NACHO permits functional heterologous expression of an insect homomeric α6 nicotinic acetylcholine receptor

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Abstract

Insect nicotinic acetylcholine receptors (nAChR) are molecular targets of highly effective insecticides. The use of chaperone proteins has been key to successful functional expression of these receptors in heterologous systems, permitting functional and pharmacological studies of insect nAChRs with particular subunit composition. Here, we report the first use of the chaperone protein, NACHO, to enable functional expression of an insect nAChR, the α6 subunit from *Apis mellifera*, in *Xenopus laevis* oocytes. This is also the first report of functional expression of a homomeric insect α6 nAChR. Using two-electrode voltage-clamp electrophysiology we show that the acetylcholine EC_{50} of the α6 receptor is 0.88 μM and that acetylcholine responses are antagonized by α-bungarotoxin. Spinosad showed agonist actions and kept the ion channel open when co-applied with acetylcholine, reinforcing the α6 nAChR subunit as a key molecular target for the spinosyn class of insecticide. The use of NACHO may provide a basis for future expression studies of insect α6 nAChRs, potentially providing a tool for the discovery of novel insecticides.

Keywords

Apis mellifera, functional expression, insecticide, NACHO, nicotinic acetylcholine receptor

Abbreviations

cysLGIC – cys-loop ligand-gated ion channel; nAChR – nicotinic acetylcholine receptor; SOS – standard oocyte solution

1. Introduction

We are delighted to be contributing a paper as part of a special issue honouring David Sattelle who has been highly influential in encouraging the next generation of scientists to research the actions of insecticides on their molecular targets.

Nicotinic acetylcholine receptors (nAChRs) are members of the cys-loop ligand-gated ion channel gene superfamily (cysLGIC), which mediate fast synaptic signalling in the nervous system (Zoli et al., 2018) and in insects they are targets of highly effective insecticides such as spinosyns (Ihara et al., 2017; Jones, 2018; Sparks et al., 2021a). As is typical for cysLGICs, nAChRs consist of five subunits arranged around a central ion channel (Zoli et al., 2018). Each nAChR subunit is encoded for by a separate gene and is denoted as an α type if two adjacent cysteine residues are present in loop C of the ligand binding site, which are important for acetylcholine binding (Corringer et al., 2000; Kao and Karlin, 1986), whilst subunits lacking these two cysteines are referred to as β. nAChRs can be

homomeric, consisting of just one α subunit type, whilst heteromeric receptors are made up of at least two different subunits.

Insect nAChR gene families have so far been found to consist of 10-19 subunits where there are core groups of subunits that are highly conserved between species (Jones et al., 2021; Jones and Sattelle, 2010). Thus, insects possess α1, α2, α3, α4, α5, α6, α7, α8 (or β2 in some Diptera species) and β1 subunits whilst the remaining subunits, which do not have clear orthologous relationships between different insects, are denoted as divergent. The α5, α6 and α7 subunits have been placed in a single core group due to their notable sequence homology with vertebrate α7 nAChR subunits (Grauso et al., 2002; Jones and Sattelle, 2010; Thany et al., 2005). As with vertebrate α7 nAChR subunits (Couturier et al., 1990), *Drosophila melanogaster* α5 or α7 subunits can form homomeric receptors in *Xenopus laevis* oocytes, although the presence of the chaperone protein, RIC-3, is required for functional expression and even then success of expression was inconsistent (Lansdell et al., 2012). The α7 nAChR subunit of another insect, the cockroach *Periplaneta americana,* was shown to express as a homomeric receptor in *X. laevis* oocytes, although very high concentrations of acetylcholine or nicotine were required to elicit a response, leading to the suggestion that co-expression with chaperone proteins may be required for more efficient expression (Cartereau et al., 2020). So far, the α6 subunit has only been found to express as part of heteromeric nAChRs along with α5, α7 and RIC-3 (Lansdell et al., 2012; Watson et al., 2010). More recently, the chaperone protein NACHO (a novel nAChR regulator) was identified, which enabled expression of the vertebrate α7 subunit in HEK cells (Gu et al., 2016). We report here the cloning of NACHO from an insect, *Apis mellifera*, and show that it enables the honey bee α6 nAChR subunit to express as a functional homomeric receptor in *X. laevis* oocytes.

