

Illuminating the structure and function of Cys-loop receptors

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Summary

1. Cys-loop receptors are an important class of ligand-gated ion channels. They mediate fast synaptic neurotransmission, are implicated in various “channelopathies” and are important pharmacological targets. Recent progress in x-ray crystallography and electron microscopy has provided a great deal of insight into the structure of Cys-loop receptors. However, data from these experiments only provide “snapshots” of the proteins under investigation. They cannot provide information about the various conformations the protein adopts during the transition from closed to open and desensitised states.

2. Voltage-clamp fluorometry (VCF) helps to overcome this problem by simultaneously monitoring movements at the channel gate (through changes in current) and conformational rearrangements in a domain of interest (through changes in fluorescence) in real time. The technique can thus provide information on both transitional and steady-state conformations and serves as a real time correlate of the channel structure and its function.

3. VCF experiments on Cys-loop receptors have yielded a wide body of data concerning the mechanisms by which agonists, antagonists and modulators act on these receptors. They have shed new light on the conformational mobility of both the ligand-binding and the transmembrane domain of Cys-loop receptors.

Introduction

Ion channels are integral components of most cell membranes and play a particularly important role in the nervous system. They can respond to wide range of both chemical (neurotransmitter, second messenger) and physical (membrane potential, mechanical force, temperature) stimuli.¹ There is currently much interest in understanding the structural mechanisms of ion channel activation and drug modulation. This review will focus on channels from the Cys-loop receptor family, a subfamily of the ligand-gated ion channel (LGIC) family.² Cys-loop receptors mediate fast neurotransmission in the nervous system. Some members of the family, including the nicotinic acetylcholine receptors (nAChRs) and serotonin type-3 receptors (5-HT₃Rs), conduct cations and thus mediate excitatory neurotransmission. Gamma-aminobutyric acid type-A and type-C receptors (GABA_ARs and GABA_CRs) and glycine receptors (GlyRs) conduct anions, thereby mediating inhibitory neurotransmission. Cys-loop receptors may comprise either homo- or hetero-

pentameric oligomers, with each subunit consisting of a large N-terminal ligand-binding domain, followed by a transmembrane domain containing four transmembrane helices.^{3,4} The ligand-binding domain is mainly composed of β -sheets, connected by flexible loops. The agonist binding site is a pocket-like structure at the interface of two subunits. It is formed by six domains: three loops from one subunit form the principal binding site (domains A-C) and three β -sheets from the adjacent subunit form the complementary binding site (domains D-F).

The ligand-binding domain is followed by the first transmembrane domain (M1), which is connected to the second transmembrane domain (M2) *via* a short intracellular loop. The M2 domain lines the water-filled pore of the channel. Importantly, structural studies by Unwin and colleagues³ have confirmed the presence of a central inward kink in the M2 helix that may form the channel gate. Although the exact location of the gate is still subject to debate, it is likely to be located either centrally or towards the intracellular end of M2. How does the M2 domain move to open the channel gate? Unwin and colleagues suggested a conformational rearrangement in the ligand-binding domain triggers a rotational movement of M2 that ultimately opens the channel gate. This mechanism is currently under debate as another study strongly argued against such a rotation and favours a simple widening of the pore.⁵ The third and fourth transmembrane domains (M3 and M4) are linked by an intracellular loop that varies dramatically in size and amino acid sequence identity from one subunit to the next. This domain contains a variety of sites that mediate channel anchoring and modulation.

All Cys-loop receptors are bound by their respective agonists within microseconds after their release into the synaptic cleft. The agonists then trigger channel opening and, depending on the ionic selectivity, excite or inhibit the postsynaptic cell. The interplay between the excitatory and inhibitory stimuli must be precisely orchestrated to coordinate complex synaptic events. For example, if Cys-loop receptors open or close too slow or close too fast, synaptic events can be strongly impaired. This is typical of the type of receptor dysfunction that causes so-called “channelopathies”, which are diseases caused by hereditary mutations resulting in improper functioning (or reduced surface expression) of ion channels. A number of such diseases are well characterised. For example, hereditary mutations in the GABA_AR γ_2 gene can give rise to epilepsy,^{6,7} mutations in the gene for the nAChR α subunit can cause a myasthenic syndrome⁸ while mutations in the

