Evidence for Dimerization of Dimers in K^+ Channel Assembly

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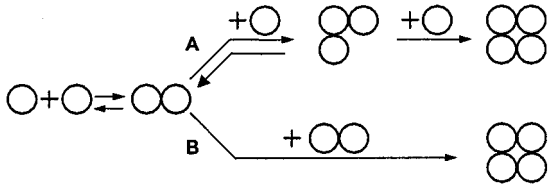
ABSTRACT Voltage-gated K^+ channels are tetrameric, but how the four subunits assemble is not known. We analyzed inactivation kinetics and peak current levels elicited for a variety of wild-type and mutant Kv1.3 subunits, expressed singly, in combination, and as tandem constructs, to show that 1) the dominant pathway involves a dimerization of dimers, and 2) dimer-dimer interaction may involve interaction sites that differ from those involved in monomer-monomer association. Moreover, using nondenaturing gel electrophoresis, we detected dimers and tetramers, but not trimers, in the translation reaction of Kv1.3 monomers.

INTRODUCTION

Potassium (K^+) channels are formed from four identical subunits (MacKinnon, 1991; Schulteis et al., 1996), presumably organized with fourfold symmetry about the central pore (Li et al., 1994; Doyle et al., 1998). These tetramers are formed in the endoplasmic reticulum (Nagaya and Papazian, 1997) and reside in the plasma membrane as irreversibly formed channels (Panyi and Deutsch, 1996). Moreover, there is some evidence to show that monomers are recruited randomly from integrated monomer pools to form functional channels (Panyi and Deutsch, 1996). Although recognition domains (Li et al., 1992; Shen et al., 1993; Xu et al., 1995) have been identified in the cytoplasmic NH_2-termini of voltage-gated K^+ channels, and association sites within transmembrane segments of these channels have been implicated as contributing to intersubunit stabilization domains (Sheng et al., 1997), the mechanism by which tetramers form is still not known. Tetramers may form by stepwise sequential addition of monomers to form dimers, trimers, and, finally, tetramers (path A, Scheme I), and/or monomers may associate to form dimers, which dimerize to form tetramers (path B).



SCHEME I

In considering Scheme I, four key questions emerge: What are the relative contributions of these pathways? Does the trimer exist? Are the interaction sites identical along the reaction pathway (e.g., Does monomer-monomer association occur by the same mechanism as dimer-dimer association?)? What are the relative kinetics along each pathway? The answers have not been determined directly for voltage-gated K^+ channels, although other oligomeric structures provide some insights. Precedent for the dimer-dimer association pathway exists in the formation of acetylcholine receptor channels (Gu et al., 1991; Saedi et al., 1991; Blount et al., 1990; however, see Green and Claudio, 1993), the T-cell receptor (Manolios et al., 1991), and in the assembly of viral membrane proteins (Doms et al., 1993). In the first case, acetylcholine will not bind until a binding site is created by subunit oligomerization. Moreover, conformational changes and folding in intermediate oligomeric states are critical to the formation of new subunit recognition sites during assembly (Green and Claudio, 1993).

The goal of this work was to determine the relative contributions of these pathways to K^+ channel assembly and to determine whether the intermediate multimeric species have different interaction conformations. To do this we have used a variety of approaches. These include expression and suppression assays in *Xenopus* oocytes of Kv1.3 current generated from wild-type (WT) and mutant subunits injected as monomers or tandem dimers and trimers, a kinetic analysis of C-type inactivation for channel populations formed from coexpressed WT and mutant subunits, as well as nondenaturing gel electrophoresis. These studies support two conclusions. First, the dominant pathway in tetramer formation is dimerization of dimers, and the steady-state concentration of trimers is relatively low. Second, dimerization is likely to use interaction sites different from those involved in monomer-monomer association.

MATERIALS AND METHODS

Oocyte expression and electrophysiology

Oocytes were isolated from *Xenopus laevis* females (*Xenopus* I, Michigan) as described previously (Chahine et al., 1992). Stage V-VI oocytes were selected and microinjected with 3–15 ng cRNA encoding for Kv1.3, tandem dimers, and tandem trimers of Kv1.3. In the case of the chimera and AV-(WT-P), we used up to 40 ng cRNA to attempt to detect expression. The mole ratio of cRNA injected for Kv1.3 channel genes to putative suppressor genes (truncated K^+ channel gene, tandem gene, or chimera