2. Materials and methods

2.1 Reagents

Calcium chloride and sodium chloride were purchased from VWR Amresco Life Sciences (Lutterworth, UK), HEPES from Melford (Ipswich, UK), magnesium chloride from Merck (Gillingham, UK) and potassium chloride from VWR Prolabo Chemicals (Lutterworth, UK). Acetylcholine chloride, collagenase type I from *Clostridium histolyticum* and spinosad were purchased from Sigma-Aldrich (Gillingham, UK), atropine from Scientific Laboratory Supplies (Nottingham, UK) whilst α-bungarotoxin was obtained from Generon (Slough, UK).

2.2 Cloning of the *A. mellifera* α6 nAChR subunit and NACHO

To identify *A. mellifera* NACHO, the honey bee genome available at NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was screened with the human or *D. melanogaster* NACHO (Gu et al., 2016) protein sequence using the TBLASTN algorithm (Altschul et al., 1990). One putative orthologue (Accession no. XP_624145) was identified.

Total RNA was extracted from whole adult *A. mellifera* using the RNeasy Mini Kit (Qiagen, UK), as previously described (Jones et al., 2006). First strand cDNA was synthesized from the total RNA using the GoScript™ Reverse Transcription System (Promega, Southampton, UK). Nested reverse-transcription PCR reactions were performed to amplify the complete coding region of *A. mellifera* α6 using PCRBIO HiFi Polymerase (PCRBiosystems, London, UK) where the forward primer (5' CAAACGGCGCAGAAGCGA 3') and the reverse primer (5' GGTGACTTGACCGGGGCA 3') were used. Amplification products from this reaction were used in a final dilution of one in 5000 as template for the second nested reaction using the forward primer (5' CGATCGGAATTCATGCTTAGCGCAAGTAGTGTATTAC 3') and reverse primer (5' CGATCGTCTAGATTGGACGATTATGTGTGGCG 3') with *ECo*RI and *Xba*I restrictions sites (underlined), respectively, that were used to clone the subunit into the pCI vector (Promega, Southampton, UK). NACHO was amplified from adult *A. mellifera* cDNA using the forward primer (5' GGAATTCATGGGCTCAGTCGTTTTGAAA) and reverse primer (5' GCTCTAGATCAATCTTGTTTCTTAGTTTTATC 3') before being cloned into pCI. Sequences of clones were verified at SourceBioscience (https://www.sourcebioscience.com/home).

2.3 Sequence analysis

The multiple protein sequence alignment was constructed with ClustalX (Thompson et al., 1997) using the slow-accurate mode with a gap-opening penalty of 10 and a gap extension of 0.1 as well as applying the Gonnet 250 protein weight matrix. The protein alignment was viewed using GeneDoc (http://nrbsc.org/gfx/genedoc/). Identity values were calculated using the GeneDoc program. Transmembrane domains were predicted using TMpred (https://embnet.vital-it.ch/software/TMPRED_form.html).

2.4 Expression of the *A. mellifera* a6 nAChR subunit in *Xenopus laevis* oocytes and two-electrode voltage-clamp electrophysiology

Functional studies of the *A. mellifera* α6 nAChR subunit along with *A. mellifera* NACHO were performed using the *X. laevis* oocyte expression system and two-electrode voltage-clamp electrophysiology. *X. laevis* were purchased from Xenopus 1, Dexter, Michigan, USA and were handled strictly adhering to the guidelines of the Scientific Procedure Act, 1986, of the United

Kingdom. Stage V and VI oocytes were harvested and rinsed with Ca^{2+} free solution (NaCl 150 mM, KCl 2.8 mM, HEPES 10 mM, at pH 7.4) before being treated with collagenase type I at 2 mg/ml for 45 minutes at room temperature with shaking at 150 RPM. The oocytes were then injected with 23 nl of pCI with the cloned cDNA at a concentration between 200-400 ng/nl. *A. mellifera* α6 and NACHO were injected at a 2:1 ratio, respectively. Injected oocytes were stored in standard oocyte solution (SOS) (NaCl 150 mM, KCl 2.8 mM, $CaCl_2$ 2mM, HEPES 10 mM, at pH 7.4) with 1X antibiotic antimycotic solution (Sigma) and neomycin 0.05 mg/ml (Sigma) at 18°C.

