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·Review·

Emerging approaches to probing ion channel structure and function

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Abstract: Ion channels, as membrane proteins, are the sensors of the cell. They act as the first line of communication with the world beyond the plasma membrane and transduce changes in the external and internal environments into unique electrical signals to shape the responses of excitable cells. Because of their importance in cellular communication, ion channels have been intensively studied at the structural and functional levels. Here, we summarize the diverse approaches, including molecular and cellular, chemical, optical, biophysical, and computational, used to probe the structural and functional rearrangements that occur during channel activation (or sensitization), inactivation (or desensitization), and various forms of modulation. The emerging insights into the structure and function of ion channels by multidisciplinary approaches allow the development of new pharmacotherapies as well as new tools useful in controlling cellular activity.

Keywords: ion channel; structure and function; mutagenesis; covalent modification; voltage-clamp fluorometry; computational chemistry

1 Introduction

Ion channels are membrane-embedded complexes forming pores in the cell membrane, and are clearly the most fundamental elements in the membranes of excitable cells. Historically, the early descriptions of ion channels simply used the concept of conductance, relying on the description of current-voltage relationships at the black-box level in the absence of any deeper understanding of the physical basis of the conductance[1]. Following the development and improvement of the patch-clamp technique by Neher and Sakmann[2] and others[3], the notion of the conducting pore as part of an ion channel with properties of permeation, selectivity, and gating[1] has been established. Since patch clamp technology provides the channel activity in real time and at the single-molecule level, it has become central to structural, functional, and regulatory studies in the ion channel field.

The subsequent era of ion channel study was molecular cloning of the physical basis of ionic conductance[1], which initiated our understanding of ion channels at the molecular level. Molecular cloning[4-7] has unraveled the primary structures of channel proteins by providing the sequence of amino-acids making up the ion channel. In addition, it has expanded our view of the type and variety of ion channels, as the human genome project has identified over 400 species of ion channel genes[8]. Furthermore, molecular cloning demonstrates the conservation of ion channel sequences and consequently provides the basis by which ion channels are classified into superfamilies. In addition to sequence homology, functional and regulatory features are also considered.

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In general, ion channels can be classified into three main groups. The first consists of the voltage-gated channels[9] such as voltage-gated sodium (Na_V), potassium (K_V), and calcium (Ca_V) channels which control neuronal signaling, muscle contraction, and hormone secretion. The second includes the extracellular ligand-gated ion channels (LGICs)[10] such as the nicotinic acetylcholine receptor (nAChR), γ-aminobutyric acid (GABA) and glycine receptor channels, most of which are activated by ligands that are neurotransmitters. The third contains the intracellular ligand-gated ion channels[11] including those activated directly by intracellular ligands such as Ca^{2+}, phosphatidylinositols or phosphoinositides, cyclic nucleotides, and other second messengers, or indirectly by the G-protein-coupled receptors. Although there are different types, all ion channels essentially function by taking a general event such as ligand binding or mechanical deformation, and transforming it, via a change in protein conformation, into a change in intracellular chemistry by permitting the flow of specific ions into or out of the cell. For voltage-gated ion channels or LGICs, changes in membrane voltage or the presence of specific ligands are converted into changes of membrane permeability through a multi-step process in which the voltage-sensing (Fig. 1A, the first to fourth membrane spanning segments termed S1–S4) or the ligand-binding domain (LBD, Fig. 1B) rearranges, and these movements then lead to the opening of a transmembrane pore (Fig. 1A, S5–S6; Fig. 1B, ion pore domain, IPD). After pore opening, a selective set of ions rapidly passes through the pore across the cell membrane. The structural and molecular mechanisms underlying the above processes occur at different levels. Events at the atomic level remain elusive, and will ultimately require multidisciplinary approaches including molecular and cellular, chemical, optical, biophysical, and computational methods to address these structural and functional aspects of ion channels.

2 Tandem construction of ion channel subunits

Molecular cloning and heterologous expression expanded our view of the types and variety of channels and provided effective ways to identify the cloned channels with their counterparts studied in native cells[1]. However, the natural expression of ion channel subunits in heterologous cells raises the important issue of the stoichiometry and assembly mechanisms of functional channels. To answer these questions without protein chemistry, researchers[14] developed a strategy using the end-to-end tandem fusion of multiple channel-coding regions into a single longer reading frame (Fig. 2). To determine the stoichiometry of K_V channels, Isacoff *et al.*[15] showed that double-length tandem constructs always form functional channels and suggested that there would be even numbers of subunits in a channel (Fig. 2A–C). However, Liman *et al.*[16] studied the constructs with 2, 3, 4, and 5 repeats and found that functional channels form with all of these constructs (Fig. 2A–D), implying that subunits from tandem constructs can join mutually to form complexes with a functional pore. Therefore, tandem linkage of ion channel subunits alone does not ensure the stoichiometry of expressed channels[17].

To be able to track subunits entering potassium channel complexes, Liman *et al.*[16] made a point mutation which resulted in a dramatically reduced sensitivity to the blocking effects of tetraethylammonium (TEA), a tangible biophysical feature distinct from the wild-type (WT) channel subunit. Coexpressing an individual copy of the TEA-insensitive subunit with a tandem composed of three (Fig. 2E) but not four copies (Fig. 2F) of WT subunits formed a channel with decreased sensitivity to blockade by TEA[16]. This suggests that a functional K_V channel is a tetramer, and most importantly, the subunits from intra- rather than inter-tandem constructs preferentially form a functional channel pore. Moreover, placing a mutated repeat at various positions within the tandem construct (Fig. 2G–J) led to a similar extent of reduced sensitivity to TEA blockade, and tetrameric constructs with varying numbers of mutant subunits (Fig. 2K–N) showed number-dependent changes in TEA sensitivity[16], both of which strengthened the notion that K_V channels are tetramers. The tandem construct strategy combined with mutagenesis (see below) that introduces a specific biophysical property has been used as a powerful method to analyze the stoichiometry of dimeric

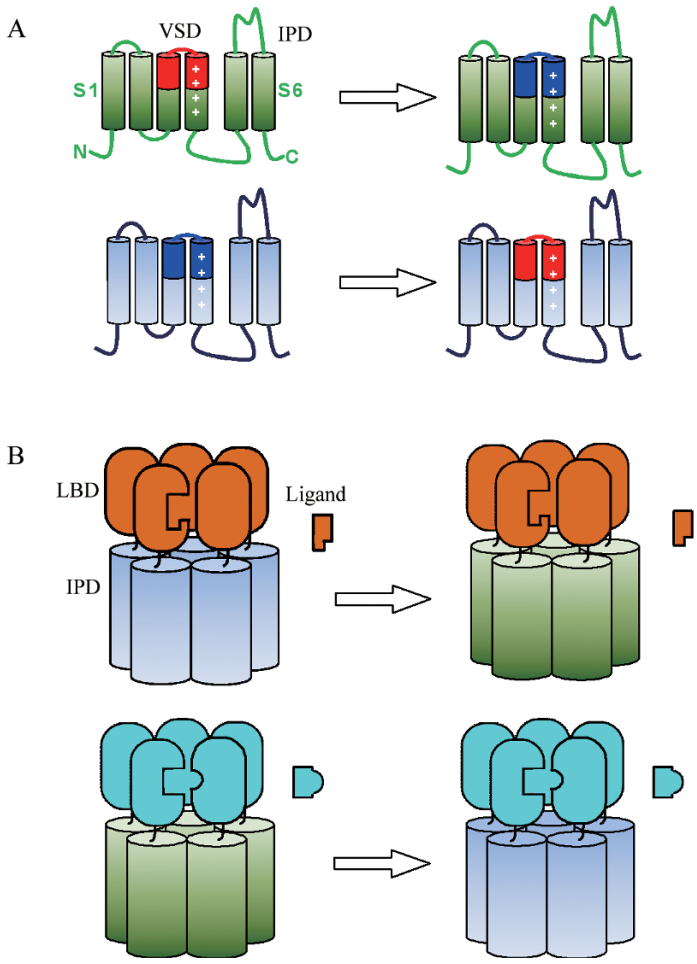

Fig. 1. Modular nature of voltage- or ligand-gated ion channels. A: Each subunit of the voltage-gated ion channel is composed of a voltage-sensing domain (VSD) corresponding to the S1–S4 segments and an ion pore domain (IPD) corresponding to the S5–S6 segments. The paddle domain (the red or blue regions) composed of S3b and S4 helices from one channel can be transplanted to another, forming a chimeric channel. Modified from Bosmans *et al.*, Nature 2008[12]. B: Structural models of ligand-gated ion channels (exemplified by the Cys-loop receptor superfamily) and the portability of the ligand-binding domain (LBD) and IPD modules. Further details in text. Modified from Magnus *et al.*, Science 2011[13]. The different colors represent the similar domains in different ion channels.

