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Review

Phylogeny of ion channels: clues to structure and function ☆

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Abstract

Voltage-gated ion channels are responsible for the electrical activity in a variety of cell types in modern-day animals. However, they represent the result of many millions of years of evolution of a family of ion channel proteins that are also found in prokaryotes and diverse eukaryotes, and probably exist in all life forms. This review traces the evolution of ion channels, with particular emphasis on the factors and evolutionary pathways that may have given rise to voltage-gated potassium (K^+), calcium (Ca^{2+}), and sodium (Na^+) channels. The review also highlights the utility of comparing phylogenetically distinct versions of the same protein as a means to better understand the structure and function of proteins.

Keywords: Calcium channel; Sodium channel; Potassium channel; Cyclic nucleotide-gated channel; Evolution; Ion channel; Jellyfish

1. Introduction

Among the innumerable proteins that support life in its many forms, voltage-gated ion channels are some of the most fascinating, partly for what they do, and partly for how they do it. The most obvious function of voltage-gated ion channels is to generate electrical activity in cells and, as such, they are responsible for many of the most overt manifestations of life — movement, the senses, and the cognitive functions of the brain. In itself, this is a remarkable biological phenomenon that has attracted generations of neurobiologists and philosophers. The manner by which voltage-gated ion channels enable electrical events to occur is also intriguing. Simply put, they undergo an extremely rapid conformational change that converts an impermeable structure into a highly permeable hole in the membrane through which ions can pass. More beguiling is the fact that while an open ion channel is able to pass ions at close to the rate of diffusion, it is still able to select one specific class of ions from among the many that are present at the opening of the pore.

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An eloquent description (Miller, 2000) of ion channels as 'superb chemists' only begins to do justice to their remarkable properties.

Although voltage-gated ion channels are seen as being critical to animals, particularly those with complex nervous systems, they are relatively old proteins, some of which are well represented in diverse prokaryotes. As a result of major genetic events such as gene duplications and more minor ones involving numerous single base mutations, a plethora of channel types has evolved from what was, presumably, a single or limited number of precursors. Various reviews have attempted to trace the evolution of ion channels (Jan and Jan, 1994; Derst and Karschin, 1998; Plummer and Meisler, 1999; Jeziorski et al., 2000a). Many are based on sequence information from relatively few ion channels. As sequence information on identified ion channels and genome information on select species has become available, our understanding of the lineage and evolutionary history of proteins such as ion channels has been changing almost continuously. For the next few years, at least, the field will likely be very fluid, particularly with respect to new potassium (K^+) channels which are being discovered almost routinely in the genomes of various prokaryotes. Therefore, rather than attempt to trace the evolutionary lineage of known ion channels, we will simply provide here a broad overview of current models and emphasize particularly important steps in that process. The more important purpose of this review is to highlight the utility of using an evolutionary approach to glean useful information about ion channel function and, by extension, about the properties of other types of proteins.

2. Overview of voltage-gated ion channels

While voltage-gated ion channels are complex proteins, the basic building block of all known ion channels is a relatively simple protein consisting of two transmembrane segments (2-TM), separated by a pore forming (P-region) loop (Fig. 1). This basic motif constitutes the entire protein in many prokaryotic channels and in the eukaryotic

