The Courtship and Marriage of K^+ Channel Subunits

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Abstract

Assembly of oligomeric membrane proteins is complex. It is even more complicated in the case of a polytopic protein such as a voltage-gated K^+ channel. However, one can engineer a particular biophysical function of such a channel to reveal the prior history of its subunits during assembly. These functional tagging experiments entail either heterologous expression of a wild-type subunit with a mutant subunit, or heterologous expression of a mutant subunit in a cell expressing endogenous wild-type channels. The method of analysis of the appropriately modified function assumes a binomial distribution for the random formation of homo- and heteromultimeric channels. Application of this general strategy to the T lymphocyte K^+ channel, Kv1.3, has revealed that subunits are recruited randomly into tetramers from mixed pools of wild-type and mutant monomers, that tetramers in the plasma membrane of the T cell do not dissociate, and that temporal, but not spatial, segregation of wild-type and mutant subunits occurs within this cell.

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Voltage-gated K^+ channels are homotetrameric membrane proteins, each subunit containing six putative transmembrane segments, S1-S6. The four channel subunits are not linked covalently, so what holds them together? And where, when, and how do channels form? We often modify structure to understand function; our approach, however, is to engineer the function of a channel to reveal its prior history, namely, to learn something about the assembly of individual subunits. In this regard the strategy and methods described below can be used to answer the following questions: Does synthesis and assembly of different channel subunits occur in the same shared compartment? Are subunits recruited randomly or preferentially? Does multimer formation occur in the plasma membrane? Are channel monomers and multimers in equilibrium in the plasma membrane? Is channel diversity

temporally or spatially regulated? To the extent that many of these events occur in the membrane, any new model of biomembranes should provide for these features of oligomer assembly.

Kv1.3, a voltage-gated *Shaker*-like K^+ channel in human T lymphocytes, opens in response to depolarization (Matteson and Deutsch, 1984; DeCoursey et al., 1984). Upon prolonged depolarization, it inactivates by a mechanism known as C-type inactivation. The wild-type homotetramer inactivates with a time constant of 200 ms and a point mutation in the S6 segment of this channel produces a mutant homotetramer that inactivates with a time constant of 4 ms, 50× faster than the wild-type channel (Panyi et al., 1995). Using this functionally-tagged subunit along with the wild-type subunit, we have shown that C-type inactivation is cooperative, in that each of the four subunits in a Kv1.3 channel contributes equal free energy to the transition from the open state to an inactivated conformation (Panyi et al., 1995). Moreover, by studying the inactivation kinetics, we have shown that simultaneous heterologous expression of wild-type and mutant subunits in a mammalian cell results in a randomly mixed pool of subunits, and therefore that the resulting population of expressed channel types can be described by a binomial distribution of tetramers containing from zero to four mutant subunits. This has allowed us to predict the inactivation kinetics for a population of such channels, which we verified by constructing tandem dimers containing a wild-type and mutant subunit covalently linked, so that the resulting tetramer had a defined 2:2 stoichiometry.

How can we use such analyses of inactivation kinetics to learn about *in vivo* assembly of native Kv1.3? We transfected Jurkat cells, which express endogenous Kv1.3, with a mutant Kv1.3 subunit. At the time of transfection there could be any number of preformed wild-type channels already in the membrane. We asked "Can heterologous and endogenous subunits mix? Do they use the same compartments for assembly, and if so what can a kinetic analysis of inactivation tell us about assembly? What distribution of channel types might we predict occurs in such an experiment?" Among the possible cases, a channel distribution that is binomial (to a constant) will occur only if tetramers are formed irreversibly from a pool of mixed monomers in the presence of preformed wild-type channels in the plasma membrane. Our results showed that endogenous subunits can mix with heterologous subunits to form channels, i.e., that synthesis and assembly of different subunits occurs in the same shared compartment, that Kv1.3 subunits are recruited randomly from integrated monomer pools, that tetramer formation occurs prior to residence of the channel in the plasma membrane, that once tetramers are inserted into the plasma membrane, thereafter they do not dissociate, and finally that regulation of endogenous K^+ channel diversity in mammalian cells is temporally, and not spatially, regulated (Panyi and Deutsch, 1996).

