

How to Validate a Heteromeric Ion Channel Drug Target: Assessing Proper Expression of Concatenated Subunits

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Native subtypes of K^+ and other ion channels are often hetero-oligomeric combinations of protein subunits. Drugs that selectively target relevant *in vivo* heteromers are valuable research tools and potential therapeutics. To recreate heteromeric channels as authentic drug targets, multiple subunits have been covalently tethered by concatenating their coding sequences. This perspective examines the linking strategies that have proven successful with many channel types and addresses some less desirable outcomes encountered. We discuss a variety of biochemical, electrophysiological, and pharmacological techniques that can assess whether constructs with concatenated subunits lead to expression of the intended uniform population of channels. Careful controls to ensure that channels are properly assembled can validate heterologously expressed concatemers as promising targets for the discovery of highly selective drugs.

Introduction

It has long been known that the ionic conduction pathways of many native channels are formed by combinatorial associations of homologous protein subunits. The time has now come to drug these tissue-specific heteromultimers. Therapeutic discovery strategies most commonly screen for ligands that bind homo-oligomers of only one channel constituent, and are liable to produce drugs that affect any tissue expressing that particular subunit. Drugs selective for tissue-specific hetero-oligomers are valuable as research tools to establish the molecular identity of native channel subtypes, and possibly as therapeutics with reduced side effects. To find heteromer-specific drugs, a means of reliably expressing a uniform population of channels with defined subunit combinations is prerequisite. For some channel types, this can be achieved by expression from a coding sequence composed of genes for multiple subunits concatenated into one open reading frame. Technically, this has been realized by removal of the 3' stop codon from initial subunits and replacement with a linker to the 5' end of another subunit's sequence. The resultant protein then has the C terminus of initial subunits covalently attached to the N terminus of following subunits. Such contiguous proteins are commonly referred to as

tethered, tandem, linked, or concatenated, the latter being our favored term. In theory, when subunits are concatenated, their proximity to each other greatly favors self-assembly, as their relative local concentration exceeds that of subunits synthesized by other ribosomes. In practice, assembly is contingent upon many factors, including the energetics of intersubunit interaction. This perspective draws primarily upon our experience with K^+ channel concatemers; it is a view toward controls and precautions that can be taken when working with such constructs to validate their use for discovery of heteromer-specific therapeutics.

Successful and Problematic Concatenation Strategies

The scientific merits of linking subunits are evidenced by the plethora of careful studies that have used the strategy. Concatenation can yield much more uniform populations of hetero-oligomers than coexpression methodologies; yet, this technique does not work perfectly for every construct. Years of work by many laboratories have shown the great advantages and occasional limitations of the use of linked constructs; here we touch on some telling examples.

Voltage-gated K^+ Channels. The pore-forming subunits of *Shaker*-related voltage-gated K^+ channel family (Kv) channels were concatenated soon after their initial cloning and functional expression (Isacoff et al., 1990). Kv proteins extend both their N and C termini into the cytoplasm and subunits linked as tetramers bear an architectural resemblance to the domain structure of voltage-gated Na^+ and Ca^+ channels. This structural relation may explain why cells have been able to express a diversity of Kv subunits stitched together into Frankenstein channels. Many constructs have reliably produced the intended linked tetramers, and extensive controls demonstrated that a uniform population of heteromeric channels containing the intended subunits can be expressed in the plasmalemma (Heginbotham and MacKinnon, 1992; Hurst et al., 1992; Kavanaugh et al., 1992; Liman et al., 1992). With some constructs, however, there was

Abbreviations used in this paper: Kv, *Shaker*-related voltage-gated K^+ channel family; CNG, cyclic nucleotide-gated; GABA_A, γ -aminobutyric acid type A; nAChR, nicotinic acetylcholine receptor.

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evidence that concatenated subunits do not always create functional channels of the desired stoichiometry, with overrepresentation of the initial subunits or underrepresentation of mutant subunits (McCormack et al., 1992a; Hurst et al., 1995). These latter studies seem to have inordinately discouraged researchers who have considered using concatemers. When read critically, these works do not invalidate other findings using linked Kv constructs, but do highlight the need to run appropriate controls to assess the uniformity and subunit stoichiometry of the channels expressed.

Cyclic Nucleotide-gated Channels. Kv channels are structurally related to cyclic nucleotide-gated (CNG) channels and have been extensively studied via concatenation. This has led to a number of useful findings and, again, some constructs were found to behave better than others. The ordering of CNG subunits in several tandem constructs appeared to affect their arrangement in the expressed channel (Gordon et al., 1996; Liu et al., 1996). However, another linkage of CNG subunits did not affect their rotational placement within channels (Morrill and MacKinnon, 1999), indicating that some concatemeric constructs control subunit assembly more tightly than others. There is at least one instance of markedly unexpected assembly of concatenated CNG subunits. An early study concluded it was possible that concatenated constructs bearing two CNG β subunits may not have inserted both β subunits into a functional channel (Shapiro and Zagotta, 1998). This cautious interpretation of results later proved prudent, as several independent assessment methods determined that CNG channels are not able to assemble with more than one pore-forming β -subunit per channel (Weitz et al., 2002; Zheng et al., 2002; Zhong et al., 2002). Thus, the tethered β subunits were not all included in the channels they were engineered to; concatemerization could not force an unnatural union of pore-forming subunits.

