

Hot on the Trail of TRP Channel Structure

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The transient receptor potential (TRP) family of proteins are generally nonselective cation channels that participate in many sensory and physiological processes. To date, no structural information at the atomic level is available for any full-length TRP channels due to difficulties encountered in overexpression, functional purification, and crystallization of eukaryotic transmembrane proteins. However, progress toward obtaining TRP protein structures has been made by combining different techniques, such as cryo-electron microscopy of entire proteins, x-ray crystallography of isolated cytosolic domains, and extensive mutagenesis combined with functional assays in heterologous expression systems. This perspective focuses on recent developments in the determination of TRP channel structure by electron cryo-microscopy and single-particle analysis, and the use of multi-resolution and “divide and conquer” approaches to solving this problem. It also discusses the implications of new methods for expression and purification for future prospects in two- and three-dimensional crystallography, as well as the use of biophysical techniques to assess the functionality of purified TRP proteins that are used for structural analysis, and to study their direct interactions with other proteins.

Introduction

Determination of ion channel structures is important for understanding basic mechanisms of gating, modulation, ion permeation, and selectivity. It holds potential for structure-based design of channel-targeted therapeutics and for understanding the structural basis of channelopathies. Despite extensive effort in many laboratories, the number of solved ion channel structures remains small because of the challenges presented for integral membrane proteins by the needs of structural methods for high level expression, high purity, and maintenance of native protein conformation and activity after removal from the membrane. These problems have proved especially challenging for eukaryotic ion channels, among which high resolution structures have been determined by x-ray crystallography for only two native channels (Long et al., 2005; Jasti et al., 2007), and one chimera from fragments of two eukaryotic potassium channels (Long et al., 2007). One channel structure, that of the nico-

tinic acetylcholine receptor, has been solved by electron crystallography at sufficient resolution to identify side chains and generate an atomic model (Miyazawa et al., 2003; Unwin, 2005).

As discussed below, TRP channels present their own challenges in addition to those presented by eukaryotic ion channels in general. The members of this family of channels have emerged as important players in several human diseases (Venkatachalam and Montell, 2007), as well as in multiple sensory modalities and signaling pathways (Ramsey et al., 2006). They are widely regarded as attractive targets for novel therapeutics (Krause et al., 2005; Nilius et al., 2007), but currently their pharmacology is nearly as undeveloped as their structural biology. We review the progress to date in understanding TRP channel structure and structure relationships, including their tetrameric organization, and discuss the prospects for combining a range of techniques to obtain further advances.

Predicted Domains, Topology, and Stoichiometry

Seven major subfamilies in the TRP family have been identified: TRPV, TRPA, TRPC, TRPM, TRPP, TRPML, and TRPN. All are predicted to have six transmembrane helices, S1–S6, per subunit, with varying sizes of cytoplasmic amino and carboxy termini, and are thought to form tetrameric assemblies (Clapham, 2003; Schindl and Romanin, 2007; Venkatachalam and Montell, 2007). Gel filtration analysis (Moiseenkova-Bell et al., 2008), blue native (low SDS) gel electrophoresis (Jahnel et al., 2001), and electrophoresis in perfluoro-octanoic acid (Kedei et al., 2001) all indicated that TRPV1 can form tetramers, although the electrophoresis assays revealed both lower- and higher-order complexes as well, depending on conditions. Sucrose gradient centrifugation of TRPV5 and TRPV6 indicated that they are predominantly tetrameric, and coimmunoprecipitation experiments suggested they can form heterotetramers as well as homotetramers (Hoenderop et al., 2003). As discussed below, the appearance of purified recombinant TRP channels in electron micrographs is consistent with homotetrameric structures. By analogy to potassium channels related to

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Abbreviation used in this paper: TRP, transient receptor potential.

the Shaker family, the ion pore is predicted to be formed by the combination of the S5 and S6 segments with the “P-loop” connecting them. Within the cytoplasmic domains, some well-known structural motifs have been identified by sequence comparisons: variable numbers of ankyrin repeats (TRPV, TRPA, TRPC, and TRPN), a “TRP” sequence of unknown structure and function found in some but not all family members, kinase domains in TRPM, and extracellular domains inserted into the transmembrane domain in TRPP and TRPML. There have been extensive studies of structure–function relationships within TRP channels, and these have been reviewed elsewhere. The focus here is on the progress made to date, as limited as it is, in determination of three-dimensional structures and on the prospects for breakthroughs in this area in the near future.

