# Recreation of Neuronal Kv1 Channel Oligomers by Expression in Mammalian Cells Using Semliki Forest Virus†

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ABSTRACT: The multiple roles of voltage-sensitive $K^+$ channels (Kv1 subfamily) in brain are served by subtypes containing pore-forming α (1.1−1.6) and auxiliary β subunits, usually in an $(\alpha)_4(\beta)_4$ stoichiometry. To facilitate structure/activity analysis, combinations that are prevalent in neurones and susceptible to α-dendrotoxin (αDTX) were reproduced in mammalian cells, using Semliki Forest virus. Infected Chinese hamster ovary cells expressed N-glycosylated Kv1.1 and 1.2 α subunits ($M_r \sim 60$ and 62 K) that assembled and bound [$^{125}$I]-αDTX with high affinity; an appreciable proportion appeared on the cell surface, with Kv1.2 showing a 5-fold enrichment in a plasma membrane fraction. To obtain 'native-like' α/β complexes, β1.1 or 2.1 ($M_r \sim 42$ and 39 K, respectively) was co-expressed with Kv1.1 or 1.2. This slightly enhanced N-glycosylation and toxin binding, most notable with β2.1 and Kv1.2. Solubilization of membranes from cells infected with Kv.1.2 and β2.1, followed by $Ni^{2+}$ chromatography, gave a purified α1.2/β2.1 complex with a size of ∼405 K and $S_{20,W} = 15.8$ S. Importantly, these values indicate that four α and β subunits co-assembled as in neurones, a conclusion supported by the size (∼260 K) of the homo-tetramer formed by Kv1.2 alone. Thus, an authentic $K^+$ channel octomer has been reconstructed; oligomeric species were also found in plasma membranes. To create 'authentic-like' hetero-oligomeric channels, Kv1.1 and 1.2 were co-expressed and shown to have assembled by the precipitation of both with IgGs specific for either. Consistently, confocal microscopy of cells labeled with these antibodies showed that the relatively low surface content of Kv1.1 was increased by Kv1.2. [$^{125}$I]-αDTX binding to these complexes was antagonized by $DTX_k$, a probe selective for Kv1.1, in a manner that mimicks the pattern observed for the Kv1.1/1.2-containing channels in neuronal membranes.

Voltage-activated (Kv1) $K^+$ channels are a diverse group of membrane proteins (*1, 2*) that perform a variety of functions ranging from control of neuronal excitability, shaping of the action potentials, determining the inter-spike interval, and indirectly regulating transmitter release (*3*). α-Dendrotoxin (αDTX)[1] and its homologues, which selectively block certain members of the Kv1 subfamily, have proved excellent tools in identifying $K^+$ currents in mammalian neurons (*4*) and were instrumental in the purification of these channels from brain (review in ref *5*). αDTX-susceptible channels were shown to be large sialoglycoproteins (∼400 K) that contain two major subunits, α ($M_r \sim 78$ K) and β ($M_r \sim 39$ K). Several genes encoding the α subunits have been cloned and classified as Kv1.1−1.6, based on their sequence homology (*6*). When expressed, each Kv1 member generates voltage-gated $K^+$ currents with distinct biophysical and pharmacological properties; for example, $DTX_k$ preferentially blocks Kv1.1 channels, whereas αDTX has a less stringent specificity with affinity for Kv1.2 > 1.1 > 1.6 (*7*−*9*). The α subunits coassemble with members of the same subfamily only, via a highly conserved N-terminal domain (NAB or TI), giving rise to homo- or hetero-oligomeric channels in vitro (*10*−*12*) and in vivo (*13, 14*). A number of subunit combinations have been identified in brain synaptic membranes (*15*−*19*) including Kv1.2 homo-oligomers and 1.1/1.2 hetero-oligomers along with other less prevalent species. The functional properties of $K^+$ channels are further modified (*20*) by the auxiliary β subunits found tightly associated with the Kv1α constituents (*21*−*24*). The initial cloning of the gene encoding the extrinsic Kvβ2 protein (*22*) led to the identification of two others (Kvβ1 and 3) (*20, 25*). Whereas the C-terminal region, common to all the β subunits (86% amino acid identity), is important for association with α subunits (*12, 26*), the extended and variable N-termini present in β1 (1.1−1.3) and β3 (β3.1) are responsible for their varied acceleration of inactivation of Kv1 α subunit currents (*20, 27, 28*). The β2.1 subunit

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[1] Abbreviations: SFV, Semliki Forest virus; DTX, dendrotoxin; CHO, Chinese hamster ovary cells; BHK, baby hamster kidney cells; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; PVDF, poly(vinylidene difluoride); PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; IgG, immunoglobulin class G; $M_r$, relative molecular weight; $IC_{50}$, concentration resulting in 50% inhibition; EDTA, ethylenediaminetetraacetic acid; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; TBS, Tris-buffered saline.

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acts as a chaperone and promotes cell surface expression of mammalian Kv1.2 (*29*) but not Shaker channels (*30*). Also, it speeds up the inactivation of the Kv1.4 current despite being devoid of the N-terminal ball, characteristic of $\beta$1 and 3 (*31, 32*).

The relatively low content of the $K^+$ channels in synaptic membranes (0.5 pmol/mg of protein) limits the amount that can be purified; moreover, the preparation isolated from brain is a heterogeneous population of channel subtypes comprised of different Kv1 $\alpha$ and $\beta$ subunits, as noted above. This restricts the scope for pharmacological, biochemical, and structural characterization of these native proteins. Although several expression systems have proved successful in generating sufficient amounts of $K^+$ channels for functional analysis (*33, 34*), these yielded inadequate quantities for investigations of their physical properties and involved expression of a single $\alpha$ subunit in the absence of their $\beta$ protein partner (*35*). Herein, the Semliki Forest virus (SFV) expression system was selected to overcome these limitations because it offers the advantage of being applicable to a broad range of mammalian cells where receptors have been expressed in high yield, glycosylated, and assembled in a functional state (*36*). Different $K^+$ channel complexes were expressed in Chinese hamster ovary (CHO) cells in adequate yield for examination of $\alpha$ subunit assembly, surface targeting, and the role of $\beta$ subunits in these processes by immunoblotting, binding of radiolabeled $\alpha$DTX, and immunofluorescence confocal microscopy. Production of naturally occurring combinations of recombinant Kv1 $\alpha$ and $\beta$ subunits, followed by their affinity chromatography, allowed determination of oligomeric size and stoichiometry of the $\alpha$ and $\beta$ subunits in the complexes.

## EXPERIMENTAL PROCEDURES

*Materials.* Chemicals, goat serum, and fluorescently labelled antispecies IgGs were purchased from Sigma Chemical Co.; glycosidases and cell proliferation kit II (XTT) were from Boehringer-Mannheim. Tissue culture plasticware was obtained from Greiner. Cell culture media, PBS/EDTA buffer, and the SFV gene expression system were supplied by Gibco BRL. Restriction enzymes were provided by Promega. ECL reagents and $Ni^{2+}$-resin were from Amersham and Novagen, respectively. pCITE-2b(+) vector was purchased from Novagen. Antibodies exclusively reactive with Kv$\beta$1.1 or 2.1 were produced in guinea pigs using synthetic peptides corresponding to residues 2−13 and 9−28, respectively, and characterized as reported (*18*). An anti-His monoclonal antibody was obtained from Qiagen. Fluoprep was obtained from BioMerieux, France.

*Construction of the pSFV $\alpha$ and $\beta$ Subunit Expression Plasmids.* pAKS Kv1.1, 1.2, $\beta$1.1, and $\beta$2.1 (kind gifts from Prof. O. Pongs) were used for the construction of pSFV plasmids. Coding sequence of Kv1.1 and 1.2 were PCR amplified using primer pairs: 5′-TGCGGATCCCATGACGGTGATGTCAG-3′ and 5′-CTGGTGCTTCTCGAGAACATCGG TCAGGAG-3′; 5′-CAACCATGGCAGTGGCTACCGGAGACCC-3′ and 5′-CACTCGAGA TCAGTTAACATTTTGATAA-3′, respectively. These included restriction sites for *Bam*HI and *Xho*I in the case of Kv1.1 and *Nco*I and *Xho*I for Kv1.2, with the exclusion of stop codons from both sequences. A 6 His-tag was introduced at the C-terminus of each by subcloning these PCR fragments into the pCITE-2b(+) vector; the resultant plasmids [pCITE-2b(+) Kv1.1 and 1.2] were sequenced. The coding sequences were then excised using *Bam*HI plus *Xba*I for Kv1.1 and *Nco*I plus *Xba*I in the case of Kv1.2. After blunt ending, these fragments were subcloned into the *Sma*I restriction site of pSFV1 vector (detailed below).

