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J Mol Biol. Author manuscript; available in PMC 2016 January 16.

Published in final edited form as:
J Mol Biol. 2015 January 16; 427(1): 190–204. doi:10.1016/j.jmb.2014.09.001.

Ion Channel Engineering: Perspectives and Strategies

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Abstract

Ion channels facilitate the passive movement of ions down an electrochemical gradient and across lipid bilayers in cells. This phenomenon is essential for life, and underlies many critical homeostatic processes in cells. Ion channels are diverse and differ with respect to how they open and close (gating), and their ionic conductance/selectivity (permeation). Fundamental understanding of ion channel structure-function mechanisms, their physiological roles, how their dysfunction leads to disease, their utility as biosensors, and development of novel molecules to modulate their activity are important and active research frontiers. In this review, we focus on ion-channel engineering approaches that have been applied to investigate these aspects of ion channel function, with a major emphasis on voltage-gated ion channels.

Introduction

Ion channels are ubiquitously expressed integral membrane proteins that eponymously control the passage of various ions (Na^+, K^+, Ca^{2+}, Cl^-) across lipid membranes in cells. The direction of ion transport through an open ion channel is governed by the electrochemical gradient for the particular ion species across the membrane in question[1]. In excitable cells such as cardiac myocytes and neurons, the activity of distinct ion channels establishes the resting membrane potential and generates action potentials to control essential biological processes including (but not limited to); muscle contraction, neurotransmitter release, and gene expression regulation. In non-excitable cells such as epithelial cells, ion channels control the flow of salt and water, and regulate cellular volume and pH.

There are approximately 400 genes that code for ion channel subunits and their alternatively spliced variants. These differ with respect to their cellular and sub-cellular localization, mechanisms of gating, ionic selectivity, modulation by accessory subunits and signaling molecules, and physiological roles. Inherited or acquired mutations in many different ion channels lead to various diseases collectively known as channelopathies[2]. Not surprisingly,

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ion channels are important therapeutic targets for a broad range of diseases from cardiac arrhythmias to cancer.

To a large extent, research direction in the ion channel field has been driven by a set of key questions. How do distinct ion channel macromolecular complexes work? What are their physiological roles, and how do they carry them out? How are they regulated? How does their dysfunction lead to disease? In this review, we discuss ion channel engineering approaches that have been essential towards addressing many of these questions. We use specific examples from the literature to provide perspective on the various approaches, discuss potential pitfalls, and envision possible future directions. To limit scope, we focus on three aspects of this broad topic. (1) Engineering ion channels to elucidate their structure-function mechanisms. (2) Engineering ion channels to probe and manipulate physiology. (3) Development of engineered ion-channel modulators.

By necessity, we are limited in the in the number of articles we can reference in this review. We apologize in advance to colleagues whose work fits within the realm of the topic but have not been referenced in this review.

Engineering ion channels to elucidate structure-function mechanisms

Though molecularly very diverse, various ion channels share several common characteristics. They are typically gated, opening and closing in response to various stimuli, including; membrane potential, neurotransmitter ligands, ions, and mechanical forces. Once open, ion channels conduct different ions with a high throughput of 10^6 – 10^7 ions/s. Channels can be highly selective for a particular ion (e.g. voltage-gated K^+, Na^+, and Ca^{2+} channels) or be non-selective (e.g. nicotinic acetylcholine receptor). Many ion channels are macromolecular complexes containing a pore-forming integral membrane protein assembled with auxiliary subunits which typically regulate channel trafficking and gating. The activity of most ion channels are not static, but can be modulated by signaling molecules and post-translational modification as a method to regulate physiology. Finally, many ion channels are targeted by small-molecules that can block, activate, or modulate channel activity. Several of these are important therapeutic agents.

Ion channel engineering approaches have been used to elucidate structure-function mechanisms governing ion channel behavior. It is worth mentioning that present day ion channel research is founded on several critical advances and powerful techniques developed over several decades. Noteworthy events include: seminal work by Hodgkin and Huxley describing the ionic conductance changes underlying the squid giant axon action potential[3]; molecular cloning of ion channel proteins[4]; development of the patch clamp method[5]; advent of ion channel heterologous expression in *Xenopus* oocytes and mammalian cell lines; advances in spectroscopic methods; acquisition of high-resolution crystal structures of ion channels; advances in molecular dynamics approaches. A historical perspective on how these and other advances have progressed ion channel research has been reviewed[6].

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Conventional site-directed mutagenesis of ion channels

One of the simplest methods used to probe ion channel structure-function mechanisms is conventional site-directed mutagenesis. In its most basic form, investigators use this approach to probe a hypothesized role played by a specific amino acid residue (or group of residues) in a specific functional property of a channel. The method typically involves the use of recombinant DNA techniques to introduce particular point mutations into the coding sequence of ion channel proteins. The plasmid containing the mutated cDNA, or an *in vitro* transcribed mRNA, is then introduced into heterologous cells which dutifully reconstitute the mutant channel for functional analyses. The researcher embarking on this path has at least two critical decisions to make— (1) which residue(s) in the channel polypeptide to mutate, and (2) what residue to mutate to.

Because ion channel proteins are typically large polypeptides in which many amino acids play a critical structural role, the choice of which residue(s) to mutate must be appropriately constrained and driven by the question under study. If the particular functional property under investigation is common to a group of channels, then residues that are important for this response might be expected to be highly conserved. This strategy has been used to investigate residues important for voltage-sensing in voltage-gated ion channels. Molecular cloning and hydropathy analyses of Na_V, K_V and Ca_V channels first indicated these polypeptides contain homologous repeats with six transmembrane spanning segments[1]. In Na_V and Ca_V channels the four homologous repeats are joined together by intracellular loops, whereas in K_v channels four individual domains associate to form the channel. The primary sequence revealed the S4 segment of each domain contains regularly spaced basic residues (arginine or lysine) which were posited to comprise the voltage sensor. In agreement with this, neutralizing S4 charged residues using site-directed mutagenesis predictably altered the voltage-dependence of ionic conductance[7]. If a crystal structure of a particular channel or its homolog is available this can serve as a powerful guide to develop hypotheses about the role of individual amino acid residues in specific channel functional properties that can then be tested by conventional site-directed mutagenesis. This approach has been used to test the roles of various residues in the gating and permeation properties of various ion channels[8; 9; 10; 11].