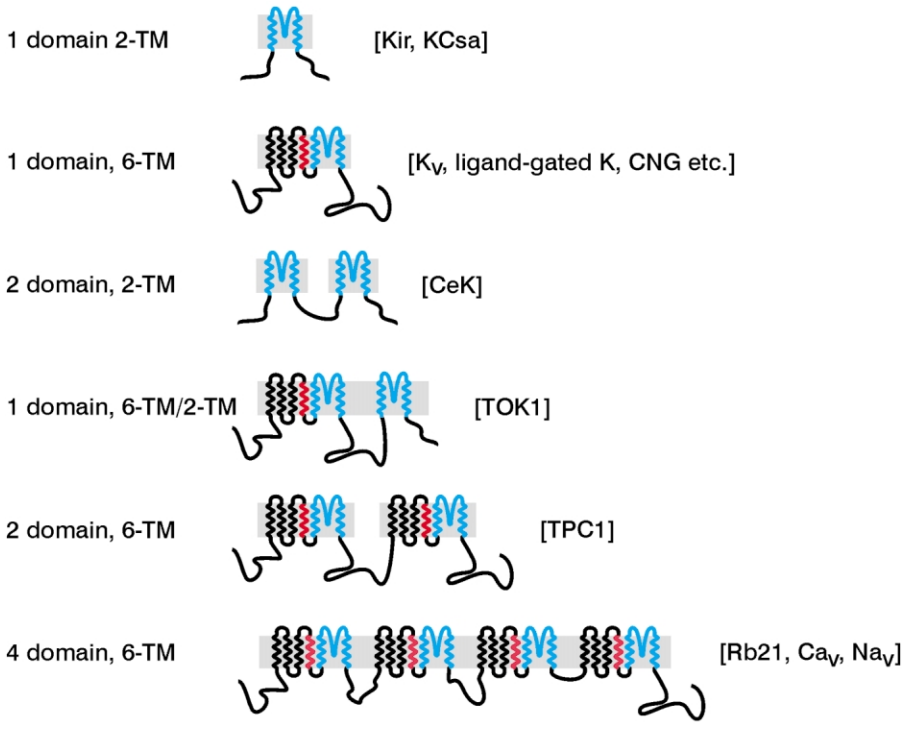

Fig. 1. Depictions of the variety of structural motifs found among diverse voltage-gated ion channels and their presumed precursor molecules. The types of channels composed of each type of subunit is listed on the right, using abbreviations that can be found in the text. In each motif, the precursor fragment composed of a pore-forming region bounded by two transmembrane segments is indicated in blue, and the voltage-sensing S4 segment is shown in red.

inward rectifier family (Kir), and, interestingly, it is thought to have undergone two rounds of gene duplication to form the early symporters (Durell et al., 1999). In some prokaryotic channels, and in the majority of eukaryotic channels, the 2-TM basic motif has been elaborated by the addition of an additional four transmembrane segments (Fig. 1), creating the six transmembrane segment (6-TM) protein most familiar to students of the field. Further elaborations include single proteins that are composed of one 6-TM and one 2-TM protein. The remainder of this review will focus on the 6-TM motif that is the structural basis for eukaryotic voltage-gated K^+, calcium (Ca^{2+}) and sodium (Na^+) channels.

Expression studies with a variety of eukaryotic 6-TM channels have permitted the mapping of specific functions of the channels to particular regions of the protein. For instance, where present, the voltage sensor has been mapped to the regularly repeated positive residues in transmembrane segment 4 (S4), and much of the ion selectivity and pharmacology of different channel types has been mapped to the P-region. In most, if not all cases, naturally occurring voltage-gated ion channels exist as multisubunit proteins composed of a pore-forming unit and from one to four accessory subunits that modulate some aspect of the pore-forming unit's function or expression. For detailed descriptions of ion channel structure and function, readers are referred to other sources (Chandy and Gutman, 1995; Goldin, 1995; Stea et al., 1995; Conley and Brammar, 1999; Catterall, 2000).

3. Potassium channels

Voltage-gated K^+ channels are the most diverse, structurally and functionally, of all voltage-gated channels. Indeed, the literature on these channels is already considerable and growing at such an enormous rate that to attempt to review the subject within the broad context of a review of voltage-gated ion channel evolution would not do the field justice. Consequently, we will only provide an overview of the subject and direct interested readers to more thorough reviews provided elsewhere (Jan and Jan, 1994; Derst and Karschin, 1998; Coetzee et al., 1999).

The 6-TM building block constitutes the basic pore-forming unit of most eukaryotic voltage-gated K^+ channels, with the functional channel being formed by the assembly of four of these subunits in the membrane. Whereas early studies identified four original members of the voltage-gated K^+ channel family — Kv1, Kv2, Kv3, Kv4 — which, respectively, correspond to the *shaker*, *shab*, *shaw* and *shal* channels originally identified in *Drosophila* (Butler et al., 1989), more recent work with mammals has identified additional varieties (Kv5,6,8,9). Furthermore, many Kv families contain several members (i.e. Kv1.1..Kv1.6; Kv2.1..Kv2.2; Kv3.1..Kv3.3; Kv4.1..Kv4.3; Kv9.1..Kv9.2), each with slightly different functional properties that in many cases can be linked to structural differences. Other forms produced by post-translational modifications and splice variants have also been identified. Because the functional tetrameric channel is formed by the assembly of four subunits, which need not be identical but can be closely related subunits or splice variants thereof, the number of potential structural combinations is enormous.

4. Calcium channels

The pore-forming unit of all known Na^+ and Ca^{2+} channels, however, is a single protein composed of four linked domains, each of which is highly homologous to a single 6-TM K^+ channel protein (Fig. 1). Like K^+ channels, Na^+ channels, and, to a greater extent, Ca^{2+} channels, show considerable structural and functional variation. The diversity of Ca^{2+} channels was evident from some of the earliest recordings of Ca^{2+} currents which revealed three broad classes — L-type currents, T-type currents, and N-type currents — many of which co-exist in the same animal if not in the same cell (Nowycky et al., 1985). Because L- and N-type currents both require large depolarizations for activation, they were classified as high voltage-activated (HVA) currents, whereas T-currents, which require only small depolarizations, were classified as low voltage-activated (LVA) currents. Subsequent pharmacological studies revealed new non-L currents, such as the P- and Q-types (for review, see Miller, 1993; Olivera et al., 1994). HVA Ca^{2+} currents can also be classified on the basis of their sensitivity to one broad class of channel blockers, the dihydropyridines. With the exception of those in invertebrates, which will be discussed later, L-type

currents are typically sensitive to dihydropyridines, whereas all others, grouped together as non-L currents, are resistant.