GlyR α_1 gene can lead to hyperekplexia, also known as “startle disease”.^{9,10}

Moreover, most Cys-loop receptors are important drug targets. GABA_ARs have long been important clinical targets for therapies directed at muscle relaxation, epilepsy, anxiolysis, sedation and anaesthesia, and are currently under consideration for indications including depression, drug addiction, psychosis and cognitive impairment.¹¹ 5-HT₃R antagonists are used clinically as anti-emetics. nAChRs play a role in nicotine addiction and are therapeutic targets for Alzheimer’s disease and Tourette’s syndrome, as well as for schizophrenia.¹² GlyRs are emerging targets for chronic inflammatory pain.¹³ Given their pathological and pharmacological importance it is highly desirable to gain a better understanding of the structure and function of Cys-loop receptors. Recent crystallographic and electron microscopy studies have made invaluable contributions to our understanding of the overall structure of these channels.^{3,14,15} The first high-resolution data was obtained from acetylcholine binding protein (AChBP), a molluscan homologue of a Cys-loop receptor ligand-binding domain.¹⁴ Most importantly, it confirmed the overall topology and the location of the ligand binding sites at the subunit interfaces as predicted by decades of functional studies. Two years later Unwin and colleagues presented electron-microscopy data derived from *Torpedo marmorata* nAChRs that provided detailed structural information on both the ligand-binding and the transmembrane domains, including the channel pore.³ Again, the data correlated well with data from previous functional studies and gave rise to the previously mentioned gating mechanism. More recently, a crystal structure of the nAChR $\alpha 1$ subunit ligand-binding domain provided crucial insights into the domains interacting with carbohydrate chains and protein toxins.¹⁵ Thus, most of the structural data available to date stems from nAChRs, with very little information available on the other members of the Cys-loop family. Also, these structures only provide still images, typically representing a closed, open or desensitised state of the channel. In other words, they present single frames of what ideally would be a molecular movie.

Consequently, the precise mechanism by which the binding of one or more ligand molecules is translated into the structural rearrangements that trigger channel opening is still not well understood.¹⁶ The voltage clamp fluorometry (VCF) technique presented here is a valuable tool to address this question in the future. The aim of this review is to 1) introduce VCF as a technique to conduct real time structure-function studies, and 2) provide an overview about the recent advances in the field of Cys-loop receptors made available by VCF experiments.

VCF – The technique

In principle, VCF is a logical extension of the scanning cysteine accessibility method (SCAM) technique.¹⁷ First, a cysteine is engineered into an otherwise cysteine-free protein. The protein is subsequently reacted with a fluorescent reporter incorporating a sulfhydryl-

reactive group, typically a methanethiosulfonate (MTS) or maleimide moiety. In the past, Alexa Fluor dyes, rhodamine or fluorescein derivatives and recently also smaller fluorophores such as bimane have been successfully used in VCF experiments. Fluorophore selection is largely a matter of trial and error. It is advisable to test different dyes at a given site to optimise the fluorescence signal, giving particular consideration to linker moieties which vary considerably in size and reactivity (MTS linkers usually react an order of magnitude faster than maleimide linkers). Furthermore, depending on the chemical microenvironment of a given labelling site, minor structural differences in the fluorophore can lead to fundamentally different fluorescence read-outs. As a result, some sites will only produce fluorescence changes with one specific dye, while a neighbouring site may yield fluorescence changes only with a different dye. As both the spectral properties and the quantum yield of these fluorophores are highly dependent on their microenvironment, they can provide a read-out of protein movements. Conveniently, they may also offer a wide dynamic range. Because the lifetime of the fluorophores excited-state is on the nanosecond scale¹⁸ but the dynamics of ion channels occur on the microsecond timescale,¹⁹ VCF can provide real-time information on protein motions (Figure 1). In fact, it has been demonstrated that VCF can resolve the fast and complex kinetics of the voltage sensor (S4 domain) movements of potassium channels.^{20,21}

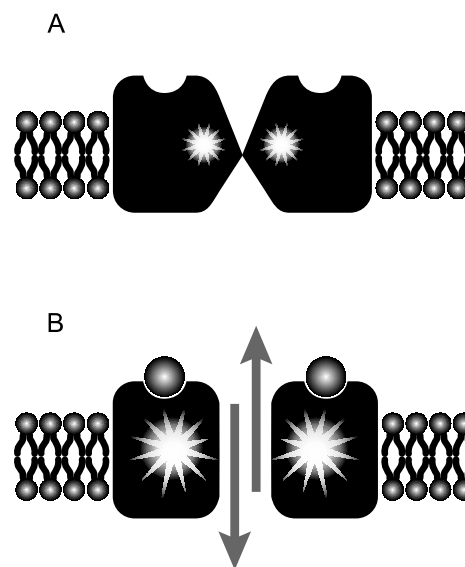


Figure 1. Site-specific fluorescent labelling allows monitoring of protein dynamics. *A. Cartoon showing a closed channel in a lipid bilayer with a fluorescent label (indicated by star flash) attached near the channel gate. The binding site is unoccupied and the fluorescence level emitted from the fluorophore is low. B. Cartoon of a ligand-bound channel with an alternative conformation near the channel gate that allows ion flux (indicated by arrows) and shows greater fluorescence (indicated by bigger star flash).*