In conventional site-directed mutagenesis, the residue of interest can be changed into any one of the other nineteen naturally occurring amino acids. The choice of the replacement amino acid is dictated by the question under study. For example, a study of mechanisms underlying specific ion channelopathies would involve the creation of ion channels with disease-causing mutations identified in humans or other species[2]. Investigation of a process believed to be regulated by phosphorylation of serine or threonine residues would typically involve replacement with phosphomimetic (aspartate or glutamate) or phospho-null (e.g. alanine) amino acids. When a discrete region of an ion channel is hypothesized or known to be involved in a particular function, scanning mutagenesis with alanine (or other appropriate amino acid depending on the study) is often used to identify the most critical residues in the region.

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Though powerful, the results of site-directed mutagenesis experiments in probing ion-channel structure-function need to be interpreted carefully. This is particularly true for mutations that lead to a loss of function. A potentially confounding issue is whether the mutation adversely affects protein folding and tertiary structure. To this point, a mutation in the $Ca_V\beta$ subunit that eliminates binding to $Ca_V\alpha1$ was originally interpreted as providing insight into the physical basis of the α_1-β interaction[12]. However, this residue is buried in the β-subunit crystal structure and likely plays a structural role in the protein rather than being directly involved in binding to $Ca_V\alpha_1$[13; 14; 15].

Substituted cysteine methods

The amino acid cysteine contains a thiol group (–SH) that reacts specifically and quickly with thiol-modifying reagents. This specific chemistry has been exploited in several ways to address structure-function mechanisms of ion channels. One approach, termed substituted cysteine accessibility mutagenesis (SCAM), has been used to identify pore-lining residues, to locate constricted regions that function as gates, and to probe voltage sensor movement in different ion channels[16; 17; 18; 19; 20]. The general strategy is to replace amino acid residues one at a time in regions hypothesized to contain pore-lining residues with cysteines. Mutant channels expressed in heterologous cells are exposed to hydrophilic sulfhydryl modifying reagents such as methanethiosulfonate-ethyltrimethylammonium (MTSET)[17]. If the introduced cysteine lines the channel pore it reacts with the hydrophilic reagent resulting in a change in measured ionic conductance. Voltage- and state-dependent changes in cysteine accessibility at different positions within the pore (or voltage sensor) can be used to make inferences about the precise location of channel gates.

In another variation, individual cysteines introduced into ion channels are labeled with fluorescent thiol-reactive reagents and different spectroscopic methods applied to query various structure-function mechanisms. Mannuzzu et al. introduced cysteines one at a time into various positions of the *Shaker* K^+ channel voltage sensor[21]. Site-specific fluorescent labeling of cysteine residues was achieved by exposing expressed channels to tetramethylrhodamine-maleimide. In voltage-clamp experiments, movement of the voltage sensor could be followed in real time due to local-environment-sensitive changes in rhodamine fluorescence. The approach was used to deduce that several S4 residues moved from a buried position to become exposed to the extracellular environment during channel gating[21]. This powerful voltage-clamp fluorimetry (VCF) approach has been utilized to monitor the voltage-dependence, kinetics, and function of individual voltage sensor domains in Kv[22; 23; 24], Nav[25; 26], and hyperpolarization-activated cyclic nucleotide-gated (HCN) channels[27].

The tendency for two cysteine residues to form a disulfide bond when they are near each other has also been exploited to probe ion channel structure-function. A commonly held model of voltage sensor operation is that basic residues in S4 are neutralized in the membrane by electrostatic interactions with negatively charged amino acids from nearby transmembrane segments. During voltage sensing, sequential interactions of positively charged arginines in S4 with acidic residues in S1, S2, and S3 facilitate the outward movement of S4. By introducing pairwise substitutions of cysteines, Catterall and colleagues

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used a disulfide locking method to provide evidence of interactions between residues in S4 and S1/S2 in the voltage-sensor domain of NaChBac, a voltage-activated bacterial Na^+ channel[28; 29]. Disulfide cross-linking of pairwise substituted cysteines combined with biochemical methods has also been utilized to map the location of auxiliary peptides with respect to pore-forming subunits in distinct K^+ channel complexes[30; 31].

Substitution with unnatural amino acids (UAAs)

Side chains of the 20 naturally occurring amino acid side chains typically differ in more ways than just one (e.g. size, charge and shape), and this can complicate precise mechanistic interpretation of the results obtained from conventional site-directed mutagenesis studies. Furthermore, the protein backbone cannot be altered using conventional mutagenesis, a caveat that prevents application of this approach to a range of relevant ion channel structure-function questions. The ability to incorporate unnatural amino acids into recombinant ion channels is a powerful extension of site-directed mutagenesis that can overcome some limitations of conventional mutagenesis. There are two main methods for incorporation of unnatural amino acids into ion channel proteins— (1) *in vivo* nonsense suppression[32; 33], and (2) ion channel semisynthesis[34].

For the *in vivo* nonsense suppression method, an amber codon (TAG) is introduced into recombinant cDNA at the desired mutagenesis site. The resulting mRNA is injected together with a synthesized tRNA bearing the CUA anticodon and an attached unnatural amino acid into a heterologous system (typically *Xenopus* oocytes). With respect to ion channels, the i*n vivo* nonsense suppression was originally applied to probe the role of aromatic residues (phenylalanine and tyrosine) in the ligand binding site of nicotinic acetylcholine receptors[35; 36]. Since then, the approach has been used to probe various aspects of ion channel structure-function. Pless et al. used unnatural amino acid substitution to examine the contribution of three S2/S3 acidic residues (Glu283, Glu293 and Asp316) as countercharges that facilitate voltage sensing in Shaker K^+ channels[37]. By replacing these residues with the synthetic neutral glutamic acid, nitrohomoalanine, they discovered that charge neutralization of Glu283, but not Glu293 or Asp316, significantly affected voltage sensing[37]. This group also used unnatural amino acid substitution to probe mechanisms of slow ‘C-type’ inactivation of K^+ channels. C-type inactivation is believed to be due to local conformational changes near the selectivity filter that are not completely understood[38]. The role of hydrogen bonds involving aromatic residue side chains in C-type inactivation was examined by replacement with synthetic amino acids that formed either weaker or stronger hydrogen bonds than the native residues. The results revealed intra- and inter-subunit hydrogen bonds that controlled the rate of C-type inactivation[39].