voltage-gated proton (H_V) channels[18], trimeric ATP-gated P2X receptor channels[19], tetrameric inwardly-rectifying potassium channels[20], and pentameric A-type GABA receptor ($GABA_AR$) ion channels[21]. Strikingly, using a triple-length tandem construct of P2X receptor subunits, Browne *et al.*[19] replaced the residue believed to be at the narrowest point of the channel, threonine (Thr)-339, with a lysine in one, two, or all three pore-forming regions and found that these mutations progressively increased the relative Cl^- permeability and reduced the unitary cation conductance regardless of which pore-forming region within the tandem was mutated. This result suggested that[19] the Thr-339 from each subunit contributes equally to the open channel permeation pathway, raising a symmetrical separation of these three pore-forming regions during the closed-open transition process of P2X receptor channels. Together, tandem construction of ion channel subunits is an effective strategy to study the stoichiometry

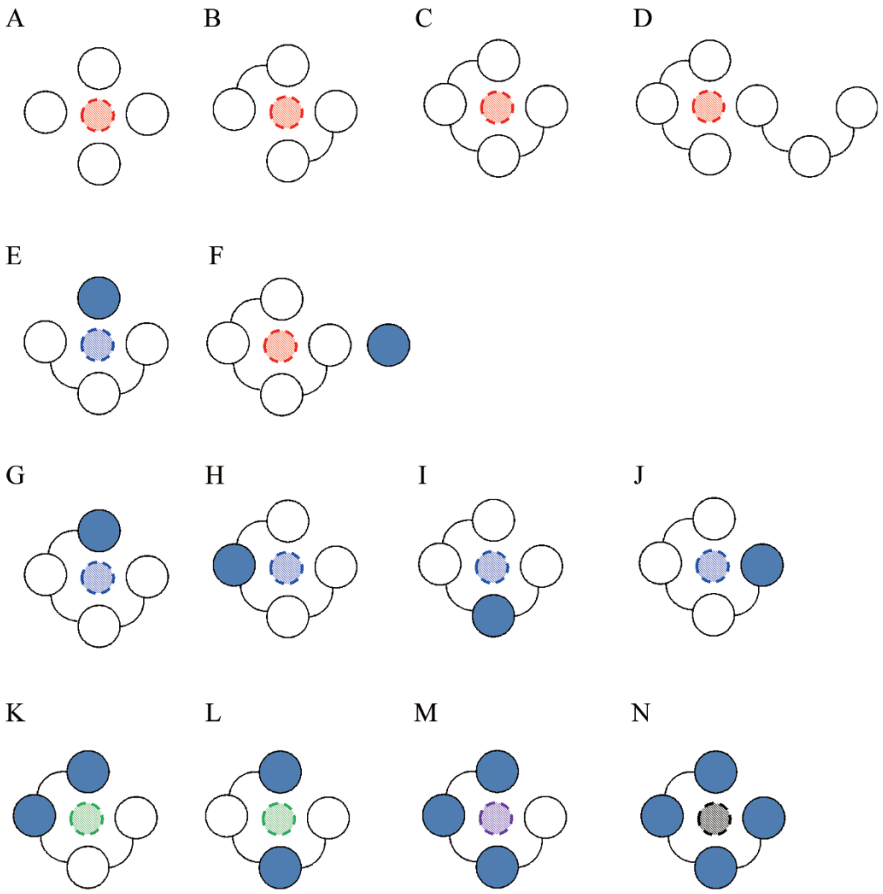

Fig. 2. Determination of the subunit stoichiometry of an ion channel by comparing channel properties of single and tandem constructs. A strategy combining the end-to-end tandem fusion of multiple channel-coding regions into a single longer reading frame (A–D) and mutation (E–N) gaining a characteristic biophysical property distinct from the wild-type (WT) channel subunit can be used to probe the channel assembly andstoichiometry (see the text). Open circles represent WT while filled circles represent mutant subunits. Dashed circles represent channel pores, for which different colors refer to different channel pore properties. Further details in text.

and assembly of ion channels.

3 Modular nature of functional domains in an ion channel

Similar to other functional proteins such as enzymes, an ion channel contains different domains or structural motifs that correspond to specific steps underlying channel activation. In voltage-gated ion channels, the voltage-sensing domain is responsive to changes in membrane voltage[22,23] and it initiates the conformational changes of the entire protein including the IPD (Fig. 1B). Within each subunit of the tetrameric K_V channel shown in Fig. 1A, there are basic residues (lysine, Lys or arginine, Arg, Fig. 3A) in the S4 region that carry positive charges[6], which are coupled with negatively-charged acidic residues in the other three transmembrane segments (S1–S3)[24], forming the S1–S4 voltage-sensing domain. As voltage sensors, that the S1–S4 domain is a functionally independent protein domain was supported by the demonstration[25,26] that a eukaryotic voltage-gated K_V channel S1–S4 region endows the minimally voltage-sensitive prokaryotic KcsA channels with strong voltage sensitivity. Moreover, bacteria can synthesize, fold properly, and insert the isolated S1–S4 voltage-sensing domain into the membrane[27,28], further

underpinning the idea that S1–S4 is an independent domain. The paddle domain, a motif composed of S3b and S4 helices (Fig. 1A, the red and blue regions), can drive activation of the voltage sensors and opening of the channel pore in response to membrane depolarization when transplanted from an archaebacterial K_V channel (K_VAP) or voltage-sensing domain proteins (i.e. *Ciona intestinalis* voltage sensor-containing phosphatase) into eukaryotic K_V channels[29]. Interestingly, the chimeric channels show the same sensitivity to tarantula toxins that are known to inhibit K_V channels by interacting with the acquired voltage sensors. Using the modular nature of paddle motifs in the voltage-sensing domains[29], Bosmans *et al.*[12] created a series of chimeric channels composed of the paddle motif from Na_V and the remaining portion from K_V channels to study the potentially differential roles of distinct amino-acid sequences in the four voltage sensors of Na_V channels. Among the four individual S3b–S4 paddle motifs within the Na_V channel, they found one unique paddle motif that slows voltage sensor activation and constitutes the selective target for tarantula and scorpion venom toxins which impede Na_V channel inactivation[12]. This study established a more specific pharmacological target within Na_V channels. The above examples demonstrate the modular nature of voltage-gated ion channels as well as the importance of motif portability in the study of their structure and function.

Coincidentally, LGICs such as the Cys-loop receptor superfamily[10] form a group of modular combinations of pharmacologically-selective LBDs and functionally diverse IPDs, both of which are independent and transportable from one channel to another (Fig. 1B). The LBD of the α7 nAChR behaves as an independent actuator module that can be transplanted onto the IPDs of other Cys-loop receptors such as ionotropic serotonin receptors (5HT3Rs)[30] or glycine receptors (GlyRs)[31], endowing the resulting chimeric channels (α7-5HT3R or α7-GlyR) with α7 nAChR pharmacology and cation or chloride conductance properties, respectively. Based on the modular property of LGICs and using chemical synthesis and protein engineering, Magnus *et al.*[13] developed a systematic toolbox of LGICs with orthogonal pharmacologic selectivity and divergent functional properties. Using these constructs, they were able to activate a range of ionic conductances in genetically-specified cell types and selectively manipulate neuronal activity in mammalian brains *in vivo*. Because ion channels, either ligand- or voltage-gated, operate mechanistically by coordinating multiple independent domains or structural motifs, probing the domains or motifs that underlie particular functional properties is an indispensable but arduous task in ion channel research. As a first step, searching for conserved or slightly modified sequence patterns using sequence homology and transplanting these sequences among different members of a superfamily[1] open new avenues for determining the relationship between the structural and functional properties of ion channels.

4 Site-directed mutagenesis

The long amino-acid sequence that makes up the channel protein presents an overwhelming impediment for unraveling the specific role of each amino-acid in channel operation[1]. Site-directed mutagenesis is a straightforward approach whereby a single amino-acid is changed at the DNA level, and the resultant mutant is then expressed in heterologous cells to test the effect of the mutation on channel function.

