Purification, Visualization, and Biophysical Characterization of Kv1.3 Tetramers*

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The voltage-gated K⁺ channel of T-lymphocytes, Kv1.3, was heterologously expressed in African Green
Monkey kidney cells (CV-1) using a vaccinia virus/T7 hybrid expression system; each infected cell exhibited
10⁴ to 5 × 10⁵ functional channels on the cell surface. The protein, solubilized with detergent (3-[cholamido-
propyl]dimethylammonio)-1-propanesulfonic acid or cholate), was purified to near-homogeneity by a single
nickel-chelate chromatography step. The Kv1.3 protein expressed in vaccinia virus-infected cells and its puri-
fied counterpart are both modified by a −2-kDa core-
sugar moiety, most likely at a conserved N-glycosylation
site in the external S1–S2 loop; absence of the sugar does
not alter the biophysical properties of the channel nor
does it affect expression levels. Purified Kv1.3 has an
estimated size of ∼64 kDa in denaturing SDS-polyacryl-
amide electrophoresis gels, consistent with its predicted
size based on the amino acid sequence. By sucrose gra-
dient sedimentation, purified Kv1.3 is seen primarily as
a single peak with an approximate mass of 270 kDa,
compatible with its being a homotetrameric complex of
the −64-kDa subunits. When reconstituted in the pres-
ence of lipid and visualized by negative-staining elec-
tron microscopy, the purified Kv1.3 protein forms small
crystalline domains consisting of tetramers with dimen-
sions of ∼65 × 65 Å. The center of each tetramer contains
a stained depression which may represent the ion con-
duction pathway. Functional reconstitution of the Kv1.3
protein into lipid bilayers produces voltage-dependent K⁺-selective currents that can be blocked by two high
affinity peptide antagonists of Kv1.3, margatoxine and
stichodactylatoxin.

Voltage-gated K⁺ (Kv) channels regulate membrane potential and thereby control many biological processes in many cell
types from bacteria to humans (reviewed in Refs. 1, 2). Four
families of mammalian genes, Kv1–Kv4, comprising 19 mem-
bers, encode a diversity of Kv channels (2). Each of these
proteins contains 500–600 amino acids, typically long N and C
termini, six putative transmembrane segments (S1–S6), and
an additional membrane-associated loop (P-region) between S5
and S6. Several functional domains have been identified by
site-directed mutagenesis in these Kv proteins (see Ref. 2), and
the outer vestibule of the pore has been topologically mapped
using structurally defined high affinity scorpion toxins as mo-
lecular calipers (3–6).

Previous attempts to overexpress and purify Kv protein for
biochemical and biophysical studies have met with limited
success. The Drosophila Shaker protein has been transiently
expressed at high levels in COS cells, but the poor transfection
efficiency (10% of cells express protein) precludes its use as a
reliable source for protein purification (7). Although expression
with the baculovirus system has been more efficient, yields
from this system have still been insufficient (8–10). In addi-
tion, the quality of the overexpressed protein is seriously com-
promised since a significant portion of this protein is localized
within inclusion bodies, rendering it insoluble in nondenatur-
ing detergent, and very little of the protein is glycosylated (7, 9,
10). Lipid-bilayer reconstitution of these purified channels for
biophysical characterization has not been demonstrated.

The goals of this study are as follows: (a) to characterize a
mammalian heterologous overexpression system for the puri-
fication of appropriately glycosylated, membrane-associated
mammalian Kv1 protein; (b) to determine the biophysical prop-
erties of purified Kv1 protein reconstituted into lipid bilayers;
and (c) to verify the tetrameric nature of the channel using
sucrose density sedimentation and negative-staining electron
microscopy. The Kv protein we have used for this analysis is
Kv1.3, which represents the type "n" channel of T-lymphocytes,
and regulates the membrane potential of these cells (11–16).
Blockers of Kv1.3, which include the high affinity peptide tox-
ins margatoxine (MgTX) and stichodactylatoxin (ShK), suppress
T-cell activation, making this channel an excellent therapeutic
target for novel immunosuppressive agents (12–16).

In this study we have exploited a heterologous vaccinia virus
(VV)-based expression system to overexpress Kv1.3 protein in
mammalian cells, where its posttranslational processing is
likely to resemble its native counterpart in T-cells. Utilizing
this expression system, recombinant Kv1.3 protein was puri-
fied to near-homogeneity from a membrane fraction, and this
protein appeared biochemically uniform, bearing a core glyco-
amide gel electrophoresis; ShK, stichodactylatoxin; RBL, rat basophilic
leukemic cells.
sylation moity and associating as an apparent tetramer. Additionally, this protein produced voltage-dependent, K⁺-selective, Mg²⁺- and ShK-sensitive currents when reconstituted into lipid bilayers and formed small crystalline domains, visible by electron microscopy, composed of 65 × 65-Å tetramers.