Kv$\beta$2.1 coding sequence was cut from the pAKS $\beta$2.1 plasmid using *Bam*HI and *Eco*RI restriction enzymes; the sites were blunt-ended before ligating into the *Sma*I restriction site of pSFV1 vector. Due to a lack of suitable restriction sites, Kv$\beta$1.1 was PCR amplified, using pfu polymerase and primers 5′-CCGGGGATCCAATGCAATGCAAGTCTCCATAGCC-3′ and 5′-GCAAAAAGGACTATAGATCCTAAGGGTCGAC-3′ to generate blunt-ended products that were ligated after DNA sequencing into the *Sma*I site of the pSFV1 vector (see below).

*Expression of $K^+$ Channel $\alpha$ and $\beta$ Subunits Using the SFV Expression System.* This entails a two-stage process; first, non-infectious viral particles containing RNA encoding the requisite subunit are made in baby hamster kidney (BHK) cells and used, after activation, to infect CHO cells in order to generate large amounts of the protein. Production of $K^+$ channel complexes was carried out essentially as described for other proteins: $P_{2X}$ and 5-$HT_3$ receptors (*36*). pSFV1 expression plasmids (containing SP6 promoter, nonstructural genes of SFV), with $K^+$ channel subunit ($\alpha$ or $\beta$) genes incorporated, were linearized by *Spe*I restrictase and capped cRNA generated by in vitro transcription using SP6 polymerase. Capped cRNA was, likewise, transcribed from the pSFV1-Helper 2 plasmid, which contained the remainder of the SFV genome (the structural genes but lacking the sequence required for packaging RNA after replication). cRNA transcripts encoding a particular Kv $\alpha$ or $\beta$ subunit were mixed in a 1:1 ratio with that produced using pSFV-Helper 2 and used to transfect BHK cells (grown to 80% confluence and harvested by trypsinization) by electroporation. The cells were plated and incubated at 37 °C for 18 h before collection of the stocks of the viral particles (only containing RNA replicated from pSFV1 $\alpha$ or $\beta$ templates because helper RNA is not packaged). Viral preparations were activated by treatment with $\alpha$-chymotrypsin (0.5 mg/mL) for 15 min at room temperature, followed by addition of its inhibitor, aprotinin (0.5 mg/mL). The titer of each virus stock was assayed using the cell proliferation kit II according to manufacturer's instructions. Activated viral particles containing the requisite $\alpha$ or $\beta$ subunit cRNAs were used individually or mixed in various combinations to infect CHO cells at 37 °C for 6−24 h before harvesting in PBS/EDTA buffer.

*Western Blotting and Immunoprecipitation of the Recombinant Proteins.* A suspension of CHO cells expressing $K^+$ channel subunits was dissolved in SDS sample buffer and subjected to SDS−PAGE in 8% gels. In some experiments, before electrophoresis the expressed $\alpha$ subunits were deglycosylated by incubation with PNGase F and neuraminidase, as recommended by the manufacturer. The proteins were electrotransferred onto a PVDF membrane, which was blocked with 5% (w/v) dried milk solution in TBS (50 mM Tris-HCl, pH 7.5, 0.15 NaCl) and probed with antibodies (3−10 $\mu$g/mL IgG) specific for Kv1 or Kv$\beta$ subunits, as detailed elsewhere (*17, 18*). Bound antibodies were detected using HRP-labeled antispecies IgGs and the ECL system. For immunopreparation experiments, cells (3 mg of protein/

mL) expressing Kv1.1, 1.2, and $\beta$2.1 were extracted under nondenaturing conditions with 1% (w/v) Thesit in buffer containing protease inhibitors, as detailed below. Aliquots of the extract were incubated overnight at 4 °C with IgG (10 $\mu$g/mL) specific for Kv1.1 or 1.2, followed by sedimentation of immune complexes after reaction with 200 $\mu$L of Sepharose coupled to protein-A or -G (for the mouse anti-Kv1.2 monoclonal); these conditions gave complete precipitation of the $\alpha$ subunits. In some experiments, the supernatants from precipitation with IgG specific for Kv1.1 or 1.2 were subjected to the same precipitation procedure but using the other antibody of the pair. In all cases, the pellets were solubilized with SDS sample buffer and Western blotting performed as above, using the antibodies specified in the figure legend.

*Purification of Plasma Membranes from CHO Cells.* Plasma membranes from CHO cells were prepared according to the procedure detailed in ref *37*. Harvested cells were washed by brief centrifugation in HES buffer (20 mM HEPES, pH 7.4, 255 mM sucrose, 1 mM EDTA) containing 1 mM PMSF before resuspension in the same buffer and homogenization by 20 passages in a glass-Teflon homogenizer at ~800 rpm. The homogenate was centrifuged at 19000*g* for 20 min, and the pellet was resuspended in HES buffer and centrifuged as above. The resultant pellet was resuspended in HES buffer, layered onto a sucrose cushion (1.3 M sucrose, 20 mM HEPES, pH 7.4, 1 mM EDTA), and centrifuged at 190000*g* for 90 min in a Beckman SW40 Ti rotor. The interface containing plasma membranes was collected and used for toxin binding (see below); also, these were solubilized in Thesit, and the extract was subjected to gel filtration followed by Western blotting of the fractions as outlined below.

*Radioiodination of* $\alpha$*DTX and Its Binding to* $K^+$ *Channnels.* $\alpha$DTX and $DTX_k$ were isolated from the venoms of *Dendroaspis angusticeps* and *Dendroaspis polylepis*, respectively; $\alpha$DTX was radiolabeled with the chloramine-T method to a specific activity of 500−700 Ci/mmol (*38*). Assay of the binding of $^{125}$I-labeled $\alpha$DTX was performed in duplicate or triplicate in 50 mM imidazole−HCl buffer, pH 7.4, containing 90 mM NaCl, 5 mM KCl, and 1 mM $SrCl_2$ as detailed elsewhere (*19*) using 1.5 nM [$^{125}$I]-$\alpha$DTX in the presence of increasing concentrations of $\alpha$DTX or $DTX_k$ and 100−200 $\mu$g protein of intact cells, purified plasma membranes or cells solubilized in extraction buffer (as described below). After being incubated at room temperature for 1 h, the channel toxin complexes were separated from free labeled toxin by centrifugation of the membrane samples through a mixture of silicone fluid and dinonyl phthalate or by gel filtration on 2-mL columns of Sephadex G-100 in the case of solubilized extract (detailed in ref *19*). The columns were washed under gravity with 25 mM imidazole−HCl, pH 7.4, 50 mM NaCl, 10 mM KCl, and 0.5 mM $SrCl_2$; reaction mixtures (0.2 mL) were loaded onto the columns and the [$^{125}$I]-$\alpha$DTX-channel complex was collected in the void volume (1.2 mL). In each case, the amount of bound [$^{125}$I]-$\alpha$DTX was quantified by $\gamma$ counting, and the saturable binding was calculated by subtraction from the total values of those obtained in the presence of 1 $\mu$M unlabeled toxin.