4.1 Feature-guided mutagenesis In the absence of detailed structural information to guide mutagenesis, selecting residues of potential functional significance based on side-chain chemistry (Fig. 3) is an excellent starting point. After the cloning of an ion channel subunit[4-7], transmembrane topology is predicted based on the presence of hydrophobic amino-acid clusters. However, the predicted membrane topology is not conclusive. In the earlier studies of ion channel membrane topology without protein chemistry, electron microscopy was successfully used as a complementary method to reveal the membrane topology of ion channels such as GlyRs[32]. Another excellent example of a well-conserved sequence pattern is the string of basic residues (Lys or Arg, Fig. 3A), each separated from the next by two hydrophobic residues in the S4 segment of Na_V, K_V, and Ca_V channels. When the first sequence of the voltage-gated channel was revealed, it was noted that the S4 seg-

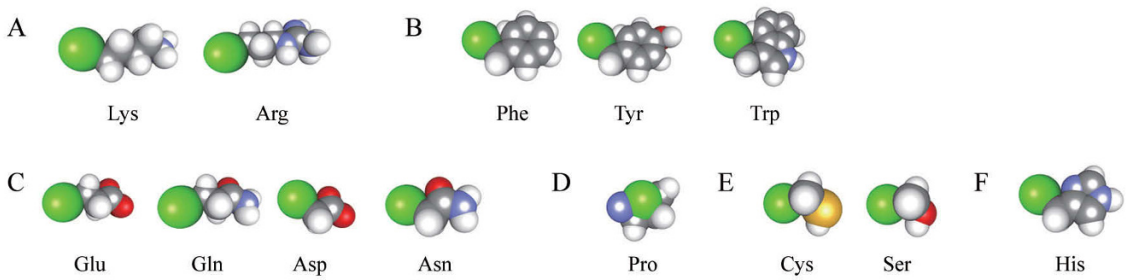

Fig. 3. Structures of side-chains of natural amino-acids in ion channel proteins. The Cα atoms (not included in side-chains) are displayed as green balls. In (D, Pro), for clarity, the N atom and the carbonyl group [displayed in the Corey-Pauling-Koltun (CPK) scheme, see below] of the protein backbone in addition to the Cα atom (green), neither included in side chains, are also displayed. The remaining part illustrating the side chain of amino-acids in ion channels are shown in the space-filling CPK scheme, in which the hydrogen atoms are displayed as white balls, carbon as black, nitrogen as blue, oxygen as red, sulfur as deep yellow, respectively. Further details in text.

ment could serve as a potential voltage sensor because of the presence of charged residues, which presumably would move within the electrical field of the membrane[5]. Studies were immediately initiated to modify the S4 sequence pattern by site-directed mutation to determine if the voltage-dependence could be affected[33,34]. Neutralization of the basic residues[33,34] indeed changed the voltage-dependence of the conductance, as expected. It was also found that mutation of uncharged residues had similar effects on the voltage-dependence of the conductance[35], suggesting that the structural integrity of S4 plays an essential role in determining the relative stabilities of the closed and open states of the channel. Moreover, negatively-charged acidic residues[24,36] in S2 and S3 have been shown to form electrostatic interactions with the positively-charged basic residues of the voltage-sensing S4 sequence. Thus, searching for signature sequences and making side-chain-guided mutations are a good starting point for probing the structure and function of ion channels.

4.2 Scanning mutagenesis Although side-chain property-guided mutagenesis has been encouraging for probing the functional roles of residues in a channel protein, it may also be somewhat fortuitous, because critical sequences can often be difficult to be identified simply based on charge and hydrophobicity. In this regard, scanning mutagenesis is an alternative strategy that allows elimination of potential interactions (loss of function) or creation of a constant interaction (gain of function) by substituting individual residues in a targeted domain one or several at a time. Because alanine (Ala) has only a methyl group as its side-chain, its insertion minimizes potential interaction with other sites without leading to an increase in backbone flexibility such as that seen with glycine (Gly). As a result, alanine-scanning mutagenesis is the most common way to remove all the potential interactions around a specific site. This scanning strategy can be used to probe multiple structural aspects of ion channel function[37-39], including activation and modulation.

Since the hydrophobic interactions between ion channel proteins and membrane lipids are thought to be generally nonspecific, it is possible to identify lipid-contacting residues in the transmembrane domains of ion channels by systematic incorporation of hydrophobic residues such as tryptophan (Trp, Fig. 3B), a residue with a large hydrophobic side-chain. Those residues that tolerate mutation to Trp are thought likely to be facing the membrane. Through tryptophan-scanning mutagenesis, the transmembrane structure[40,41], the molecular mechanism of channel regulation[42,43] by hydrophobic molecules, and even the gating mechanisms[42,44] of various types of ion channels have been revealed. In addition, to distinguish between water-accessible positions and positions adjacent to membrane lipids or within the protein interior, residues in transmembrane domains can be mutated individually to aspartate (Asp, Fig. 3C). Due to the energy cost of burying a charge in a hydrophobic environment, an amino-acid with a charged

side-chain should not be tolerated at lipid-facing or interior positions. Based on the above scanning mutagenesis strategy, Collins *et al.*[45] established the first structural model of inwardly-rectifying potassium channels and provided strong evidence supporting the notion that the membrane-spanning segments are α-helices.

Proline (Pro) is the only α-imino acid present in proteins. Its presence in a protein always dramatically decreases the flexibility and changes the orientation of the carbon backbone (Fig. 3D). Consequently, proline-scanning mutagenesis can be used to probe gating machinery by generating constitutively active ion channels[46].

Cysteine (Cys, Fig. 3E) has a thiol group as the side-chain that can be covalently modified by thio-oxidants. In ion channels and other proteins, two adjacent and appropriately orientated cysteines often form a disulfide bond, which plays an important structural role[10,47,48] or even acts as a physiological sensor[49] for reducing factors. In cysteine-scanning mutagenesis, Cys is introduced as a single amino-acid replacement in a channel protein that had been engineered to remove all native cysteines[50,51]. Cysteine mutagenesis together with the covalent modification induced by methane thiosulfonate (MTS) reagents[52] makes up the substituted-cysteine accessibility method (SCAM). SCAM can be used to determine the solution accessibility of particular sites, which provides an effective approach to identify residues in the membrane-spanning segment that line the pore of a channel[53,54], determine differences in the structures of the membrane-spanning segments in different functional states of the channel protein[55-57], map the electrostatic potential in the membrane-spanning domains, and define the size of a channel or ligand binding-site crevice[58].

4.3 Proton transfer Histidine (His, Fig. 3F) is a characteristic residue associated with pH changes because of its positively-charged imidazole functional group. The imidazole side-chain of histidine has a *p*Ka of ~6.0, and, overall, the amino-acid has a *p*Ka of 6.5, meaning that, at physiologically relevant pH values (~7.2), a minimal pH shift changes the average charge. When the pH is below 6, the imidazole ring is mostly protonated and therefore positively-charged. As a result, histidine residues mediate many types of channel activation or regulation by acidic or alkaline pH. By virtue of its protonability, histidine-scanning mutagenesis can be used to monitor the movement of specific channel domains such as the voltage sensor upon voltage changes. The accessibility of each residue in and around the sensor when mutated to histidine, measured by the response to pH changes, can be used to reflect movements of the voltage sensor in response to a change in the membrane potential. To expose the movement of voltage sensors such as the S4 segment in K_V channels upon voltage changes, each basic residue can be individually replaced with a histidine. If the histidine-substituted residue participates in the formation of the voltage sensor, then the gating charge displaced by the sensor will take account of the histidine charge. The accessibility of histidine residues to the bulk solution can then be monitored by pH-dependent changes in the gating currents evoked by membrane potential pulses. Because histidine accessibility can be detected by labeling with protons, its very confined local environment can be resolved and the proton-labeling introduces minimal interference with voltage sensor motion. Using the histidine-scanning mutagenesis strategy, Starace *et al.*[59] identified the residues that move in the transmembrane electrical field in response to membrane voltage changes in K_V channels.

In addition to His and Lys, the residues Arg, Asp, and Glu (Fig. 3A, C) are also ionizable in response to different ranges of pH change. In the channel protein, charges on ionizable side-chains control single-channel conductance[60,61], ion selectivity[62-64], open-channel block[65,66], gating[67], and voltage sensing[36,68]. Using the ionizable Lys, His, and Arg, Cymes *et al.* developed a single-channel proton-transfer technique[69,70] to probe the conformation of the pore opening state. In this electrophysiological approach, a Lys, Arg, or His (Fig. 3A, F) is systematically introduced at different positions in the transmembrane helices forming the channel pore. As a result, single-channel recording reflects current fluctuations between two levels: one corresponding to the 'neutral' pore and the other to the pore carrying an extra unit of charge. Exemplified in

the case of a single basic residue engineered into a cation-selective pore, the 'deprotonated pore' is expected to have roughly the same conductance as the WT channel, whereas the 'protonated pore' carrying a positively-charged residue is expected to conduct cations at a lower rate. Accordingly, the occurrence of protonation-deprotonation events in an open channel should be seen as oscillations between two discrete current sizes. Consequently, the proximity of the protonatable group to the long axis of the pore can be measured as the ratio of these two current values. The larger the extent of the block, the closer the engineered ionizable side-chain is to the long axis of the pore. In addition, the kinetics of the current fluctuations manifest the rates of proton transfer as well as the *p*Ka of the side-chain within the specific microenvironment. The more the *p*Ka value declines, the poorer the positive-charge-stabilizing property of the side-chain's microenvironment compared to that of bulk water[71-73]. By applying this methodology to the pore-lining M2 transmembrane α-helix[69] as well as the entire M1 and M3 segments[74] of the nAChR, and taking into account the existing closed channel structure, Cymes *et al.* revealed that the pore dilation that underlies channel opening involves only a subtle rearrangement of these three transmembrane helices.