**MATERIALS AND METHODS**

**Reagents, Cell Lines, Viruses**

*Restriction Enzymes and Reagents*—The VV transfer vector, pTM1, was a gift from Dr. Bernard Moss (National Institutes of Health, Bethesda). Reagents used in these experiments included the following: restriction enzymes, CHAPS, and imidazole (Boehringer Mannheim), Sequenase (U. S. Biochemical Corp.), chelating-Sepharose and Mono Q columns (Pharmacia, Uppsala, Sweden), sodium cholate, tunicamycin C2 analog, tetraethylammonium, and protease inhibitors (Sigma). [³⁵S]Methionine (1175Ci/mmol) was purchased from DuPont NEN. Margatoxin (MGTX) was purchased from BACHEM (King of Prussia, PA), and staichodactylotoxin (ShK) and kalixotoxin (KTX) from Peptides International (Louisville, KY).

**Antibodies**—A horseradish peroxidase-labeled mouse monoclonal antibody (Ab) specific for a 12-amino acid epitope derived from gene 10 of bacteriophage T₇ (T₇-Tag, also referred to as anti-gene 10 Ab) was purchased from Novagen (Madison, WI), and Texas Red-conjugated donkey anti-mouse IgG was obtained from The Jackson Laboratories (Bar Harbor, ME).

*Cell Lines and Vaccinia Viruses*—African Green monkey kidney cells (CV-1), rat basophilic leukemia (RBL) cells, the WR strain of VV, and CV-1 or RBL cells were doubly infected with 5–10 multiplicity of infection of VV:Kv1.3 and VV:T7 (see 19); in addition, this protein produced voltage-dependent, K⁺ channel activity and associating as an apparent tetramer. Additionally, this protein produced voltage-dependent, K⁺-selective, Mg²⁺- and ShK-sensitive currents when reconstituted into lipid bilayers and formed small crystalline domains, visible by electron microscopy, composed of 65 × 65-Å tetramers.

**Generation of VV-Kv1.3 Construct and Recombination into Vaccinia**—We generated the pTH1 vector by inserting a 144-base pair NcoI/HindIII fragment (the HindIII site was blunt-ended by a fill-in reaction) from the pTREHis vector (a kind gift from Dr. Leonard Wittwer of the Invitrogen Corp., Sorrento Valley, CA) into the pTM1 vector (17, 18) at NcoISau3A sites. The 144-base pair insert contained sequences coding for an initiator methionine followed by a hexahistidine repeat, a serologically detectable epitope from bacteriophage T7 (gene 10), an enterokinase cleavage site, and a multiple cloning site. The coding region was flanked by a T7 promoter and terminator, and the 5′-noncoding region was derived from asephalomyocarditis virus which permissive for efficient initiation of protein production of mouse Kv1.3 gene was inserted into pTH1 as a 2-kilobase BglII/EcoRI fragment excised from the pMK3T construct, (11), and the integrity of the construct was confirmed by dideoxy sequencing. We recombined the Kv1.3-pTH1 plasmid into VV to generate the VV-Kv1.3 recombinant using standard methods (19).

**Assessment of Levels of Kv1.3 Expression in CV-1 Cells**

**Electrically Active Cells**—CV-1 or RBL cells were doubly infected with 5–10 multiplicity of infection of VV-Kv1.3 and VV:T7 (see 19); in these cells, T7 RNA polymerase synthesis controlled by the VV early p7.5 promoter leads to T7-mediated transcription and Kv1.3 protein production. After 2–16 h, these cells were subjected to either patch clamp or immunofluorescence analysis or were harvested for protein production. After 2–16 h, these cells were subjected to either patch clamp or immunofluorescence analysis or were harvested for protein production. After 2–16 h, these cells were subjected to either patch clamp or immunofluorescence analysis or were harvested for protein production. Additional incubation on ice for 30 min, the supernatant was aspirated, and the cell pellet was gently removed from the microcentrifuge tube and infiltrated with 50% polyvinylpyrrolidone in 0.1 M phosphate buffer, pH 7.4, containing 2.3 M sucrose overnight at 4 °C. Thick 0.5-μm crosssections of the pellets were prepared (21) and then incubated with the anti-gene 10 Ab or control mouse IgG (1:1,000) for 1 h. Following incubation with 5 μg/ml Texas Red-conjugated donkey anti-mouse IgG. Exposure-matched fluorescence micrographs were taken on a Zeiss FXA microscope.