Expression Systems and Purification

In general, ion channels are expressed at very low levels in the membranes in which they occur naturally, and TRP channels are not exceptions to this rule. Therefore, finding a system for expressing them at high levels is a prerequisite for structural methods. Bacterial overexpression is the most efficient and economical approach in most cases for obtaining milligram quantities of protein, but to date there have been no reports of successful use of bacteria for expression of full-length TRP channels. Bacteria have proven useful, however, for producing large quantities of soluble fragments from the cytoplasmic domains of TRP channels, including the ankyrin repeats from TRPV1, TRPV2, and TRPV6 (Jin et al., 2006; McCleverty et al., 2006; Lishko et al., 2007; Phelps et al., 2008), the α -kinase domain of TRPM7 (Yamaguchi et al., 2001), and C-terminal cytoplasmic coiled-coil domain of TRPM7 (Fujiwara and Minor, 2008). It seems likely that additional cytoplasmic fragments may yield to this approach. The cytoplasmic domains of TRP channels make up most of their mass, so solving fragment structures by x-ray crystallography or nuclear magnetic resonance may provide high resolution structures of most of the protein, which could then be fit into lower resolution structures of the full-length proteins determined by electron microscopy (see below).

Mammalian cell culture has been used to express numerous full-length TRP channels. In most cases, the amounts produced have been sufficient for measuring currents through the channels, but not nearly enough for structural methods. However, in the cases of TRPC3 (Mio et al., 2005, 2007) and TRPM2 (Maruyama et al., 2007), transfection of mammalian cells and purification in the detergent dodecyl maltoside have been used to obtain sufficient amounts of protein for electron microscopy.

Another alternative is to use eukaryotic microbes, such as the methylotrophic yeast, *Pichia pastoris*, or the budding yeast, *Saccharomyces cerevisiae*, for expressing TRP channels. The *Pichia* system has been used successfully for

aquaporin (Nyblom et al., 2007) and potassium channels (Long et al., 2005, 2007; Tao and Mackinnon, 2008), but so far no success has been obtained for TRP channels. In contrast, *S. cerevisiae* has proven to be a useful and versatile system for expressing TRP channels in functional form. The first report of expression of a TRP channel in yeast was for TRPV1 (Moiseenkova et al., 2003). The functionality of the protein was demonstrated by ligand-triggered Ca^{2+} influx detected with the fluorescent Ca^{2+} -indicator dye, Fura-2. A recent study made use of these observations to establish a screen for mutants in TRPV1 with altered function by selection in budding yeast (Myers et al., 2008). Subsequent improvements (Moiseenkova-Bell et al., 2008) upon these early efforts, including the use of a carboxyl-terminal epitope tag taken from rhodopsin and an immuno-affinity column of immobilized monoclonal antibody 1-D4 (MacKenzie et al., 1984), allowed one-step isolation of detergent-solubilized TRPV1 essentially free of contaminating proteins. This method allowed purification of milligram amounts of the protein for functional and structural studies. Gel filtration chromatography is a useful method for identifying conditions, such as the type of detergent, which maintain the protein in monodisperse form, and for assessing the subunit stoichiometry of the purified channel. In the case of TRPV1, a single major peak corresponding to a tetramer was observed upon gel filtration in the detergent decyl maltoside (Moiseenkova-Bell et al., 2008).