Oocytes were tested for responses 3-5 days after injection, using two-electrode voltage clamp, with borosilicate glass microelectodes filled with 3 mM KCl (resistance 0.5-2 MΩ) and an Oocyte Clamp OC-725C amplifier (Warner Instruments, CT, USA). Oocytes were clamped at -100 mV and continuously perfused with SOS at a flow rate of 10 ml/min. Responses were recorded on a flatbed chart recorder (Kipp & Zonen BD-11E, Delft, The Netherlands). Oocytes were selected for experiments if responses were consistent (within 95% of each other) for two or more applications of 3 μM acetylcholine. Acetylcholine EC_{50} concentration was determined using agonist concentration response curves, which were generated by challenging oocytes to different concentrations of agonist in SOS, with 5 min between challenges. Curves were calculated by normalizing the current response to maximal response (3 μM acetylcholine). Spinosad (10 μM) and α-bungarotoxin (100 μM) were initially dissolved in DMSO (BDH Laboratory supplies, Poole, UK) then at working concentrations in a 1:1000 ratio in SOS. The working DMSO concentration did not affect electrophysiological readings. For co-application with atropine (1 μM), α-bungarotoxin (0.1 μM) or spinosad (60 μM), oocytes were pre-incubated with the compound in SOS for 10 min followed by a combination of the compound and 3 μM acetylcholine.

2.5 Data analysis

Analysis of the acetylcholine concentration response curve was performed using Graphpad Prism 6 (GraphPad Software, La Jolla, CA United States). The concentration of agonist required to evoke 50% of the maximum response (EC_{50}) and the Hill coefficient (nH) were determined using the nonlinear regression analysis provided.

3. Results

3.1 Cloning of *Apis mellifera* α6 nAChR subunit and NACHO

The *A. mellifera* α6 subunit (Jones et al., 2006) was amplified by reverse-transcriptase PCR and cloned into the pCI plasmid. The clone used in subsequent expression studies possessed the exon 8a splice variant and the N164D, I181M and T184A RNA A-to-I editing isoforms (Jones et al., 2006).

A. mellifera NACHO was identified from the honey bee genome using TBLASTN then successfully amplified by reverse-transcriptase PCR and cloned into the pCI plasmid. Its open reading frame consists of 471 nucleotides encoding 156 amino acid residues (Fig. 1), which shares 60% and 32% sequence identity with *D. melanogaster* and human NACHO, respectively. As previously noted for NACHO from human and *D. melanogaster* (Gu et al., 2016), *A. mellifera* NACHO contains four putative transmembrane domains (Fig. 1).

3.2 Functional expression of the *A. mellifera* α6 nAChR subunit in *Xenopus laevis* oocytes

Xenopus laevis oocytes were injected with the pCI plasmid encoding the *A. mellifera* α6 nAChR subunit. Using two-electrode voltage-clamp electrophysiology, a response to the application of acetylcholine up to 100 μM concentration was not observed in 300 oocytes from 10 different animals (Fig. 2A). However, when the α6 subunit was co-injected with *A. mellifera* NACHO responses to acetylcholine were detected in a concentration-dependent manner (Fig. 2A). No response was observed in oocytes injected with NACHO alone (Fig. 2A) showing that the presence of this chaperone along with *A. mellifera* α6 was required to detect functional expression. The size of response was small with I_{max} of around 15 nA and expression was detected in 33% of the oocytes tested. Despite the small current response sizes, an acetylcholine concentration response curve was generated (Fig. 2B) from which an EC_{50} of 0.88 ± 0.50 μM and a hillslope of 1.00 ± 0.21 were determined (n=5 oocytes from 3 different animals). In order to ensure that the response to acetylcholine was not caused by muscarinic acetylcholine receptors endogenous to the oocytes, atropine, an inhibitor of muscarinic acetylcholine receptors, was applied at 1 μM concentration. This did not ablate the response (Fig. 3A), demonstrating that the currents observed were not muscarinic and is likely due to the injected α6 subunit.