The nAChR superfamily has evolved to generate both cation- (i.e. nAChR and 5-HT3R) and anion-selective members (i.e. $GABA_AR$ and GlyR), raising a fundamental question about the structural basis of this difference. Using the single-channel proton-transfer technique to estimate the protonation state of native ionizable side-chains, it was found that[75] anion-selective-type sequences favor, while cation-selective-type sequences prevent, the protonation of the conserved, buried basic residues at the intracellular entrance of the pore. That is, the tunable charge state of the ring formed by a buried basic side-chain from each subunit is an essential feature of these channels' versatile charge-selectivity filter. On the whole, these examples clearly demonstrate that estimating the *p*Ka values of basic and acidic side-chains using electrophysiological recordings[70] is a robust approach to studying the structure and function of ion channels.

4.4 Unnatural amino-acid mutagenesis As a powerful extension of site-directed mutagenesis, unnatural amino-acid mutagenesis makes possible the site-specific incorporation of synthetic amino-acids, enabling detailed structure-function studies as well as the incorporation of optical probes (see below) into ion channels[76]. This method uses nonsense-suppression methods for the incorporation of unnatural amino-acids into proteins expressed in living cells[77]. Following earlier work in the biology of nonsense suppression, which reported the general method for the biosynthetic incorporation of unnatural amino-acids[78,79], the protocol has been adapted for use with heterologous expression systems such as the *Xenopus* oocyte[77,80]. The basic method[76] for *in vivo* nonsense suppression first entails mutating a codon of interest to the amber stop codon, TAG, using conventional site-directed mutagenesis, followed by *in vitro* transcription of UAG-containing messenger RNA (mRNA). Separately, a suppressor transfer RNA (tRNA) containing the appropriate anticodon (CUA) is prepared and chemically acylated with an unnatural amino-acid. The tRNA and mRNA are then coinjected into a *Xenopus* oocyte. Protein synthesis and surface expression are carried out by the oocyte, permitting subsequent functional studies.

With this technique, together with functional studies, a more detailed understanding of ligand-channel interactions with chemical-scale precision at the level of single atoms and bonds becomes possible[76]. In particular, the cation-π interaction, a potent and general noncovalent binding force in ligand recognition of LGICs, was identified. The cation-π interaction[81] is an electrostatic attraction between a cation and the negative electrostatic potential associated with the face of a simple π system, and it acts as an important component contributing to the secondary structure of ion channel proteins[82,83]. In biological recognition, the π system is typically provided by the aromatic side-chain (Fig. 3B) of phenylalanine (Phe), tyrosine (Tyr), or Trp, while a wide range of cations includes simple inorganic ions (e.g. Na^+ and K^+), protonated amines (RNH_3^+), and quaternary ammoniums. In some cases, the roles are reversed such that the protein provides the cation (e.g. Arg,

Lys, or His; Fig. 3A, F) and the ligand provides the π system. In the former case, fluorination of the aromatic residues in the channel protein such as by incorporation of a 5,6,7-trifluorotryptophan (F3-Trp) in place of Trp, presumably perturbs the negative electrostatic distribution along the face of the simple π system, and dramatically reduces the potential cation-π interaction. This strategy has been used to map a strong cation-π interaction[84] in brain but not muscle nAChRs that mediates the differential action in response to nicotine. Overall, unnatural amino-acid mutagenesis constitutes a complementary approach for studies of ion channels with considerable molecular precision in a physiologically relevant cellular environment.

4.5 Mutant cycle Single-site mutagenesis provides strong implications for the role of one residue in channel function while double-mutant cycles[85] determine the strength of energetic interactions between residue pairs or between protein-ligand complexes. A double-mutant cycle involves WT protein, two single mutants and the corresponding double mutant protein. If the change in free energy associated with a structural or functional property of the channel protein upon a double mutation differs from the sum of changes in free energy due to the single mutations, then the residues at the two positions are recognized as coupled. The interactions between these residues reflected by such energetic coupling can be either direct or indirect. In the absence of coupling between residues, it is possible to predict the mutational effects by assuming their additivity. By measuring the additivity of two mutations at separate sites as well as mapping the energetic interaction properties, investigators have established many strong residue-residue interactions occurring in multiple steps underlying channel operation, including such processes as coupling[86,87] between agonist binding and channel gating, and voltage sensing[88] in ligand- and voltage-gated ion channels, respectively. Moreover, multidimensional mutant cycle analysis can be used to measure higher-order cooperativity between intramolecular or intermolecular interactions[89]. Although the mutant cycle calculation is a powerful tool for measuring the strength of different types of stabilizing interactions in channel proteins, complementary methods such as obtaining the 3-D structures of channel proteins are also needed for validation of the calculated results.

5 Chemical modulation and modification of ion channels

Almost every ion channel can be activated or modulated by specific chemical factors. Small-molecule screening, including image-based high-throughput screening, to find new compounds that control channel function is a major strategy for probing the structure and function of ion channels. For the systematic identification and evaluation of potassium channel modulators, a compound selection procedure has been developed by combining the specific chemical bleaching of surface channels with time-course measurements of activity recovery by nonradioactive rubidium flux assays[90]. This technique has been tested for the identification of inhibitors of potassium channels with either acute (*via* inhibition of channel activity) or chronic (*via* regulation of trafficking and/or half-life of channel proteins) mechanisms[91]. By using a similar strategy, researchers identified[92] a new opener, zinc pyrithione, which activates KCNQ M currents by interacting with the channel protein to cause an increase of single-channel open probability. In contrast, to discover new modulators of anion-selective ion channels such as GlyRs, the anion-sensitive yellow fluorescent protein (YFP) is an optimal strategy for primary screening, followed by automated electrophysiology which is useful for confirming hits and quantitating the actions of identified compounds[93]. Through the application of YFP mutants in high-throughput screening on tens of thousands of different compounds, novel modulators of the cystic fibrosis transmembrane conductance regulator[94,95] as well as new ligands of GlyR and $GABA_AR$ channels[96] were identified.

Identification of modulators of ion channels is not the end, but just the beginning of promoting structure-function studies of ion channels and probing specific domains conferring modulation. Using the power of small-molecule screening[97], we recently identified[98-101] a group of small molecules as exemplified by 2-guanidine-4-methylquinazoline (GMQ, Fig. 4A, B) that are effective activators of