*Immunofluorescence Microscopy of CHO Cells Expressing Various Kv1 Subunits*. CHO cells were plated at 50% confluence in 35/10 mm sterile Petri dishes for 16 h before viral infection (see Experimental Procedures). After 16 h of infection with recombinant SFV encoding Kv1.1, 1.2 or both, the cells were quickly washed and fixed with 4% paraformaldehyde in PBS for 10 min on ice and then washed in PBS. Nonspecific binding sites were blocked for 20 min with 3% (v/v) normal goat serum in PBS before permeabilization of the cells with 0.05% (v/v) Triton X-100 and incubation for 2 h at room temperature with anti-Kv1.1 (10 $\mu$g IgG/mL) and monoclonal against Kv1.2 (100-fold dilution of the ascites) or His-tag (1:100 dilution). After being washed (5 times), samples were incubated for 1.5 h in the dark with secondary fluorescein- or rhodamine-conjugated antispecies IgG (1:100 dilution). Cells were then washed (5 times), mounted with a drop of Fluoprep in the middle of Petri dishes immediately before being covered by a 13-mm coverslip, and analyzed using confocal laser microscopy. Samples were imaged with a laser scanning microscope (Zeiss 510) mounted on an upright microscope (Axioplan-2 Zeiss) and operated with the manufacturer's software (LSM 510 version 1.49.44) running on Windows NT 4.0 operating system (Microsoft, U.S.A.). The 488- and 543-nm lines of an argon ion and helium−neon ion laser, respectively, were used for excitation with intensity minimized to 4% of 25 mW and 30% of 30 mW power, respectively. Images were collected using an oil-immersion objective (plan-Neofluar, 40×/1.3) and separated with a combination of an FITC-type narrow band-pass filter block (505−530 nm) and a long-pass rhodamine-type block (≥560 nm). Images were processed batch-wise in Photoshop v5.0, and the same adjustments ($\gamma$ levels for presentation purposes) were applied to all images. For estimation of the relative levels of expression of Kv1.1 or Kv1.2 alone and the two together, low magnification (×10) fluorescence images of large populations of the different cells were analyzed using the Image Pro Plus (V4; Datacell) and the same optical parameters. The mean density of fluorescence per cell for the different combinations ($n$ = 502 for each type) was calculated.

*Purification and Characterization of the* $K^+$ *Channel Complexes.* CHO cells expressing Kv1.1 or 1.2, with and without $\beta$1.1 or 2.1, were homogenized with a glass-Teflon homogenizer; a post-nuclear, high-density membrane fraction was prepared by differential centrifugation and solubilized in 1% (w/v) CHAPS or octyl glucoside or 4% (w/v) Thesit in 62.5 mM imidazole−HCl, pH 7.4, 0.5 M KCl, 5 mM EDTA, 0.5 mM PMSF, 25 $\mu$g/mL soybean trypsin inhibitor, 2.5 mM benzamidine, and 20 $\mu$g/mL bacitracin. Purification of the solubilized channel complexes was achieved by affinity chromatography on $Ni^{2+}$-charged resin, in accordance with the manufacturer's instructions. Fractions containing purified channel were pooled, concentrated ~15-fold by ultrafiltration using a Centricon 30 concentrator, and dialyzed against TBS supplemented with 50 mM KCl and 0.1% Thesit. The purity of the resultant preparations was checked by SDS−PAGE on 8% gels, with silver staining (*15*) and/or immunoblotting, as above.

*Hydrodynamic Studies of the Channel Complexes*. Gel chromatography of the purified $K^+$ channels was performed on a Superose 6HR column (10 mm × 30 cm; Pharmacia) equilibrated with TBS supplemented with 50 mM KCl and 0.1% Thesit using a BioLogic system (Bio-Rad). Centrifugation of the channel complexes and standard proteins of

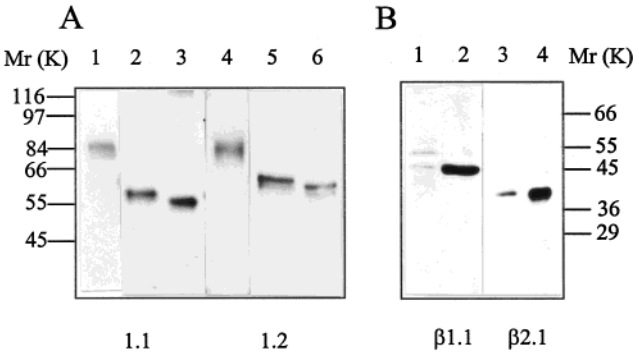


FIGURE 1: Immunoidentification of recombinant Kv1 and Kv$\beta$ subunits. SFV-infected CHO cells (30 $\mu$g of protein) expressing Kv1.1 (A, lanes 2 and 3), Kv1.2 (A, lanes 5 and 6), $\beta$1.1 (B, lane 2), or $\beta$2.1 (B, lane 4) and rat synaptic membranes (A, lanes 1 and 4; B, lanes 1 and 3; 50 $\mu$g protein) were subjected to SDS−PAGE. Prior to electrophoresis, the Kv1.1 and Kv1.2 samples were incubated for 24 h at 32 °C in the absence (A, lanes 2 and 5) and the presence (A, lanes 3 and 6) of neuraminidase and PNGase F, as detailed by the manufacturer. After electrotransfer onto PVDF membrane, the proteins were labeled with the subunit-specific antibodies specified and visualized using HRP-conjugated antispecies IgGs in conjunction with ECL reagents. The positions of standard proteins are shown.

known sedimentation coefficients was carried out in sucrose gradients prepared in $H_2O$ and $^2H_2O$, essentially as described elsewhere (*39*). Linear 5−20% (w/v) gradients (12.4 mL) of sucrose were prepared in SW40 Ti Beckman ultracentrifuge tubes that contained a 0.5-mL cushion of 50% (w/v) sucrose solution. Aliquots (150 $\mu$L) of concentrated channel preparation or a mixture of the standard proteins were loaded onto the top of the gradients. Centrifugation was performed for 17 h at 220000*g*. In all cases, distribution of the channels was monitored in the fractions by immunoblotting using the ECL detection system; the developed films were scanned and digitized using Photoshop V5.0, ScionImage and Sigma Plot 4.1 software. Assay of the standard proteins and analysis of the results were performed as described previously (*39*).

## RESULTS

*SFV Provides an Effective Means of Generating $K^+$ Channels in Mammalian Cells.* BHK cells were used to generate a high titer of viral particles whereas CHO cells proved more suitable for producing recombinant $K^+$ channel subunits; for this purpose, CHO cells also have the advantage of possessing very little outward $K^+$ current (*40*). Western blotting demonstrated the expression of $K^+$ channels in the infected cells, which reached a maximum level between 12 and 24 h. Antibodies against Kv1.1 or 1.2 each detected a broad band ($M_r \sim$ 60 and 62 K) or doublet depending on the amount of protein loaded (Figure 1A). As the respective molecular weights of Kv1.1 and 1.2 core proteins are ~56.3 and 56.7 K (*7*), the apparent sizes observed indicate that some post-translational modification had occurred but to lesser extents than for their native counterparts, which gave $M_r \sim$ 80 K (Figure 1A), as reported previously (*15, 41*). The levels of glycosylation were examined by incubating the expressed subunits with neuraminidase and PNGase F. Immunoblotting revealed a decrease in size of 2−3 K for both subunits (Figure 1A) to values that approach the molecular masses deduced from their nucleotide sequences.

Because Kv$\beta$ subunits are co-assembled with α subunits in vivo (see Introduction) and in vitro (*29, 42*), expression and characterization of these auxiliary proteins were undertaken. CHO cells infected with recombinant SFV encoding Kv$\beta$1.1 or 2.1 were analyzed by Western blotting, employing isoform-specific antibodies raised against the variable N-termini of the $\beta$ subunits (*18*). Recombinant Kv$\beta$1.1 and 2.1 gave $M_r \sim$ 42 and 39 K, respectively (Figure 1B), similar to the size of their neuronal counterparts except that for brain synaptic membranes a second larger band was observed when stained with $\beta$1.1 specific IgG. Similar analysis of control cells infected with only an α subunit (Kv1.1 or 1.2) failed to detect $\beta$1.1 or 2.1 proteins (Figure 2A,B). Although mRNAs for $\beta$ subunits have been found by others in the CHO cells (*43*), these must either be absent from the cell line used herein or not translated.