These experiments suggest a general strategy for exploring the history of channel subunits during assembly. This strategy proceeds in two steps. As outlined in Figure 1, the first test determines the relative affinities of mixed subunits. Wild-type (WT) and mutant (MUT) subunits are heterologously co-expressed in a cell devoid of the channel in question, and the resulting channel population either conforms to or fails to conform to a binomial distribution. If it conforms, this means that WT and MUT subunits are recruited randomly and independently with the same probability. Failure means that subunits are not selected randomly, but since they are present at the same time in the same heterologous cell compartment, failure means there is some cooperativity, positive ("like prefers like"), or negative ("like avoids like"). This will be manifest as an excess of homomultimers or an excess of heteromultimers, respectively, compared to a binomial distribution.

The second test determines whether subunits are recruited from integrated or segregated monomer pools and what must be the nature of the segregation. It should be used after verifying that the first test shows no cooperativity. MUT subunits are heterologously expressed in a cell which already has endogenous WT subunits. A binomial distribution can be interpreted as evidence that random recruitment of subunits occurred from an

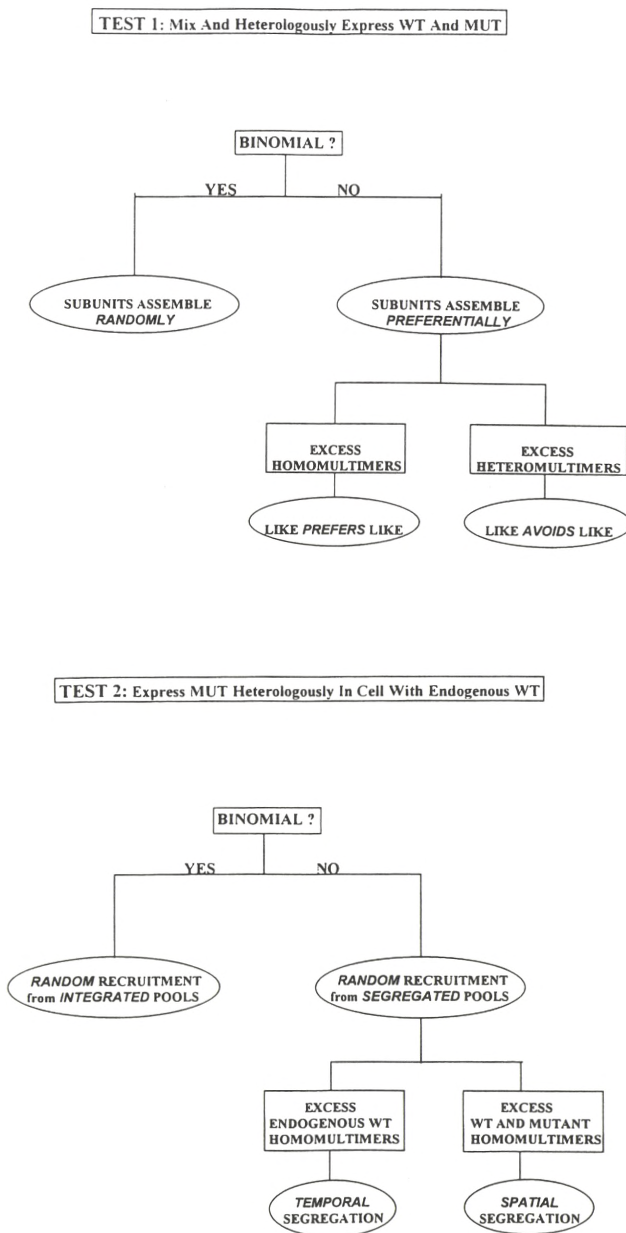

Figure 1. Prior history of channel subunits. Flow diagram illustrating tests for subunit preferences in association (Test 1) and for segregation of subunits (Test 2). The results (rectangles) and interpretations (ovals) of heterologous co-expression of wild-type (WT) and mutant (MUT) subunits in a cell are shown in Test 1. The results and interpretations of heterologous expression of MUT in a cell already expressing endogenous WT are shown in Test 2, where it is known that WT and MUT subunits show no preferential association.

integrated pool of subunits. This test can only fail the binomial distribution if there is some segregation, assuming that these subunits did not fail the first test, i.e. did not show cooperativity. Segregation will be manifest either as an excess of endogenous WT channels or as an excess of both WT and MUT channels compared to the binomial distribution. The first case indicates temporal segregation of subunits (i.e., multimers were formed irreversibly at different times), while the second indicates spatial segregation (i.e., WT and MUT homomultimers were formed in spatially separate compartments).