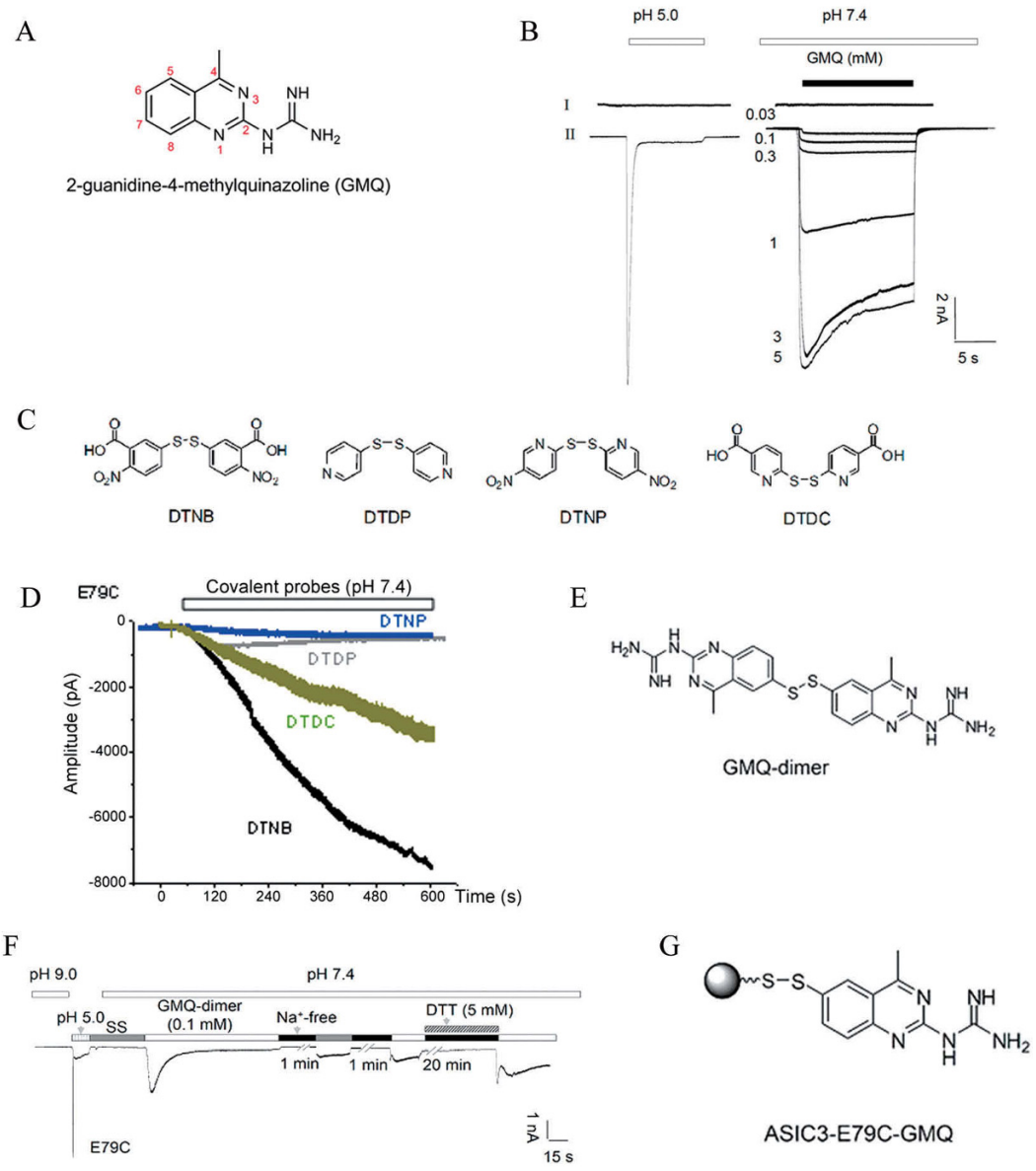

Fig. 4. Chemical modulation and modification of ion channels. A: Chemical structure of 2-guanidine-4-methylquinazoline (GMQ). B: GMQ activates ASIC3 channels in a sustained manner independent of marked acidosis (right panel), without the rapid desensitization observed in proton-gated channel activation (left panel). C: Chemical structures of covalent probes 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and its analogs. D: Representative effects of different probes on the cysteine-mutant of ASIC3 channels ($ASIC3^{E79C}$). E: Chemical structure of GMQ dimer. F: Effects of GMQ-dimer on $ASIC3^{E79C}$ channels. GMQ dimer induces $ASIC3^{E79C}$ channel activation *via* a covalent reaction. Subsequent administration of GMQ dimer does not induce additional current due to the formation of E79C-S-S-GMQ complexes. The reducing agent 1,4-dithiothreitol (DTT, 5 mmol/L) partially restores channel activity by breaking the disulfide bond in the E79C-S-S-GMQ complex, rendering the channel responsive to a subsequent GMQ dimer application. G: Putative configuration of covalent linkage of GMQ to E79C site. Reprinted from Yu *et al*., Neuron 2010[98].

acid-sensing ion channels (ASICs), one type of channel that has long been considered to be an extracellular proton-gated cation channel. Using GMQ as a probe combined with site-directed mutagenesis, we uncovered[98] a nonproton ligand-sensing domain involving Glu-423 and Glu-79 in the extracellular region that is essential for the nonproton

activation of ASIC3 channels. In addition, we revealed that both 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) and its analogs, induce covalent modification (Fig. 4C, D) of the E79C mutant but not WT ASIC3 channels[98]. Covalent linkage of GMQ by treatment with a GMQ-dimer (Fig. 4E–G) causes $ASIC3^{E79C}$ channel activation, supporting the notion that this motif is sufficient for channel activation. This example provides new insights into channel mechanisms and potential natural ligands for ASICs[99,102], and also demonstrates the power of chemical modulation and modification in probing the function of ion channels[101-105].

In addition to small molecules, antagonistic or agonistic venom peptide toxins, another group of channel modulators, have been used to probe specific functional domains underlying channel operation. Many well-known venom peptide toxin inhibitors including those binding to voltage-gated Na_V, Ca_V, and K_V channels as well as ligand-gated nAChRs and N-methyl-*D*-aspartate receptors can act as standard tools to probe channel function (see above). Moreover, series of venom peptide toxins have been recently identified as activators of sensory ion channels including the capsaicin receptor (TRPV1)[106,107] or ASICs[102] to induce pain, which opens up new opportunities for probing novel structural elements implicated in the activation of these sensory receptors in response to diverse stimuli.

By using the selectivity of venom peptide toxins for ion channels, a novel strategy, termed "tethered toxins", was recently developed to characterize neuronal circuits[108]. Routinely, peptide toxins do not exist as cell-surface anchored molecules. Nevertheless, the prototoxin lynx1, consisting of an N-terminal secretory signal followed by the lynx1 peptide and a C-terminal glycosyl phosphatidyl inositol (GPI) anchor, enables the peptide to be secreted and tethered to the surface of the expressing cells *via* the GPI anchor. A design using the lynx1 family scaffold directs bioactive peptides to the secretory pathway, where the signal sequence is cleaved and the GPI targeting sequence is substituted by a covalent bond to GPI, anchoring the bioactive peptide to the extracellular side of the plasma membrane. The high degree of specificity with which venom peptide toxins bind to ion channels makes them ideal for deciphering the connections between cell types, and for reversibly manipulating the activity of selected cell subtypes[108].

6 Crystal structures

Crystallization of an ion channel protein to obtain its 3-D structure is the clearest approach for mapping the atomic basis of channel operation. The first ion channel crystal structure was acquired from a bacterial potassium channel called KcsA from *Streptomyces lividans* by the MacKinnon group[109]. This represents another landmark in ion channel research that initiated a new epoch in the study of the structure-function relationship. By learning from the structure of a bacterial potassium channel homolog that has only two transmembrane segments and a pore loop, the MacKinnon group[110] depicted the location of almost every atom in the ion channel. The structure has a beauty of its own, with the four subunits[111] intertwined *via* the inner transmembrane segment (corresponding to S6 of eukaryotic K_V channels) making a closed gate in the predicted intracellular side[112]. A wide aqueous cavity residing at the center of the membrane constitutes the channel pore. The pore loop forms the selectivity filter with the carbonyl groups of the Gly-Tyr-Gly-Asp motif lining the pore and the dipoles of pore helices pointing into a central lake enclosed by the second transmembrane segments. From this structure, one can actually see that, in the selectivity filter, several K^+ ions are coordinated by the carbonyl groups in a row. This pore architecture clearly established the physical principles underlying the selective ion conduction of potassium channels.

Up to now, considerable numbers and types of ion channel crystal structures[8,27,47,48,113-117] have become available due to great technological improvements (Fig. 5). Moreover, the 3-D structure can be obtained not only from crystals, but also by other methods such as nuclear magnetic resonance (NMR) spectroscopy. In contrast to crystal structures that provide snapshots with atomic details, NMR determines the structural and functional dynamics by visualizing the channel protein in different states[118]. Furthermore, cryo-electron microscopy (cryo-EM), a form of

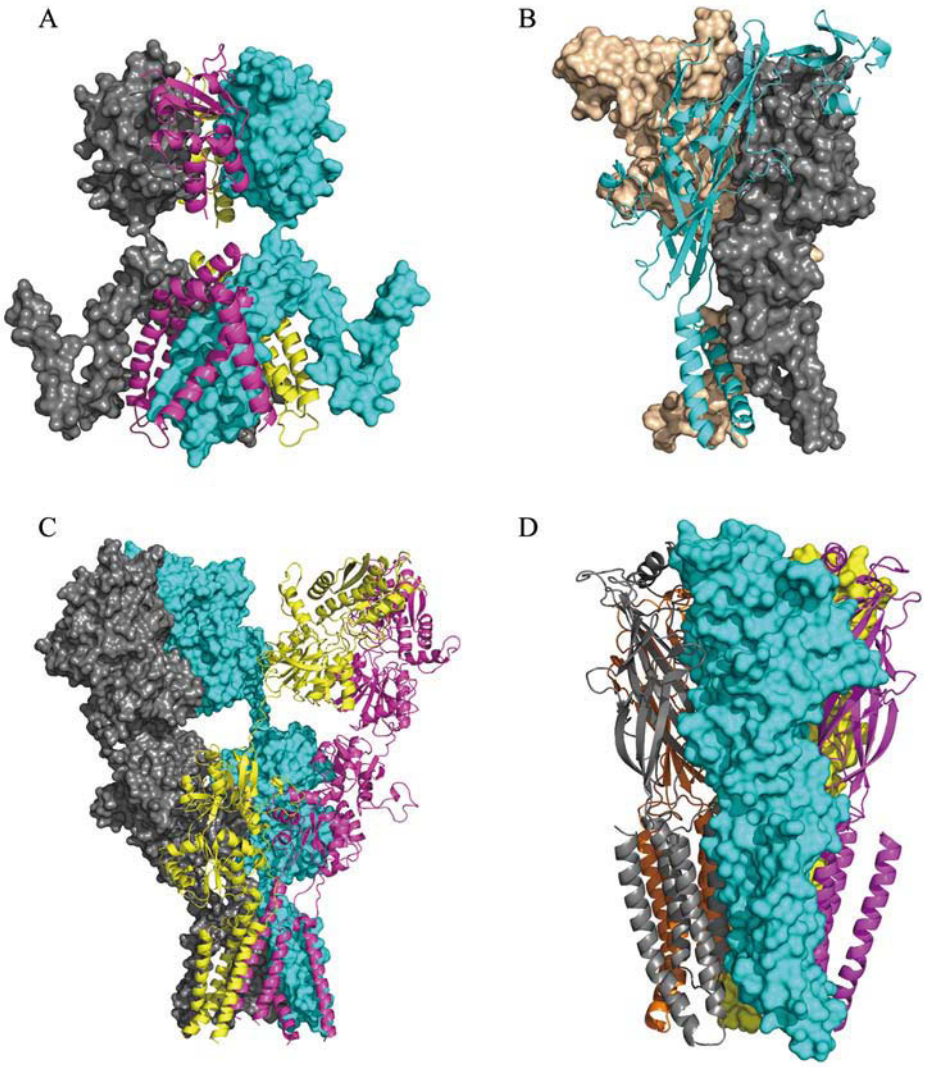

Fig. 5. Crystal structures available for voltage- (A) and ligand-gated (B–D) ion channels. Shown are the crystal structures of a voltage-gated potassium channel (A, rat Kv1.2-β subunit complexes, PDB code:2a79)[113], an ATP-activated P2X4 receptor (B, zebrafish P2X4, trimer, PDB code: 3H9V)[115], an AMPA-subtype glutamate receptor ion channel (C, rat GluR2 receptor, tetramer, PDB code: 3KG2)[116], and a glutamate-gated chloride channel (D, *Caenorhabditis elegans* GluClα, pentamer, PDB code: 3RIF)[117]. Figure was made with PyMol.