*Co-expression of Kv1α and β Subunits.* Recombinant SFV encoding one α or $\beta$ subunit were mixed and used to infect CHO cells; in pilot experiments, conditions were optimized for co-expressing multiple subunits and, once established, used throughout this study. Immunoblots of CHO cells expressing Kv1.1 and $\beta$1.1 or 2.1 (Figure 2A) showed

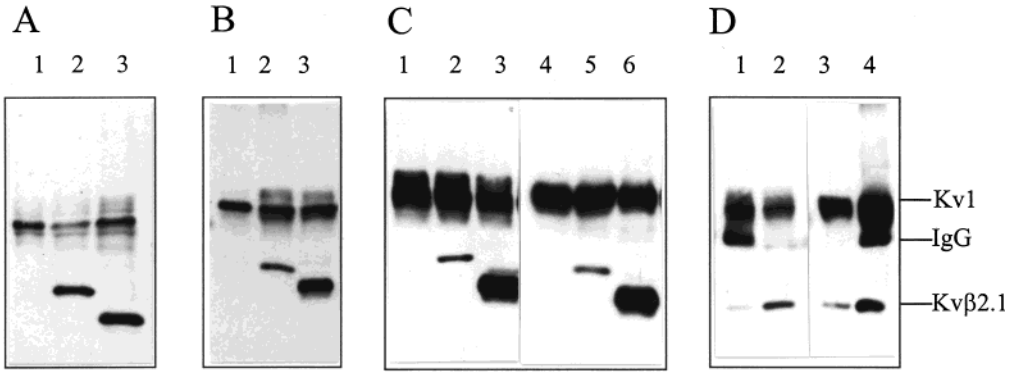


FIGURE 2: Co-expression of Kv1 and Kv$\beta$ subunits in CHO cells. Kv1.1 (A), Kv1.2 (B), or both (C) were expressed in the absence (lane 1) and the presence of $\beta$1.1 (lane 2) or $\beta$2.1 (lane 3). A sample (10 $\mu$g of protein) of each was subjected to SDS−PAGE and blotted, as in Figure 1, with mixtures of anti-Kv$\beta$1.1 and 2.1 antibodies plus IgG specific for Kv1.1 (A and C, lanes 1−3) or Kv1.2 (B and C, lanes 4−6). (D) A solubilized Thesit extract of cells expressing Kv1.1, 1.2, and $\beta$2.1 was incubated for 16h at 4 °C with 10 $\mu$g/mL IgG specific for Kv1.1 (lanes 1 and 3) or Kv1.2 (lanes 2 and 4), followed by sedimentation of the resultant channel−antibody complexes by reaction with protein A- (lanes 1 and 3) or protein G-Sepharose (lanes 2 and 4). After extracting the pellets in sample buffer, SDS−PAGE and Western blotting were performed, as before, using anti-Kv1.1- (lanes 1 and 2) or 1.2-IgG (lanes 3 and 4) plus antibodies specific for $\beta$2.1 in both cases. The positions of Kv1 and $\beta$2.1 subunits plus the cross-reactive IgG chain are indicated.

Table 1: [$^{125}$I]-αDTX Binding to CHO Cells Expressing $K^+$ Channel α Subunits Alone or in the Presence of Either $\beta$1.1 or 2.1[a]

| CHO cells infected with $K^+$ channel genes | binding of [$^{125}$I]-αDTX to CHO cells $B_{max}$ (pmol/mg of total protein) | |
|---|---|---|
| | intact cells | solubilized |
| Kv1.1 | 0.30 ± 0.02 | |
| Kv1.1 + $\beta$1.1 | 0.36 ± 0.02 | |
| Kv1.1 + $\beta$2.1 | 0.36 ± 0.06 | |
| Kv1.2 | 1.20 ± 0.05 | 5.59 ± 0.30 |
| Kv1.2 + $\beta$1.1 | 1.30 ± 0.03 | 6.20 ± 0.02 |
| Kv1.2 + $\beta$2.1 | 1.70 ± 0.03 | 6.10 ± 0.01 |
| Kv1.1 + 1.2 | 1.10 ± 0.01 | 7.34 ± 0.04 |
| Kv1.1 + 1.2 + $\beta$1.1 | 1.20 ± 0.03 | 6.40± 0.50 |
| Kv1.1 + 1.2 + $\beta$2.1 | 1.50 ± 0.02 | 9.10 ± 1.50 |

[a] CHO cells or detergent extract (100−200 μg of protein) were incubated at 22 °C for 1 h with 1.5 nM [$^{125}$I]-αDTX in the absence or presence of 1 μM unlabeled αDTX to calculate nonsaturable binding. In each case, the latter values were subtracted from the totals to give the content of saturable sites (±SEM) shown for triplicate assays that were carried out as outlined in Experimental Procedures.

minimal changes in the expression level of the α subunit, although $\beta$2.1 gave a slight increase and created small amounts of a few larger isoforms of Kv1.1. The latter change is possibly due to further glycosylation as this auxiliary protein has been implicated in such a role (*29*). Notably, co-expression of Kv1.2 with $\beta$1.1 or 2.1 raised the yields of this α subunit more substantially and caused the appearance of a higher molecular weight isoform (Figure 2B). These effects of the $\beta$2.1 subunit could have arisen from co-assembly with Kv1.1 or 1.2, as demonstrated below by purification of the α−$\beta$ complexes formed, via the His-tag on each α subunit. On the other hand, there appeared to be only limited association of $\beta$1.1 with either α protein (data not shown).

*Cellular Distribution of Recombinant Kv1 Channels: Effect of β Subunits on Their Targeting to the Cell Surface.* αDTX selectively binds to the extracellular pore forming region of Kv1.1 or 1.2 (*7, 44*). As toxin only binds to the oligomerized $K^+$ channel (*45*), the content of αDTX sites is a direct measure of the amount of properly assembled channels produced. Moreover, the binding of [$^{125}$I]-αDTX to intact cells reflects the content of channels on the plasmalemma while the value measured in a solubilized extract gives the total quantity of active channels. In this way, the influence of $\beta$ subunits on the synthesis and surface targeting of the channels could be monitored. CHO cells expressing the respective α subunits were carefully detached from the flasks, and binding assays were performed immediately using [$^{125}$I]-αDTX. The $B_{max}$ value calculated for intact CHO cells infected with Kv1.1 showed that an appreciable amount of Kv1.1 was inserted into the surface membrane (0.3 pmol/mg of the total cellular protein); the presence of $\beta$1.1 or 2.1 augmented this level slightly (Table 1). Similar experiments using Kv1.2 showed a significantly higher content of toxin sites on the cell surface (1.2 pmol/mg of total protein) than seen with Kv1.1. Notably, a purified plasma membrane fraction gave a 5-fold enrichment of toxin-binding sites per milligram of protein. Co-expression of Kv1.2 with either $\beta$ subunit substantially increased the level of toxin binding to intact cells (Table 1); $\beta$2.1 proved more effective in this respect, consistent with the demonstrated association of these two subunits (see later). After solubilization of the cells, there was a large increase in the content of [$^{125}$I]-αDTX sites, indicating that a large fraction of the toxin-binding channels did not reach the plasma membrane (Table 1) but had folded in an active form. No major influence of the $\beta$ subunits was apparent on the content of toxin binding sites in the solubilized extracts (Table 1), presumably because they only enhance cell surface expression of α subunits by helping their glycosylation and correct folding. Distribution of the channels expressed in CHO cells was also monitored by antibody labeling and confocal microscopy (Figure 3A). Weak labeling was seen for Kv1.1 precluding demonstration of its precise location (Table 1), the bulk of the labeling of this channel in the fluorescent images resided within the cell. Kv1.2-producing cells showed intense staining with its monoclonal antibody; specificity of this was established by the absence of fluorescence in cells expressing Kv1.1 (Figure 3A). Much of the signal for Kv1.2 resided intracellularly, but some was detected on the plasmalemma (Figure 3A). Cotransfection with $\beta$2.1, or $\beta$1.1 to a much lesser extent, caused an elevation in the content of Kv1.2 on the surface (data not shown); this is in accord with the role of $\beta$2.1 as a chaperone (*29*).

*Co-assembly of Kv1.1 or 1.2 with β2.1 Demonstrated by Purification of α/β Oligomers.* To determine the oligomeric and subunit properties of the channels formed upon expressing Kv1.1 or 1.2 with and without $\beta$2.1, the proteins were purified in each case from a post-nuclear high-density membrane preparation from the requisite CHO cells. Initially, the extent of extraction of the membrane-bound channels by several nondenaturing detergents was estimated by comparing the band intensities on immunoblots for equal amounts of protein from the detergent extracts and resultant pellets (data not shown). CHAPS and Thesit proved most efficient, each solubilizing more than 75% of the channels present; as this value is similar to that found for bovine synaptic membranes, there was no evidence for insoluble subunits being produced using the SFV system. The solubilized channels were purified by affinity chromatography on $Ni^{2+}$-charged resin. Distribution of the α subunit in fractions eluted from the column with 0.3 M imidazole was assessed by dot-immunoblotting, and those containing the channels were pooled, concentrated, and subjected to Western blotting (Figure 4). When Kv1.1 or 1.2 was co-expressed with $\beta$2.1, blotting each of the resultant purified samples with the requisite subunit-specific IgGs detected $\beta$2.1 and the respective co-associated α subunit partner (Figure 4). If either Kv1.1 or 1.2 was expressed alone, only the expected α subunit was found, consistent with above-noted failure to detect endogenous $\beta$2.1 in CHO cells by immunoblotting (Figure 2A,B). This series of experiments demonstrated formation of the appropriate α/$\beta$ complexes because the observed co-purification of $\beta$2.1 relied on assembly with the α subunits that contained the His-tag.