As molecular cloning techniques were applied to studies of Ca^{2+} channels, it became apparent that the functional variation among Ca^{2+} currents correlates with significant structural diversity. To date, 10 different families of voltage-gated Ca^{2+} channel pore-forming units have been recognized. These were originally identified alphabetically ($\alpha 1_{A-I,S}$), but as the number of channels grew, and raised the threat of there being an $\alpha 1_L$, with the associated risk of confusion with L-type currents, a numeric grouping similar to that used for K^+ channels was adopted (Ertel et al., 2000). This system breaks Ca^{2+} channel types into HVA and LVA channels, with the HVA channels further subdivided as L- and non-L: L-type channels, $\alpha 1_{S,C,D,F}$, are now called $Ca_V1.1$, 1.2, 1.3, and 1.4, respectively; non-L-channels, $\alpha 1_{A,B,E}$, have become $Ca_v2.1$, 2.2., and 2.3, respectively; and the three LVA channels $\alpha 1_{G,H,I}$, are now $Ca_V3.1$, 3.2, and 3.3, respectively.

5. Sodium channels

Prior to the application of molecular cloning techniques to Na^+ channels, the basic dogma was that whereas there must be many different K^+ and Ca^{2+} channels to account for the diversity of currents that had been recorded from different tissues, there were probably only one or a few Na^+ channels. Na^+ currents from organisms as diverse as mammals and jellyfish were remarkably similar — all were fast, transient currents that served to rapidly depolarize the cell's membrane in order to propagate the signal down the length of an axon, or to trigger other events (e.g. contraction, secretion). The only noticeable differences between different Na^+ currents were a few pharmacological oddities — TTX-resistance in some instances (Anderson, 1987), and the finding that some Na^+ channels could be blocked by rare channel blockers including certain cone shell toxins (Hasson et al., 1995). When sequence data became available, it became evident that there are a variety of different Na^+ channels, and it is now recognized that mammals have 11 different Na^+ channel α subunit genes (for review, see Plummer and Meisler, 1999). However, all mammalian Na^+ channels are remarkably similar. Whereas the many mammalian HVA Ca^{2+} channels (L- vs. non-L) share as little as 30–35% identity (Stea et al., 1995), the most different mammalian Na^+ channels show over 75% identity to each other (Catterall, 2000). Multiple Na^+ channels are also found in invertebrates, but to a more limited extent; two Na^+ channels have been cloned from both *Drosophila* (Salkoff et al., 1987; Loughney et al., 1989) and squid (Sato and Matsumoto, 1992; Rosenthal and Gilly, 1993).

6. Evolution of voltage-gated ion channels

The pore-forming units of all known 6-TM channels consist of four identical or highly homologous domains. In K^+ channels, each domain is a separate protein, whereas the pore-forming units of Na^+ and Ca^{2+} channels consist of a single protein encompassing all four domains. These facts argue strongly that the Na^+ and Ca^{2+} channels evolved from K^+ channels. This premise is supported by the observation that domains I and III of Na^+ channels are more similar to one another than they are to domains II and IV which are, themselves, similar to one another (Strong et al., 1993). This suggests that the four-domain precursor protein that gave rise to Na^+ and Ca^{2+} channels arose through two rounds of gene duplication of a single-domain 6-TM channel, which initially converted the single domain into a two-domain protein, which then duplicated in its entirety to form the four-domain channel.

A survey of the distribution of Ca^{2+} and Na^+ currents in lower eukaryotes reveals that, for the most part, protozoans use Ca^{2+} as the inward charge carrier; the lone exception known to date is the Heliozoan *Actinocoryne contractilis* (Febvre-Chevalier et al., 1986). Purely Na^+-dependent action potentials are not common until early metazoans, including cnidarians. This distribution of currents prompted Hille (1984) to speculate that Na^+ channels evolved from Ca^{2+} channels in parallel with the evolution of the first nervous systems. The selection of Na^+-selective channels would arise from the fact that neurons differ from protozoa inasmuch as the former can produce large numbers of action potentials, often at high frequency. If Ca^{2+} were the only charge carrier, toxic levels of intracellular Ca^{2+} would likely accumulate during neural activity. At first, comparisons between the structure of Na^+ and

Ca^{2+} channels, including those in lower invertebrates, provided little support for this model. However, with the cloning of the first LVA Ca^{2+} channel, the link became very evident (Spafford et al., 1999), and some current evolutionary trees show Na^+ channels evolving from LVA channels. This is not altogether surprising since the types of current produced by LVA channels — fast, transient currents — are very similar to those produced by Na^+ channels.