**Solubilization of Membrane Protein**—CV-1 cells, coinfected with VV:Kv1.3 and VV:T7, were harvested 24 h postinfection using Versene buffer (phosphate-buffered saline, 2 mM EDTA, 0.0015% phenol red). Following centrifugation, cells were resuspended in 10 volumes of hypotonic lysing buffer (2 mM KCl, 30 mM Tris, pH 7.4, and a mixture of protease inhibitors). Cells were Dounce-homogenized (Kontes pestel A) and centrifuged at 750 × g for 10 min. The low-speed pellet was retained, and the supernatant was subjected to ultracentrifugation at 108,000 × g for 40 min to collect small cellular membranes. The low- and high-speed pellets were combined and solubilized for 1 h with complete cells (300 μg/ml tunicamycin, 0.1% Triton X-100, 0.1% phosphatidylcholine, 50 mM imidazole, 1 mM iodoacetamide, 5 μg/ml aprotinin, 1 μg/ml pepstatin A, 0.2 mM phenylmethylsulfonyl fluoride, and 1 μg/ml leupeptin, and containing either 2% CHAPS or 1.5% cholate). Insoluble material was removed by centrifugation at 106,000 × g for 15 min at 4 °C.

**Electrophysiology**—We generated the pTH1 vector by inserting a 144-base pair NcoI/HindIII fragment (the HindIII site was blunt-ended by a fill-in reaction) from the pTREHis vector (a kind gift from Dr. Leonard Wittwer of the Invitrogen Corp., Sorrento Valley, CA) into the pTM1 vector (17, 18) at NcoISau3A sites. The 144-base pair insert contained sequences coding for an initiator methionine followed by a hexahistidine repeat, a serologically detectable epitope from bacteriophage T7 (gene 10), an enterokinase cleavage site, and a multiple cloning site. The coding region was flanked by a T7 promoter and terminator, and the 5′-noncoding region was derived from asephalomyocarditis virus which permissive for efficient initiation of protein production of mouse Kv1.3 gene was inserted into pTH1 as a 2-kilobase BglII/EcoRI fragment excised from the pMK3T construct, (11), and the integrity of the construct was confirmed by dideoxy sequencing. We recombined the Kv1.3-pTH1 plasmid into VV to generate the VV-Kv1.3 recombinant using standard methods (19).

**Dual Infection of CV-1 Cells**—CV-1 or RBL cells were doubly infected with 5–10 multiplicity of infection of VV-Kv1.3 and VV:T7 (see 19); in these cells, T7 RNA polymerase synthesis controlled by the VV early p7.5 promoter leads to T7-mediated transcription and Kv1.3 protein production. After 2–16 h, the cells were subjected to either patch clamp or immunofluorescence analysis or were harvested for protein purification.

**Assessment of Levels of Kv1.3 Expression in CV-1 Cells**

**Electrophysiology**—Patch-clamp experiments were carried out in either whole-cell or outside-out patch configuration. The external mammalian Ringer solution contained in mM: 140 KCl, 2 CaCl₂, 1 MgCl₂, 5 HEPES, pH 7.4. The internal pipette solution contained in mM: 140 KCl, 2 CaCl₂, 5 HEPES, pH 7.4, with 4% paraformaldehyde, and placed on ice for 10 min. A firm cell pellet was made by spinning the cells at 10,000 × g for 10 min. After an additional incubation on ice for 30 min, the supernatant was aspirated, and the cell pellet was gently removed from the microcentrifuge tube and infiltrated with 50% polyvinylpyrrolidone in 0.1 M phosphate buffer, pH 7.4, containing 2.3 M sucrose overnight at 4 °C. Thick 0.5-μm crosssections of the pellets were prepared (21) and then incubated with the anti-gene 10 Ab or control mouse IgG (1:1,000) for 1 h. Following incubation with 5 μg/ml Texas Red-conjugated donkey anti-mouse IgG. Exposure-matched fluorescence micrographs were taken on a Zeiss FXA microscope.