The availability of larger quantities of expressed Kv1.2, with and without $\beta$2.1, allowed these purified preparations to be visualized on the gels by silver staining (Figure 4). The isolated Kv1.2/$\beta$2.1 complexes contained two prominent bands with $M_r$ ~62 and 39 K, corresponding to those stained by their respective binding of antibodies specific for Kv1.2 and $\beta$2.1 (Figure 4). The absence of any major additional proteins indicates the purity of the two channel preparations and lack of degradation, although the Kv1.2/$\beta$2.1 sample contained a minor component ($M_r$ ~50 K) that seems to be

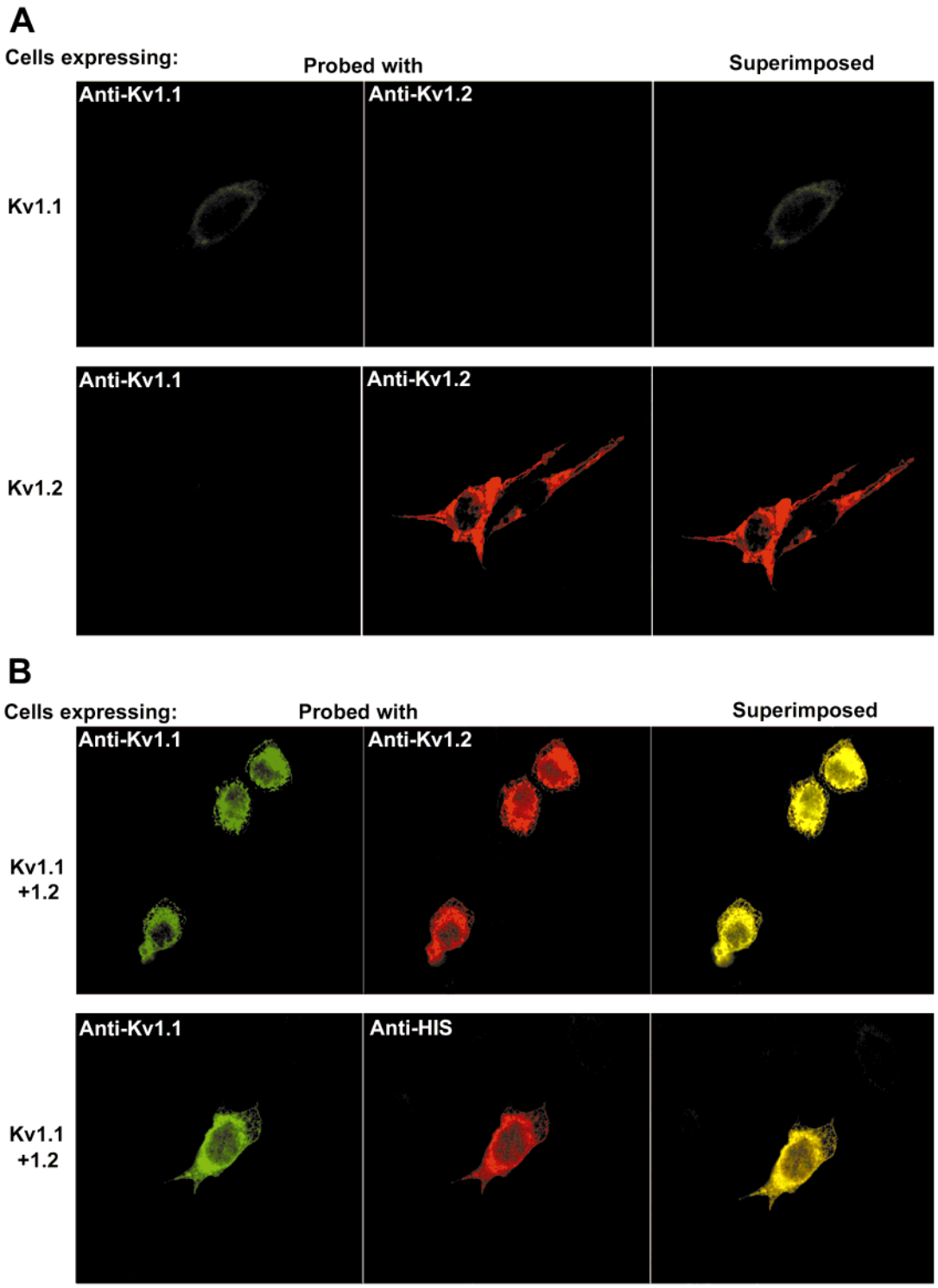


FIGURE 3: Immunofluorescent micrographs of CHO cells expressing $K^+$ channels. Three lots of CHO cells infected for 16 h with recombinant SFV encoding Kv1.1 or Kv1.2 alone (A) or both together (B) as specified were fixed, permeabilized with Triton X-100, labeled with the antibodies noted in the panels, and examined by confocal microscopy. The individual and superimposed fluorescent images are shown. Note the plasma membrane labeling on cells expressing Kv1.2 or expressing Kv1.1 and 1.2 revealed by anti-Kv1.2 (A), anti-Kv1.1, Kv1.2, and His (B); this corresponded to the outline of the cells' surfaces, delineated by phase contrast images (not shown). Details are given in Experimental Procedures.

a contaminant because of not being stained by either of the antibodies as well as its absence from the preparation when Kv1.2 was expressed alone. The yield of purified complexes reached 80−150 $\mu$g from 15 mg of membrane protein.

*Evidence for Formation of an* $(\alpha)_4(\beta)_4$ *Oligomer That Mimicks Authentic Octomeric* $K^+$ *Channels from Measurement of Its Physical Properties.* Molecular masses and stoichiometry of $\alpha$ and $\alpha/\beta$ subunits in the purified Kv1.2 and Kv1.2/$\beta$2.1 channels, respectively, were determined by gel filtration in conjunction with sedimentation analysis, using the bouyant density method in which the samples are centrifuged on sucrose gradients prepared in $H_2O$ and $^2H_2O$. Fractions obtained from each procedure were analyzed by immunoblotting with anti-Kv1.2 antibody; the ECL devel-

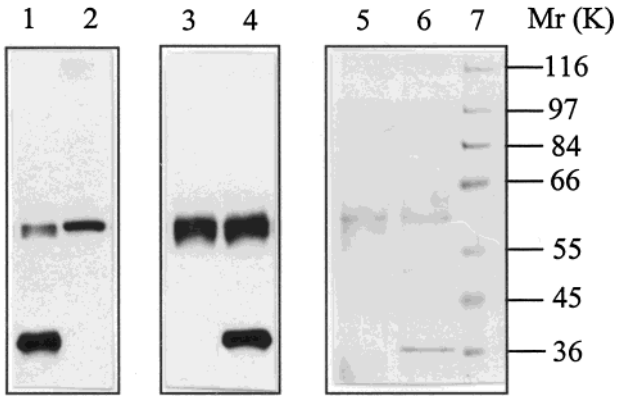


FIGURE 4: Western blotting of recombinant $K^+$ channels purified from the membranes of cells expressing α and α/*β* subunits. Kv1.1 and 1.2 expressed alone (lanes 2, 3, and 5) or in the presence of *β*2.1 subunit (lanes 1, 4, and 6) were solubilized in Thesit and purified on $Ni^{2+}$-charged affinity columns via His-tag on the α subunits only, as detailed in Experimental Procedures. Aliquots of the purified preparations were subjected to SDS−PAGE, followed by blotting and developing with antibodies specific for Kv1.1 and *β*2.1 (lanes 1 and 2) or Kv1.2 and *β*2.1 (lanes 3 and 4), or silver stained (*5*−*7*). Positions of the molecular mass markers are shown in lane 7.