One significant and particularly intriguing question in the lineage of voltage-gated ion channels is the timing of the two gene duplications that gave rise to the four-domain protein, particularly with reference to the change in ion selectivity that ultimately gave rise to the first Ca^{2+} channel(s). Artificially created four-domain, 6-TM K^+ channel constructs prepared by connecting four identical (symmetrical) single-domain K^+ channel subunits, can produce functional K^+ channels (Liman et al., 1992). However, although a two-domain hybrid of one 6-TM domain and one 2-TM domain (TOK1), and a family of two-domain, 2-TM proteins (CeK family) have been found (Salkoff and Jegla, 1995), there are no known native two-domain K^+ channels of the 6-TM type, and no four-domain K^+ channels at all. This indicates either that the precursor channel had changed its selectivity prior to the first gene duplication, or that there was some selective pressure against multi-domain K^+ channels. Based on the structure of known voltage-gated K^+ channels in comparison to Na^+ and Ca^{2+} channels, it has been suggested (Salkoff and Jegla, 1995) that symmetry of the pore may be critical for K^+ ion selectivity (i.e. each of the four loops that constitute the lining of the pore should be identical or nearly identical). However, although the pores of extant Na^+ and Ca^{2+} channels are asymmetrical, that asymmetry presumably evolved over time through mutations. The original two-domain protein formed from the first round of gene duplication would have had two identical domains, and, if symmetry were essential to its function as a K^+ channel, mutations that disrupted that symmetry would, presumably, have been selected against. Similarly, the second round of gene duplication would have produced a protein in which domains III and IV were identical to I and II and, once again, mutations that produced undesirable asymmetry would have been selected against. Thus, if symmetry were a requirement for a K^+ channel, there is no a priori reason that a two- or four-domain channel has to be any less symmetrical than four identical one-domain, 6-TM proteins.

An alternative explanation for the origin of the four-domain 6-TM channels is that they and the one-domain, 6-TM K^+ channels both share a common origin (Fig. 2). The most likely candidate for this precursor is something akin to modern cyclic nucleotide-gated (CNG) channels, which are one-domain, 6-TM proteins. When first cloned, these channels were not immediately recognized as members of the voltage-gated ion channel family, but subsequent analysis revealed that they have a region similar to the S4 voltage sensor of K^+, Ca^{2+} and Na^+ channels, and a region that resembles the pore region of voltage-gated K^+ channels (for review, see Yau and Chen, 1995). CNG channels are now considered to be members of the voltage-gated ion channel family. Some extant CNG channels are activated by changes in membrane potential (Gauss et al., 1998; Krieger et al., 1999), and others display some voltage sensitivity in their responses to cyclic nucleotides. Furthermore, they are permeable to both monovalent and, to a greater extent in the case of the cGMP channel of vertebrate rods, divalent cations (Yau and Chen, 1995). Thus, they fulfill the requirements for a precursor of Ca^{2+} channels inasmuch as they have rudimentary voltage sensitivity and are permeable to Ca^{2+}.

One argument against the CNG channel being the precursor for four-domain 6-TM channels is that most modern four-domain channels do not have obvious cyclic nucleotide binding sites. However, it is conceivable that other ligand-activated channels with properties similar to those of the CNG channels may have been present, and one of these may have served as the precursor. In this context, it is interesting to note the recent discovery that the Ca^{2+} modulation of a diverse array of ion channels, including CNG channels and Ca^{2+} channels (Levitan, 1999), is mediated by way of constitutively bound calmodulin. This apparent conservation of the calmodulin binding site on these channels may be a direct link to the identity of the common ancestor of modern day voltage-gated ion channels. Interestingly, if something akin to an early CNG- or calmodulin-gated channel with properties similar to those of modern CNG channels were the precursor, then the voltage sensitivity of one-domain K^+ channels