A ligand-binding study involving a subunit chimera containing the N-terminal extracellular domain of a *D. melanogaster* nAChR fused to the C-terminal domain of the 5-hydroxytryptamine receptor, $5HT_{3A}$, implicated the insect α6 nAChR subunit in forming high-affinity α-bungarotoxin binding sites (Lansdell and Millar, 2004), the study of which has proven instructive in elucidating the different nAChR subtypes in insect tissues (Taillebois et al., 2018). We therefore tested the action of α-bungarotoxin on *A. mellifera* α6 expressed in *X. laevis* oocytes. α-bungarotoxin showed antagonistic actions, blocking the acetylcholine response of the α6 subunit, which was reversible as shown by recovery of the response after a 10 minute SOS wash (Fig. 3B).

Since the insect α6 nAChR subunit is considered a major molecular target of spinosyn insecticides (Sparks et al., 2021a), we tested the actions of spinosad on *A. mellifera* α6 expressed in *X. laevis* oocytes. We found that by itself, spinosad showed agonist actions (Fig. 3C) where 10 μM of spinosad resulted in approximately 30% of the maximal acetylcholine response (3 μM). It was not possible to conduct a full concentration response curve due to the low currents exhibited by spinosad application. Co-application of spinosad with acetylcholine led to the activation of the α6 homomer evoking an inward current equal to acetylcholine alone, however, the channel remained open and recovery did not occur after 10 minutes of SOS washing (Fig. 3D).

4. Discussion

The use of chaperone proteins has proven critical in permitting the heterologous expression of insect nAChRs thereby enabling the functional/pharmacological characterization of receptors of particular subunit composition. Thus, the use of three proteins (RIC-3, UNC-50 and TMX3) have enabled the robust expression in *X. laevis* oocytes of heteromeric receptors consisting entirely of insect or crustacean nAChR subunits, these being α1, α2, α3, α8, β1 and β2 subunits (Ihara et al., 2020; Rufener et al., 2020). Also, RIC-3 has enabled expression of other insect nAChR subunits, these being α5, α6 and α7 (Lansdell et al., 2012; Watson et al., 2010). Unlike *D. melanogaster* α5 and α7 nAChR subunits, the fruitfly α6 has not been shown to form functional homomeric receptors in heterologous expression systems despite its notable sequence homology with vertebrate α7 nAChR subunits (Grauso et al., 2002; Jones and Sattelle, 2010), instead appearing to contribute to heteromeric receptors consisting of α5+α6 or α5+α6+α7 nAChRs (Lansdell et al., 2012; Watson et al., 2010). We show here that the α6 subunit from *A. mellifera* can form a homomeric receptor when co-expressed with another chaperone protein, NACHO (Gu et al., 2016). As with *D. melanogaster* α5, α6 and α7 nAChR subunits co-expressed with RIC-3 (Lansdell et al., 2012; Watson et al., 2010), success of expression was inconsistent. Nevertheless, we determined that the *A. mellifera* α6 nAChR has a higher sensitivity to acetylcholine, as shown by an EC_{50} of 0.88 μM (Fig. 2B), than *D. melanogaster* α5 or α7 homomeric receptors (EC_{50}s of 8.8 μM and 6.7 μM, respectively) but a lower sensitivity than the heteromeric α5+α6+α7 nAChR that has an EC_{50} of 13.5 nM (Lansdell et al., 2012). Recently, it was shown that co-expression of *D .melanogaster* NACHO with *Bombus terrestris* RDL influenced GABA EC_{50} (Smelt et al., 2021). It would thus be of interest to determine whether the EC_{50}s of α5, α7 or α5+α6+α7 are altered by co-expression with NACHO.

We found that the *A. mellifera* α6 homomeric nAChR was sensitive to the antagonistic actions of α-bungarotoxin (Fig. 3B) highlighting that this subunit contributes to high-affinity α-bungarotoxin binding sites. The use of α-bungarotoxin has been used to identify nAChR types involved in honey bee behaviour. For example, α-bungarotoxin sensitive nAChRs have been implicated in long term memory during tactile and olfactory learning (Dupuis et al., 2012). It would be of interest to see whether the α6 subunit forms part of this receptor subtype.