In the ion channel semisynthesis approach, a short portion of the channel (<60 amino acids) which contains the site for UAA incorporation is chemically synthesized and then ligated to the remainder of the protein which is bacterially expressed and purified[34; 40]. This method has been used to probe features of the K^+ channel selectivity filter and its role in C-type inactivation. The selectivity filters of K^+ channels contain four ion-binding sites (S1-S4) formed from the protein backbone carbonyl oxygens and threonine side chains of the K^+ channel signature sequence T-V-G-Y-G[34; 40; 41]. Based on the property that C-type

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inactivation is dependent on the permeant ion, Matulef et al. used protein semisynthesis to introduce amide-to-ester substitutions in the protein backbone of the selectivity filter to examine how altering ion occupancy at specific sites affected inactivation[42]. An amide-to-ester substitution that eliminated ion binding to the S2 site prevented C-type inactivation of KcsA, explicitly linking ion occupancy of this site to the inactivation process. By contrast, eliminating ion binding to S1 did not prevent inactivation[42]. Another study examined the proposal that a constricted conformation of the selectivity filter that is evident in crystal structures of KcsA at low K^+ or in the open state was responsible for C-type inactivation[43]. Protein semi-synthesis was used to replace the first conserved glycine in the selectivity filters of KcsA and KvAP with D-Alanine, a substitution that prevented constriction of respective selectivity filters. This unnatural amino acid substitution did not prevent inactivation, suggesting the constricted selectivity filter conformation does not represent the C-type inactivated state[43].

Kalstrup and Blunck used the *in vivo* nonsense suppression method to introduce intrinsically fluorescent unnatural amino acids into different regions of Shaker K^+ channels and used VCF to interrogate dynamics of sites associated with voltage sensing and pore opening[44]. This approach enables the use of VCF to probe dynamics of regions that would otherwise be inaccessible using cysteine-substitution methods. In another study, incorporation of photoresponsive unnatural amino acids was used to create ion channels regulated by light. Kang et al. used nonsense suppression to introduce 4,5-dimethoxy-2-nitrobenzyl-cysteine (Cmn) into the pore of Kir2.1 to generate a photoactivatable inwardly rectifying K^+ channel (PIRK)[45]. The incorporated Cmn blocks the Kir2.1 channel pore; exposure to UV light irreversibly removes the 4,5-dimethoxy-2-nitrobenzyl group to restore ionic conduction. An important advance by this group is the development of methods to express ion channel incorporating unnatural amino acids in mammalian cells, including neurons[45; 46].

Chimeric channel analyses

Beyond single-residue mutagenesis, generation of chimeric channels is an important approach for investigating ion channel structure-function mechanisms. This method exploits the modular nature of ion channels in which distinct well-defined domains underlie different functions. An example of this is the separation of S1-S4 and S5-S6 into voltage sensor and pore domains, respectively, in voltage-gated ion channels. Swartz and colleagues used a chimeric approach to examine structure-function relationships among distinct voltage sensors from different voltage-dependent ion channels across species. They generated a series of chimeric channels in which various portions of S1-S4 from archaebacterial voltage-activated K^+ channel (KvAP), Nav1.2a, or the voltage-sensing domain proteins, Hv1 and Ci-VSP, were swapped into eukaryotic Kv2.1 channels. Using this strategy they discovered that a 'paddle' motif comprised of S3b and S4 helices is a portable element that preserves voltage-dependent gating and transfers unique pharmacological sensitivities to toxins to chimeric Kv2.1 channels[47; 48].

The chimeric approach has also been useful in identifying domains responsible for channel modulation by auxiliary subunits or intracellular modulatory proteins[49; 50; 51]. As an example, in high-voltage activated Ca_V channels (Ca_V1 and Ca_V2) association between

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pore-forming α_1 and cytosolic β subunits is necessary for effective channel trafficking to the cell surface[52; 53]. $Ca_V\beta$ subunits bind to an 18-residue conserved sequence (termed the α_1 interaction domain, or AID) in the I-II loop of $Ca_V\alpha_1$ proteins[54]. Fang and Colecraft used a chimeric approach to determine the combination of α_1-subunit intracellular loops and N-/C-termini that were necessary to reconstitute β-dependent regulation of $Ca_V\alpha_1$ trafficking[51]. A series of 25 chimeras was generated in which all possible permutations of $Ca_V1.2$ α_{1C} intracellular loops were transferred into the β-independent $Ca_V3.1$ α_{1G} subunit. The strategy revealed that $Ca_V\beta$-dependent regulation of channel surface trafficking was an emergent property that required at least four $Ca_V\alpha_1$ intracellular loops, with the I-II loop and C-terminus being essential[51].

Using tagged proteins to study ion channel trafficking

Many ion channels must target to the cell surface to accomplish their functions. In addition to biophysical defects, one potential mechanism by which inherited or acquired mutations in ion channels can lead to disease is by compromising channel trafficking to the cell surface. Moreover, altering surface trafficking of ion channels frequently underlies channel regulation by auxiliary subunits, post-translational modifications, modulatory proteins, therapeutic molecules, and cellular activity. Hence, an ability to quantitatively measure relative surface density of ion channels is an important component of many studies.

One of the simplest protein engineering methods used to monitor channel subcellular localization is fusion with a fluorescent protein such as green fluorescent protein (GFP). GFP and other related fluorescent proteins have a high fluorescent quantum yield, are relatively small, and are usually biologically inert making them useful tools to monitor protein subcellular localization. Combined with confocal microscopy, this approach has been used to identify differential sub-cellular localization of L-type vs non-L-type calcium channels in dysgenic myotubes[55]; infer that protein kinase A promotes surface trafficking of cardiac Nav1.5 channels[56]; determine that PI3 kinase increases surface density of $Ca_V2.2$ channels[57]. A caveat of this approach is that in many cases fluorescent protein-tagged ion channel pore-forming subunits display fluorescence signal distributed throughout the cell. Combined with the relatively limited spatial resolution of confocal microscopy it is often not possible to determine and quantify the fluorescence signal that emanates specifically from plasma membrane surface channels.