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1.3/3.1) was 1:1, 1:2, or 1:4, depending on the purpose of the experiment. Whenever a comparison was made, i.e., in the suppression and comparative expression experiments, we recorded the control and experiment from the same batch of oocytes from the same frog, always within a 2-h recording session. K^+ currents from cRNA-injected oocytes were measured with two-microelectrode voltage clamp using a OC-725C oocyte clamp (Warner Instrument Corp., Hamden, CT) after 15–72 h, at which time currents were 2–10 μA. This level of expressed current was optimal for observing suppression. Electrodes (<1 MΩ) contained 3 M KCl. The currents were filtered at 1 kHz. The bath Ringer's solution contained (in mM) 116 NaCl, 2 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, and 5 HEPES (pH 7.6). The holding potential was -100 mV. Some data are presented as box plots, which represent the central tendency of the measured current. The box and the bars indicate 25–75 and 10–90 percentiles of the data, respectively. The horizontal line inside each box represents the median of the data. Other data sets are represented as mean $\pm$ SEM. To determine steady-state inactivation, we recorded from oocytes held for 2.5 s at voltages from -100 mV to -10 mV (10-mV steps), then at -100 mV for 0.1 ms, and finally at a test voltage of $+50$ mV for 45 ms. Between stimuli the oocytes were held at -100 mV for 50 s.

Recombinant DNA techniques

Standard methods of plasmid DNA preparation, restriction enzyme analysis, agarose gel electrophoresis, and bacterial transformation were used. All isolated fragments were purified with "Geneclean" (Bio 101, La Jolla, CA), recircularized using T4 DNA ligase, and then used to transform DH5α^{TM} or XL1-blue competent cells (BRL, Gaithersburg, MD). The nucleotide sequences at the 5′ ends of all NH_2-terminal deletion mutants, at the 3′ ends of all C-terminal deletion mutants, and at the linkage sites between tandem constructs were confirmed by restriction enzyme analysis or by DNA sequence analysis (Sequenase Version 2.0 DNA Sequencing Kit; USB, Cleveland, OH).

Plasmid constructs

Each tandem linkage lacks the first four amino acids in the amino terminus of the added subunit. Each construct containing a subunit that lacks the complete pore region ((Kv1.3-P), referred to as (WT-P) in the Results) contains the first five putative transmembrane segments and lacks the terminal half of the pore region through the carboxy terminus. The pGEM9zf(−)/Kv1.3-Kv1.3 tandem dimer (referred to as WT-WT in the Results) was made by isolating an *Eco*RI/*Mse*I blunt-end digested fragment (~1.8 kb) from pGEM9zf(−)/Kv1.3 and ligating it into partially *Sma*I-digested/*Eco*RI-digested pGEM9zf(−)/Kv1.3. The pGEM9zf(−)/Kv1.3-Kv1.3-Kv1.3 tandem trimer (referred to as WT-WT-WT in the Results) was made by ligating a partially *Pst*I-digested/*Hin*dIII-digested fragment (~2.2 kb) from pGEM9zf(−)/Kv1.3-Kv1.3 into partially *Pst*I-digested/*Hin*dIII-digested pGEM9zf(−)/Kv1.3-Kv1.3 (~5.8 kb). The pRc/CMV/Kv1.3-Kv1.3(A413V) (referred to as WT-AV in the Results) was made by ligating a partially *Apa*I/*Bst*EII-digested fragment (~2.8 kb) from pGEM9zf(−)/Kv1.3-Kv1.3 into partially *Apa*I-digested/*Bst*EII-digested CMV/Kv1.3(A413V) (Panyi et al., 1995). The pGEM9zf(−)/Kv1.3(A413V)-(Kv1.3-P) (referred to as AV-(WT-P) in the Results) was made by ligating an *Eco*RI/*Pml*I-digested fragment from pALTER-1/Kv1.3(A413V) into an *Eco*RI/*Pml*I-digested pGEM9zf(−)/Kv1.3(T1$^-$)-(Kv1.3-P), which was derived from ligation of a partially *Pst*I-digested/*Eco*RI-digested fragment from pGEM9zf(−)/Kv1.3(T1$^-$)-Kv1.3 into an *Eco*RI/*Pst*I-digested pGEM9zf(−)/Kv1.3(S3-S4-S5) (Tu et al., 1996). The pRc/CMV/Kv1.3-Kv1.3-Kv1.3(H399Y) (referred to as WT-WT-HY in the Results) was made by ligating a partially *Eco*NI-digested/*Bgl*II-digested fragment (~4.8 kb) from pRc/CMV/Kv1.3-Kv1.3-Kv1.3 into a *Eco*NI/*Bgl*II-digested pRc/CMV/Kv1.3(H399Y). The pRc/CMV/Kv1.3-Kv1.3-Kv1.3 was derived from an *Eco*RrI/*Hin*dIII-digested fragment isolated from pGEM9zf(−)/Kv1.3-Kv1.3-Kv1.3 and triple-ligated with an *Eco*RI/*Pvu*I-digested fragment and a *Pvu*I/*Hin*dIII-digested fragment, each of which were previously isolated from pRc/CMV. The pGEM9zf(−)/Kv1.3-Kv1.3-(Kv1.3-P) (referred to as WT-WT-(WT-P) in the Results) was made by ligating an *Eco*RI/*Pml*I-digested fragment from pGEM9zf(−)/Kv1.3(T1$^-$)-(Kv1.3-P) into partially *Pml*I-digested/*Eco*RI-digested pGEM9zf(−)/Kv1.3-Kv1.3-Kv1.3. The pGEM9zf(−)/Kv1.3-Kv3.1 chimera was made by ligating a *Bst*BI blunt end-digested/*Hin*dIII-digested fragment from pRc/CMV/Kv3.1 into an *Aat*II blunt end-digested/*Hin*dIII-digested fragment from pGEM/Kv1.3. The pRc/CMV/Kv1.3(H399Y) (referred to as HY in the Results) was made by mutating the histidine to tyrosine at position 399, using the PLATER-1 mutagenesis system (Promega, Madison, WI) and verified by DNA sequence analysis (Sequenase Version 2.0 DNA Sequencing Kit, USB). The mutant insert (1.8 kb) was cloned into a pRc-based plasmid containing a CMV eukaryotic promoter sequence (5.4 kb), yielding the pRc/CMV/Kv1.3(H399Y) plasmid. The S1-S2-S3 construct contains base pairs 441–941. It has 30 amino acids before S1 and 11 amino acids after S3. The S3-S4-S5 construct contains base pairs 843-1180, starting from S3 and ending 27 amino acids after S5 (Tu et al., 1996). Table 1 lists the above-mentioned constructs that were used to generate the data presented in the Results.