transmission EM where the sample is studied at cryogenic temperatures (generally that of liquid nitrogen), can visualize the structures of ion channels and other proteins in their native state[119] but at the nanometer level. Fast-scanning atomic force microscopy (AFM) offers very high resolution on the order of down to fractions of a nanometer using scanning probe microscopy. Time-lapse AFM imaging of ion channels in different functional states can provide details of the global conformational changes underlying the state transitions of ion channels[120,121].

With the growing body of structures for ion channels, we can imagine that if there are two or more structural models for one type of ion channel, it will give rise to a reasonable atomic description of a channel transition between its various functional states. While they are highly detailed, the 3-D structures are in fact snapshots of highly dynamic proteins such as the acid-sensitive ASIC channel[47,48]. What happens in free solution between these

static frames can only be speculated on. In that regard, the crystal structural determination of ion channels is not an end, but just a beginning to understanding the structure (dynamics)-function relationship at the atomic level, which will ultimately depend on comprehensive analyses combining a variety of different methods.

7 Optical methods

Patch-clamp recording provides the most sensitive method of single-molecule functional analysis[1], which represents the final output of the entire channel protein. To reveal the dynamic motions of local and specific sites within channel proteins, other methods such as optical techniques are required[122,123]. Optical techniques can be used to monitor the structural rearrangements that take place within ion channels[122]. In some cases, an engineered channel containing a small light-sensitive module can allow light to directly control the ion channel activity[124,125]. This approach is beyond the scope of the present review. To passively monitor the structural rearrangements of a channel in real time, incorporation of small organic fluorophores or large fluorescent proteins into specific regions of interest is required.

7.1 Site-directed fluorescence labeling (SDFL) Photolabeling refers to a variety of methods by which light-dependent reagents are incorporated into biological targets such as membrane-bound ion channels[126]. This technique makes use of the reasonable chemical stability of most photochemical reagents in the absence of light, permitting them to be targeted to a distinct compartment or component prior to their activation by specific wavelengths of light. Usually, the photoreactive probes are radiolabeled. After photoincorporation of the probe into the targeted proteins, the photolabeled amino-acids can be identified by microsequencing of protein fragments isolated from proteolytic digests. Using this method, the specific sites or domains for agonists, antagonists, or modulators of LGICs can be identified[127,128]. As a probe, the fluorescence is directly influenced by motions or mutations in an interacting region of the channel protein, so this technique can be used to probe the structural and functional properties of ion channels. The nAChR, an allosteric channel protein undergoing the interconversion among several different conformational states, is a good example that has been intensively studied by this time-resolved photolabeling approach[129]. As a result, many photolabeling experiments targeting different sites have unambiguously revealed not only the tertiary and quaternary rearrangements in the cholinergic binding pocket as well as in the pore lumen, but also the structural determinant and the functional linkage between the binding of agonist and the channel gating[129].

Besides the nonspecific photolabeling methods, SDFL, a modified branch of SCAM (see 4.2 Scanning mutagenesis) using thiol-specific photosensitive reagents, is also widely used to measure membrane protein topology[130] and structural rearrangements corresponding to different functional states[131,132]. Strikingly, these measurements were the first to reveal experimentally the outward movement of the S4 voltage sensor during channel activation, and they helped to define the molecular constituents of the pore-lining portions of voltage-gated channels[133-135]. Moreover, as described above, site-directed incorporation of fluorophore probes into ion channels can also be accomplished by unnatural amino-acid mutagenesis (see 4.4 Unnatural amino-acid mutagenesis).

7.2 Voltage-clamp fluorometry (VCF) Expanded from the SDFL approach, an optical measurement[136,137] of VCF was developed. In VCF, the fluorescent probe can either be a conjugated fluorophore (Fig. 6A) or a fluorescent protein[138] (Fig. 6C) introduced into the target ion channel. A targeted ion channel protein is engineered to include a single reactive cysteine residue and is selectively labeled with an organic thiol-reactive fluorophore. After tagging, the fluorescence can be monitored with a photomultiplier tube, photodiode, or charge-coupled device (CCD) camera, while the functional state of the channel is controlled simultaneously using the patch clamp technique. As the spectral properties and quantum yield of the channel-bound fluorophore are tightly influenced by the local environment, changes in ion channel structure near the fluorophore are reported as a dimming or brightening of the fluorescence (Fig. 6B). In addition, as the excited-state lifetime of