Subsequently, additional TRP channels have been expressed in *S. cerevisiae* with the 1-D4 epitope tag, and TRPV2, TRPY1-4 (Moiseenkova-Bell, V., L. Stanciu, I. Serysheva, B. Tobe, Y. Zhou, and T.G. Wensel. 2007. 51st Annual Biophysical Society Meeting. Abstr. 2626), TRPM8, and TRPA1 all behave well in this system, allowing for purification of sufficient quantities of protein for electron microscopy or setting up crystallization trials.

Electron Microscopy in Negative Stain

Electron microscopy has been a useful tool to study ultrastructures of cells and tissues. Recently, it has evolved into a powerful technique to determine structures of biological macromolecules. The highest resolution structures have been obtained using two-dimensional crystals, but unfortunately conditions for forming these for TRP channels have not yet been reported. Alternatively, single-particle analysis (van Heel et al., 2000; Frank, 2002; Chiu et al., 2005; Jiang and Ludtke, 2005) uses thousands of projection images, ideally with more or less randomly distributed orientations, to obtain sufficient data for three-dimensional reconstructions. Proteins provide relatively little contrast against a water background, so these data are most easily obtained if the image contrast is enhanced with the use of negative stains, such as uranyl acetate. This approach has been applied to several ion channels, including TRPM2 (Maruyama et al., 2007), TRPC3 (Mio et al., 2005), and TRPV1 (Moiseenkova, V.,