This method of analysis assumes a binomial distribution for the random formation of heteromultimeric channels. For a multimer of N subunits, the fraction of channels with exactly m mutant subunits will be

$$B(N,p,m) = \frac{N!}{m!(N-m)!}p^m(1-p)^{N-m} \qquad (1)$$

where p is the fraction of mutant subunits in the membrane. The wild-type homomultimer is represented by $m = 0$, whereas $m = N$ represents the homomultimeric mutant channel. If the biophysical properties of each member of this population are known, it is possible to estimate both p and the validity of the underlying assumption, namely that WT and mutant subunits assemble randomly. Biophysical properties that can be quantified in this way include the kinetics of inactivation, the affinity of an open-channel blocker, and the single-channel conductance. Any functional property of the channel can be used for this purpose. The major criterion that must be met, however, is that an order of magnitude difference in the chosen functional parameters for the wild-type and mutant subunits must exist, regardless of which parameter is being studied, whether it be time constants of gating, binding constants of some ligand, or single-channel conductances.

Three similar equations (Figure 2) may be used to fit the data obtained from a cell expressing both endogenous and heterologous subunits, depending on the biophysical parameter to be measured. In the case of gating kinetics, $I(t)$ is the current at time t and $Y_m(t)$ is a function describing the gating kinetics for a channel with m mutant subunits (see example below). In the case of blocker affinity, $I([bk])$ is the current in the presence of blocking agent, $I(O)$ is the current in the absence of blocking agent, bk is the blocker molecule, $[bk]$ is the blocker concentration, and $F_{unbk,m}([bk])$ is the fraction of unblocked current for a channel with m mutant subunits. In the case of single-channel conductance, i_m is the single-channel current for a channel with m mutant subunits. The conclusions from our T-cell studies have led us to ask a whole new set of questions. If subunit recruitment is random and subunits diffuse to find their correct partners, then what are the recognition signals for assembly? Do recognition and assembly occur in a membrane compartment? If the tetramer never dissociates, then what are the stabilization interactions holding the tetramer together? And finally, if the cell must regulate the time of subunit expression, and possibly the kinetics of synthesis and assembly of channel proteins in order to produce separate channel isoforms in the same spatial compartment, then what are the mechanisms of kinetic control of K^+ channel diversity?

Neither these mechanisms nor the interacting surfaces across subunit boundaries are known. Pfaffinger and Li, as well as my laboratory, have found recognition domains in the N-terminal cytoplasmic tail of K^+ channels, referred to as T1 ("first tetramerization") domains, that are known to tetramerize *in vitro* and to confer subfamily specificity (Li et al., 1992; Shen et al., 1993; Shen and Pfaffinger, 1995; Tu et al., 1995; Xu et al., 1995). But this finding does not preclude other recognition signals nor inform us about the stabilization interactions. In fact, we made a deletion mutant of Kv1.3 that lacks the first 141 amino acids (Kv1.3($T1^-$)), and in oocytes it produces currents whose biophysical properties are identical to those produced by full-length Kv1.3 (Tu et al., 1995; 1996). We have interpreted this finding to mean that there are association sites in the central core of Kv1.3 that provide sufficient stabilization interactions for channel assembly.

To probe for these putative interaction sites across subunit boundaries, our approach has been to test the ability of a series of hydrophobic Kv1.3 peptide fragments to suppress Kv1.3 cur-

$$\text{Gating Kinetics:} \quad I(t) = \sum_{m=0}^{N} B(N,p,m)Y_m(t)$$

$$\text{Affinity of Channel Blocker:} \quad \frac{I([bk])}{I(0)} = \sum_{m=0}^{N} B(N,p,m)F_{unbk,m}([bk])$$

$$\text{Single Channel Conductance:} \quad \frac{\textit{number of openings to level } i_m}{\textit{total number of openings}} = B(N,p,m)$$

$$B(N,p,m) = \frac{N!}{m!(N-m)!}p^m(1-p)^{N-m}$$

Figure 2. Read-outs of functionally-tagged subunits. Equations that describe gating kinetics, open-channel block, and single-channel conductance. In each case, $B(N,p,m)$ represents the binomial distribution, as described in the text, along with the functions and symbols used in these equations.

rent when they are heterologously co-expressed with Kv1.3($T1^-$) subunits in Xenopus oocytes, a so-called dominant negative suppression strategy. We have experimentally demonstrated several prerequisites in order to use this strategy to infer putative interaction sites (Tu et al., 1996; Sheng and Deutsch, 1997; Sheng et al., 1997). Thus, we can interpret suppression by a specific peptide as evidence that it competes with full-length subunits for self-association sites involved in assembly. Our results from both electrophysiological experiments and from immunoprecipitation experiments identify specific intramembrane association (IMA) sites in Kv1.3 and support a model in which association between subunits occurs between transmembrane segments in the plane of the lipid bilayer (Tu et al., 1996; Sheng et al., 1997). Furthermore, our studies show that synthesis and integration of interacting proteins into microsomal membranes occur rapidly and that the protein-protein interaction itself is the rate-determining membrane-delimited step in association (Sheng et al., 1997).