Pentameric Ligand-gated Channels. The ionotropic “Cys-loop” receptors are channels composed of five subunits that each have their N and C termini on the extracellular side of the plasmalemma, making these channels attractive targets for concatenation. Thorough and careful work has validated strategies to engineer linked subunits of the γ -aminobutyric acid type A (GABA_A) receptor (for review see Minier and Sigel, 2004), and fully concatenated pentameric GABA_A channels have been functionally expressed in mammalian cells and *Xenopus* oocytes (Baur et al., 2006). While some constructs produced the intended heteromers, others expressed poorly or behaved strangely. Intersubunit linker length has been shown to be important: linkers that are too short may physically constrain channels and lead to lowered GABA sensitivity; whereas those overly long lead to degenerate assembly (Baumann et al., 2001). With linkers

of just the right length, certain orderings of subunits express a higher proportion of functional channels and these combinations have been identified as candidates for native GABA_A subtypes. Concatemers have also been used to constrain nicotinic acetylcholine receptor (nAChR) stoichiometry (for review see White, 2006). Linkage of nAChR subunits has sometimes resulted in receptors that function well, while other concatemers exhibit bizarre behavior indicative of inappropriate assembly (Groot-Kormelink et al., 2004). The linking of human nAChR subunits has even led to the tethered proteins covalently linking one complex to another, mimicking the dimeric receptor structure found in *Torpedo* (Zhou et al., 2003). Pentameric concatemers of nAChR subunits were found to assemble into functional channels, and have even been shown to prevent inclusion of coexpressed monomeric subunits (Groot-Kormelink et al., 2006). This exclusionist behavior was similar to that of successfully concatenated Kv1 channels (Liman et al., 1992) and further validated the linking strategy for nAChR channels.

Generalizable Findings with Concatemers

The above studies are mentioned to highlight past triumphs, and pitfalls to be wary of, when endeavoring to use this auspicious approach. Analysis of published findings suggest it is unwise to assume a priori that the translation product of everything encoded in a concatenated DNA will be incorporated into a functional ion channel. Some lessons that have emerged from these studies are as follows.

(a) Work with nature! Intersubunit interfaces do not allow all heteromeric assemblies, but if concatemers are designed to imitate subunit combinations that occur in vivo, then the odds of assembly as planned are high.

(b) Although the majority of concatemeric channels may form correctly, assembly energetics could leave a fraction with an unexpected subunit stoichiometry.

(c) Every concatemer is different and, thus, must be individually validated.

(d) Do not let a few examples of meddlesome concatemers turn one off from a valuable tool.

Approaches to Validate the Uniformity and Stoichiometry of Expressed Concatemers

The key question to be asked by a concatemer validator is: are the expressed channels a uniform population comprised of the expected subunits? Researchers have at their disposal a wide variety of tools to address this issue. Which controls are chosen and what level of heterogeneity can be accepted are, of course, specific to each study's tolerances. For any validation, a useful test is to rearrange subunits in different expression constructs and assess the impact of positioning. Beyond this, there is a variety of means to assess the success of a concatenation strategy; it is worth contemplating the

utility and limitation of the different techniques available. These can be subcategorized by discipline into biochemical, electrophysiological, and pharmacological methods—we believe the best validation strategy is to use a combination of all three.

Biochemical Validation—Are the Surface Proteins Intact? A basic assessment of the size of an expressed channel can be surprisingly informative. Expressed protein may be truncated in such a manner that, if recognized, would alter interpretation of results. Controls indicating that concatenated proteins expressed are of the expected size can allay concerns about truncated concatemers. The findings of Annette Nicke and colleagues (Nicke et al., 2003) should strike fear in the hearts of those who trust concatemers to remain intact in the plasma membrane. Their attempted biochemical validation of concatenated P2X₁ receptors found that, despite the predominance of full-length concatemeric protein in *Xenopus* oocytes, the surface channels were comprised of degraded monomeric and dimeric constituents. Our laboratory has chosen to work with Kv1 concatemers expressed in HEK 293 cells, as Western blots indicate the predominant protein produced remains full length (Sokolov et al., 2007). In contrast, some breakdown of linked Kv1 channel proteins has been seen in blots of transfected COS-7 and CHO cells. However, examination of biotinylated surface channel protein in CHO cells indicated that the plasma membrane channels are intact (Fig. 1). It seems, thankfully, that the opposite of the concatenated P2X₁ phenomenon occurs when expressing linked Kv1 subunits. In fact, no biochemical evidence exists in the literature (or our laboratory) to indicate significant amounts of degraded or truncated Kv1 concatemer on the surface of any mammalian cell. Even if degraded concatemers were to be found, it is possible that channels assembled as intact concatemers could be later nicked by proteases to lose their covalent connection, yet remain as channels of the expected stoichiometry. A convincing validation of this situation could prove difficult; although evidence could be obtained through electrophysiological and pharmacological means.