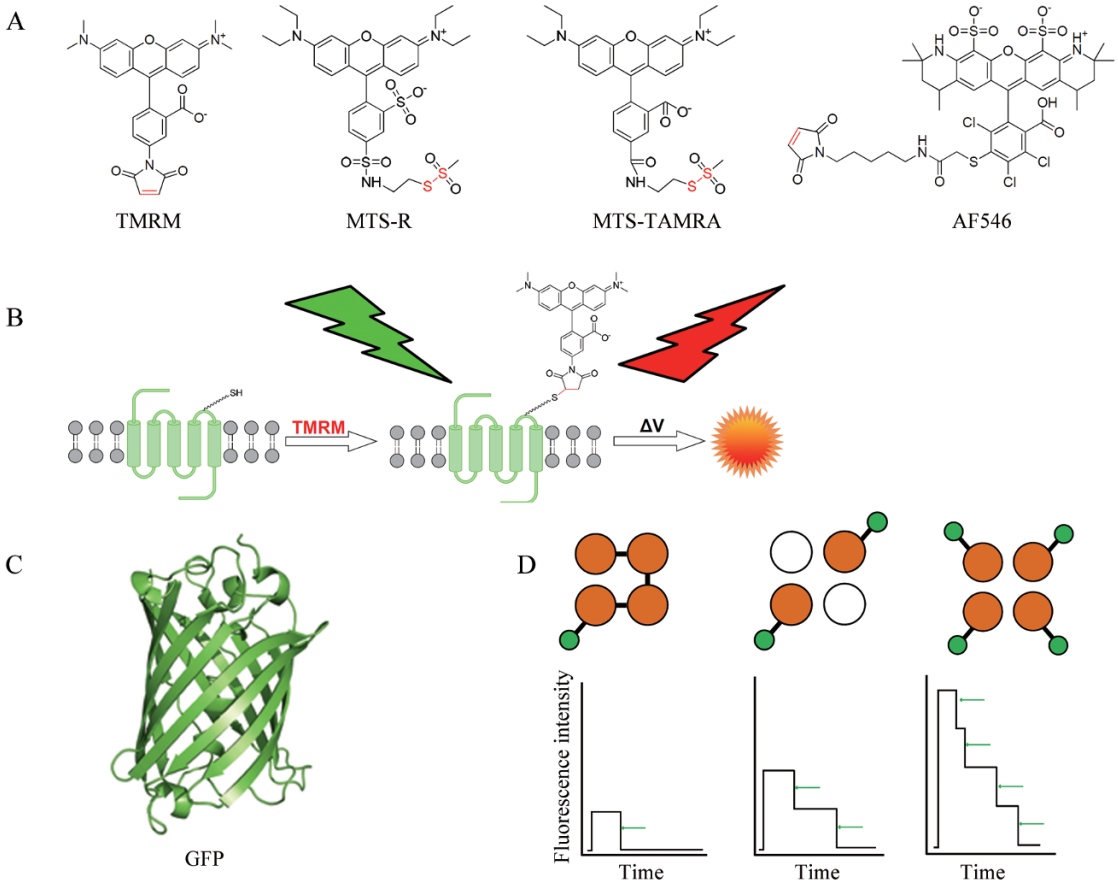

Fig. 6. Optical techniques for monitoring the conformational changes associated with channel activation and subunit numbers in native membrane-bound proteins. A: Chemical structures of probes for site-directed fluorescence labeling. TMRM, tetramethylrhodamine-6-maleimide; MTS-R, sulforhodamine methanethiosulfonate; MTS-TAMRA, 2-((5(6)-tetramethylrhodamine) carboxylamino) ethyl methanethiosulfonate; AF546, Alexa Fluor 546C5 maleimide. The red part of each molecule indicates the reactive site responsible for labeling the cysteine sites on the channel protein. B: Principles of voltage-clamp fluorometry (VCF). TMRM is covalently attached to a cysteine introduced at a specific site of a subunit of an ion channel. TMRM absorbs a high-energy green photon and emits a lower-energy red photon. While a change in voltage occurs (ΔV), the intensity of TMRM fluorescence increases or decreases depending on the conformational state of the channel. Modified from Gandhi *et al*., Trends Neurosci 2005[122]. C: Crystal structure of green fluorescence protein (GFP) (PDB code:1gfl)[138]. The structural model was made with PyMol. D: An optical method for subunit counting in membrane-bound proteins. Bleaching steps of a single fluorescent protein complex reveal the number of GFP-labeled subunits. Different labeling schemes for membrane protein complexes that include 1 (left), 2 (middle), or 4 (right) subunits fused to GFP are shown in upper panels, respectively. The expected fluorescence emission patterns for the labeling schemes are shown in the corresponding lower panels. The arrows indicate the bleaching steps. Modified from Ulbrich & Isacoff, Nat Methods 2007[142].

most fluorophores is on the nanosecond scale[139] while the fastest ion-channel motions are thought to occur on the microsecond scale[140], time-resolved VCF therefore can give a real-time readout of the channel protein kinetics. Moreover, because a VCF experiment can be designed so that a fluorophore reports structural changes only near its attachment site, the optical signal naturally signifies the contribution of different parts of the protein to discrete steps of complex kinetic pathways[141]. On the whole, these time-resolved fluorescence measurements essentially answer questions about which parts of the channel protein move as well as how these motions are associated with transitions among different functional states.

The VCF method was pioneered with voltage-gated

channels, and has greatly advanced our understanding of how membrane potential regulates channel activity. In conjunction with SCAM, fluorescence measurements revealed that the S4 segment of the K_V channel moves outwards through the membrane in response to depolarization[136,141,143]. VCF measurements showed that this motion occurs in a series of sub-steps, implying that S4 ratchets through the membrane one charge at a time. These initial steps take place independently in each subunit[144-146] and are followed by a final combined motion of all four subunits, driving the gates to open or close the pore[147]. Similar optical approaches also demonstrated that S4 control has the remarkable property of reverse-coupling to gating in the pacemaker hyperpolarization-activated cyclic nucleotide-gated channels, as hyperpolarization opens these channels by moving S4 inwards[148,149], which stands in stark contrast to what happens in depolarization-gated K_V and Na_V channels.

Different from K_V channels that are homotetramers, Na_V channels are composed of four non-identical pore-forming domains formed by a single polypeptide chain, raising the possibility of differential functions for these domains. VCF measurement revealed that these domains are activated in a sequential manner[150], which is consistent with the observed subunit-specific pharmacological roles within the entire Na_V channel[12]. Similar observations were obtained for the differential roles of different subunits in LGICs such as nAChRs[151]. Besides the subunit-specific conformational changes revealed by VCF, environment-sensing fluorescence has also shown that LGICs, such as $GABA_A$Rs and GlyRs, are activated at different rates by ligands that have distinct actions. Each ligand seems to place the receptor ion channel in a distinct conformation[152,153]. Overall, the application of VCF to ion channel studies will shed more light on the conformational dynamics associated with channel operation.

7.3 Fluorescence resonance energy transfer (FRET)

While VCF measurements provide structural insights into channel motions at the single amino-acid level, the FRET method is a distance-dependent measurement that provides an optical ruler to measure the channel motion. FRET is a process by which light energy absorbed by a donor fluorophore is transferred to a nearby acceptor fluorophore with an absorption spectrum that overlaps the emission spectrum of the donor[154]. The efficiency of energy transfer falls off with the sixth power of the distance between the donor and acceptor molecules, making FRET an extremely sensitive reporter of proximity. A combination of the discovery of green fluorescent protein (GFP)-based FRET pairs, for example the cyan fluorescent protein (CFP) and YFP pair, and the rapid advances in fluorescence microscopy has resulted in the enthusiastic adoption of FRET approaches to a wide spectrum of biological studies of molecular colocalizations, specific protein-protein interactions, and conformational rearrangements. Because of the large size of the fluorescent-protein donors and acceptors, their FRET efficiencies cannot be used to calculate atomic distances. However, they can identify the movements of protein subdomains if these movements are sufficiently large, and they can robustly report the association and disassociation interactions of proteins in complexes. FRET between fluorescent proteins has suggested that rotation and expansion of the N and C termini drive the two-transmembrane domain (2-TM) inward-rectifier potassium channel (GIRK) to open following Gβγ-protein binding, by bending and possibly rotating the second transmembrane segment[155]. A similar strategy revealed the gating rearrangements in the cytoplasmic domains of a 6-TM channel that is gated by internal cyclic nucleotides[156,157] and a 2-TM P2X2 receptor channel gated by external ATP[158]. With respect to studying protein complexes, FRET has been used to determine the subunit stoichiometry of the epithelial sodium channel (ENaC)[159], transient-receptor potential channels[160], and cyclic-nucleotide-gated channels[161]. In addition, because of their small size, organic fluorophores acting as donor and acceptor molecules can be used to calculate protein motion at the Angstrom level once factors such as fluorescence anisotropy and spectral overlap are taken into account. Protein conformational changes deduced from FRET measurements using organic fluorophores provided evidence for the helical rotation of S4 during the activation of K_V channels[162,163].

Of particular interest in applications of the FRET

technique is a recently developed probe used to perform ratiometric Cl^- imaging. Based on YFP mutants with Cl^--sensitivity (see also 5 Chemical modulation and modification of ion channels), Kuner and Augustine[164] developed a genetically-encoded ratiometric YFP-based Cl^- indicator, termed Clomeleon, which consists of two fluorescent proteins, CFP and a variant of YFP (Topaz fluorescence protein, TFP), connected with a polypeptide linker. In principle, binding of a Cl^- anion to TFP reduces its emission, leading to a decrease in FRET efficiency; a process that can be visualized as a reduction in the ratio of fluorescence emission between the TFP acceptor and CFP donor fluorophores. Analysis of emission spectra of this construct revealed that the fluorescence intensity depends on Cl^- concentration. Moreover, the presence of the isosbestic point in normalized spectra allows the use of this indicator as a ratiometric probe for estimation of Cl^- concentration. As a result, Clomeleon has been used for measurements of intracellular Cl^- concentration in cultured hippocampal neurons[164] as well as in cells in brain slices[165]. Overall, the genetically-encoded Cl^- probe offers a complementary means of screening pharmacological agents, analysis of Cl^- homeostasis and functions of Cl^--selective channels under various physiological and pathological conditions.

In addition, more and more advanced optical methods are emerging in the study of the structure and function of ion channels. Total internal reflection fluorescence microscopy can be used to obtain simultaneous and independent recordings *via* imaging of single-channel Ca^{2+} flux[166]. This optical single-channel recording is applicable to diverse voltage- and ligand-gated Ca^{2+}-permeable channels and has potential for high-throughput functional analysis[167]. Thus, this technique is anticipated to be developed as a useful adjunct to patch-clamping for single-channel studies, with capabilities including simultaneous readout from multiple channels, high-resolution mapping of channel location, and mobility that is inaccessible by electrophysiological means. More recently, Ulbrich and Isacoff[142] developed a single-molecule technique for determining the subunit number and stoichiometry of ion channel proteins by observing the bleaching steps of GFP fused to a channel protein of interest in live cell membranes (Fig. 6D). The compositions of ion channel proteins with known[142] or unknown[168,169] stoichiometries were resolved by this method. In the long run, optical monitoring of channel conformation provides a unique method allowing investigators to relate structural rearrangements in proteins to the resultant physiological processes.

8 Computational prediction of channel dynamics

Once the structure of an ion channel is available, the structural dynamics underlying the gating mechanism must next be established. For potassium channels, the structural basis of ion selectivity was demonstrated by crystal structures revealing that carbonyls replace the hydration water of the K^+ ion[110]. Na^+ does not fit as well in the cage because it is smaller and does not permeate as well as K^+. However, Roux and his colleagues[170], who are computational chemists, argued that the size of the cage is expected to easily change due to thermal vibration of the backbone chain, and that it would therefore have no difficulty in accommodating Na^+ as well as K^+, which would negate the selectivity. After performing molecular dynamic free-energy computations of the potassium channel crystal structures[110], the computational chemists[170] discovered that the repulsion between the carbonyls that coordinate the ion provides a hidden energy term preventing them from drawing close enough to coordinate a smaller ion like Na^+. This computational study demonstrates the important roles of local dynamic and electrostatic effects in addition to steric factors in determining ion channel selectivity and underscores the power of molecular dynamic free-energy computations in studying the structural and functional aspects of ion channels.