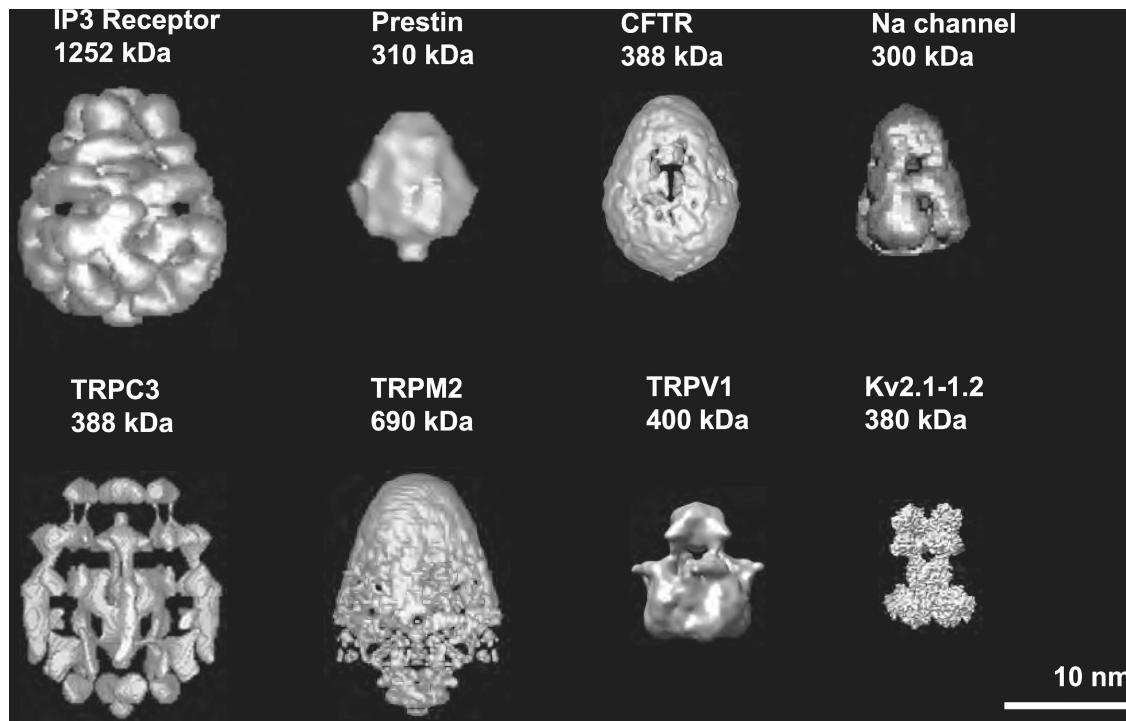


Figure 1. Comparison of reported structures from electron microscopy of TRP channels and other membrane proteins. Negative stain structures, resolution: Prestin, 20 Å (Mio et al., 2008a); CFTR, 20 Å (Mio et al., 2008b); TRPM2, 37 Å (Maruyama et al., 2007); TRPC3, (Mio et al., 2005). Cryo-electron microscopy structures: Na channel, 19 Å (Sato et al., 2004); InsP₃ receptor, 20 Å (Sato et al., 2004); TRPC3, 15.3 Å (Mio et al., 2007); TRPV1, 19 Å (Moiseenkova-Bell et al., 2008). Fourier shell correlation >0.5 is used as the resolution criterion for electron microscopy structures. X-ray structure: Kv2.1-1.2, 2.4 Å (Long et al., 2007).

Z. Zhang, B.N. Christensen, and T.G. Wensel. 2005. 49th Annual Biophysical Society Meeting. Abstr. 551), with examples shown in Fig. 1. Results from TRPM2 and TRPC3 suggest that each is bullet-shaped, with a dense bullet-head domain, interpreted as the transmembrane channel domain, and a more open but larger putative cytoplasmic domain. From the TRPC3 results, the height of the protein was calculated to be 235 Å, and the top view had a width of 200 Å; for TRPM2, the height of the protein reported to be 250 Å, and the top view had a width of 170 Å. In their overall shapes, these proteins resemble other membrane protein structures determined using this methodology, including prestin (Mio et al., 2008a), and the cystic fibrosis transmembrane conductance regulator, CFTR (Mio et al., 2008b). The CFTR diameter was previously estimated as 9.0 nm, based on images of freeze-fracture replicas (Eskandari et al., 1998). In interpreting these results, it is worth bearing in mind that at best negative stain provides the structure of a stain-filled “cast” around the part of the protein from which it is excluded, which therefore has limited resolution, that the staining conditions can distort the structure, and that because the contrast comes from the stain, not the protein, lipid aggregates are difficult to distinguish from protein. The TRPM2 and TRPC3 structures were determined using an automatic particle-picking algorithm that has not been extensively tested.

Electron Microscopy in Vitreous Ice

An alternative approach to structure determination by electron microscopy is the use of samples captured without stain or fixative in vitreous ice. Although the limited image contrast obtained using this method presents challenges for proteins <500 kD, the hardware and software available have been improving steadily, so that now structures of noncrystalline specimens at a resolution close to 4 Å can be obtained under favorable conditions (Ludtke et al., 2008). Structures in this resolution range have not yet been determined for TRP channels, but progress has been made in determining lower resolution structures. Recently (Moiseenkova-Bell et al., 2008), electron cryo-microscopy and single-particle analysis were used to determine the structure of TRPV1 to a 19-Å resolution (Fig. 2). The structure is fourfold symmetric and consists of two well-defined domains. One of these is compact and of appropriate volume and length (along the axis of symmetry; presumably the direction of the transmembrane vector) to be the transmembrane domain. This domain measures 40 Å (length) by 60 Å (diameter) and was interpreted as containing the ion channel pore and associated transmembrane helices. The voltage-gated potassium channel Kv 1.2 has a similar six-loop topology in its transmembrane domain to that predicted for TRP channels, and its structure, as determined by x-ray crystallography (Long et al., 2005),