Electrophysiological Validation—A Concatenated Product Is More than the Sum of its Parts. Characterization of ionic currents is an indispensable part of any channel validation strategy and can be used to assess the uniformity of expressed channels. Electrophysiological recordings can indicate the presence of subunits that endow channels with a particular property (e.g., inactivation, desensitization, ionic selectivity, altered voltage sensitivity), although properties of parental homomers are not always reflected in their heteromeric offspring. A striking example: while the N-type inactivation associated with Kv1.4 currents has been a hallmark of coassembly

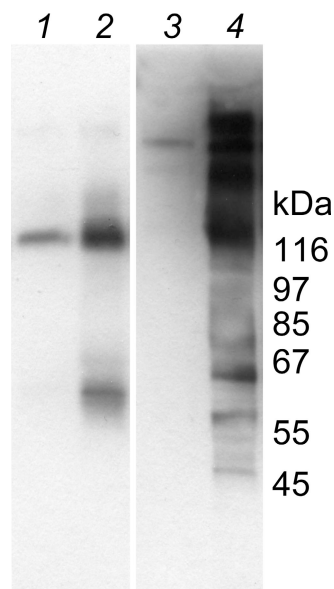


Figure 1. Surface biotinylation of heteromeric Kv1 concatemers expressed in CHO cells. Concatenated constructs of Kv1.1-1.2 or Kv1.1-1.2-1.2-1.2 (Akhtar et al., 2002) were expressed CHO cells by electroporation of RNA transcripts from a Semliki Forest virus vector. After 48 h, electroporated cells were harvested in PBS buffer containing 5 mM EDTA. Cell surface biotinylation was performed with sulfo-NHS-LC-biotin (Pierce Chemical Co.), as recommended by the manufacturer. Membrane proteins solubilized with 2% Triton X-100 were precipitated using streptavidin-agarose (Pierce Chemical Co.). Bound proteins were dissolved in lithium dodecyl sulfate sample buffer (Invitrogen) and SDS-PAGE and Western blotting with anti-Kv1.2 antibody were performed, as outlined elsewhere (Akhtar et al., 2002). Lanes: 1, biotinylated Kv1.1-1.2; 2, total extract from cells expressing Kv1.1-1.2; 3, biotinylated Kv1.1-1.2-1.2-1.2; and 4, total extract from cells expressing Kv1.1-1.2-1.2-1.2.

with other Kv1 subunits, fast inactivation is absent in heteromers comprised of Kv1.4 and Kv1.6 (Roeper et al., 1998).

For the analysis of voltage-gated channels, their conductance–voltage (g-V) relation is a standard metric and has been used to validate the proper assembly of concatemeric proteins. A g-V plot well fit by a single Boltzmann distribution is consistent with a uniform population of channels, while a g-V too complex to be fit by a single Boltzmann can be reflective of multiple channel types. One should be aware that the complex gating of a uniform channel population could also lead to a complicated g-V relation. As an example, some Kv channels have a voltage-dependent flicker (Hoshi et al., 1994) that causes their open probability to continue increasing at positive voltages beyond that predicted by a single Boltzmann distribution. In consequence, published g-V relations are often trimmed to voltage ranges over which the Boltzmann equation fits nicely. This being kept in mind, the midpoint and slope of these fits have been very useful measurements. Parameters of Boltzmann fits have been used to assert that subunit

order in the concatemeric coding sequence affected the stoichiometry of expressed Kv channels (McCormack et al., 1992b), and the ability of a single Boltzmann, rather than the sum of multiple, to fit g - V relations has been used to assess whether or not channel subunits coassemble (Lee et al., 1994).

Ion channel kinetics are often well described by simple equations that can be used to assess the uniformity of a channel population. Current recordings that can be fit with a single exponential function, or the product of multiple exponentials, are reflective of conformational change occurring with the same energetics throughout an entire channel population, and suggest that the underlying channels are similar. In contrast, mixed populations of channels with different kinetics will require fitting with the sum of multiple functions. Currents from concatemers that were distinct from those of their parental homomers and have kinetics that can be fit with exponential decay have indicated expression of a uniform population of heteromers (Sokolov et al., 2007).