An alternative approach that circumvents this drawback involves introducing a short epitope tag (such as hemagglutinin, myc, or FLAG) into an extracellular facing region of ion channel subunits which can then be recognized by a cognate antibody in non-permeabilized cells. This method has been used to selectively label surface $Ca_V1.2$ channels in heterologous cells and neurons[58; 59], and to determine impact of cardiac arrhythmia-causing mutations on surface trafficking of Kv7.1 channels[60]. A variation of this method is to introduce a 13-residue high-affinity bungarotoxin binding site into an extracellular site of a channel[61; 62]. Surface channels can then be probed with fluorophore-conjugated bungarotoxin[63]. Alternatively, the BBS epitope expressed on the cell surface can be detected using sequential exposure to biotinylated bungarotoxin and streptavidin-conjugated quantum dot (Fig. 1) [51; 62]. This takes advantage of the high extinction coefficient and quantum yield

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of quantum dots, and the fact that they are resistant to photobleaching. When combined with a high throughput method to measure fluorescence signals, such as flow cytometry, these surface epitope labeling approaches provide a robust way to quantitatively characterize relative surface expression of ion channels[51; 62].

Using engineered ion channels to probe and manipulate cell physiology

The use of heterologous expression systems combined with recombinant DNA methods has proven powerful in investigating fundamental aspects of the structure-function of voltage-gated ion channels especially relating to mechanisms of voltage sensing, ionic conductance and permeation, and trafficking. Nevertheless, in organisms, voltage-gated ion channels are found in excitable cells such as cardiac myocytes and neurons which have a more complicated cyto-architecture and intracellular environment than is found in heterologous cells. Hence, insights into the physiological roles of particular ion channels typically require experiments in native cells. Studying engineered ion channels in this setting can be useful in elucidating how specific ion channel structural elements contribute to physiological effects (structure-physiology relationships). Beyond structure-physiology relationships, engineered ion channels have been developed as biosensors to probe various aspects of cellular physiology. In this section we discuss examples of using engineered ion channels to evaluate structure-physiology relationships and as biosensors to probe cellular physiology.

Engineering ion channels to probe structure-physiology relationships: Animal models

Knockin animals offer perhaps the most stringent method to determine structure-physiology relationships of voltage-gated ion channels. This approach has been used with some success to create and investigate animal models of disease caused by mutations in specific ion channels. Several knockin mice models have been generated to study disease mechanisms including: $Ca_V2.1$ for familial hemiplegic migraine and spinocerebellar ataxia[64; 65; 66]; $Ca_V1.2$ for autistic traits observed in Timothy syndrome[67; 68]; and $Na_V1.4$ for hypokalemic periodic paralysis[69]. Beyond disease, knockin mice have also been used to address more fundamental aspects of voltage-gated ion channel structure-physiology relationships. For example, knockin mice expressing mutant $Ca_V1.2$ channels— S1512A/S1570A and S1928A — were used to investigate a hypothesized role of phosphorylation of these residues in $Ca_V1.2$ regulation by calmodulin kinase II (CaMKII) and protein kinase A (PKA), respectively[70; 71]. Knockin $Ca_V1.2$ S1928A mice displayed cardiac L-type currents that were still strongly up-regulated by PKA, definitively settling a debate about the importance of Ser1928 phosphorylation for β-adrenergic regulation of cardiac Ca_V 1.2[71; 72; 73]. Despite the overall strengths of the knockin mice approach, there are also certain disadvantages that prevent the routine use of this method for structure-physiology studies. Principally, it is expensive and time-consuming to generate a single knockin mouse. Furthermore, the knocked in channel may cause neonatal lethality which can limit mechanistic insights into the mutation[74; 75]. Some of these limitations can be circumvented with the use of transgenic models which are less expensive and quicker to make. A potential complication here is that since the native channels are typically still present strategies need to be in place to distinguish between exogenous and endogenous channels. One method to achieve this is to introduce point mutation(s) that render the exogenous channel insensitive to a small-

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molecule antagonist. This way, the function of the exogenous channel can be studied in the presence of a drug that silences native wild-type channels. Another possible confounding factor is uncontrolled expression of the exogenous protein, potentially leading to over-expression artifacts. This can be attenuated with the use of promoters that permit adjustable protein expression levels. As an example of this approach, the Marx laboratory has generated transgenic mice expressing doxycycline-inducible expression of dihydropyridine-resistant $Ca_V1.2$ channels in cardiac myocytes. They have used this system to investigate the role of phosphorylation sites proposed to underlie PKA modulation of cardiac $Ca_V1.2$ channels[76].

Cellular studies

Ion channel structure-physiology studies in primary cells such as cardiac myocytes and neurons are an important bridge between heterologous expression systems and whole-animal studies. Such primary cells retain unique cyto-architectural features and an intracellular milieu that is unattainable in heterologous cells. Compared to whole-animal studies, experiments in primary cells are more economical and less time-consuming making them ideal for comprehensive hypotheses-driven structure-physiology investigations. The drawback is that primary cells are typically more complicated to culture and transfect compared to heterologous cells. Primary adult cardiac myocytes represent a good example of the challenges that may be encountered when working with primary excitable cells. These terminally differentiated cells do not divide and can only be maintained in culture for a relatively short time (3 to 5 days) before they de-differentiate and lose their rod-like morphology[77]. Moreover, adult cardiac myocytes are resistant to transfection using conventional calcium-phosphate-precipitation or lipid-based methods, making it necessary to use viral vectors to achieve genetic manipulation[78].