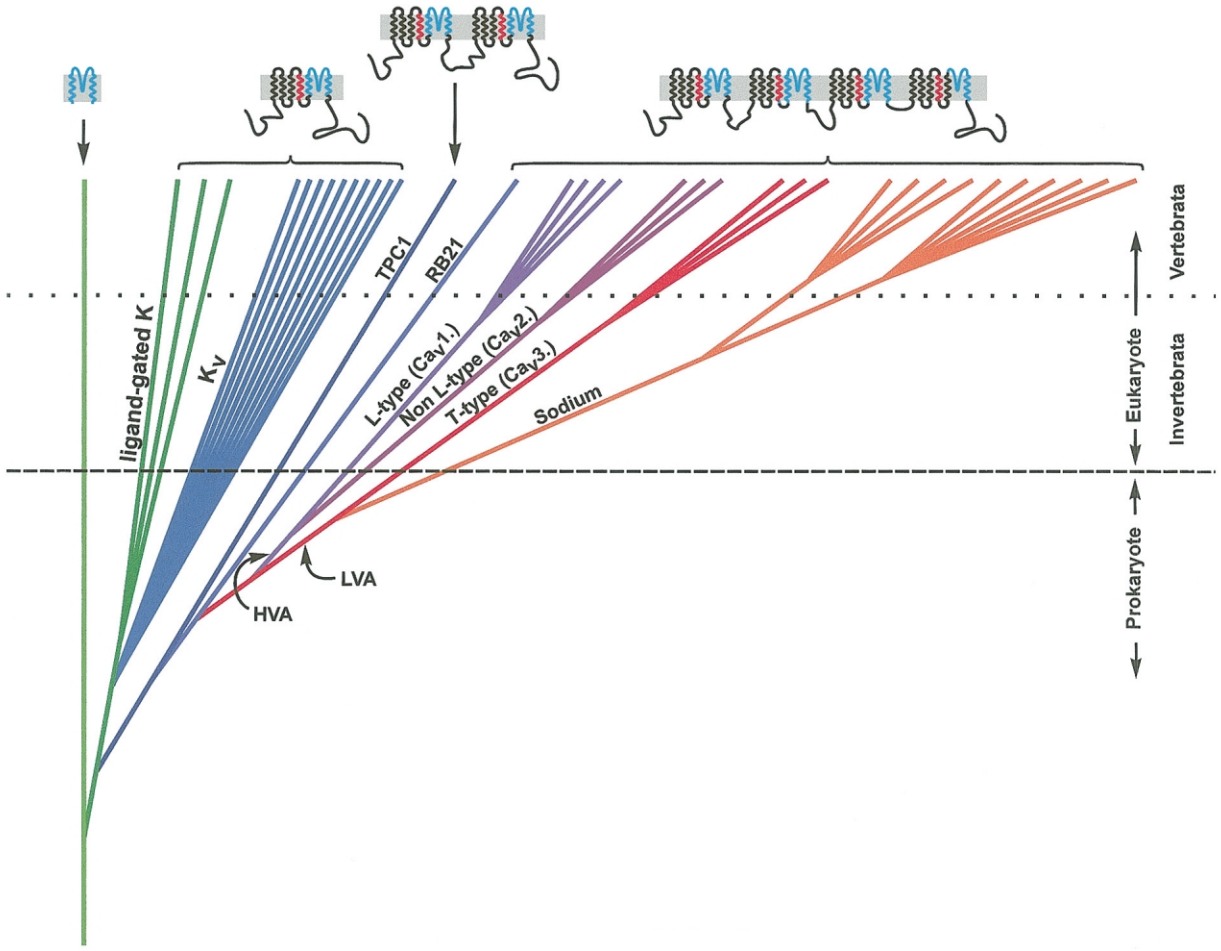

Fig. 2. A hypothetical phylogeny of voltage-gated ion channels. The model predicts that voltage-gated ion channels evolved over time (*Y*-axis not to scale) from a prokaryote 2-TM channel. Following the addition of four more domains, an early, ligand-gated, 6-TM protein gave rise to the voltage-gated K^+ family (Kv) and extant ligand-gated K^+ channels, and then, following two rounds of gene duplication, formed a four-domain 6-TM channel. Early four-domain channels were likely non-selective, but some are assumed to have developed calcium selectivity, giving rise to LVA and HVA calcium channels. Sodium channels are thought to have evolved from LVA channels.

and modern four-domain channels may reflect convergent evolution of the S4 region as a voltage sensor that triggers the conformational change associated with channel opening.

The selectivity of the two- and four-domain channels that resulted from the two rounds of gene duplication was likely to be similar to that of the precursor channel. As noted above, it appears that Ca^{2+}-selective voltage-gated ion channels preceded Na^+-selective ones. Although CNG channels are generally thought to be non-selective overall, they are typically more selective for divalent cations than they are for monovalent ones (for review see Yau and Chen, 1995), suggesting that they, or a related channel, represent the precursor of Ca^{2+} channels. Considerable work has been done by various groups to elucidate the structural basis of ion selectivity, and the general conclusion that has emerged is that single amino acid mutations can be sufficient to radically alter the selectivity of the channel (e.g. Mikala et al., 1993; Yang et al., 1993). Thus, although a change in ion selectivity has a major effect on ion channel function, the structural underpinnings of that change can be very minor. Nevertheless, it is not altogether unreasonable to assume that the earliest two- and four-domain 6-TM channels may have been relatively non-

selective, perhaps akin to CNG channels, and that they fine tuned their ionic selectivity to meet specific demands.

