Genomics, cys-loop ligand-gated ion channels and new targets for the control of insect pests and vectors

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Short Title

Insect cys-loop ligand-gated ion channels

Abstract

Cys-loop ligand-gated ion channels (cysLGICs) play roles in the nervous system. They consist of five subunits arranged around a central ion channel with each subunit being encoded for by a separate gene. In insects, the cysLGIC superfamily commonly consists of 21-25 genes giving rise to several receptor classes such as nicotinic acetylcholine receptors and GABA receptors. Insect cysLGICs are of interest as they are the target of insecticides. Analyses of genome sequences have identified cysLGIC gene superfamilies from different species including crop pests, disease vectors and beneficial insects. This review explores recent studies that have pushed forward our knowledge about this superfamily and considers the potential of developing improved strategies to control insect pests whilst sparing non-target organisms.

Highlights

- *Aedes aegypti* cysLGIC superfamily is the most recent one to be characterised
- Some insect cysLGICs may perform non-neuronal roles
- Insect glycine receptor subunits have potentially been discovered
- Differences in insect cysLGICs may be exploited to develop improved insecticides

Introduction

Cys-loop ligand-gated ion channels (cysLGICs) make up a superfamily of receptors, for which the best-known role is to mediate the actions of neurotransmitters (sometimes referred to as agonists) in sending signals throughout the nervous system. CysLGICs act as molecular switches, which change conformation upon binding to an agonist to allow a net influx of ions into the cell [1]. They consist of five subunits arranged around a central ion channel (Figure 1). Each subunit has four transmembrane domains (TM1-4) and possesses an N-terminal extracellular domain containing the characteristic Cys-loop motif consisting of two disulphide bond-forming cysteines separated by 13 amino acid residues. In insects, neurotransmitters known to act on cysLGICs in this way include acetylcholine (ACh), γ-aminobutyric acid (GABA), glutamate and histamine. The neurotransmitter binding site is located at the interface of two adjacent subunits and is formed by six distinct regions (loops A-F [2]) in the N-terminal extracellular domain with loops A, B and C being contributed by one subunit and loops D, E and F by another. CysLGICs can exist either as homomers, where all five subunits are the same, or as heteromers consisting of at least two different subunits. The subunit composition determines the functional and pharmacological properties of the cysLGIC, thus receptor diversity is generated by multiple subunit-encoding genes in a given organism. As will become apparent throughout this review, insect cysLGICs are of interest as they are involved in various aspects of nervous system function as well as being the molecular targets of highly effective insecticides.

Sequencing of insect genomes have allowed for detailed comparisons of cysLGIC gene families from diverse species (Table 1). From Table 1, it can be seen that the number of cysLGIC genes are similar from one species to another, ranging from 21 to 26 genes. Many subunits are highly conserved in different species, which is highlighted by recently characterised cysLGIC superfamilies from the tiger mosquito, *Aedes aegypti* (BJ Matthews *et al*., unpublished), the bumble bee *Bombus terrestris* [3] and the pea aphid *Acyrthosiphon pisum* (del Villar and Jones, unpublished) [4], which are shown in a phylogenetic tree in Figure 2. The cysLGICs have been grouped according to the neurotransmitter they respond to. These groups are briefly considered in turn below with specific focus on recent developments.

Nicotinic acetylcholine receptors

Nicotinic acetylcholine receptors (nAChRs) mediate the fast actions of acetylcholine (ACh). Subunits are denoted as α subunits due to the presence of two adjacent cysteine residues in loop C, which are important for ACh binding [5]. nAChR subunits lacking these two cysteines are referred to as β. To be functional, nAChRs require at least two of the subunits to be α. As shown in Figure 2, *A. pisum*, *Ae. aegypti* and *B. terrestris* possess 11, 15 and 11 nAChR subunit genes, respectively. Insects have core groups of subunits (α1-8, β1) that are highly conserved between species and therefore, presumably, play important roles [6]. Interestingly, *A. pisum* lacks an α5 subunit (Figure 2). The aphid cysLGIC gene superfamily is the most primitive one characterised to date, thus it has been speculated that the α5 subunit is the newest member of the insect core group of subunits appearing in more highly evolved species [4]. nAChRs have been long known to play important roles in the insect nervous system. For example, a recent study showed, using RNA interference, that α1, α4, α5, and α6 (but not α3) nAChR subunits are involved in olfactory memory processing in *Drosophila* [7].

nAChRs are the molecular targets of neonicotinoid insecticides [8,9], three of which (imidacloprid, clothianidin and thiamethoxam) will be banned, most likely by the end of 2018, from outdoor use by the European Union amidst fears that they are contributing to the alarming decline of

bees and other non-target organisms (BBC Science and Environment News; URL: https://www.bbc.co.uk/news/science-environment-43910536). Several other classes of insecticides, such as spinosyns, sulfoxamines and butenolides, act on nAChRs [9]. Also, natural peptides found in venom, such as that of the funnel-web spider, can act on insect nAChRs, with a higher selectivity than on vertebrate nAChRs [10].