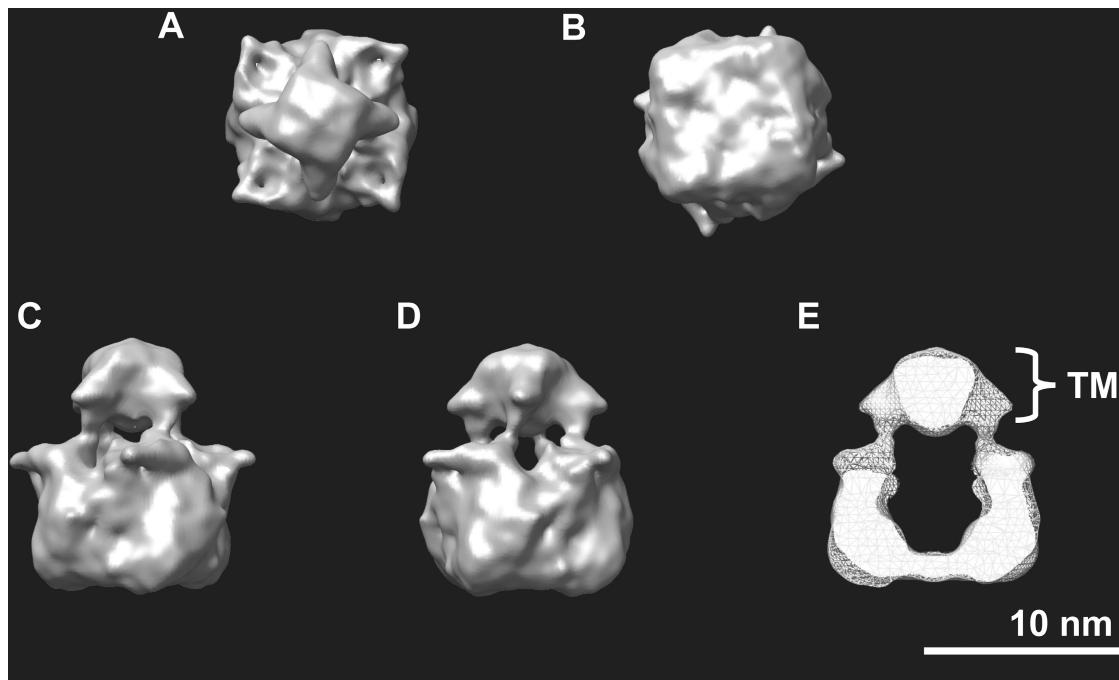


Figure 2. Structure of TRPV1 at a 19-Å resolution from single-particle analysis and electron cryomicroscopy (Moiseenkova-Bell et al., 2008). Semitransparent orthogonal surface views are shown from (A) the “top” (the side suggested to face the extracellular milieu), (B) the “bottom” (the side suggested to be cytoplasmic), and (C and D) two “side” (perpendicular to the fourfold symmetry axis) directions. In E, a cutaway view is shown to reveal the empty cavity within the basket-like cytoplasmic domain. A bracket indicates the proposed transmembrane domain.

fits well into the putative transmembrane domain of the TRPV1 structure. In addition, a large basket-like domain hangs from the transmembrane domain by fairly thin connecting densities. This domain has sufficient volume to contain both the N- and C-terminal cytoplasmic domains. Its overall dimensions are 100 Å (diameter) by 75 Å (length along symmetry axis), but its surface encloses a large central cavity of unknown function. The structure of the ankyrin repeat domain of TRPV1 (Lishko et al., 2007), from its N-terminal region, fits well into four shoulder-like domains in the putative cytoplasmic domain near the proposed membrane surface, although this placement must be considered hypothetical until a higher resolution structure is obtained. Preliminary results from TRPV2 suggest its overall structural architecture resembles that of TRPV1 (Moiseenkova-Bell, V. L. Stanciu, I. Serysheva, B. Tobe, Y. Zhou, and T.G. Wensel. 2007. 51st Annual Biophysical Society Meeting, Abstr. 2626).