Several studies have utilized viral expression of modified voltage-gated ion channels in cardiac myocytes to investigate various aspects of channel function in this native setting. Adenoviral mediated expression of ion channel pore-forming subunits has been used to: identify differences in the sub-cellular localization and surface mobility of GFP-$K_V2.1$ and YFP-$K_V1.4$ in adult rat atrial and ventricular cardiomyocytes [79]; decipher trafficking and gating mechanisms underlying loss of K^+ in adult rat cardiomyocytes current due to an LQT2 mutation in Kv11.1 (T421M)[80]; identify an important role for the $Ca_V1.2$ distal C-terminus in PKA up-regulation of L-type calcium in adult guinea pig ventricular myocytes[81]. Viral-mediated expression of ion channel auxiliary subunits or modulatory subunits has also proven useful in revealing aspects of their role in cardiac physiology. For example, over-expressing $Ca_V\beta$ subunits in cardiomyocytes is sufficient to markedly increase L-type channel currents, suggesting this subunit may be limiting for Ca_V 1.2 functional expression in the heart[82; 83]; eliminating Ca^{2+}-dependent inactivation of $Ca_V1.2$ channels by over-expressing a mutant CaM that does not bind Ca^{2+} (CaM_{1234}) yielded cardiac myocytes with ultra-long action potentials, revealing an essential role for $Ca_V1.2$ CDI in controlling action potential duration[84].

Viral vectors suitable for short-term gene expression studies in cardiomyocytes have a practical packaging capacity of 4 – 8 kb. This size limitation hinders structure-physiology

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studies of large proteins such as the full-length $Ca_V1.2$ α_{1C} subunit in primary cardiac myocytes. This issue has been addressed using a split-intein protein transsplicing method (Fig. 1)[78]. The approach harnesses the properties of split-inteins, which are naturally occurring protein-splicing elements found in archaeal, eubacterial, and eukaryotic genes [85]. When attached to two different polypeptides (termed exteins), trans-acting split-inteins can rapidly associate to form an active intein that uses a self-catalytic reaction to splice the two exteins together with a peptide bond while excising itself out of the resulting protein sequence[86; 87]. The split DnaE intein from the cyanobacterium *Nostoc punctiforme* (Npu) was used to reconstitute full-length $Ca_V1.2$ *in situ* from two separate halves. The split-intein-tagged $Ca_V1.2$ fragments readily incorporated into adenovirus and reconstituted dihydropyridine-resistant channels in cardiomyocytes. Similar to endogenous L-type calcium channels, intein-spliced $Ca_V1.2$ targeted to dyads, triggered Ca^{2+} transients, associated with caveolin-3, and supported PKA regulation of excitation-contraction coupling[78]. This approach can now be used to address several outstanding questions related to structure-physiology relationships of $Ca_V1.2$ α_1 and other large proteins in adult cardiac myocytes (Fig. 1).

Engineered ion channels as biosensors

Several studies have exploited various properties of ion channels to engineer them as probes for biologically relevant signaling molecules and events. We discuss here three examples where engineered ion channels have been used as biosensors to: (1) measure local Ca^{2+} signals in Ca_V channel nano/micro-domains; (2) estimate the local calmodulin (CaM) concentration around Ca_V channels; and (3) report on changes in membrane potential.

Probing local Ca^{2+} in Ca_V channel nano-domains

Ca_V1 and Ca_V2 channels open and close in response to membrane depolarization and repolarization, respectively, to translate electrical signals into Ca^{2+} influx that drives biological responses. Ca^{2+} ions inflowing through individual Ca_V channels typically act on effector proteins that are situated within tens of nanometers of the channel pore, i.e. within the Ca_V channel nano-domain. This is the case for the Ca^{2+}-induced Ca^{2+} release that underlies cardiac muscle contraction[88]; triggering presynaptic vesicle release for neurotransmission[89]; Ca_V channel coupling to Ca^{2+}-activated K^+ channels that regulate neuronal excitability[90; 91]; and activation of local enzymes such as CaM kinase II and calcineurin to regulate gene expression[92; 93]. Given its biological importance, there is great interest in accurately measuring Ca_V channel nano-domain Ca^{2+} signals. However, this is difficult because of the highly localized nature and fast millisecond kinetics of the Ca_V channel nanodomain Ca^{2+} signal[94]. Small organic Ca^{2+} indicators that have fast response characteristics are diffusely distributed when introduced into cells making it difficult to accurately measure nanodomain Ca^{2+} even with high-resolution imaging methods. Fusing a genetically-encoded Ca^{2+} indicator, TN-XL, to $Ca_V2.2$ channels permitted local Ca^{2+} signals to be measured using TIRF microscopy[95]. However, the slow on and off kinetics of genetically-encoded Ca^{2+} indicators limit their capacity to report on the millisecond Ca^{2+} transients that occur in Ca_V channel nanodomains. Tour et al. developed a hybrid synthetic fast Ca^{2+} indicator, Calcium Green FlAsH, capable of genetic targetability by virtue of a biarsenical moiety that can interact with a small tetracysteine motif introduced into target

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proteins[96]. By incorporating a tetracysteine motif in the intracellular N-terminus of $Ca_V1.2$ α_1-subunit these authors were able to (under high buffering conditions) observe fast Ca^{2+} transients that tracked the whole-cell Ca^{2+} current. A complication of the approach was that some of the observed fluorescence signal emanated from non-conducting channels[96].

Ca_V1 and Ca_V2 channels are subject to feedback regulation by Ca^{2+} ions (Ca^{2+}-dependent inactivation and facilitation, CDI and CDF, respectively) that is mediated by calmodulin (CaM) tethered to the C-termini of $Ca_V\alpha1$ subunits that acts as a resident Ca^{2+} sensor[97; 98; 99; 100; 101]. Tadross et al used CDI of engineered $Ca_V1.3$ channels as a sensitive indicator to probe nanodomain Ca^{2+} of active channels[102]. By calibrating and comparing CDI as a function of either bulk cytosolic Ca^{2+} concentration (obtained by intracellular Ca^{2+} uncaging) or time-averaged unitary current flux, they found that the nano-domain Ca^{2+} amplitude (due to Ca^{2+} influx through the channel) was 10-fold higher than predicted from theoretical reaction-diffusion equations. The boost in local Ca^{2+} could be modeled by a decreased diffusion co-efficient for Ca^{2+} ions in the nano-domain compared to free aqueous diffusion[102].