Recent studies have highlighted particular nAChR subunits as being the targets of certain insecticides. For instance, the neonicotinoids nitenpyram and imidacloprid, stimulated dopamine release in nerve cords of *Drosophila melanogaster* larvae by acting as agonists on nAChRs [11]. This dopamine release was significantly lower in *Drosophila* with mutations in the α1 and β2 nAChRs subunits, indicating that these two subunits are important for the actions of both neonicotinoids. The β1 subunit has also been implicated as an important neonicotinoid target since the R81T mutation is associated with neonicotinoid resistance in aphids [12] although the mutation can affect neonicotinoids differentially depending on whether the insecticide has a cyano or nitro chemical group [13]. The use of RNAi and patch-clamp electrophysiology on cockroach (*Periplaneta americana*) dorsal unpaired median neurons indicated that the α3 and α8 subunits may also form part of nAChRs targeted by imidacloprid [14].

Another class of insectcides, spinosyns, acts on a different nAChR subunit, α6, as indicated by recent findings that there were significantly more truncated forms of the α6 subunit in the flower thrips, *Frankliniella occidentalis*, which were resistant to spinosad [15]. Also, altered α6 mRNA, which lacked exon 3, was found at higher levels in the tomato leaf miner, *Tuta absoluta*, that were resistant to spinosad [16]. The CRISPR/Cas9 system capable of making desired changes in genome sequences [17] was used to show that the G275E point mutation in α6 of *Drosophila* flies confers decreased sensitivity to spinosad [18], demonstrating this subunit as being important for spinosyn action.

It remains to be seen which nAChR subunits are targeted by other insecticide classes. It will be of interest to see whether different insecticide classes act on the same handful of subunits or whether the whole nAChR gene family can effectively be targeted. Genome editing with CRISPR/Cas9 or the use of model organisms such as *D. melanogaster* with the function of whole genes effectively removed may help to answer this question. Indeed, *D. melanogaster* with a knockout of the α1 nAChR gene was resistant to imidacloprid and nitenpyram [19]. Consistent with this, it was found that RNAi of α1 in *Leptinotarsa decemlineata* decreased sensitivity to imidacloprid and thiamethoxam [20]. Perhaps similar approaches on all nAChR subunits may aid in elucidating which subunits are important for the actions of the various insecticide classes.

In addition to the core nAChR subunits, insects possess at least one divergent subunit that shows low sequence similarity to all other known nAChR subunits [6]. Unlike core group subunits, analogous divergent subunits in different insects are difficult to assign. It is common for each insect species to possess a different set of divergent nAChR subunits. For example, *B. terrestris* possesses two divergent subunits (Figure 2) whilst *Ae. aegypti* has a notably large set consisting of two α and four β subunits. Less is known about the function of divergent subunits than core ones and it would be of interest to see if they perform species-specific roles. Recently, it has been shown that the divergent subunit from *Locusta migratoria* (β3) is part of a high affinity binding site for imidacloprid [21], highlighting the possibility that divergent nAChR subunits may be targets of insecticides, at least in some species. Divergent nAChR subunits may, therefore, be of interest as targets to control certain insect pests.

GABA-gated ion channels

The subunit RDL (resistance to dieldrin) is commonly thought of as *the* insect GABA receptor although RDL can co-assemble with LCCH3 whilst LCCH3 and GRD can co-assemble to form GABA-gated cation channels [22]. RNAi of *Rdl* in a subset of neurons in *D. melanogaster* results in overconsumption, showing that this subunit is required for proper control of consumption [23], highlighting the important role RDL plays in the nervous system.