oped films were scanned and quantified using digitization software. Gel filtration of the purified Kv1.2 oligomer and Kv1.2/*β*2.1 complexes or standard proteins of known Stokes' radii was performed on a Superose 6HR column. Each of the $K^+$ channel preparations yielded one major peak; their elution positions (Figure 5A) were used to determine the Stokes' radius: 6.4 and 7.4 nm for Kv1.2 and 1.2/*β*2.1 oligomers, respectively (Table 2). Centrifugation of these complexes on sucrose gradients in $^2H_2O$ (Figure 5B) and $H_2O$ gave one distinct peak for each protein in both cases, with the Kv1.2/*β*2.1 samples sedimenting further down the gradients than the Kv1.2 homo-oligomer (Figure 5C). As expected, the profiles observed for the two $K^+$ channels preparations and marker proteins were shifted to earlier fractions in the $^2H_2O$ gradient as compared to those in the other medium (Figure 5C). The linear relationship obtained, when the respective positions of the markers on the two gradients were plotted against their known $S_{20,w}$ values (Figure 5C), allowed determination of the sedimentation coefficients for Kv1.2 and Kv1.2/*β*2.1 as 10.9 and 15.8 S, respectively (Table 2). Using the procedure detailed by (*46*), the slopes of the latter plots for the standards in $^2H_2O$ and $H_2O$ were used to derive the partial specific volumes of each of the two channel preparations (Table 2). In turn, the oligomeric size of Kv1.2 channel was calculated to be ∼260 K as compared to a value of ∼405 K for the Kv1.2/*β*2.1 complex, after allowance had been made for the contribution of bound detergent (Table 2). On the basis of the known size of the constituent subunits, these molecular weights can be accounted for by the association of four α subunits in a Kv1.2 homo-tetramer plus four *β*2.1 subunits in the case of the larger hetero-oligomer. Thus, an octomeric channel has been reconstructed recombinantly with an $(\alpha)_4(\beta)_4$ stoichiometry, matching that for $K^+$ channels isolated from brain (*21*). In addition to the above-noted analysis of the recombinant channels in a partially purified membrane preparation, a plasma membrane fraction from CHO cells expressing Kv1.2 and *β*2.1 was separated by sucrose gradient centrifugation (see Experimental Procedures). After detergent extrac-

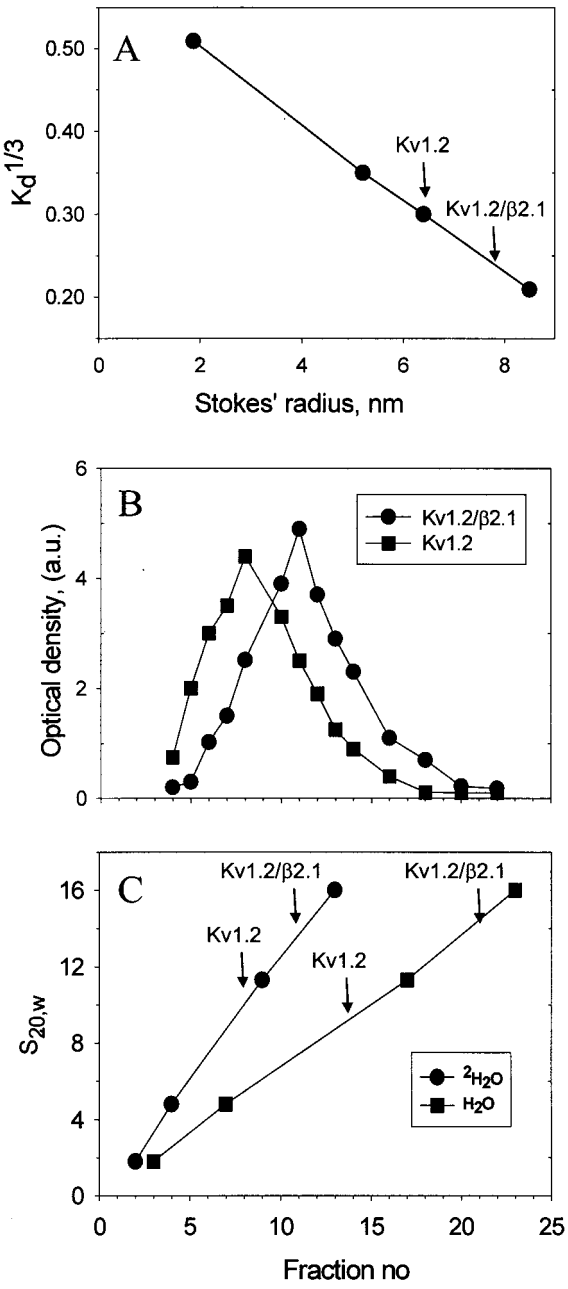


FIGURE 5: Analysis of the physical parameters of purified Kv1.2 and Kv1.2/*β*2.1 channel complexes from the membrane fraction of infected CHO cells. (A) Gel filtration on a Sepharose 6HR column. The channels were detected in eluted fractions by SDS−PAGE and immunoblotting using anti-Kv1.2 antibody; the resultant ECL films were densitometrically scanned to obtain the intensities of reactive bands. The column was calibrated with standard proteins of known Stokes' radii (nm: cytochrome C, 1.9; catalase, 5.2; apoferritin, 6.4; and thyroglobulin, 8.6), which were detected after elution by monitoring $A_{280\,nm}$. $V_i$ and $V_o$ of the column were determined using tryptophan and blue dextran. Using these values and the eluted positions of the standard proteins, their partition coefficients were calculated and plotted against Stokes' radii. Arrows indicate the values obtained for the two $K^+$ channel complexes. (B) Sedimentation profiles of the two channel preparations after centrifugation through linear (5−20%) sucrose gradients in $^2H_2O$. The content of Kv1.2 in fractions from gradients was quantified as above. (C) Calibration of the gradients by centrifugation of standard proteins with known $S_{20,w}$ values: cytochrome C, 1.8; liver alcohol dehydrogenase, 4.8; catalase, 11.3; and *β*-galactosidase, 16S. The standard proteins were monitored in fractions from the sucrose in $^2H_2O$ and $H_2O$ gradients as described in ref *39*. Peak positions of the channel complexes are indicated by arrows.

Table 2: Physical Properties of Recombinant Purified $K^+$ Channels[a]

| | Kv1.2 | Kv1.2/$\beta$2.1 |
|---|---|---|
| apparent $S_{20,w}$ ($H_2O$) | 9.6 | 14.4 |
| apparent $S_{20,w}$ ($^2H_2O$) | 9.8 | 13.8 |
| $S_{20,w}$ (S) | 10.9 | 15.8 |
| partial specific vol (mL/g) | 0.74 | 0.75 |
| Stokes' radius (nm) | 6.4 | 7.4 |
| mol mass of protein/detergent complex (K) | 307 | 503 |
| mol mass of protein complex (K) | 260 | 405 |

[a] Indicated values were determined by gel filtration and sucrose gradient centrifugation, as described in Experimental Procedures. The $K^+$ channels were monitored by immunoblotting followed by scanning and digitization of the developed films. Partial specific values of Thesit were taken to be 0.91 mL/g and that of the acceptors as 0.70 mL/g; see ref *21*.

tion of these purified membranes, gel filtration on a Superose 12 HR column yielded a single peak of $K^+$ channel, after blotting of the fractions with IgG specific for each subunit, of the appropriate size for $(\alpha)_4(\beta)_4$. This indicates the existence of an octomer in the plasmalemma, as found above.