The choice of the fluorescent light source depends mainly on the spectral properties of the dye in use, but generally mercury, xenon and halogen lamps provide good results for a range of dyes. For most VCF applications a standard epifluorescence microscope, fitted with an appropriate filter set and a powerful objective will be sufficient. Photodiodes, photomultiplier tubes (PMTs) or fast charge-coupled devices (CCDs) can be used for fluorescence detection (Figure 2). A key consideration in VCF experiments is the minimisation of background light levels. The experimental set up should thus be effectively shielded from light. More importantly, background fluorescence should be kept to a minimum. As fluorophore molecules can attach to other (native) membrane components present in the cell membrane or non-specifically incorporate into the membrane itself, the labelling procedure has to be carefully optimised by varying dye concentration (typically 5-50 μM), labelling time (around 30 s for MTS-linked fluorophores and 30-60 min for maleimide-like fluorophores) and temperature (4-20°C). In some cases it may be necessary to react the cells with a non-fluorescent sulfhydryl-reactive compound 12-24 hours before the experiment to ensure that most endogenous cysteines are blocked and that only newly synthesised protein is available for labelling. For this purpose, N-ethylmaleimide (NEM) or compounds typically used for SCAM studies, such as MTSET or MTSES can be used.

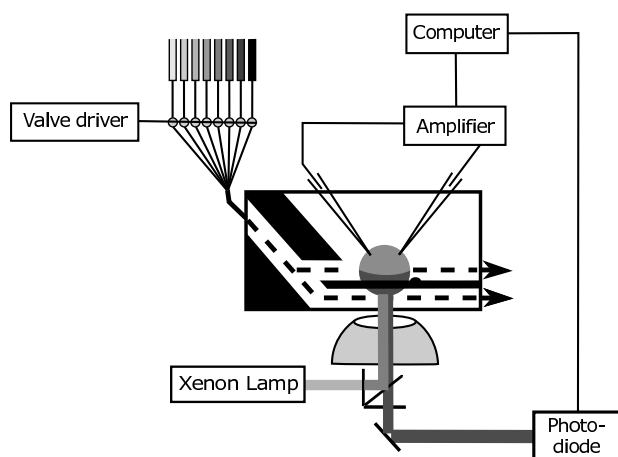


Figure 2. Typical experimental set up for VCF experiments on ligand-gated ion channels. The perfusion is operated by an automatic valve driver that constantly perfuses the oocyte in the chamber as indicated by the arrows. A xenon lamp provides the excitation light that illuminates the animal pole of the oocyte. The photodiode detects the fluorescence and feeds data into the computer that simultaneously receives input from the amplifier performing the two-electrode voltage clamp. Modified from Chang & Weiss.⁵¹

VCF experiments with ligand-gated ion channels impose additional technical challenges, as they require

constant perfusion and fast solution changes. This requires a more rigid positioning of the oocyte to minimise artefact-inducing movements of the oocytes and the realisation of a reliable and fast solution exchange. However, VCF experiments with ligand-gated ion channels still have a much lower time resolution than those with voltage-gated ion channels as voltage can be changed in the microsecond time frame whereas complete solution exchange to an oocyte is difficult to achieve in less than a few hundred milliseconds. Thus, VCF has yet to be successfully used to give real time information about domain movements in ligand-gated ion channels. Typically, *Xenopus laevis* oocytes have been used in VCF experiments. They offer a large surface area, easy handling, generate very little auto-fluorescence and provide high expression levels. Performing VCF experiments in mammalian cells is more desirable but significantly more difficult as the much smaller surface area of mammalian cells leads to reduced signal-to-noise and signal-to-background ratios. Only one study has accomplished this goal to date,²² using a technically sophisticated set up including a laser and a total internal reflection fluorescence or semiconfocal microscopy.^{23,24}

A valuable extension of the VCF approach is the combined use of spectrophotometers and CCD cameras. This allows real time monitoring of the spectral emission of the fluorophore in use. This can be very useful, because many organic fluorophores change their spectral properties depending on the hydrophobicity of their environment. In case of rhodamine derivatives, the emission peak is blue-shifted when exposed to a more hydrophobic environment. The use of spectrophotometers thus allows to discern between (de)quenching events caused by changes in position of nearby quenching groups and changes in the hydrophobicity of the microenvironment of the fluorophore.^{25,26} In other words, measuring spectra provide additional information about the structural basis of the conformational change.