One question that remains is whether or not any two- or four-domain presumably non-selective channels continue to exist. In the case of two-domain channels, two candidates have recently been cloned, one (TPC1) from rat kidney (Ishibashi et al., 2000), the other from the plant *Arabidopsis*. Although TPC1 is similar in structure to Ca^{2+} and Na^{+} channels and aligns best with domains III and IV of those channels, Na^{+} and Ca^{2+} channels are more similar to one another than they are to TPC1, suggesting that the divergence of TPC1 preceded that of Na^{+} and Ca^{2+} channels. The *Arabidopsis* channel, which has only been reported as Genbank Accession number AAD11598, has fewer positive charges in the S4 region, and shares 26% identity with TPC1. Neither has been expressed, so their mode of activation and selectivity is unclear. A few four-domain channels with properties consistent with a lineage dating back prior to the Ca^{2+} channel/Na^{+} channel divide have recently been identified. The first (RB21) was found in rat and human tissue (Lee et al., 1999). Although it has not been expressed, its unusual ionic selectivity was inferred from the ring of amino acids around the pore of this channel (EEKE), a motif which causes an otherwise Ca^{2+} selective channel to become impermeable to divalents and to select for monovalent cations instead (Parent and Gopalakrishnan, 1995). Homologous channels were revealed by database screening and include two from the *C. elegans* genome and a putative Ca^{2+} channel from the yeast *Shizosaccharomyces pombi*. When these channels are compared phylogenetically with other known channels, they are found to cluster as a distinct group separate from both Ca^{2+} and Na^{+} channels (Lee et al., 1999).

The identity of the earliest Ca^{2+} channel is unclear, but it is likely that the emergence of the great diversity of Ca^{2+} channel types was a relatively early event and not an exclusively vertebrate phenomenon. The original classification of Ca^{2+} currents into L- and non-L types, and the subsequent separation of N-type currents into N, P, and Q, was derived from the known action of specific pharmacological agents on those currents. Those responses were seen almost exclusively in vertebrate cells, and for many years, it was felt that Ca^{2+} channels in invertebrates, which were frequently insensitive to many of the channel blockers in question, were 'different.' However, with the application of molecular cloning to invertebrates and the subsequent expression of some of the cloned channels, it has become evident that classifications based on pharmacology, which can be altered by as little as a single amino acid change, are not necessarily valid. For example, the jellyfish *Cyanea capillata* possesses a Ca^{2+} channel, *Cy*Ca_v1, which based on the structure is clearly an L-type channel (Jeziorski et al., 1998). Currents gated by this channel following expression in *Xenopus* oocytes are essentially insensitive to dihydropyridines, like non-L channels, highlighting the need to be cautious in defining a channel's classification based exclusively on its pharmacology. Interestingly, *Cyanea* possesses a second α_1 subunit (*Cy*Ca_v2) which based on structure is a non-L channel. However, *Cy*Ca_v1 is more similar to mammalian L-type channels (51%) than it is to *Cy*Ca_v2, which itself is more similar to mammalian non-L channels (59%) (Fig. 3). These facts suggest that the L-/non-L divide is very old and may well have predated the emergence of eukaryotes. Indeed, a search of various microbial genomes, using RB21, the four-domain

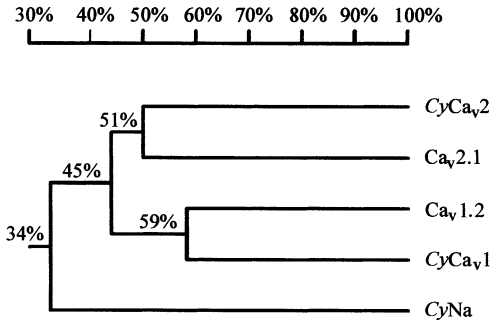

Fig. 3. A tree depicting the percent identity between an L-type (*Cy*Ca_v1) and a non-L-type (*Cy*Ca_v2) channel from the jellyfish *Cyanea capillata*, two equivalent mammalian channels ($Ca_v1.2$ and $Ca_v2.1$), and the jellyfish Na^{+} channel (*Cy*Na). The analysis, which was conducted using ClustalW (Thompson et al., 1994), as implemented in DNAMAN, v. 4.0 (Lynnon Biosoft), and restricted to domain III of each of the channels, reveals that the two jellyfish sequences are more similar to their mammalian counterparts (51 and 59%, respectively) than they are to each other (45%), indicating that the L-/non-L type divide greatly preceded the emergence of the Cnidaria. The two calcium channel families are more similar to one another than they are to the outlying jellyfish Na^{+} channel (*Cy*Na) (34%), highlighting the difference between these widely different four-domain, 6-TM channels, even in the earliest eukaryotes with a nervous system.

6-TM sequence with unpredictable ionic selectivity (Lee et al., 1999) as a template (R. Greenberg, unpublished), yields at least one bacterial sequence with high levels of identity, and it would not be altogether surprising if future work identifies complete 4-domain 6-TM channels in some prokaryotes. However, with the completion of the *C. elegans* and *Drosophila* genome projects, it is clear that neither species has the same diversity of Ca^{2+} channels as mammals; five Ca^{2+} channel homologs have been identified in *C. elegans* and four in *Drosophila* (Littleton and Ganetzky, 2000), suggesting that much of the mammalian diversity occurred later. The same is true to a greater extent for Na^+ channels — as indicated earlier, *Drosophila* has only two Na^+ channels whereas *C. elegans* appears to have lost all Na^+ channel genes.