*Generation of Authentic $K^+$ Channels Combinations.* To create Kv1.1/1.2 hetero-multimeric channels that are prevalent in synaptic membranes (*17*−*19*), Kv1.1 and 1.2 were co-expressed in CHO cells. SDS extracts of the cells contained substantial quantities of both subunits (Figure 2C); notably, a much larger yield of Kv1.1 was obtained per unit of protein than when expressed alone (cf. Figure 1A). Accordingly, the $B_{max}$ value observed for [$^{125}$I]-αDTX binding to intact cells expressing Kv1.1 and 1.2 was much higher than that obtained for Kv1.1 alone (Table 1). Confocal microscopy of these batches of cells, after labeling with IgGs specific for each subunit, confirmed the surface co-expression and -localization of both Kv1.1 and 1.2 (Figure 3B) with an elevated level of staining for Kv1.1 being apparent as compared to the subunit alone (Figure 3). Calculation of the mean fluorescence densities for these cells ($n = 502$ for each set) showed that Kv1.2 increased the overall expression of Kv1.1 by 4-fold; a slight increase (∼20%) in the level of Kv1.2 was also noted. Moreover, the staining pattern for the two α subunits was very similar (Figure 3B). The additional expression of $\beta$1.1 or 2.1 with the two α subunits resulted in the production of the requisite three subunits for each combination as demonstrated by blotting (Figure 2C). Further evidence for surface co-expression of both α subunits was provided by labeling Kv1.1 with its specific IgG while visualizing both Kv1.1 and 1.2 anti-His antibody. The apparent co-localization observed, particularly on visible stretches of plasma membrane (Figure 3B), is consistent with hetero-oligomeric formation although not demonstrated directly. Thus, to establish conclusively that hetero-oligomers were generated, a detergent extract of cells infected with Kv1.1, 1.2 and $\beta$2.1 was incubated with IgG specific for Kv1.1 or 1.2, and the resultant precipitates were blotted with either of these antibodies and IgG specific for $\beta$2.1. In each case, the sedimented pellets showed reactivity with the three sets of subunit-specific IgGs (Figure 2D), demonstrating that hetero-oligomers were formed that contained Kv1.1, 1.2 and $\beta$2.1 co-assembled into a naturally occurring combination. In fact, sequential quantitative precipitation with the IgGs specific for each α subunits, as used reliably before (*17*), revealed that the vast majority of Kv1.1 and 1.2 had co-assembled because little (<10%) of each subunit remained free (data not shown). Also, the presence of each $\beta$ subunit boosted the level of channels on the plasma membrane, as reflected by the increase in [$^{125}$I]-αDTX binding to the intact cells (Table 1). The pattern of change was similar to that observed for Kv1.2 alone, with $\beta$2.1 causing a larger elevation in the content of cell surface channels capable of binding toxin. These collective results established that Kv1.2 co-assembled with Kv1.1 and $\beta$2.1, as occurs in neurons. Despite the upregulation of Kv1.1 by Kv1.2 and the chaperoning effect of the $\beta$ subunit observed, the bulk of the channels expressed using these various combinations remained intracellular, but the large increase in the content of [$^{125}$I]-αDTX binding sites after solubilization with detergent (Table 1) indicates that at least a proportion of the proteins were correctly folded and assembled.

*Binding of [$^{125}$I]-αDTX to the Recombinant $K^+$ Channels and Patterns of Inhibition by $DTX_k$ Resemble the Behavior Seen with Neuronal Membranes.* The biological activities of the recombinant channels were assessed by comparing their binding of [$^{125}$I]-αDTX with that for $K^+$ channels in brain synaptic membranes, which are largely composed of Kv1.2 homomer and Kv1.1/1.2 heteromer plus $\beta$2.1 (see Introduction). The curves obtained for antagonism by αDTX of the binding of 1.5 nM [$^{125}$I]-αDTX to Kv1.1 or 1.2 in the cells were close to that observed for synaptic membranes (Figure 6A,B); the similarity of the $IC_{50}$ values calculated (1.1, 2.2 and 1.9 nM, respectively) show that both of the recombinant channels bound αDTX with high affinity like their native counterparts, indicative of the expressed channels having been correctly folded. Also, it is notable that co-expression with $\beta$1.1 or 2.1 had insignificant influence on the affinity of the Kv1.2 channel for binding αDTX (Figure 6B), consistent with data published for $\beta$2.1 (*29*). In the case of $K^+$ channels obtained by expressing Kv1.1 and 1.2, the binding of 1.5 nM [$^{125}$I]-αDTX was antagonized by unlabeled αDTX (Figure 6A) and gave an $IC_{50}$ of 4 nM, approximating that seen with Kv1.1 or 1.2 alone (Figure 6B). To gain some insight into the nature of the oligomers mediating the binding of [$^{125}$I]-αDTX, antagonism by $DTX_k$ was examined because this homologue only displays high affinity for Kv1.1-containing channels (*47*). As expected, $DTX_k$ inhibited [$^{125}$I]-αDTX binding to the Kv1.1 homomer much more effectively than the interaction with Kv1.2 (Figure 6C). Notably for the Kv1.1/1.2 channels, $DTX_k$ antagonized [$^{125}$I]-αDTX binding with a potency intermediate between that observed for Kv1.1 and for 1.2. This is indicative of the formation of hetero-oligomeric complexes, ratifying the coprecipitation of both Kv1.1 and 1.2 from a solubilized extract of these cells, using IgG specific for either subunit (cf. Figure 2D). Moreover, the inhibition pattern largely resembles the competition curve observed with $DTX_k$ for rat synaptic membranes, which contain a mixture of oligomers (*19*). Thus, it proved possible to generate recombinant channels in CHO cells that mimic authentic hetero-oligomeric combinations.

## DISCUSSION

With the long-term goal of elucidating the molecular basis of the functional diversity of Kv1 channels, many groups have characterized the electrophysiological properties of $K^+$ currents produced by α subunits expressed individually, or

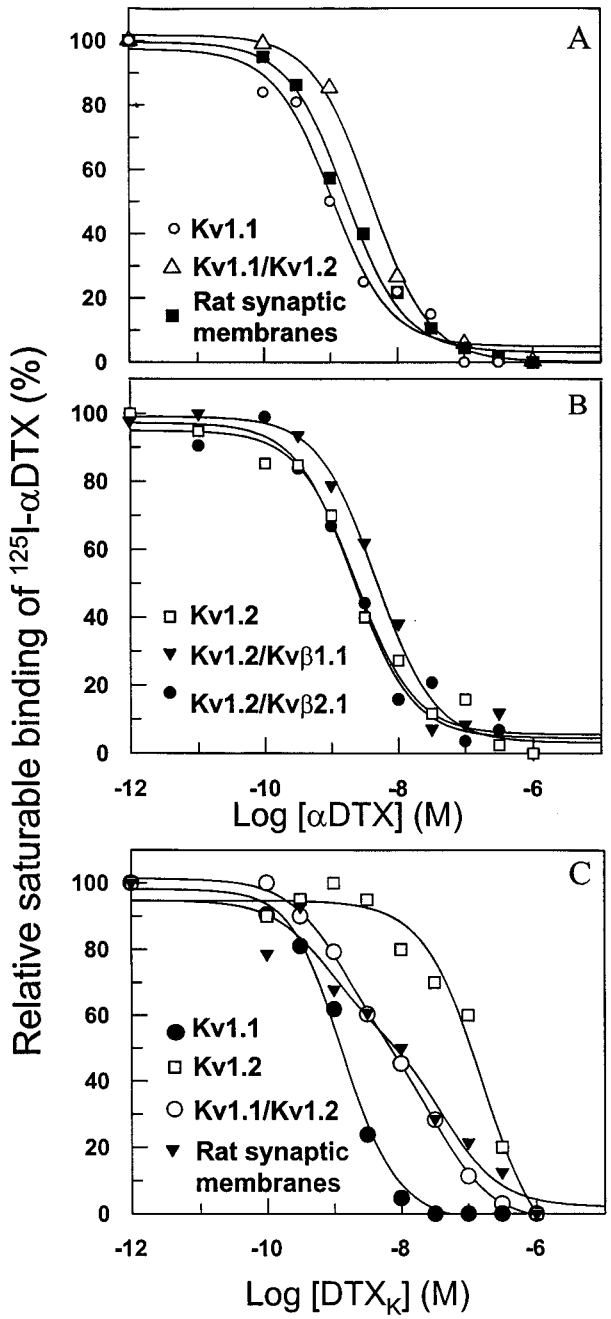


FIGURE 6: Inhibition of [$^{125}$I]-αDTX binding to the recombinant and native $K^+$ channels by αDTX and DTX$_k$. CHO cells (100 $\mu$g of protein) expressing the subunits indicated and rat synaptic membranes [100 $\mu$g of protein; purified as described previously (*17*)] were incubated with 1.5 nM [$^{125}$I]-αDTX at 22 °C for 1 h in the absence and the presence of various concentrations of αDTX (A and B) or DTX$_k$ (C). Binding was assayed by rapid centrifugation of the membrane-bound $K^+$ channel−[$^{125}$I]-αDTX complex through oil with quantitation of the bound toxin in the pellet by $\gamma$-counting. Values for nonsaturable binding measured in the presence of 1 $\mu$M αDTX (A and B) or DTX$_k$ (C) represented 25 and 35%, respectively, of the total binding and have been subtracted from the values plotted. The data presented represent the mean values from duplicate determinations, which showed less than 8% error.

less frequently in pairs, with and without one of the $\beta$ subunits. Although several functional domains have been elegantly pinpointed, there is a paucity of detailed information relating the biochemical properties of each oligomeric species to the biophysical parameters of the requisite $K^+$ currents. This unsatisfactory situation has arisen from the impracticalities of analyzing the precise subunit structure of $K^+$ channels assembled from multiple subunits on the cell surface of oocytes, normally used for such expression. This contrasts with other membrane-bound proteins concerned with synaptic transmission (e.g., nicotinic acetylcholine receptors) where their precise physical dimensions, including the positioning of the four different subunits within the pentamer, and structural changes underlying activation of the ion channel have been measured (*48*). To reach this state of knowledge of $K^+$ channel multimers, many of the subunit combinations occurring in brain have been identified (see Introduction). Now these need to be recreated by recombinant means and purified—worthwhile tasks undertaken herein—to provide material suitable for much needed investigations on structure/activity relations.

