21 Å. KChIP2 incorporation was found to create an ~36 × 115 Å, intracellular fenestrated rotunda: 4 peripheral columns that extend down from the membrane-embedded portion of the channel to enclose the central Kv4.2* "hanging gondola" (a platform held beneath the transmembrane conduction pore by 4 internal columns) (21, 31, 42, 43). To reach the pore from the cytosol, ions pass through one of four external fenestrae to enter the rotundal vestibule and then cross one of four internal windows in the gondola. The location of KChIP2 subunits in the structure is lateral to the gondola platform and does not overlap with the sub-platform locale for the Kvβ2 subunits apparent in crystal structures (33). It is as yet unknown whether a single channel complex can carry more than one type of accessory subunit and if subunit stoichiometry is thereby varied. These issues are under study by the methods described here.

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