Probing CaM concentration in the Ca_V channel nanodomain

Ca_V channel CDI has also been used as a sensor to estimate CaM concentration in the $Ca_V1.2$ nano-domain[103]. Here, a Ca^{2+}-insensitive mutant CaM (CaM_{1234}) fused to the $Ca_V1.2$ C-terminus via short polyglycine linkers (G_2 and G_{12}) completely eliminated CDI by out-competing endogenous wild-type CaM for binding to the pre-IQ/IQ region in $Ca_V1.2$ C-terminus. Progressively lengthening the linker led to increasing recovery of CDI as the effective concentration of CaM_{1234} was reduced allowing endogenous CaM to gain a competitive foothold. By plotting strength of CDI as a function of linker length, and using a polymer chain statistics algorithm to calculate the effective concentration of CaM_{1234}, the authors estimated a 2.5 mM concentration of wild-type CaM in the $Ca_V1.2$ nanodomain (Fig. 2). This is orders of magnitude higher than the estimated ~50 nM bulk free CaM concentration in HEK 293 cells[104].

Genetically-encoded membrane potential reporters

Genetically-encoded membrane voltage sensors are a sought after enabling tool for neuroscience and other applications[105]. In principle, such sensors could be selectively expressed in a targeted population of cells and permit measurement of cellular activity with high spatial and temporal resolution. Because of their inherent voltage-sensing properties, voltage-gated ion channels are natural candidate templates for engineering genetically-encoded voltage sensors. In a first attempt to achieve this, a modified GFP was inserted in frame just after S6 of a non-conducting mutant of Shaker K^+ channels. The resulting construct, named FlaSh, when expressed in *Xenopus* oocytes responded to step depolarizations with fluorescence changes that displayed slow on and off kinetics[106]. Later, insertion of GFP into skeletal muscle voltage-gated Na^+ channel intracellular II-III loop yielded SPARC (sodium channel protein-based activity reporting construct) which produced much faster (but smaller) fluorescent signal responses with changes in membrane potential[107]. However, FlaSh and SPARC were ineffective at detecting membrane voltage changes in mammalian cells in part because they are poorly targeted to the plasma

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membrane[105; 108]. Recent advances in genetically encoded voltage sensor development have utilized fluorescent proteins fused to the autonomous voltage-sensing domain of the non-ion channel protein *Ciona intestinalis* voltage-sensor containing phosphatase (Ci-VSP). Successful Ci-VSP-based sensors have been generated with either a single fused fluorescent protein (ArcLight, ElectricPK)[109; 110] or two tandem fluorescent proteins in which signal changes are reported by FRET (Mermaid, SFV2.1)[111]. In general, sensors with a single fluorescent protein fused to Ci-VSP yield membrane-potential dependent signal changes with the fastest on and off kinetics and are thus capable of tracking fast action potentials with the highest fidelity. ArcLight, which contains a mutated super ecliptic pHluorin fused to Ci-VSP has been used to track neuronal action potentials and sub-threshold electrical events[109]. ElectricPK features a circularly permuted eGFP fused to Ci-VSP and has also been used to track hippocampal neuron action potentials[110].

Engineered ion channel inhibitors and modulators

Molecules that selectively block or modulate the activity of specific ion channels are important therapeutics or essential research tools. Two general problems that dog the use of many small-molecule ion channel modulators are lack of targetability to specific cell populations and limited selectivity/specificity for particular ion channel isoforms. These two issues can collaborate to produce off-target effects that can limit therapeutic applications or confound interpretation of experimental results. We discuss here three engineering approaches that have been developed to address some of the limitations of small-molecule ion channel modulators: (1) tethered toxins; (2) engineering toxins for improved channel selectivity; and (3) genetically-encoded intracellular channel inhibitors.

Development and applications of tethered toxins

Animal venoms contain numerous peptide neurotoxins some of which are potent ion channel blockers. Specific animal toxins have proven invaluable tools to decipher biological functions and structural features of distinct ion channels, to probe and manipulate neuronal circuits, and as therapeutics[112; 113]. One drawback of using toxins in living organisms is that they cannot be restricted to a particular cell population since they are soluble. A tethered toxin approach has been developed that helps overcome this limitation[114; 115]. The method was inspired by the prototoxin, lynx1, an endogenous modulator of nAChRs in mammalian central nervous system[116]. The open reading frame of lynx1 contains a secretory signal sequence, a cysteine-rich region with homology to secreted snake venom neurotoxins, and a hydrophobic C-terminus domain with a consensus site for GPI anchor addition. This basic design has been exploited to design tethered toxins with specificity for different ion channels. The general design principle is to fuse in frame a secretory signal; the toxin of interest; a linker sequence; and either a GPI anchor or single-pass transmembrane sequence. Additional variations include the incorporation of fluorescent proteins or epitope tags that permit visual detection of the tethered toxin[114]. Early proof-of-concept experiments showed that several tethered bungarotoxins and conotoxins specifically and strongly inhibited particular nAChR isoforms in *Xenopus* oocytes and in zebrafish muscle *in vivo*[115]. Similarly, the tethered conotoxins MrVIA and MVIIA selectively and completely blocked co-expressed recombinant $Na_V1.2$ and $Ca_V2.2$ currents, respectively[115]. Tethered conotoxin

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MVIIA and spider agatoxin IVA blocked $Ca_V2.2$ and $Ca_V2.1$ channels, respectively, and inhibited neurotransmission in cultured neurons and *in vivo*[117]. Furthermore, transgenic mice with tethered MVIIA expressed in nociceptive neurons displayed reduced sensitivity to inflammatory and neuropathic pain[117]. The powerful tethered toxin technology could potentially be improved by designing in the ability to acutely regulate channel activity with a secondary signal. A proof-of-concept of this capability has been achieved with the development of lumitoxins, which graft light-mediated regulation into the basic tethered toxin design[118]. The modular architecture of lumitoxins incorporates the in-frame fusion of: an ion channel targeting toxin; a light-oxygen-voltage photoswitch domain from *Avena sativa* (LOV2-Jα); a 26-residue linker; and a single-pass transmembrane domain from platelet derived growth factor receptor. Channel-specific lumitoxins for Kv1.1, Kv1.2 and Shaker were developed which displayed relative channel block in the basal (dark) state; exposure to 455 nm blue light quickly actuated the photoswitch domain to relieve channel block, resulting in larger K^+ currents[118].