**Visualisation, Biochemistry, and Physiology of Kv1.3 Tetramers**

**Silver staining of SDS-PAGE gels was performed by the diamine staining method (24). Quantification of protein was determined by A₅₉₀ by the biocinchonic assay (Pierce) and by quantitative chemiluminescence blotting (using anti-gene 10 Ab).**

**Glycosylation of Kv1.3**

For [³⁵S]Methionine labeling, dually infected CV-1 cells were incubated in methionine-deficient minimum Eagle’s medium with 10% dialyzed fetal bovine serum, and 100 μCi of [³⁵S]Methionine (1175 Ci/mmol) in the presence or absence of 1 μg/ml tunicamycin (C2 analog). After 12 h, cells were washed, harvested, and solubilized in RIPA buffer (150 mM NaCl, 1% Nonidet P-40, 0.1% SDS, 50 mM Tris, pH 8.0). To immunoprecipitate the Kv1.3 protein, cell nuclei were first pelleted and the supernatant precleared with 5 mg of protein-A Sepharose beads. One microtiter of the anti-gene 10 Ab was then added and incubated for 1 h at 4 °C, and then 5 μg/ml fluorescein-labeled goat anti-sea horse A-Sepharose beads were added for another hour. The protein A-Sepharose was washed with RIPA buffer three times and resuspended in 30 μl of sample buffer (see above). Samples were analyzed by fluorography following SDS-PAGE separation on a 10% polyacrylamide gel.

In *vitro* glycosylation was performed using N-glycosidase F (Boehringer Mannheim). Samples were diluted in buffer containing 35 mM...
sodium phosphate, pH 7.0, and 5 mM EDTA, followed by the addition of SDS to a final concentration of 0.2%. After 1 h at room temperature, an equal volume of phosphate/EDTA buffer with 5% Nonidet P-40 was added along with 0.4 units of N-glycosidase F, and the mixture was incubated at 57°C overnight. Samples were separated by SDS-PAGE on a 10% polyacrylamide gel, transferred to polyvinylidene difluoride membrane, and immunoblotted as described above.

**Determining the Multimeric Nature of Kv1.3**

Sucrose Density—One-half milligram of each protein standard (apo-ferritin, alcohol dehydrogenase, bovine serum albumin, carbonic anhydrase) and ~10 μg of purified Kv1.3 were layered on top of a 5-50% sucrose gradient (volume of ~12 ml) containing 20 mM Tris, pH 7.4, 50 mM KCl, 0.2 mM phenylmethylsulfonyl fluoride, and either 1.0% CHAPS or 0.6% cholate. Samples were centrifuged for 14 h at 220,000 × g (20°C) and 10-drop fractions were subsequently collected. To determine which fractions contained Kv1.3, 10 μl of each sample was filtered onto nitrocellulose using a slot blot apparatus and immunoblotted as described above. The scanned image of the negative was then analyzed with NIH Image (24) to quantitate the optical density of each spot. To visualize the protein standards, 15 μl of each fraction was loaded with an equal volume of Laemmli buffer, run on a 10% SDS-polyacrylamide gel, and the gel then stained with Coomassie Blue.

The mass of Kv1.3 was estimated as described by Martin and Ames (25). Briefly, the sedimentation rates of proteins on continuous sucrose gradients are related to molecular masses by the equation S1/S2 = (M1/M2)^1/3, where S1 and S2 are the distances traveled by two proteins, and MW1 and MW2 are their molecular masses (25). We solved this equation for Kv1.3 relative to each protein standard and plotted each value against the molecular mass of the standard; the slope of the resulting line represents the estimated mass of Kv1.3.

**Negative Staining EM of “Crystalline” Kv1.3 Protein**—The purified Kv1.3 protein in CHAPS was reconstituted with dimyristoyl phosphatidylcholine at a lipid-to-protein ratio of 1:1 (w/w) and a protein concentration of 1 mg/ml, and the detergent was slowly removed by dialysis (26, 27). For electron microscopy, a carbon-coated electron microscope grid was glow-discharged for 2 min prior to the application of 2 μl of a Kv1.3 crystalline patch. The sample was kept on the grid for about 1 min before blotting with filter paper to remove excess buffer and left to air-dry. Samples were then coated with 1.5% uranyl acetate.

**Biophysical Characterization of Lipid Bilayer Reconstituted and Purified Kv1.3 Protein**

Purified Kv1.3 was reconstituted into small unilamellar vesicles using previously published methods (28). Briefly, PC/PE (2:1) solubilized in 60 mM CHAPS or cholate was added to 100-μl aliquots of purified Kv1.3 to obtain a protein-to-lipid ratio of 1:1000, and then dialyzed overnight in a Slide-a-lyzer (Pierce) dialysis chamber against 3 liters of buffer containing 100 mM KCl and 50 mM Hepes, pH 7.4. Aliquots were stored at ~20°C prior to use.