These results are consistent with the endoplasmic reticulum membrane itself facilitating K^+ channel tetramerization even when subunits are expressed at low levels, thus permitting efficient and rapid oligomerization relative to non-membrane assembly compartments (Helenius et al., 1992). We propose that Kv1.3 contains intersubunit IMA sites, located between S1 and S5, which may serve a specific role in tetramer assembly. Moreover, the S1-S2-S3 segments may be important for tetramer stabilization. The voltage-gated K^+ channel protein has multiple functions (Figure 3) that can be assigned to discrete structural domains, which may be created by the primary, secondary, tertiary, and/or quaternary conformations of the protein. The subunit stoichiometry of each of these functions has yet to be deter-

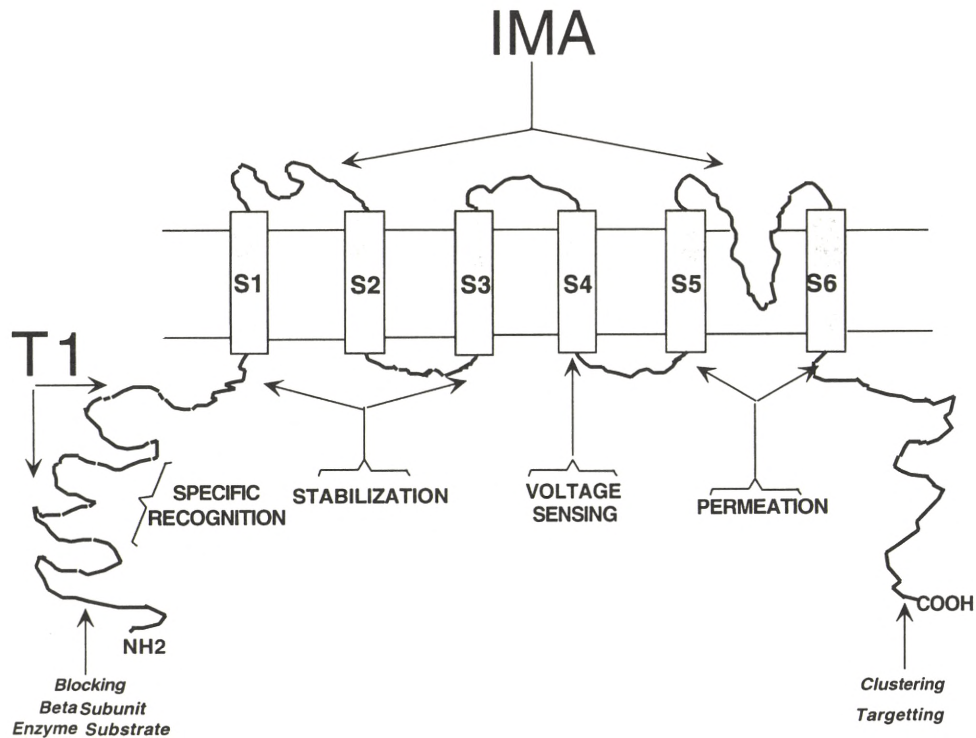

Figure 3. Modular functions of a voltage-gated K^+ channel.

mined. For instance, we know that all four subunits participate in voltage sensing, in pore formation, and in the binding sites for a variety of channel blockers, but what is the subunit participation in recognition? Do all four T1 domains participate? Do IMA sites from all four subunits participate equally in tetramer stabilization? And finally, do tetramers form by stepwise addition of monomers or, as we propose, do tetramers arise from association of two dimers, using different interaction mechanisms and IMA sites from those used in the initial monomer-monomer interaction? Where and at which stage in tetramer formation are T1 and IMA domains critical? The major question that these studies pose for our new model of biomembranes is how does the bilayer accommodate the random aspects of channel assembly and the modes of spatial and temporal segregation involved in oligomerization of polytopic membrane proteins.

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