The presence of such diversity in all channels raises two major questions — when did it occur, and why? The timing of the duplications will probably become evident as genome information and additional sequence information become available, but the question of why there is so much diversity is intriguing.

The enormous diversity is best exemplified by K^+ channels. Most organisms, including some of the earliest such as sea anemones (Anderson, unpublished), jellyfish (Jegla et al., 1995), and even *Paramecium* (Jegla and Salkoff, 1995), possess multiple K^+ channel subtypes. *C. elegans* alone has 76 different K^+ channels, and *Drosophila* has 27 (Littleton and Ganetzky, 2000). This inevitably begs the question of why so much diversity is required, particularly when one bears in mind that K^+ channels are heteromultimers whose functional properties vary according to the types of subunits that create the functional channel (Covarrubias et al., 1991). Is the diversity necessary to produce channels with exactly the right functional properties to meet defined needs, or is it simply a developmental oddity resulting from the segregation of different channel types in different tissues or developmental stages, under the control of different promotors, thus making them available for independent evolution? The fact that single cells can express multiple K^+ or Ca^{2+} channels (Nowycky et al., 1985; Butler et al., 1989) at the same time suggests that part of the diversity is indeed required to meet different functional needs. However, although different variants of the same channel type do gate currents with slightly different macroscopic properties, the impact of these differences on the amount of charge that crosses the membrane during the time course of, for example, a 5-ms action potential may not be significant. If that impact is negligible, then it would suggest that much of the diversity reflects the evolution of tissue- or developmentally-segregated channel subtypes, which may be governed by the need to merely maintain ion selectivity and other broad properties, as opposed to selection of channel variants to meet very specific functional needs.

7. The use of evolutionary information for structure / function analysis

Evolutionary information of the type presented above can be viewed in two ways, either as an interesting side bar to on-going studies of modern day ion channels or, more constructively, as a means by which to gain useful information that is directly applicable to our understanding of ion channel function. Following the major breakthrough in our understanding of ion channel structure achieved by MacKinnon and his colleagues with the crystallization of KcsA (Doyle et al., 1998), a 2-TM K^+ channel from *Streptomyces*, most neurobiologists would likely argue that they fully appreciate the utility of ion channels in lower organisms. However, what is not commonly perceived is that the use of evolutionary information can provide other, perhaps more subtle, information that is often exceedingly difficult to obtain from more traditional approaches. Indeed, as the search for other ion channels in prokaryotes and lower eukaryotes continues, aided by various genome projects, it may prove possible to generate crystals for other types of ion channels, thereby providing much needed information about the tertiary structure of more complex channels. However, if we are to fully understand the structure/function relationships of ion channels and critical issues such as how various modulatory subunits interact with the pore-forming units, then other approaches are needed. Here, comparisons between phylogenetically distinct ion channels can yield useful information that would be otherwise difficult to obtain. The remainder of this review will discuss two instances where this phylogenetic approach can significantly improve the signal-to-noise ratio inherent in sequence information,

thereby simplifying the investigator's task of assigning structure to function.

The β subunit of voltage-gated Ca^{2+} channels is a cytoplasmic protein that modulates the function of the α_1 subunit in a variety of ways, including the level of Ca^{2+} channel expression, the rate of inactivation, the voltage-dependency of activation (for review see, Stea et al., 1995), and the rate of recovery from inactivation (Jeziorski et al., 2000b). The function of β subunits seems to have been conserved across many millions of years of evolution. Co-expression of a β subunit from the jellyfish *Cyanea capillata* with the jellyfish α_1 subunit (*Cy*Ca_v1) reveals that the β subunit modulates the α_1 subunit in exactly the same manner as occurs with mammalian channels. That is, it increases the magnitude of current carried by the α_1 subunit, accelerates the rate of inactivation, and produces a negative shift in the I/V relationship of that current (Jeziorski et al., 1999). This conservation of function among β subunits is highlighted by the fact that *Cy*Ca_v1 can be modulated in the same way by co-expressed mammalian β subunits.

The β subunit of a functional Ca^{2+} channel binds to the α_1 subunit at a site termed the alpha interaction domain (AID) (Pragnell et al., 1994).

