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Ito channels are octomeric complexes with four subunits of each Kv4.2 and K+ channel-interacting protein 2.

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Mammalian voltage-gated K\(^+\) channels are assemblies of pore-forming \(\alpha\)-subunits and modulating \(\beta\)-subunits. To operate correctly, Kv\(\alpha\)-subunits in the heart and central nervous system require recently identified \(\beta\)-subunits of the neuronal calcium sensing protein family called K\(\alpha\) channel-interacting proteins (KChIPs). Here, Kv4.2-KChIP2 channels are purified, integrity of isolated complexes confirmed, molar ratio of the \(\beta\)-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\(\alpha\)-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 \(I_{\text{CaL}}\) 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, neurocalcin, and hipocalcin) (15); the roles of visinin-like proteins are now expected for other channels employing neuronal calcium sensing \(\beta\)-subunits.

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Materials and Methods

**Molecular Biology—Human Kv4.2 and KChIP2 were expressed in prat, a dual purpose vector with a cytomegalovirus promoter for mammalian expression and a T7 promoter for cRNA synthesis. Charybdotoxin (CTX)-sensitive Kv4.2 (Kv4.2*) was produced by plaque-forming unit-based mutation (Stratagene, La Jolla, CA); the pore segment containing mutations was isolated with NruI and BamHI encoding a linker and the epitope (RVPDGDPDETSQVAPAX). cRNA was synthesized after linearization with NotI using an mMessage mMachine kit (Ambion, Austin, TX). cRNAs were quantified by spectroscopy and gel electrophoresis. The gene variants used in this study were human Kv4.2 (accession number AF009258), KChIP2 (accession number AF199588), and Kv1.3 (accession number NM_002232).

Electrophysiology and Data Analysis—Oocytes from Xenopus laevis frogs were defolliculated by collagenase and injected with 0.2 ng of Kv4.2 or Kv4.2* and 0.4 ng of KChIP2 cRNA. For CTX block studies less RNA was injected for mixed complexes so macroscopic current was like that for \(\alpha\)-subunits alone. Currents were studied at 12–24 h. Whole-cell currents were measured by two electrode voltage clamp (Geneclamp 500, Axon Instruments, Union City, CA). Electrodes were filled with 3 M KCl and had resistance of 0.1–1.2 \(\Omega\)t. Data were sampled at 1 kHz. Data recording was performed using Clampex v8.0 and assessed with Clampfit v8.0, Excel, and Origin 6.0. All experiments were performed at room temperature. Standard bath solution was ND-96: 96 mM NaCl, 2 mM KCl, 1 mM MgCl\(_2\), 1.8 mM CaCl\(_2\), 5 mM HEPES/NaOH, pH 7.5) and supplemented with 0.1% bovine serum albumin for CTX studies. CTX binding kinetics were examined with voltage pulses to 40 mV for 500 ms from a resting membrane voltage of ~80 mV repeated 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 \( V_c \) were calculated by fitting the conductance-voltage relationship to a Boltzmann function. Similarly, inactivation \( V_{1/2} \) and \( V_c \) were calculated by fitting the normalized current-voltage relationship to a Boltzmann function.

Expression and Purification with COS7 Cells—Kv4.2* was co-expressed with KChIP2 in COS7 cells using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA) and 2.5 and 5 \( \mu \)g 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 dithiothreitol, 0.2 mM leupeptin/pepstatin, 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 centrifugation at 100,000 \( \times \) 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/pepstatin, 1 mM EDTA, 20 mM HEPES-KOH, pH 7.4 with Complete protease inhibitors (Roche Applied Science). Protein was then eluted (E) in wash buffer with 100 mM NaCl and 0.2 mg/ml 1D4 peptide (Yale University Reck Facility). 1D4 monoclonal antibody was purchased (NCC, 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—\( ^3 \)H-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 \( ^3 \)H-CTX (36 nm) that took place in the presence of 1 \( \mu \)M non-radioactive CTX. Test groups measured total \( ^3 \)H-CTX binding and specific binding was determined by subtracting the nonspecific from the total binding. All steps were performed at 4 °C. Cells were exposed to \( ^3 \)H-CTX in binding buffer (20 mM KCl, 10 mM NaPi, 100 mM sucrose, and 1 mg/ml bovine 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 \( \mu \)l binding buffer, centrifuged, and after removal of supernatant, suspended in 100 \( \mu \)l 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 \( \mu \)l elution of purified material was exposed to 36 nm \( ^3 \)H-CTX with or without 1 \( \mu \)M unlabelled CTX. As above, the two samples were used to assess nonspecific and total binding of \( ^3 \)H-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 \( \mu \)l wash buffer with only 100 mM NaCl by centrifugation. The filter was then placed in a liquid scintillation vial and studied in a scintillation counter as above. The dose–titration assay was performed in a similar manner with 25 \( \mu \)l aliquots of purified channel complex. 0, 0.45, 1.33, 4, 12, or 36 nm \( ^3 \)H-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 \( \mu \)g, E2–4) was layered on a 12 ml 10–40% (w/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 \( \mu \)g 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 for 15 h at 35,000 rpm and 4 °C 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 Coomas-