VCF offers a range of advantages over existing techniques as it can give information about 1) the temporal resolution of domain movements during gating 2) whether all subunits behave in a similar way 3) whether different ligands produce different conformational changes, and 4) it can provide information about conformational changes that are electrophysiologically silent.

Zagotta and colleagues have developed an extension to this technique known as patch-clamp fluorometry (PCF) to gain insight into the structural rearrangements of cyclic nucleotide-gated channels.^{27,28} PCF uses electrophysiological recordings from excised inside-out patches. This method permits the fluorescent labelling of intracellular domains and offers the advantage that electrophysiological and fluorescence recordings come from the same population of receptors. PCF has been reviewed extensively elsewhere.²⁹

VCF of ligand-gated ion channels

The technique was first used to gain insights into the

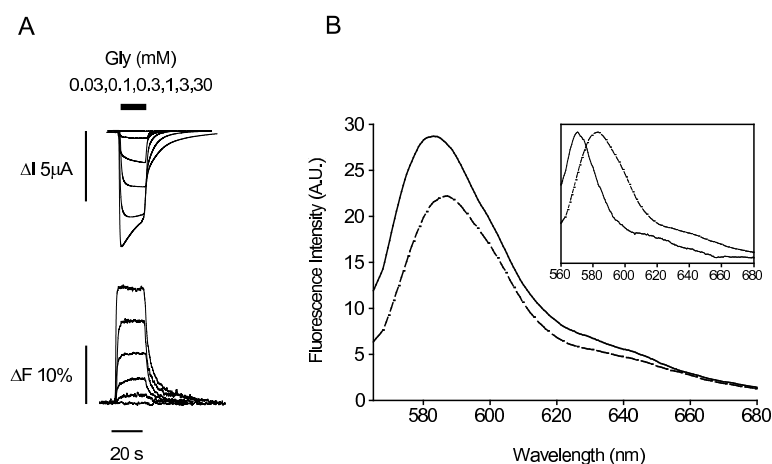


Figure 3. Rhodamine-methanethiosulfonate (MTSR)-labeled GlyR $\alpha 1^{R271C}$ oocytes show an increased quantum yield and changes in their spectral properties when activated by glycine. **A.** Glycine-evoked current (upper panel) and fluorescence (lower panel) recordings from MTSR-labeled GlyR $\alpha 1^{R271C}$ oocytes. Black bar indicates duration of glycine application. **B.** Spectral emission from MTSR-labeled GlyR $\alpha 1^{R271C}$ oocytes before (dashed line) and during (solid line) the application of 30 mM glycine (spectra averaged from 5 cells). Inset shows normalised difference emission spectra from MTSR-labeled GlyR $\alpha 1^{R271C}$ oocytes recorded before and during application of glycine. Spectra recorded in the absence of glycine were subtracted from spectra recorded in the presence of glycine to obtain difference emission spectra. Modified from Pless et al.²⁶

structural rearrangements of *Shaker* K⁺ voltage-gated channels by the Isacoff laboratory.³⁰ It has since provided a wealth of information on potassium channels, especially on movements of the voltage sensor and its neighbouring domains.^{20,21,30-37} It was subsequently applied to various other membrane proteins, including hyperpolarisation-activated cyclic nucleotide-gated channels,^{38,39} sodium channels,^{22,40-42} Na⁺/K⁺-ATPase,^{43,44} Na⁺Pi co-transporter^{45,46} and glutamate transporters.^{47,48} The work on voltage-gated ion channels has been extensively reviewed elsewhere.^{49,50} Together, these studies have shown that VCF can provide real time information on protein movements at single amino acid resolution³⁶ and, owing to its wide dynamic range, shows great versatility in the investigation of extracellular and transmembrane segments of ion channels.