Bilayers were formed in Teflon chambers divided into two compartments, each having a volume of 2 ml. The compartments were separated by a 20-μm-thick Teflon partition with a ~200-μm hole pretreated with approximately 0.5 μl of squalene (Atomergic Chemicals Corporation, Plainview, NY) (29, 30). With the aqueous solutions below the hole, 10 μl of 5 mg/ml phosphatidylethanolamine (Avanti Polar Lipids, Birmingham, AL), dried under argon, and dissolved in pentane (Aldrich) was spread over the aqueous phase. To form the bilayers, the solutions in the compartments were slowly raised above the level of the hole. Following bilayer formation (monitored by capacitance increase), an aliquot of reconstituted Kv1.3 protein was added to the high KCl (cis) side of the cell, and both chambers were stirred by magnetic fields to promote vesicle interaction with the bilayer. The bilayer was voltage-clamped by an AXOPATCH 200 A controlled by an 80286 computer driving an Interactive Microwave (State College, PA) ADALAB interface board controlled by locally written software.

**RESULTS AND DISCUSSION**

**Biophysical Characterization of Kv1.3 Channels in VV-infected CV-1 Cells**—We performed a detailed comparison of the functional properties of the Kv1.3-pTH1 channels expressed in CV-1 cells with channel “fingerprints” of the cloned Kv1.3 channel in mammalian cells (Table I) and Xenopus oocytes (11) and the native channel in mouse T-cells (31). These experiments were aimed at determining whether the 37-amino acid N-terminal tag altered the biophysical and pharmacological properties of the Kv1.3 channel.

**Voltage Dependence**—Patch-clamp analyses of outside-out patches from dually-infected CV-1 cells at 12 or 24 h postinfection revealed large K+-selective outward currents (Fig. 1). The outward currents appear to represent a single population of K+ channels that activate at depolarizing potentials with a V1/2 of ~31 mV (Fig. 1A and Table I). This value is characteristic of Kv1.3 channels heterologously expressed in Xenopus oocytes (11) or mammalian cells (20), and of the native channels in mouse or human T-cells (30).

**Deactivation and Inactivation**—The time course of inactivation of recombinant Kv1.3 is similar to that of Kv1.3 expressed in mammalian cells (Table I). Like their native counterparts (3, 11, 20), the Kv1.3-pTH1 channels exhibit cumulative inactivation during 200 ms depolarizing pulses to +40 mV from a holding potential of ~80 mV, repeated once every second (Fig. 1, B and C). Channel deactivation provides another convenient property to distinguish between diverse K+ channel types. The kinetics of K+ channel closing can be determined by first opening the channels with a 15-ms conditioning pulse to +40 mV and then forcing the channels to close by repolarizing to different potentials (Fig. 1D). The time constant (τ) of the resultant “tail” currents is similar to that of the native Kv1.3 channel (Table I).

**Pharmacology**—The pharmacological profile provides a further test for defining Kv1.3-pTH1 channels. As shown in Fig. 1, E and F, and in Table I, the Kv1.3-pTH1 channels, like their native Kv1.3 counterparts, are moderately sensitive to tetraethylammonium and highly sensitive to the peptide toxins ChTX and MgTX, and ShK (Table I). Our results indicate that the biophysical and pharmacological properties of the Kv1.3-pTH1 channels are indistinguishable from those of native Kv1.3 channels (11) and that the 37-amino acid N-terminal tag does not perceptibly alter channel function. By determining the peak current amplitudes and membrane capacitances of these patches and by comparing their membrane capacitances with those of whole cells, we estimate that between 1 × 10⁴ and 5 × 10⁵ Kv1.3 channels are expressed at the cell surface of CV-1 cells 24 h postinfection (data not shown).

**Visualization of Kv1.3 Protein in CV-1 Cells by Immunofluorescence Microscopy**—Kv1.3 protein is detectable in CV-1 cells at 4 h postinfection, and the intensity of staining increases significantly over the next 8 h (Fig. 2). Although the protein is widely distributed in the cell, staining is not visible at any time within the nucleus. A similar pattern is seen in RBL cells,
Nearly all cells show high expression of Kv1.3 both at the membrane and intracellularly. Intense fluorescence at the edge of the cell (arrowhead) is consistent with Kv1.3 being expressed at the cell membrane. Although at 12 h the protein appears to be primarily localized at or near the cell surface (data not shown), the biophysical and immunofluorescence experiments clearly demonstrate that a substantial amount of Kv1.3 protein is produced by the heterologous VV-expression system.