Engineering toxins for improved specificity and potency

In many cases, the therapeutic potential of particular venom toxins is limited by their lack of specificity for a target ion channel of interest. In such cases, toxins may be engineered to improve specificity and potency for the desired target. As an example, autoimmune diseases such as multiple sclerosis involve activated memory T cells which exhibit a selective up-regulation of $K_V1.3$ channels that control membrane potential and Ca^{2+} signaling in these cells[119]. Agents that selectively block Kv1.3 channels on T lymphocytes are possible therapeutics for autoimmune diseases[120; 121]. A 35-amino-acid polypeptide from the sea anemone *Stichodactyla helianthus*, ShK, potently blocks Kv1.3 and Kv1.1 with IC_{50}s in the low picomolar range[122]. Complementary mutagenesis of ShK and Kv1.3 combined with mutant cycle analyses revealed two residues in ShK, lys22 and tyr23, are essential for potassium channel block[122]. Using such structure-function information, several derivatives of ShK have been engineered with improved selectivity for Kv1.3 over Kv1.1, including; ShK-Dap[22] in which the unnatural amino acid diaminopropionic acid is substituted for the critical lys22, and ShK-170 which features an L-phosphotyrosine attached to Arg1 of ShK via an aminoethyloxyethyloxy-acetyl linker[122; 123]. Both these engineered toxins blocked proliferation of memory T lymphocytes and suppressed hypersensitivity with limited off-target toxicity in animal models.

In a different approach, a combinatorial strategy was utilized to develop a toxin with selectivity for Kv1.3[124]. The library was based on kaliotoxin-1 (KTx), a scorpion toxin that potently blocks Kv1.3, but also inhibits Kv1.1 and Kv1.2. A combinatorial library containing 11,200 de novo toxins was generated from 31 known or predicted α-KTx toxins in a manner that preserved scaffold architecture maintained by conserved disulfide bonds. The toxins were presented by phage display and affinity selected on a purified target. This approach led to the isolation of mokatoxin-1 which blocks Kv1.3 with nanomolar affinity, but bound poorly to Kv1.1 and Kv1.2. Consistent with this, mokatoxin-1 inhibited induced cytokine production from isolated human T lymphocytes, but had no off-target effects on guinea pig ileal strips that contain native Kv1.1 and Kv1.2 channels[124].

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Genetically-encoded intracellular ion channel blockers

Recently, a new approach to develop cytosolic genetically-encoded ion channel blockers and modulators has been demonstrated for Ca_V1 and Ca_V2 family channels. The method was inspired by the RGK (Rad, Rem, Rem2 and Gem/Kir) GTPases, a four-member family of Ras-like G-proteins that constitutively and strongly inhibit Ca_V1 and Ca_V 2 channels[125; 126; 127; 128]. RGK protein inhibition of Ca_V channels was found to have a dual requirement— direct anchoring of the G-protein to the plasma membrane, and binding of the G-protein to the cytosolic β subunit in the Ca_V channel complex[129; 130; 131; 132]. These findings led to a hypothesis that membrane-targeted RGKs indirectly pull on the α_1-subunit I-II loop via the associated $Ca_V\beta$ in a manner that is transmitted to close the channel pore. Testing this hypothesis led to the discovery that diverse cytosolic proteins that bind $Ca_V\alpha_1$-subunits can be converted into Ca_V channel inhibitors with tunable selectivity, potency and kinetics by anchoring them to the plasma membrane[131]. The method has been termed channel inactivation induced by membrane-tethering an associated protein (ChIMP) (Fig. 3).

An advantage of genetically encoded Ca_V channel inhibitors, shared with tethered toxin technology, is that they can be expressed in a locally restricted manner thereby limiting off-target side effects[133]. As an example of this, restricted expression of the RGK protein Gem in the atrioventricular (AV) node reduced AV nodal conduction and heart rate in a porcine model of atrial fibrillation[134]. Another important advantage of genetically encoded Ca_V channel inhibitors is that they may be engineered to selectively block Ca_V channels based on their sub-cellular localization within a single cell. In adult mammalian ventricular myocytes, it has been postulated that there are at least two functionally distinct populations of $Ca_V1.2$ channels— those targeted to dyadic junctions engage in Ca^{2+}-induced Ca^{2+} release that underlies EC coupling; those targeted to caveolae that signal through local effectors to regulate gene expression[92]. Dysfunction of caveolae-targeted $Ca_V1.2$ channels has been proposed as a molecular mechanism for pathological cardiac hypertrophy. In agreement with this, a caveolae-targeted Rem selectively blocked hypertrophic Ca^{2+} signaling in cardiac myocytes without affecting contraction[135].

Summary

This review has focused on three aspects of ion-channel engineering, with an emphasis on voltage-gated channels: (1) modifying ion-channel parts to understand how they function, (2) tailoring ion channels to probe cell biology and physiology, and (3) engineering novel modulators of ion channels. There has been remarkable progress in understanding structure-function mechanisms of ion channels over the last few decades driven by continual advances in new technologies to modify and probe these proteins. The pace of discovery will continue as structures of more ion channels become available, and with advances in computational methods to model channel behavior. There is ample room for development of new approaches to modulate ion channels with unprecedented specificity at the molecular, sub-cellular, and cellular levels in whole organisms. Such approaches are needed to expand the available toolbox for probing the complicated structure-physiology relationships of distinct ion channels, and also to fully realize the potential of these proteins as therapeutic targets.

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Acknowlegdements

This work was supported by NIH grant RO1GM107585 (to H.M.C.). HMC is an Established Investigator of the American Heart Association.

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Highlights

- Ion channels facilitate passive movement of ions across biological membranes and are essential for life.
- Ion-channel engineering approaches help elucidate structure-function mechanisms of these proteins.
- Engineered ion channels are important tools for probing and manipulating cell biology.
- Engineered ion channel modulators are essential research tools and therapeutics.