In vitro translation

Capped cRNA was synthesized in vitro from linearized templates, using Sp6 or T7 RNA polymerase (Promega). Proteins were translated in vitro with [^{35}S]methionine (2 μl/25 μl translation mixture;~10 μCi/μl Dupont/NEN Research Products, Boston, MA) in the absence of microsomal membranes for 60–180 min at 30°C (Fig. 2) or in the presence of canine microsomal membranes for the indicated times and temperatures (Fig. 6), in rabbit reticulocyte lysate, according to the Promega Protocol and Application Guide.

Gel electrophoresis and fluorography

Electrophoresis was performed on a C.B.S. Scientific gel apparatus, using 7.5% SDS-polyacrylamide gels made according to standard Sigma protocols (Sigma Technical Bulletin, MWM-100). SDS in the sampling buffer, running buffer, and gel was 2%, 0.1%, and 0.1%, respectively. Native (nondenaturing) conditions were used in some experiments, in which case no SDS was present in the gel, and only 0.1% SDS was in the sampling buffer and running buffer. Gels were soaked in Amplify (Amersham Corp., Arlington Heights, IL) to enhance ^{35}S fluorography, dried, and exposed to Kodak X-AR film at -70°C. Typical exposure times were <36 h. Quantitation of gels was carried out directly with a Molecular Dynamic PhosphoImager (Sunnyvale, CA).

TABLE 1 Summary of Kv1.3 constructs

WT	Wild-type monomer
AV	Monomer with the A413V mutation in S6; speeds inactivation
HY	Monomer with the H399Y mutation in the pore; slows inactivation
WT-P	Monomer lacking terminal half of pore through C-terminus; nonfunctional
Chimera	Monomer containing S2 through C-terminus from Kv3.1; nonfunctional
WT-WT	Tandem dimer
WT-AV	Tandem dimer
AV-(WT-P)	Tandem dimer
WT-WT-WT	Tandem trimer
WT-WT-HY	Tandem trimer
WT-WT-(WT-P)	Tandem trimer
S1-S2-S3	Truncated peptide Kv1.3 fragment
S3-S4-S5	Truncated peptide Kv1.3 fragment

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