Insect GABA receptors are the targets of several insecticide classes including cyclodienes, phenylpyrazoles and isoxazolines [24]. An alanine-to-serine mutation in the second transmembrane domain (A2'S) underlies resistance to cyclodienes, such as dieldrin [25-27]. A mutation at this site, either to serine or another residue such as glycine, has been commonly found in insecticide-resistant insects of diverse species. In some cases, mutations at other sites in RDL have been identified although always together with the TM2 mutation [26-28]. The mutation at the A2 site, therefore, is commonly used as a diagnostic marker for resistance. Recent studies have shown that mutation at TM2 can reach high levels in insect populations. For example, 77-100% of *Anopheles sinensis* mosquitoes collected from different geographical locations across Guangxi, China, had the A2'S mutation [28]. Despite the prevalence of this mutation, RDL is still a target of interest for insect control as certain classes of insecticides, such as isoxazolines, can still act on the GABA receptor with the A2' mutation [29].

RDL can be expressed as a homomeric receptor in heterologous expression systems such as *Xenopus laevis* oocytes giving the impression that it is a relatively simple receptor. This impression, however, is likely to be false as alternative splicing and RNA A-to-I editing of *Rdl* transcripts can considerably increase receptor diversity [27]. Recently, it has been shown that RNA A-to-I editing, which can alter up to six amino acid residues in *An. gambiae* RDL, can give rise to at least 24 different RDL isoforms [30]. Furthermore, it was shown that editing at just one site can affect the potency of ivermectin, highlighting that RNA editing may affect sensitivity to insecticides in a species-specific manner.

Glutamate-gated chloride channels

Glutamate-gated chloride channels (GluCls) are the targets of macrocyclic lactones, such as ivermectin and abamectin [31], as was well as promising new insecticide classes, an example of which are okaramines [32]. Recent studies of insect GluCls have reinforced our knowledge concerning resistance mechanisms and the importance of GluCl as an insecticide target. For example, RNAi of GluCl in *Bemisia tabaci* resulted in increased tolerance to abamectin [33] whilst mutations in TM3 of *Plutella xylostella* GluCl or *M. domestica* GluCl affected abamectin [34] or ivermectin actions [35], respectively.

Histamine-gated chloride channels

Compared to other cysLGICs, histamine-gated chloride channels (HisCls) have been studied to a lesser degree. However, a recent study that expressed HisCl1 (otherwise known as HCLB) and HisCl2 (HCLA) from *M. domestica* in *Xenopus* oocytes enhanced our knowledge concerning the pharmacological properties of these ion channels [36]. Both subunits responded to histamine whilst only HisCl1 was activated by GABA and to a lesser degree by choline, serotonin, tyramine and dopamine. HisCl1 was not affected by insecticides such as fipronil and fluralaner and was only marginally activated by ivermectin. Given that HisCl1 is involved in important neuronal processes such as sleep regulation [37] HisCls may represent targets for future insecticides with novel pharmacological properties.

pH-sensitive chloride channels

As is the case for HisCls, our recent knowledge about pH-sensitive chloride channels (pHCls) comes from expressing these ion channels in *Xenopus* oocytes. Ivermectin was found to activate or block *B. mori* pHCl in a pH-dependent manner whilst fipronil had no effect [38].

Insect Group 1 of cysLGIC subunits

Similar to divergent nAChRs, there are cysLGIC subunits in a group denoted Insect Group 1 of cysLGIC subunits [39] that show low sequence homology to other subunits and therefore it is difficult to assign them orthologous relationships between different species. Furthermore, the number of subunits falling into this group varies between species [40]. In *Drosophila*, these subunits are CG6927, CG7589 and CG11340 whilst in other species they have been called CLGC (cys-loop ligand-gated ion channel) [39] (Figure 2). The function of these subunits was unknown until recently where CG6927, CG7589 and CG11340 were found to be expressed in non-neuronal tissues with possible roles outside of neurotransmission including regulating fluid secretion and mediating the response to osmotic stress [40,41]. CG11340 is capable of forming an ion channel in *Xenopus* oocytes that is sensitive to pH but not to several neurotransmitters including acetylcholine, GABA, glutamate, glycine, histamine, serotonin, dopamine, tyramine and octopamine [41]. The low levels of conservation of Group 1 cysLGIC subunits raises the prospect of these subunits as potential targets of insecticides that act preferentially on pest species. *D. melanogaster* with knockout of CG11340 were viable showing that this subunit would not be an effective insecticide target [40]. However, CG7589 appears to be a more promising target as RNAi of this subunit resulted in reduced viability [40].