**Single-step Purification of Membrane-associated Kv1.3 Protein**—We solubilized Kv1.3 protein from the membrane fraction, but unlike the case for Shaker expressed in Sf9 cells, where a significant proportion of the protein was lost as insoluble aggregates in inclusion bodies (7–10), the majority of our protein was solubilized in nondenaturing detergent. The purified Kv1.3 protein was solubilized by CHAPS or cholate and stained poorly with silver, as has been reported for the Shaker protein (9, 10). Use of a higher concentration of SDS (7.5%) in the SDS-PAGE running buffer, however, enhanced the visualization of the protein in silver-stained gels. Ni2+-chelate chromatography greatly enriched a 64-kDa protein, visible on the silver-stained SDS-polyacrylamide gel (Fig. 3, lane 2), consistent with the expected size for Kv1.3. Immunoblotting with the anti-gene 10 Ab confirmed this protein to be Kv1.3 (Fig. 3, lane 3). Similar results were obtained for cholate-solubilized protein (data not shown).

Table II shows results from one representative experiment. Total membrane protein was quantified by two independent methods which gave consistent results, namely the bicinchoninic assay and absorbance at 280 nm. Quantitative immunodot blots using anti-gene 10 antibody were used to estimate the relative yield of Kv1.3. From 115 mg of total solubilized membrane protein, we recovered 340 μg of gene 10-reactive protein; this represents ~23% of the total immunologically reactive material in the solubilized membrane fraction and an enrichment factor of ~90-fold. These experiments were repeated several times (n > 10) with yields varying from 10 to 60 μg/107 cells for CHAPS- or cholate-solubilized protein.

Kv1.3 Is Glycosylated and the Purified Protein Appears Relatively Homogeneous in This Respect—An N-glycosylation consensus site (NX(ST)) is present in the S1–S2 extracellular loop of most Kv1 family proteins including Kv1.3 (2, 9, 10, 32–34); four additional N-glycosylation motifs are present in the N and C termini of Kv1.3, but since these are predicted to be located intracellularly they are unlikely to be utilized (2). To determine whether Kv1.3 is glycosylated in this expression system, we immunoprecipitated [35S]methionine-labeled Kv1.3 from VV-infected CV-1 cells cultured in the presence or absence of tunicamycin, an inhibitor of N-linked glycosylation; the Kv1.3 analyzed here is derived from both intracellular and cell surface protein. Tunicamycin treatment resulted in a small (~2–3 kDa) but reproducible shift in the migration of Kv1.3 in SDS-PAGE gels (Fig. 4, top); tunicamycin also inhibited incorporation of [14C]glucosamine into Kv1.3 (data not shown). Treatment with N-glycosidase F of whole-cell solubilized protein from dually VV-infected cells, followed by Western blotting with the anti-gene 10 Ab to detect Kv1.3, revealed a similar reduction in size of the Kv1.3 protein (Fig. 4, bottom left). These data indicate that the purified Kv1.3 protein, both from intracellular and cell surface compartments, is relatively homogeneous and bears a single N-linked core sugar moiety which contributes 2–3 kDa to the mass of the protein. The faint smear seen above the major 64-kDa band may represent Kv1.3 modified by other processes.

![Image](image-url)
FIG. 3. Purification of Kv1.3 protein. Proteins were solubilized in CHAPS, separated by nickel-chelate chromatography, then analyzed on a 10% SDS-polyacrylamide gel, and visualized by silver staining. 1st lane, total solubilized membrane protein prior to purification. 2nd lane, protein after nickel chelate chromatography. 3rd lane, Western blotting with the anti-

TABLE II
Recovery of total protein and immunoreactive Kv1.3 from vaccinia-infected CV-1 cell membranes

| Fraction          | Total protein | Kv1.3 by immunoblot |
|-------------------|---------------|---------------------|
|                   | BCA          | A<sub>280</sub> | mg | mg |
| Solubilized       | 100          | 130             | 1.40 |
| membranes         |               |                 |     |
| Ni<sup>2+</sup>   | 0.28         | 0.25            | 0.34 |
| fraction          |               |                 |     |

FIG. 4. Kv1.3 expressed in VV-infected cells, and purified Kv1.3 is appropriately N-glycosylated. Top, VV-infected CV-1 cells were incubated with 100 μCi of [35S]methionine in the presence or absence of 1 μg/ml tunicamycin for 14 h. The cells were then lysed; the Kv1.3 protein was immunoprecipitated by the anti-gene 10 Ab, and the resulting protein was run on an SDS-PAGE gel and autoradiographed. Bottom, Western blot analysis using the anti-gene 10 Ab of total solubilized cellular protein (left) or purified Kv1.3 (right) incubated for 12 h at 37 °C in the presence or absence of N-glycosidase F.

for example phosphorylation (35).