More recently, VCF has been also used to investigate ion channels from the Cys-loop family. The initial study on the GABA_AR⁵¹ was followed by studies on the nAChR,^{25,52} GABA_AR⁵³ and GlyR.²⁶ Most of the investigations to date have concentrated on the ligand-binding domain of these receptors. These studies have established that residues in loops A, E and C of the ligand-binding domain undergo agonist-induced movements that lead to increases in fluorescence.⁵¹⁻⁵³ The results from one of these studies⁵¹ seems to indicate a small difference in the size of the fluorescence response in loop E, depending on the agonist used. This could indicate that loop E adopts agonist-specific conformations. Loops A-F are thought to be crucial elements for agonist binding. The authors of the studies concluded that the movements in loops A, E and C are likely to represent the closure of the agonist-binding

pocket upon agonist binding that is thought to trigger channel opening.^{51,53} The idea of a pocket-like binding site that contracts upon agonist binding has also been proposed in a crystallographic study of an AMPA-sensitive glutamate receptor, a member of the ligand-gated ion channel superfamily, but not the Cys-loop family.⁵⁴ Interestingly, competitive antagonists alone were found to induce distinct conformational changes on their own in loop A and E. This is likely to reflect a mechanism by which competitive antagonists do not merely occupy the ligand-binding site but induce a discrete conformation, thereby “actively” stabilising the closed state of the channel. Loop C, however, was found to respond with similar structural changes to both agonists and antagonists in GABA_CRs.⁵¹ This apparent lack of discrimination between agonists and antagonists is surprising as loop C is thought to be a key element in triggering channel activation (reviewed in Sine & Engel⁵⁵). Taken together, these results show a previously unexpected conformational flexibility in the response to the binding of agonist and antagonist molecules in the ligand-binding domain of Cys-loop receptors.

The transmembrane domains of Cys-loop receptors have received much less attention with only two VCF studies available to date.^{25,26} Both studies focus on the extracellular end of M2 and show large, agonist-induced fluorescence changes of 10 - 20%. As shown in Figure 3 the considerable magnitude in fluorescence change at the GlyR $\alpha 1$ R271C (or R19'C) position enabled a detailed spectral analysis.²⁶ The ~10 nm blue-shift in the emission peak clearly demonstrates that the fluorophore moves to a more hydrophobic environment in the channel open state. As expected, the competitive GlyR antagonist strychnine

did reverse agonist-induced conformational changes but did not induce a distinct structural change on its own at this site.

The extracellular end of M2 is located midway along the agonist-induced “conformational wave” that extends from the ligand-binding site to the channel gate.⁵⁶ It is hence an ideal starting point to address the question if different agonists (with the same or at least overlapping binding sites) open the channel by the same mechanism. Although glycine, taurine and β -alanine share the same binding site in the GlyR ligand-binding domain, they were found to induce distinct conformational changes at the extracellular end of M2, even in a mutant where taurine and β -alanine were converted into antagonists.²⁶ This has important implications because both the binding and the effector site (the channel gate) are the same for these agonists, and yet they employ distinct signal transduction pathways within the protein. Can GlyRs be activated by mechanisms different to those induced by agonists binding in the “classical” binding site? Addressing this question the unusual GlyR agonist ivermectin was tested on the α 1 GlyR incorporating the R271C mutation to the external region of the M2 domain. The binding site of ivermectin is not known, but is unlikely to overlap with the glycine binding site.⁵⁷ Interestingly, Pless *et al.* showed that ivermectin activates GlyRs by a mechanism that does not lead to conformational changes at the extracellular end of M2, possibly suggesting an activation mechanism triggered by the intracellular part of M2.²⁶ Together with the data obtained for taurine and β -alanine, this demonstrates that movements at the top of M2 are not necessarily reflected by movements of the channel gate and *vice versa*. These findings shed new light on the conformational dynamics of Cys-loop receptors, as other studies^{56,58-61} assumed the same conformational changes for different agonists. This may partially explain the disagreements among different models for Cys-loop receptor activation. However, further studies are needed to elucidate the models in more detail.

Conclusion and outlook

VCF has been shown to be a very powerful technique in structure function studies on various different ion channel types, including members of the Cys-loop family. VCF studies to date have demonstrated an unexpectedly large conformational variability in both the ligand-binding and the transmembrane domains of Cys-loop receptors. The technique will no doubt lead to further breakthroughs in the field. To make full use of the potential of this technique it will, however, be necessary to address a few issues: ideally, the perfusion should be accelerated, intracellular domains should be made accessible for labelling and finally it would be desirable to use mammalian cells in VCF experiments instead of *Xenopus* oocytes. A promising new technique to at least overcome the two latter problems is the incorporation of unnatural amino acids, that will allow the use of genetically encoded fluorescent amino acids in the future.⁶²⁻⁶⁴ This approach promises very low background fluorescence and the ability

to incorporate the fluorescent reporter group at almost any position throughout the protein. It will undoubtedly be very interesting to follow the future developments of this powerful combination of electrophysiology and fluorescence, as it nicely complements the information gained from decades of functional studies and the ever increasing number of crystal structures available.

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