CG8916 and CG12344

These two subunits remain the least characterised of the insect cysLGIC superfamily. Analysis of RNA in specific cell types identified CG8916 as being highly expressed in *Drosophila* T4/T5 neurons along with LCCH3 [42]. CG8916 clusters with subunits gated by GABA (Figure 2) so perhaps it assembles with GRD, LCCH3 or RDL to diversify GABA receptor function. More recently, RNAi studies implicated CG12344, GRD and CG7589 in mediating responses to glycine in *Drosophila* dorsal neurons identifying for the first time potential insect cysLGIC glycine receptors analogous to that found in vertebrates [43].

Challenges and Future Prospects

The upcoming ban of neonicotinoid insecticides in Europe stresses the importance of developing insecticides that are selective for pest species whilst sparing beneficial insects and non-target organisms. The characterisation of complete cysLGIC gene superfamilies from insects ranging from crop pests, disease vectors to pollinators (Table 1) highlights differences that perhaps can be exploited in developing ways to specifically target a particular species. The obvious differences lie in the divergent subunits which show low sequence homology between species. Targeting these subunits may offer a route to control particular pests. However, the fact that they are divergent indicates that they have not been constrained by evolution and therefore may not be performing a vital role. Gene targeting approaches to knock out or silence these subunits, such as by CRISPR/Cas or RNAi, may help to determine their importance for a given insect and thus evaluate their potential as an insecticide

target. Even the more conserved subunits have sequence differences between species that can be exploited. Knowledge of these differences in conjunction with the use of three-dimensional models will likely prove useful in adopting a rational approach to developing compounds that bind more specifically to a cysLGIC from a pest species. However, at present, 3-D models of insect cysLGICs based on structures from other organisms such as vertebrates (E.g. the $\alpha4\beta2$ nAChR [44]), molluscs (acetylcholine binding protein AChBP [45]) or nematodes (GluCl [46]) are being relied upon [47]. The low sequence conservation limits the accuracy of these homology models. For example, the extracellular N-terminal domain of the *A. pisum* $\alpha1$ nAChR subunit shares 48% and 25% sequence identity with the human $\alpha4$ nAChR subunit and molluscan AChBP, respectively. Determining the crystal structure of actual insect cysLGICs would greatly improve the accuracy of 3-D models. Differences in cysLGIC sequences between species may also be exploited for more novel approaches to insect control, such RNAi [48], where sequences particular to pests could be targeted to silence specific subunits.

Whilst genome sequences have helped us in identifying the full complement of cysLGIC subunits in diverse insect species, we still know very little about the receptors themselves. For instance, how do the dozen or so nAChR subunits in a given insect combine together to form the various subtypes of nicotinic acetylcholine receptors? Does the insect GABA receptor just consist of the RDL subunit? Does GRD form either GABA or glycine receptors depending on which subunits it combines with? A major challenge in characterising the insect cysLGICs, therefore, would be to determine how the different subunits assemble to make up native receptors *in vivo*.

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Table 1. Insect species that have their complete cysLGIC superfamily described

Species	Order	Significance	cysLGIC subunit gene number	Reference/s
Aedes aegypti	Diptera	Disease vector	25	(BJ Matthews *et al.,* unpublished)
Apis mellifera	Hymenoptera	Pollination, honey production	21	[49,50]
Acyrthosiphon pisum	Homoptera	Crop pest	22	[4], (del Villar and Jones, unpublished)
Bombus impatiens	Hymenoptera	Pollination	21	[3]
Bombus terrestris	Hymenoptera	Pollination	21	[3]
Drosophila melanogaster	Diptera	Genetic model organism	23	[50,51]
Musca domestica	Diptera	Disease vector	23	[52]
Nasonia vitripennis	Hymenoptera	Biological control of insect pests	26	[53]
Tribolium castaneum	Coleoptera	Pest of stored food	24	[39]

FIGURE LEGENDS

Figure 1. Structure of a cys-loop ligand-gated ion channel (cysLGIC). Schematic representation of a heteromeric receptor consisting of two different subunits (dark and light grey). The peptide layout of two subunits are shown highlighting the Cys-loop (two white circles connected by a white double line) and four transmembrane domains. The six binding loops (A-F) that contribute to ligand binding are shown and two neurotransmitter (N) molecules are bound. The five subunits that make up the receptor are arranged around a central ion-permeable channel. The ions passing through the channel depend on the type of cysLGIC.

Figure 2. Tree showing the cys-loop ligand-gated ion channel (cysLGIC) gene families of *A. pisum* (pea aphid), *Ae. aegypti* (tiger mosquito) and *B. terrestris* (bumble bee). Based on their high amino acid sequence similarity, the cysLGIC subunits cluster together into groups according to their receptor type.