Another TRP channel structure determined by electron cryomicroscopy, that of TRPC3 (Mio et al., 2007), is strikingly different from both the TRPV1 structure and the TRPC3 structure determined in negative stain (Mio et al., 2005) (Fig. 1). Its overall molecular envelope exceeds in size that of the InsP₃ receptor determined by the same group (Sato et al., 2004), although the latter has more than three times the mass (1,250 kD) of TRPC3 (388 kD). The structure, as reported, is very open and

mesh-like, with many thin connections and no obvious compact domain of sufficient length to span the bilayer. As with the negative stain studies on TRPC3 and TRPM2, an automated particle-picking procedure was used. Because of low contrast, such algorithms tend to be even less reliable for cryo-electron microscopy images of moderately sized proteins than they are for negative stain data. It is not obvious why the structure of TRPC3 looks so different from any of the other TRP channels determined by electron microscopy, but it is conceivable that inclusion of lipid aggregates among the images may influence the results.

Functional Reconstitution and Assays of Purified Proteins and Fragments

It is desirable that any structural work on TRP channels be performed on proteins as close to their native and functionally active states as possible. Structural analysis requires TRP channels to be purified in detergent solutions, and channels in micellar form can be used for direct binding studies to detect interactions with small molecules and other proteins. Properties requiring insertion into a lipid bilayer can be performed by reconstitution into vesicles or by incorporation of purified protein into a planar bilayer in a recording chamber. For example, methods were recently described for functional reconstitution and assays of ligand-gated ion flux with detergent-purified TRPV1 (Moiseenkova-Bell et al., 2008)

by the addition of phospholipids and detergent removal by dialysis to yield TRPV1 reconstituted in unilamellar phospholipids vesicles.

X-Ray Crystallography of Fragments

In lieu of crystals of full-length TRP channels, a promising approach is the crystallization of fragments that are experimentally more tractable. Recently (discussed in detail in a Perspective by Rachelle Gaudet in this issue [p. 231]), progress has been made in the determination of the structures of the N-terminal cytoplasmic portion that contains ankyrin repeat domains for TRPV2, TRPV1, and TRPV6 channels (Jin et al., 2006; McCleverty et al., 2006; Lishko et al., 2007; Phelps et al., 2007, 2008). The structure of the α -kinase domain of TRPM7 (Yamaguchi et al., 2001), and the structure of the C-terminal cytoplasmic coiled-coil domain of the same protein (Fujiwara and Minor, 2008), were also determined. It seems likely that structures of carboxyl-terminal cytoplasmic domains, or perhaps engineered constructs linking the N- and C-terminal domains (without the transmembrane segments), will be forthcoming. Such structures enhance the value of lower resolution structures determined by techniques such as electron cryo-microscopy or spectroscopic techniques because as their resolutions improve, the latter can be used to determine the relative alignment and positioning of the various domains determined at higher resolution.

Future Prospects

Although initial progress in determining the three-dimensional structures of TRP channels has been slow, it is likely to pick up speed. Expression systems and purification procedures have been worked out for both full-length proteins and soluble domains, and it is likely that these methods will be extended to additional TRP channels and their domains. The amounts of some full-length TRP channels obtained by expression in budding yeast are sufficient for extensive crystallization trials. Alternative expression systems may also bear fruit. In addition, there is reason for optimism that some TRP channels will be amenable to two-dimensional crystallization, which would open up the possibility of using electron crystallography to obtain higher resolution structures than those obtained by single-particle analysis. Meanwhile, the resolution of the latter is sure to improve with advancements in technology and collection of more data on TRP channels and their complexes. Additional complementary structural techniques, such as fluorescence resonance energy transfer, nuclear magnetic resonance, and electron paramagnetic resonance are likely to provide additional structural constraints for enhancing our understanding of TRP channel structures and their relationships to channel function. An interesting alternative approach to determine channel structures embedded in bilayers, which might be applicable to reconstituted TRP chan-

nels, is the combination of single-particle analysis and random conical tilt images of freeze-fracture replicas (Lanzavecchia et al., 2005).

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