Fortunately, only a small number of the possible tetrameric combinations of Kv1 α subunits with $\beta$ partners seem to be present in rat (*19*) or human (*18*) brain. In this study, we concentrated on Kv1.1 and 1.2 because these are the most prevalent in the adult nervous system as constituents of the major species identified (Kv1.2 homo- and 1.1/1.2 hetero-oligomers) (*16, 17, 19*). Moreover, Kv1.1 mutations are associated with seizures, episodic ataxia, and impaired memory (*49, 50*). Additionally, Kv$\beta$2.1 was studied because this is a component of most $K^+$ channel complexes (e.g., Kv1.2 homomer, Kv1.1/1.2 hetero-multimer) found in brain (*51*), whereas the importance of Kv$\beta$1.1 in mediating fast inactivation of most Kv1 subfamily members (*20, 52*) warranted its inclusion in this investigation.

Initially, the pore-forming α subunits were expressed individually in the SFV system. A very high proportion of the cells (>95%) were shown to be infected under the conditions used, according to a standard colorimetric assay (*53*). It seems that the number of viral particles infecting any one cell varied because a large range of flourescent intensities was obtained upon microscopical analysis of antibody labeling of the expressed channels. The $M_r$ values for Kv1.1 and 1.2, before and after treatment with glycosidases, equate to those reported for Kv1.1 and 1.2 expressed in mouse L and COS cells, respectively (*10, 29*). The minimal reduction observed in the sizes of each α subunit upon N-deglycosylation indicates that at least some core glycosylation had occurred in the endoplasmic reticulum but not additional extension in the Golgi; yet these exhibited high-affinity binding of αDTX. After detergent extraction of the membrane and affinity purification of the Kv1.2 channels, analysis by gel filtration and buoyant density sedimentation gave a Stokes' radius of 6.4 nm and an oligomeric size of 260 K. The latter corresponds to the value predicted for four copies of the α subunit ($M_r$ ~62 K), indicating the formation of a tetramer as also established for Kv1.3 (subunit and oligomeric sizes of 64 and 270 K) (*54*) and deduced from electrophysiological measurements on other α subunits (*45, 55*). As $K^+$ channel blocking toxins bind only to the tetrameric proteins (see Results), the observed labeling with [$^{125}$I]-αDTX of intact cells expressing Kv1.1 or 1.2 channels represents the fraction that had assembled and inserted in the plasma membrane. Consistently, the content of saturable [$^{125}$I]-αDTX binding sites/mg of protein was found to be increased very substantially in isolated plasma membranes relative to that for intact cells.

Supportive evidence for targeting of a proportion of the Kv1.1 or 1.2 to the cell surface was provided by confocal microscope images after labeling with subunit-specific IgGs that have been shown by electron microscopy to bind to oligomeric $K^+$ channels in the plasmalemma of central neurons (*56*).

The other constituent of authentic $K^+$ channels, the $\beta$ subunits, expressed well in SFV-infected CHO cells with $\beta$1.1 and 2.1 comigrating on SDS−PAGE with their equivalents from rat synaptic membranes. Their apparent sizes are somewhat smaller than those predicted for the core proteins; this may have arisen from phosphorylation because both contain consensus sites for protein kinases A or C, and the native proteins are readily phosphorylated in vitro (*22*). In view of $\alpha/\beta$ complexes being found in brain, as noted above, it was pertinent to express these two subunit types together. When $\beta$2.1 and Kv1.1 or 1.2 were co-expressed, the observed co-purification of each pair documented their assembly, consistent with the tight interaction known to occur via the NAB domain common to the $\alpha$ subunits (see Introduction). Co-translational association of $\alpha/\beta$ subunits is indicated by the noted effects of $\beta$2.1 on N-glycosylation of Kv1.2, consistent with the findings in ref *29*. Also, in accordance with $\alpha/\beta$ assembly occurring at a much earlier stage than pore formation (*57*), $\beta$2.1 exerted no change in the interaction of $\alpha$DTX with Kv1.2. For both Kv1.1 and 1.2, $\beta$2.1 caused an appreciable increase in surface expression as revealed by [$^{125}$I]-$\alpha$DTX binding to the intact cells and confocal microscopy. Similar experiments with $\beta$1.1 revealed minimal influence on Kv1.1 or 1.2 and consistently gave no significant level of co-assembly. This is possibly due to the inability of $\beta$1.1 to oligomerize and, thus, has reduced capability to assemble with $\alpha$ subunits whereas the efficient oligomerization of $\beta$2.1 promotes its interaction with Shaker channels through multiple sites (*58*).

The abundance of Kv1.2/$\beta$2.1 oligomer in synaptic membranes of bovine and human brain (*17, 18*) warranted an investigation of its physical properties. As a substantial quantity of this complex could be expressed in CHO cells and solubilized in detergent, complete purification of Kv1.2/$\beta$2.1 complex was achieved for the first time. As expected, its determined Stokes' radius of 7.4 nm is smaller than that (8.6 nm) reported for the mixture of $\alpha$DTX-sensitive $K^+$ channels isolated from mammalian brain (*21, 39*) because of the observed incomplete glycosylation of the recombinant Kv1.2 subunit relative to its native form. The oligomeric size of 405 K—calculated for this recombinant $\alpha/\beta$ complex by sedimentation analysis after allowing for the contribution of bound detergent—corresponds closely to the molecular weight (408 K) of 4$\alpha$ and 4$\beta$ subunits; thus, the SFV expression system has produced an octomer like the authentic $(\alpha)_4(\beta)_4$ $K^+$ channels. Notably, purified plasma membranes were found to contain a Kv1.2/$\beta$2.1 complex of the same apparent oligomeric size.

Creation of hetero-oligomers resembling combinations present in neurons was achieved by co-expressing Kv1.1 and 1.2, which gave a dramatic increase in the yield of Kv1.1 apparently as a result of co-assembly as demonstrated by immunoprecipitation; the lower expression of Kv1.1 relative to Kv1.2 together with the elevated level when the two were expressed could explain the absence of Kv1.1 homomeric $K^+$ channels from brain and the prevalence of Kv1.1/1.2 complex. As judged by the content of [$^{125}$I]-$\alpha$DTX sites obtained for intact cells or a detergent extract, inclusion of $\beta$1.1 or 2.1 raised the expression of these two $\alpha$ subunits in accord with their demonstrated assembly. Notably, the co-immunoprecipitation observed when IgGs specific for Kv1.1 or 1.2 were used in sequence revealed that virtually all Kv1.1 was incorporated into oligomers with Kv1.2. Likewise, the minimal amount of monomeric Kv1.2 detected indicated that it also prefers to associate with Kv1.1 rather than to homotetramerize; the basis of this interesting behavior requires further in-depth investigations. Antagonsim by $\alpha$DTX of [$^{125}$I]-$\alpha$DTX binding to intact cells revealed that the 1.1/1.2 hetero-oligomer gave a profile resembling that for rat synaptic membranes where this oligomer is a major constituent. Notably, $DTX_k$ exhibited a less extended inhibition curve for Kv1.1/1.2 channels than the established biphasic pattern of antagonism seen with brain membranes (*19*). The latter reaffirms the ability of $DTX_k$ (not possessed by $\alpha$DTX) to distinguish subtypes of native channels that apparently have different contents of Kv1.1 (*19*), the only subunit with high-binding affinity for $DTX_k$. The presence in the recombinant channels of a lower proportion of hetero-oligomers in which Kv1.1 is the most abundant subunit could explain the less pronounced inhibition at lower $DTX_k$ concentrations than seen with synaptic membranes. Clearly, production of oligomers with defined stoichiometries of different subunits—an arduous task being attempted by tandem linkage of their constructs (*55*)—would be ideal for examining such interaction with toxins as well as defining the biophysical properties of these channels.

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