Absence of the core sugar moiety resulting from tunicamycin treatment of CV-1 cells did not perceptibly alter the biophysical properties of Kv1.3 nor did it change expression levels (data not shown). As expected, the purified Kv1.3 protein also demonstrated the 2–3-kDa shift following treatment with N-glycosidase F (Fig. 4, bottom right), indicating that the majority of purified Kv1.3 is glycosylated as is its native counterpart in CV-1 cells.

The closely related Shaker channel is also glycosylated in its S1–S2 loop by at least two moieties that increase the mass of the protein by 3 and 6 kDa, respectively, and is functionally unaffected by deglycosylation (9). In contrast, voltage-gated sodium channels are extensively modified by carbohydrate, which accounts for 30% of their mass, and deglycosylation causes significant shifts in the voltage dependence of activation and enhances the frequency of reversible transitions to subconductance states (36).

Purified Kv1.3 Is a Tetramer with an Approximate Mass of 270 kDa and Dimensions of 65 × 65 Å—Biophysical studies on the Shaker channel have suggested that functional Kv proteins are tetrameric (37). Earlier EM studies on a single-particle preparation of Shaker protein revealed tetramers with dimensions of 80 × 80 Å, although the mass of the complex was not determined (8). We have estimated the mass of our purified Kv1.3 protein by sedimentation and have utilized electron microscopy to visualize the protein in a near-crystalline array.

Purified Kv1.3 was centrifuged on a continuous 5–50% sucrose density gradient. Positions of the molecular mass standards are shown. The peak represents the migration of Kv1.3 protein. Bottom, migration of Kv1.3 protein was compared with the migration of molecular weight standards, and its mass was estimated by the method of Martin and Ames (25).
Consistent with the channel's being highly 
K\textsuperscript{+}-selective, the  
salts in our lipid bilayer system is inverted, a high potassium con-
centration (250 mM) being present at the outer surface of the 
channel. At negative potentials the probability of opening is significantly 
greater than at positive potentials, suggesting that the exter-
nal side of the channel is located on the side of the bilayer to 
which vesicles were added (Fig. 7A). Thus, the K\textsuperscript{+} distribution 
in our lipid bilayer system is inverted, a high potassium con-
centration (250 mM) being present at the outer surface of the 
channel; negative voltages in the bilayer therefore correspond 
to depolarization in cells. The reconstituted channels begin to 
open at voltages negative to +60 mV (see +40 mV trace in Fig. 
7A); in cells this would correspond to activation at voltages positive to −60 mV, similar to the Kv1.3 channel expressed in mammalian cells or *Xenopus* oocytes (11, 20; see Table I). Consistent with the channel's being highly K\textsuperscript{+}-selective, the 
reversal potential measured in asymmetric solutions (250 
K\textsubscript{Cl}/25 K\textsubscript{Cl}) was −58 mV (Fig. 7B), and changed minimally when 
Na\textsuperscript{+} was added to the inside (250 K\textsubscript{Cl}/25 K\textsubscript{Cl} + 225 Na\textsubscript{Cl}, Fig. 7C).

As an independent test of function, we examined the recon-
stituted channel's sensitivity to the peptide toxins MgTX and 
ShK. Native Kv1.3 channels are blocked with high affinity by 
both these toxins (3, 20, 38), but toxin potency is reduced as the 
salt concentration increases, presumably due to electrostatic 
shielding or competition for a common binding site between K\textsuperscript{+} and the 
toxin. In order to maintain the stability of the bilayer, 
all the toxin binding experiments were performed at salt con-
centrations (100 K\textsubscript{Cl}/10 K\textsubscript{Cl}) higher than those used in cell sys-

tems, making it necessary to use a higher external concentra-
tion of MgTX (300 nM) and ShK (100 nM) in our experiments. As 
shown in Fig. 7, D and E, the Kv1.3 currents in bilayers were 
completely blocked by MgTX and ShK. Taken together, our 
data indicate that the purified Kv1.3 protein reconstituted into 
lipid bilayers forms voltage-dependent K\textsuperscript{+}-selective channels 
that are potently blocked by these peptide toxins. These results 
extend earlier reports on the reconstitution of membrane frac-
tions containing Shaker channels (33).