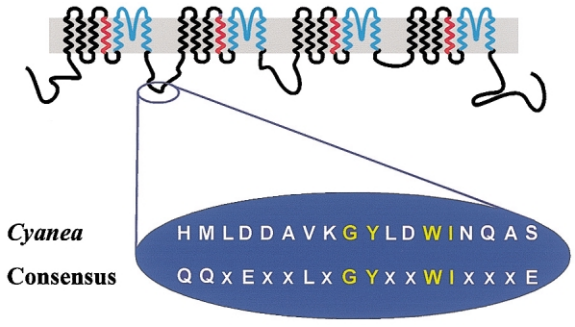

Fig. 4. An illustration of the utility of using broad phylogenetic sequence comparisons to improve the signal-to-noise ratio inherent in more restricted sequence alignments. Here, the region of the cytoplasmic loop that connects domains I and II of calcium channel α_1 subunits, and has been implicated in binding to β subunits, is compared between *Cyanea* and a consensus sequence. The consensus was derived from an alignment of sequences of α_1 subunits from mammals, the marine ray, *Drosophila*, and the fly, *Musca*. In the consensus sequence, residues that differ in at least one sequence, including conserved substitutions, are represented by 'X'. Residues that are identical in both the consensus and the *Cyanea* sequence are colored yellow. Of the four identical residues, three (Y, W, I) are reported to be most essential for interactions between α_1 and β subunits (De Waard et al., 1996).

This site was mapped to the linker between domains I and II of the α_1 subunit and subsequently to a nine amino acid stretch in this region. Using a combination of immunoprecipitation and site-directed mutagenesis of each of the residues in this stretch, Campbell and his colleagues concluded that three amino acids were critical to that interaction (Fig. 4) (De Waard et al., 1996). The AID in the jellyfish α_1 subunit is rather poorly conserved overall (Jeziorski et al., 1998) — only four of the amino acids are identical to those in an α_1 subunit consensus sequence derived from various mammalian, vertebrate and invertebrate sequences. Interestingly, three of those four residues are amino acids critical for the α_1/β subunit interactions (De Waard et al., 1996). The point to be made here is that when function is conserved, as is the case with the α_1/β interaction, alignments across large phylogenetic distances can help reveal the signal that is concealed within the noise that is inherent in alignments that exclude important phylogenetic variation.

The potential of this approach is evident in another facet of the α_1/β interaction. To date, four classes of mammalian β subunits have been identified (β_{1-4}), with each class being composed of up to four splice variants (e.g. β_{1a-c}, β_{2a-d}). The degree of amino acid identity within the four classes of β subunits is sufficiently high that in alignments of mammalian β subunits (Fig. 5A), large areas of very high identity predominate. Such high levels of identity or conservation make the assignment of function to particular regions of the subunit exceedingly difficult.

When the jellyfish β subunit is added to the alignment, together with one from the house fly *Musca* (Grabner et al., 1994), which produces exactly the same kind of modulatory effects on α_1 subunits, much of this conservation is lost (Fig. 5B), and the amount of identity decreases from 55 to 34% in the central conserved core and from 47 to 39% overall (Fig. 5C). Because the function of the different β subunits is conserved, we know that the remaining regions of identity are those that are critical to the observed functions. In this context, it is interesting to note that islands of regularly repeated charged residues, reminiscent of the S4 regions of pore-forming channels, remain. Given that some of the modulatory actions of β subunits (e.g. the hyperpolarizing shifts in the I/V relationship) may derive from interac-

Fig. 5. A further example of information that can be revealed by aligning sequence information from functionally conserved but phylogenetically distant proteins. (A) Consensus β subunit, obtained through an alignment of human β_1, β_2, β_3, and β_4. Identical residues are marked with dark blue, conservative substitutions are indicated in light blue, with '+' or '−' indicating the charge on the conserved residue, and 'p' and 'n' indicating a conserved polar or non-polar residue, respectively. (B) The residues that are no longer conserved when β subunits from the fly *Musca* and the jellyfish *Cyanea* are added to the alignment. (C) The consensus after inclusion of the fly and jellyfish sequence. Identical or functionally conserved residues are marked yellow, positively charged residues are marked red, negative ones blue. Note that this approach highlights regions of conserved charge, such as the nine negatively charged residues at the carboxyl terminal of the conserved central core, and the preceding span of 10 positive charges.

tions between charged residues, this broad phylogenetic alignment may, in essence, be highlighting the regions in question.

The basis of the approach described above is to try to match structural conservation with functional conservation. The complement to this would

be to try to correlate structural differences with either the loss or acquisition of new function. Given that, overall, the structural differences between phylogenetically diverse channels tend to be far greater than the similarities, this approach may be more difficult. However, in many phylogenetically distant channels, there are very often islands of high identity such as transmembrane segments and pore regions. If a specific function can be mapped to one of those regions using other approaches, then in channels that lack that particular function (i.e. drug sensitivity), structural differences in that otherwise conserved region may reveal critical components of a particular function, for example, a critical component of a drug's binding site. Thus, both approaches have the potential to yield very useful information that would be difficult to acquire otherwise and, as such, give new applications for comparative studies.

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