**Table I**

<table>
<thead>
<tr>
<th>Kv4.2</th>
<th>Kv4.2*+KChIP2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Activation ( V_{1/2} ) (mV)</td>
<td>-24.3 ± 0.6</td>
</tr>
<tr>
<td>Activation ( V_c ) (mV)</td>
<td>13.7 ± 0.3</td>
</tr>
<tr>
<td>( I_{peak} ) at 40 mV (( \mu )A)</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Inactivation ( V_{1/2} ) (mV)</td>
<td>-62.2 ± 0.8</td>
</tr>
<tr>
<td>Inactivation ( V_c ) (mV)</td>
<td>6.1 ± 0.2</td>
</tr>
<tr>
<td>Inactivation ( \tau_{inact} ) (ms)</td>
<td>204 ± 20</td>
</tr>
<tr>
<td>Inactivation ( \tau_{off} ) (ms)</td>
<td>30.5 ± 4.2</td>
</tr>
<tr>
<td>( A_{on/off} )</td>
<td>0.48</td>
</tr>
<tr>
<td>( R_{on/off} )</td>
<td>0.52</td>
</tr>
<tr>
<td>Recovery ( \tau_{rec} ) (ms)</td>
<td>192 ± 8</td>
</tr>
<tr>
<td>n (no. of oocytes)</td>
<td>5</td>
</tr>
</tbody>
</table>

**Figure 1.** A CTX-sensitive variant of Kv4.2, a. alignment of the pore loop region of human Kv4.2 (residues 343–385 shown) and the toxin-sensitive mutant (Kv4.2*), changes reproduce residues in Kv1.3. b, wild-type Kv4.2 (open square) and Kv4.2*KChIP2 channels (filled squares) are insensitive to CTX (arrows), 15 and 45 nm applied, respectively. Kv4.2* (open circle) and Kv4.2*KChIP2 channels (filled circles) are blocked by 1.67 mM CTX (arrows). Channels expressed in oocytes studied by two-electrode voltage clamp with depolarization from -100 to 40 mV for 500 ms every 20 s (see Materials and Methods). c, CTX block of channels formed by Kv4.2* (open circles) and Kv4.2*KChIP2 (filled circles). Data represents unblocked fractional current as a function of CTX concentration and is fit to: \( f = \frac{1}{(1 + ([CTX]/K_i)^h}) \). Fits yield \( K_i \) = 0.35 ± 0.04 and 0.76 ± 0.03, with coefficients h = 0.89 and 1.02, respectively. Each point is mean ± S.E. for 3–10 oocytes.
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 conduction 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 ($K_i$) 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 $K_i = 0.76 ± 0.03$ nM (Fig. 1, b and c). As CTX is external and KChIP2 subunits are intracellular, the increase in $K_i$ observed on KChIP2 incorporation was unexpected. Relaxation to equilibrium blockade upon acute toxin application revealed KChIP2 to have little effect on the toxin on-rate ($K_{on} = 18.1 ± 1.4 \mu M^{-1} s^{-1}$ versus $K_{on} = 24.5 ± 2.3 \mu M^{-1} s^{-1}$ with KChIP2, n = 8–10) but to destabilize bound toxin, increasing its off-rate −3-fold ($K_{off} = 8.0 ± 0.6 \times 10^{-3}$ s⁻¹ versus $K_{off} = 22.9 ± 2.6 \times 10^{-3}$ 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 (ta slow) and fast (ta 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 α-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 ([3H]-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 = 4) 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, because a single high affinity toxin site is apparent. Because KChIP2 has 252 residues and a predicted mass of 28 kDa this result argues that complexes contain between 1 and 6 KChIP2 subunits (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 channels over time, binding of [3H]-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 [3H]-CTX with high affinity. Binding studies with four separate preparations Kv4.2*-KChIP2 were well fit to a single-site function reveals Keq = 4.8 nM (Fig. 4b). 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 (~316–456 kDa). The predicted mass for an octameric complex with 4 Kv4.2* subunits and 4 KChIP2 subunits is 400 kDa.

To accurately assess the subunit composition of purified Kv4.2*-KChIP2 complexes, the two subunits were separated by SDS-PAGE, identified by staining with Coomassie Brilliant
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.2 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 I₅Na (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 a 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, KCNM1-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 predicted 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 RCNQ1 α-subunits to form cardiac IK₅Ca channels (39, 40); a 2:4 ratio is therefore expected for other channels with MinK-related peptides (MRIP1–4) (20). How many DPXPP monomers (another single span β-subunit) assemble with Kv4.2* (41) is yet to be determined.

In a companion study,2 Kv4.2*-KChIP2 complexes purified

### REFERENCES


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Kv4.2-KChIP2 Channels Have 4 Subunits of Each Type