Since the probability of protein-carrying vesicle fusion with 
the bilayer is unknown, bilayer conductance cannot be used as 
a quantitative assay for the degree of purity of Kv1.3 protein. 
However, reconstitution of membrane proteins nearly always 
involves the use of detergents that can destabilize the bilayer, 
a situation which dictates a narrow range of experimental 
conditions where the amount of protein added to the bilayer is 
adequate to observe channels but not enough to break the 
bilayer. If the purified material contained only a small fraction 
of functional Kv1.3 channels along with a large proportion of 
nonfunctional but membrane-associated proteins, adding suf-

cient material to observe currents would likely result in a 
broken bilayer or in a noisy record. Such noisy records or 
broken bilayers are not seen with any greater frequency after 
the addition of reconstituted Kv1.3 protein compared with con-

![A](image1.jpg) ![B](image2.jpg) ![C](image3.jpg) ![D](image4.jpg) ![E](image5.jpg)

**FIG. 6.** Kv1.3 tetramers visualized by electron microscopy. 
Electron micrograph of Kv1.3 protein reconstituted with dimyristoyl 
phosphatidylcholine at a low lipid-to-protein ratio. The image shows 
many small crystalline domains of Kv1.3 protein negatively stained 
with uranyl acetate (above). The well defined square-shaped objects 
seen at higher magnification (below), one of which is indicated by an 
arrow, appear to represent tetramers of Kv1.3. A centrally located 
stain-filled depression can be seen in many tetramers and is presumed 
to represent the location of the ion conduction pathway. Scale bars are 
shown for each image.

**FIG. 7.** Biophysical properties of purified Kv1.3 reconstituted 
into lipid bilayers. A, single channels at different voltages. The applied potentials are shown in the record. B, C, ionic selectivity. 
Ramp protocol was from −100 mV to +100 mV (B and C). The trace in 
B displays a reversal potential of +58 mV. Traces in C display a 
reversal potential in 250 KCl (front) and 25 mM KCl (rear), which was 
altered minimally by the addition of 100 and 225 mM NaCl to the 
rear-side of the bilayer. D and E, block by MgTX and ShK. Ramp 
protocol as above for B and C. The addition of 300 nM MgTX (D) and 100 
nM ShK (E) to the front-side of the bilayer completely blocks the 
channel. A–C, the lipid bilayer separated solutions containing 250 mM 
KCl, 10 mM HEPES, pH 7.4, at the “front” of the bilayer, and 25 mM 
KCl, 10 mM HEPES, pH 7.4, at the “rear.” D and E, the front and rear 
solutions contained 100 and 10 mM KCl, respectively.
quantitative functional assay such as radiolabeled toxin binding was not possible, since the addition of CHAPS or cholate to TVV-infected cell membranes containing Kv1.3, prior to any purification step, markedly reduced $^{125}$I-MgTX binding, similar to the rapid decay in $^{125}$I-ChTX binding reported with solubilized Shaker protein (7). Collectively, our data are consistent with a significant proportion of the purified protein being functional.

Conclusion—Using a modified VV-based system, we have successfully expressed $10^4$ to $5 \times 10^5$ functional Kv1.3 channels on the surface of each infected CV-1 cell, and the membrane-associated Kv1.3 protein is readily solubilized in non-denaturing detergents (either CHAPS or cholate). Reconstitution of purified protein into lipid bilayers produces Kv1.3-like currents; to our knowledge these experiments represent the first biophysical characterization of a purified Kv protein. By combining biochemical and electron microscopic approaches, we have demonstrated that the purified Kv1.3 protein complex has a mass of ~270 kDa and forms small crystalline domains in lipid membranes, consisting of well-defined tetramers with horizontal dimensions of 65 $\times$ 65 Å; a central stain-filled density seen in these tetramers may represent the location of the channel pore. These results extend our earlier work using scorpion peptide toxins to map the pore of Kv1.3 (3, 39), which revealed the existence of a 30-Å wide and 4–6-Å deep external vestibule which narrows to ~9 Å at the external entrance to the ion conduction pathway. Future studies with two-dimensional crystals may provide a higher resolution structure of the Kv1.3 tetramer.

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