For the LGICs, computational chemistry can also provide important structural information when the channel's crystal structure is available. To consolidate the basis of our finding on the nonproton ligand-sensing domain[98] in ASIC3 channels described above, we recently established its atomic basis by using the agonist GMQ as a probe along with an interactive computational-experimental ap-

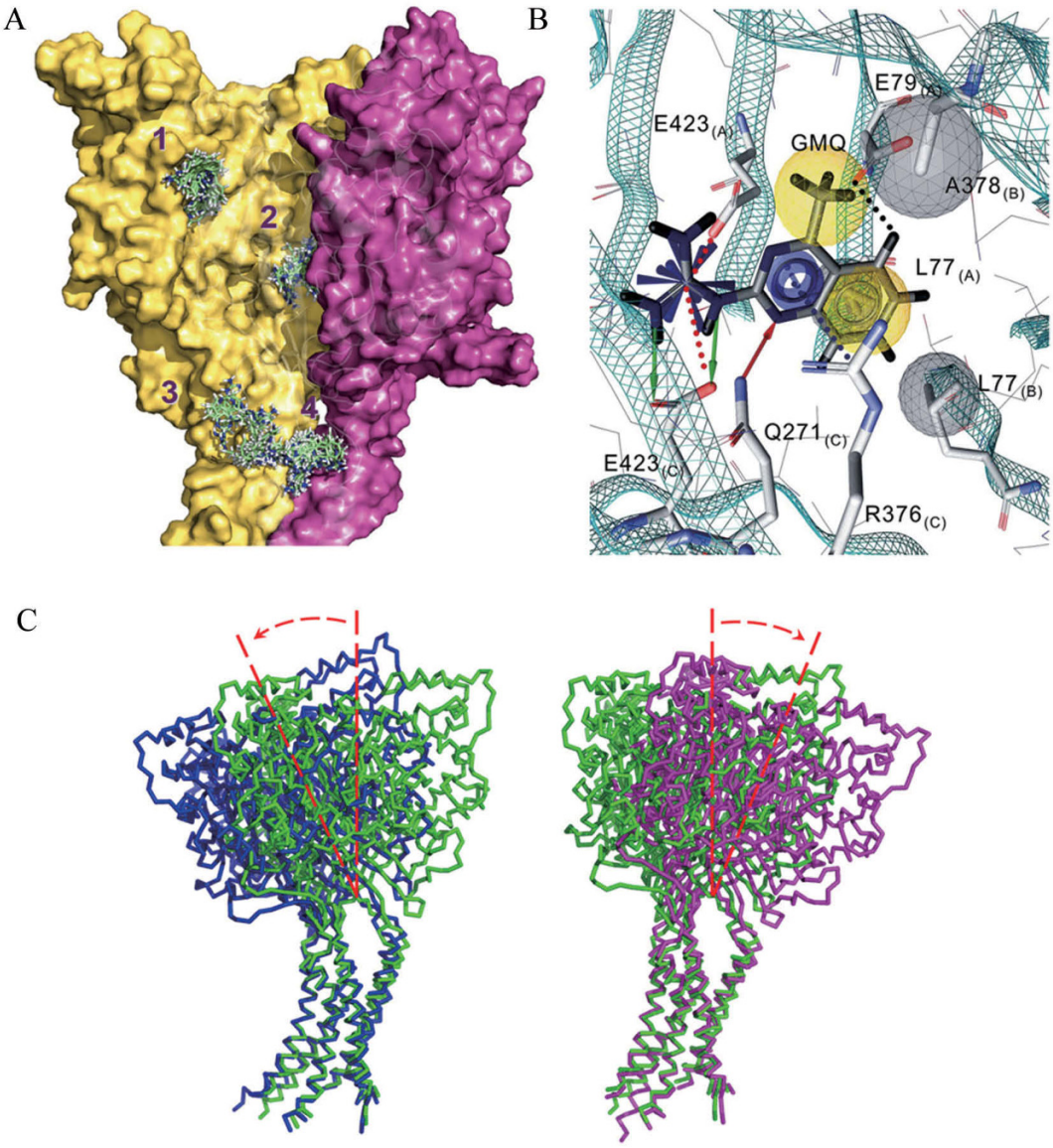

Fig. 7. Computational approaches to investigating the structural mechanisms underlying channel activation. A: Identification of putative ligand (GMQ)-binding sites in ASIC3 channels using a cavity-searching algorithm as well as in silico (ligand-protein) docking. Accordingly, four putative GMQ-binding sites were identified. Site 1 has been previously implicated in pH sensing in ASICs and is located in the cleft between the "thumb" and "finger" domains; site 2 is in the interface of the three subunits, namely the cavity lined by Glu-79 and Glu-423 in the palm domain; site 3 is in the interface of any two subunits; and site 4 is formed by a cluster of acidic residues in the post-TM1 and pre-TM2 regions. Further details in Ref. [171]. B: Construction of ligand (GMQ)-binding mode using in silico docking. The residues interacting with GMQ are displayed as sticks for emphasis. The green and red arrows indicate H-bond contact between GMQ and Glu-423 or Gln-271. The black, red, and blue dotted lines indicate charge-assisted H-bond, electrostatic, and cation-π interactions between residues and GMQ, respectively. The residues of ASIC3 (gray spheres) and groups of GMQ (yellow spheres) implicated in the hydrophobic interactions are displayed as surface spheres. All the ligand-ASIC3 interactions were auto-detected by Ligandscout 2.02 (trial version), except for the charge-assisted H-bond. Reprinted from Yu *et al*., J Biol Chem 2011[171]. C: General motions of ASIC1 channel protein as detected by normal mode analysis. Shown are the rocking motions between the extracellular domains. Two snapshots at harmonic periods one-quarter (blue) and three-quarters (magenta) of mode 2 were superimposed on the initial structure (green) by fitting all the Cα atoms of the transmembrane domain. The arrows show the direction of the rocking motions. Reprinted from Yang *et al*., PLoS Biol 2009[177].

proach[171]. We first constructed a 3-D model of ASIC3 at neutral pH based on the crystal structure of the chicken ASIC1 channel[47] using homology modeling[172,173] and molecular dynamics (MD) simulation[174,175] approaches. Using the acquired 3-D model, we then systemically examined the potential GMQ-binding sites (Fig. 7A) using a cavity-searching algorithm as well as *in silico* (ligand-protein) docking[176] in combination with mutagenesis and functional studies. By using these approaches, we established a GMQ binding mode and revealed the residues playing key roles in forming the GMQ-sensing domain (Fig. 7B). We then used *ab initio* and quantum mechanics/molecular mechanics (QM/MM) interaction energy calculations as well as mutagenesis to verify the critical role of the above GMQ-binding residues in regulating ASIC3 function[171]. Collectively, we used a range of complementary techniques to depict the structural details of the GMQ-sensing domain at the atomic level, laying the foundation for further investigations of the gating mechanisms of these ion channels. From a methodological point of view, this study[171] can be used as a template for the study of the ligand-binding sites of other LGICs.

Because the current MD methods are limited to addressing the local movements of proteins, normal mode analysis (NMA) as a complementary approach[178-180] is efficient for predicting the collective dynamics and inherent flexibilities in biological macromolecules. This approach has already been applied to studying the structural dynamics-function relationship of several ion channels, such as the prokaryotic large-conductance mechanosensitive channel MscL[181], KcsA potassium channel[182,183], as well as the eukaryotic nAChR channel[184-187]. By using the NMA approach, we have recently shown[177] that a series of collective motions among the domains and subdomains of trimeric ASIC1 channel protein (Fig. 7C) correlate with its acid-sensing function, indicating that the structure of the closed, desensitized state of the ASIC1 channel protein[47] intrinsically tends to undergo a twisting motion to open the gate[188]. This study reinforces the notion that the "twist-to-open" motion is a common mechanism for gating membrane proteins with an inherent cylindrical symmetry[189].

9 Concluding remarks

The progress in studying the structural and functional aspects of ion channels has been series of strides, each stride followed by the steady emergence of new approaches. Multidisciplinary approaches including molecular and cellular, chemical, optical, biophysical, or computational, can be applied to map the structural and functional aspects of ion channels. Progress in these different areas is leading us to a more complete atomic description of many types of ion channels. A true understanding of an ion channel at the molecular level will only occur when the kinetics and steady state of the channel function[190] can be predicted from the dynamics of the structure[1]. We are far from this objective, but there is no doubt that achieving this goal will require a synergy between structural, functional, and spectroscopic techniques merged with extensive computational analysis. Beyond this goal lie the problems related to the *in vivo* physiological roles of the different structural motifs mediating each corresponding channel property. For that purpose, the generation of site-directed genetically-modified animals and the corresponding physiological assessments[191] are needed. Moreover, the growing knowledge of structure and function of ion channels by multidisciplinary approaches allows the development of new pharmacotherapies and new ways to control cellular activity.

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