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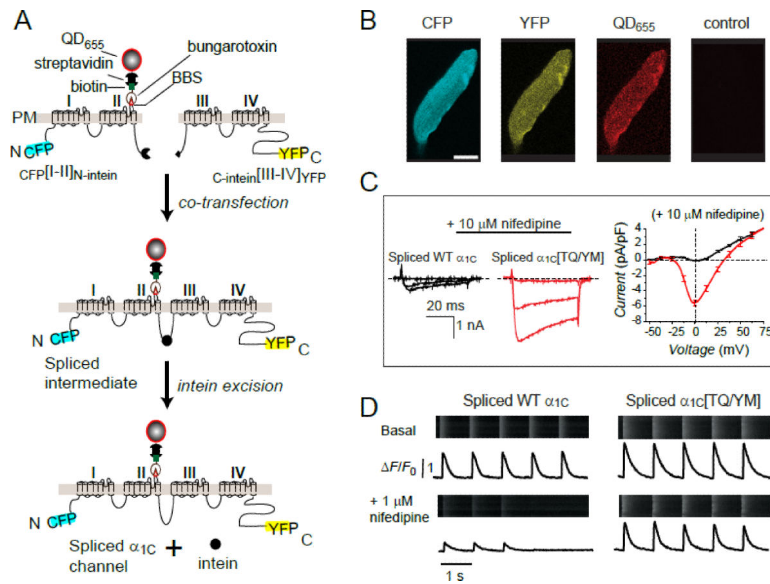

Figure 1. Functional expression of engineered $Ca_V1.2\alpha_{1C}$ in adult cardiomyocytes using a split-intein ligation strategy

(A) Strategy for generating full-length α_{1C} from two halves using split-intein protein transsplicing. A 13-residue bungarotoxin binding site (BBS) inserted into the extracellular domain II S5-S6 linker enables detection of surface channels with bungarotoxin-biotin and streptavidin-conjugated quantum dot. (B) Fluorescent detection of split-intein ligated α_{1C} expressed in an adult rat cardiomyocyte. (C) *Left* and *middle*, $Ca_V1.2$ currents in myocytes expressing either intein-spliced wild-type α_{1C} (black) or a dihydropyridine-resistant mutant, α_{1C}[TQ/YM] (red), in the presence of 10 μM nifedipine. *Right, I-V* curves in 10 μM nifedipine for intein-spliced WT (black) and DHP-resistant (red) α_{1C} channels with 2 mM Ca^{2+} as charge carrier. (D) Impact of nifedipine on Ca^{2+} transients from cardiomyocytes expressing either intein-spliced wild-type (*left*) or dihydropyridine-resistant (*right*) α_{1C}.

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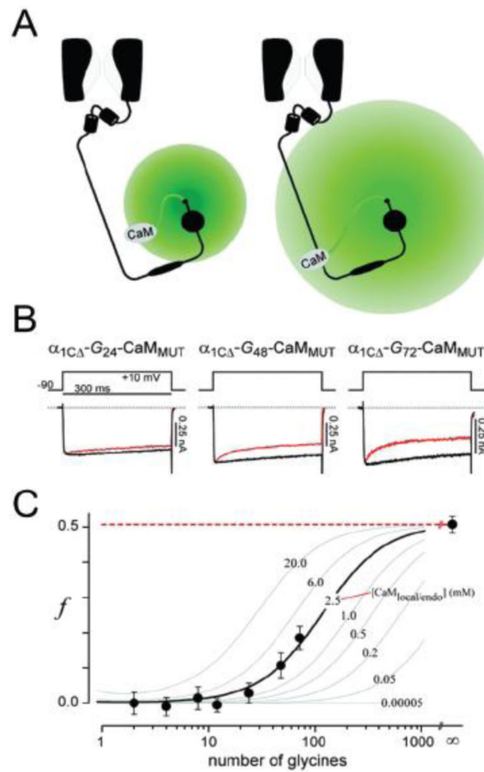

Figure 2. Ca_V1.2-CaM chimeras used as biosensors to estimate local CaM concentration
(A) Schematic showing concept of how extending linker length would be expected to decrease the effective local concentration around the tether site. (B) Ca^{2+}-dependent inactivation (CDI) observed in Ca_V1.2-CaM_{1234} chimeras with varying glycine linker lengths. (C) CDI strength (*f*) of Ca_V1.2-CaM_{1234} chimeras plotted as a function linker length (●). Family of continuous lines was generated from polymer chain statistical theory using different estimates for endogenous local CaM concentration [$CaM_{local/endo}$]. Data were best fit with an estimated [$CaM_{local/endo}$] of 2.5 mM and $r = 7$Å (black trace).

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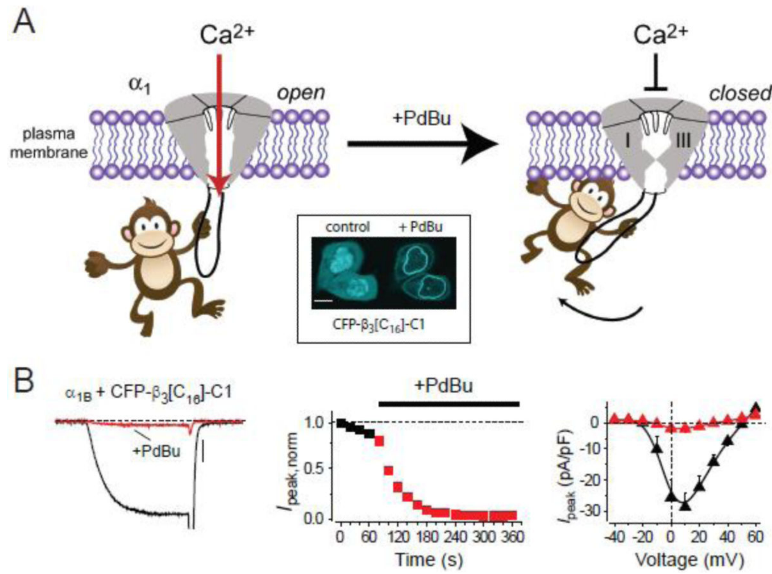

Figure 3. Channel inactivation induced by membrane tethering an associated protein (ChIMP)

(A) Cartoon showing concept of ChIMP. A cytosolic protein associated with a cytoplasmic domain of a $Ca_V\alpha_1$ subunit is permissive for ionic conductance (*left*). Directly anchoring the cytosolic protein to the plasma membrane induces a conformational change that closes the channel (*right*). *Inset*, phorbol-12,13-dibutyrate (PdBu)-induced membrane translocation of a C-terminus truncated CFP-tagged $Ca_V\beta_3$ fused to the C1 domain of protein kinase C (CFP-β_3[C16]-C1). (B) Conversion of $Ca_V\beta_3$ into a PdBu-inducible Ca_V2.2 channel inhibitor using the ChIMP concept.