Pharmacological Validation—Are Dose–Response Profiles Sensical? Drug effectiveness can provide insights into the make-up of expressed channels. Equations from stochastic physics determine the theoretical concentration dependence of ligand binding and can be useful for validating the uniformity of concatemers. A ligand that binds to any population of identical channels with a 1:1 stoichiometry should obey a standard Langmuir binding isotherm, and the binding curve should be fitted by a Hill equation with a slope of one. The Hill equation can be a powerful tool for demonstrating uniformity, as subpopulations of binding sites with different affinities will produce a multiphasic dose–response profile that will shallow the slope of attempted fits. A Hill slope steeper than predicted can be a consequence of significant depletion of dilute concentrations of drugs from solutions. For K^+ channels, external TEA dose–response can be a telling means to assess channel stoichiometry, as the ligand–channel interaction is quite well understood. Each subunit appears to contribute a constant energy regardless of its positional arrangement, and detailed electrophysiological measurements of K^+ current inhibition by external TEA have been a primary method to validate assembly of Kv1 concatemeric constructs (Heginbotham and MacKinnon, 1992; Kavanaugh et al., 1992; Liman et al., 1992).

While detailed dose responses to drugs can be useful in assessing channel uniformity, the limited lifetime of most electrophysiological recordings can make such measurements difficult with slow-acting drugs. The development of techniques to measure displacement of high-affinity radioligands from ion channels has provided an efficient means to measure drug binding. A benefit of working with certain Kv1 channels is the avail-

ability of labeled pore-blocking peptide toxins such as ^{125}I - α -dendrotoxin, ^{125}I -dendrotoxin_k (Wang et al., 1999a), and ^{125}I -hongotoxin₁ Y19F/A37Y (Koschak et al., 1998), which have slow dissociation rates sufficient to allow retention of bound radioligand during washes. The precise structures of dose–response profiles obtained with this technique have been quite revealing, and multiphasic radioligand displacement profiles have been used to expose the heterogeneity of some Kv1 concatemers (Middleton et al., 2003). Although researchers should remember that heteromers do not always retain the pharmacology one might predict from their monomeric substituents, drugs can be used to demonstrate a subunit's inclusion in channels. For example, the Kv1.1-specific dendrotoxin_k from mamba snake venom has bound all Kv1 heteromers tested to date that contain at least one Kv1.1 subunit. Therefore, dendrotoxin_k binding to heteromeric channels verifies the presence of Kv1.1, and has provided a means of validating the uniformity of concatemers (Akhtar et al., 2002).

How to Find a Heteromer-specific Drug

To validate recombinant ion channels as therapeutic targets, the big question is: which subunit combinations are relevant *in vivo*? The clearest conclusions concerning the identity of native Kv1 heterotetramers have been gleaned from successive immunoprecipitation experiments, a technique that proved to be capable of definitively identifying the underlying oligomer when channels were composed of four different α subunits. Interestingly, out of the vast number of potential heteromers, a handful of Kv1 subunit combinations were seen to be predominant in mammalian brain (Shamotienko et al., 1997; Koschak et al., 1998; Coleman et al., 1999; Wang et al., 1999b). At the International Centre for Neurotherapeutics, we have embarked on a program seeking drugs specific to concatemeric recreations of these native Kv1 heterotetramers.

Does it ever seem queer that the K^+ channel pore-blocking peptides from venomous creatures use an asymmetrical scaffold to bind to fourfold symmetric channels? This might seem less strange when one considers that heteromers may be the *in vivo* targets of most venom peptides. The majority of studies using these toxins have been conducted on homomeric channels merely because homomers are more straightforward for researchers to express. By virtue of binding along the central axis of K^+ permeation, pore-blocking peptide toxins present a unique interaction face to each channel subunit. The subunit-spanning design imbues the peptides with an exquisite ability to distinguish heteromers. A derivative of the ShK anemone toxin serves as a poignant example; it has been found to displace radioligand from heteromeric channels containing both Kv1.1 and Kv1.2 at concentrations too dilute to bind either parental homo-tetramer (Middleton et al., 2003). This finding is

remarkable, because it is one of the few instances where the affinity of a pore-blocking peptide toxin has been determined for a defined heterotetramer (also Akhtar et al., 2002), and begs the question of what other heteromer-specific toxins are harbored in the poison glands of creatures worldwide.

As a parting note, we mention another promising route to the discovery of drugs imbued with heteromeric specificity. Large, symmetric ligands with arms designed to reach out to individual subunits have proven to be high-affinity ligands for homomeric CNG (Kramer and Karpen, 1998) and Kv channels (Gradl et al., 2003). A fascinating venture would be to break the symmetry of these lead molecules and attach subunit-specific chemical moieties to enable selective targeting of heterooligomeric proteins. Therein lies a real challenge with enormous potential impact!

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