Several studies have highlighted the insect α6 nAChR subunit as being the molecular target of spinosyns (Ihara et al., 2017; Sparks et al., 2021a). These include the identification of the G275E mutation in the α6 subunit of spinosad resistant pests (Bao et al., 2014; Chen et al., 2021; Puinean et al., 2013; Silva et al., 2016). Also, introduction of the G275E mutation or knockout of the α6 subunit resulted in decreased sensitivity to spinosad (Perry et al., 2021; Perry et al., 2007; Zimmer et al., 2016; Zuo et al., 2021) whilst introduction of α6 rescued the spinosad susceptibility phenotype in *D. melanogaster* (Perry et al., 2015). Our findings reinforce the insect α6 nAChR subunit as being a key target for spinosad. We found that spinosad shows agonist actions on the *A. mellifera* α6 nAChR (Fig. 3C) and keeps the ion channel open when activated by acetylcholine (Fig. 3D) in accord with the modulatory mode of action noted for spinosyns by the Insecticide Resistance Action Committee (Sparks et al., 2021b).

In summary, we have shown for the first time successful functional expression of an insect nAChR in a heterologous system due to the addition of NACHO. Since the insect α6 subunit is a known target for an effective class of insecticides, heterologous expression of this receptor may provide a useful screening tool for the discovery of novel insecticidal compounds, especially if NACHO enables expression of α6 from pest species. Site-directed mutagenesis introducing the G275E mutation will improve the screen further in identifying compounds unaffected by resistance mutations currently in the field (Bao et al., 2014; Chen et al., 2021; Puinean et al., 2013; Silva et al., 2016). The current amplitudes of *A. mellifera* α6, however, are in the nA range, which are considerably smaller than those of the vertebrate α7 homomeric nAChR and the insect homomeric GABA receptor, RDL, which generate μA responses when expressed in *X. laevis* oocytes (Couturier et al., 1990; Taylor-Wells et al., 2017). This indicates that conditions may need to be modified to achieve optimal expression. *A. mellifera* α6 and NACHO were always injected into oocytes at a 2:1 ratio. Perhaps altering this ratio may increase the number of oocytes expressing a detectable nAChR and/or enhance the response such as has been found for co-expressing different amounts of RIC-3 with vertebrate or nematode nAChRs (Ben-David et al., 2016; Bennett et al., 2012). Additional factors may be required for optimal expression of *A. mellifera* α6 in *X. laevis* oocytes. Thus, it

remains to be determined whether co-expression of the insect α6 nAChR subunit with other chaperone proteins such as RIC-3, UNC-50 and TMX3, in addition to NACHO, will result in more robust expression thereby facilitating further functional characterization of this receptor. Current responses in the μA range have also been achieved for insect nAChRs consisting of several subunits (α1, α2, α3, α8, β1 and β2) expressed with chaperone proteins (RIC-3, UNC-50 and TMX3) (Ihara et al., 2020; Rufener et al., 2020). Perhaps robust expression of the insect α6 nAChR subunit requires co-expression with other subunits, such as α5 and α7 (Lansdell et al., 2012; Watson et al., 2010), in addition to several chaperone proteins.

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383 **Figure legends**

384 **Fig. 1.** Protein sequence alignment of NACHO from *A. mellifera*, *D. melanogaster* (Accession number
385 QWT44801) and *H. sapiens* (NP_067650). The four transmembrane regions (TM1-TM4) are indicated
386 and the grey and black shading highlight the degree of amino acid conservation.

387 **Fig. 2.** Reponses to acetylcholine in *X. laevis* oocytes expressing the *A. mellifera* α6 nAChR subunit
388 and NACHO. A. Representative current traces showing no response to 3 μM acetylcholine when
389 oocytes were injected with either water, *A. mellifera* α6 alone or *A. mellifera* NACHO alone.
390 Responses to acetylcholine in a concentration dependent manner (0.001 – 30 μM) were observed
391 only when oocytes were injected with both *A. mellifera* α6 and NACHO. B. Acetylcholine
392 concentration response curve. Data are normalized to 3 μM acetylcholine and have a mean EC_{50} of
393 0.88 ± 0.50 μM from 5 oocytes from 3 different batches of eggs.

394 **Fig. 3.** Reponses to atropine, α-bungarotoxin and spinosad in *X. laevis* oocytes expressing the *A.*
395 *mellifera* α6 nAChR subunit and NACHO. A. The response to 3 μM acetylcholine (ACh) was not
396 affected by a 10 minute pre-incubation with atropine. B. The response to 3 μM acetylcholine was
397 blocked by a 10 minute pre-incubation with α-bungarotoxin (α-BTX). Recovery from this block was
398 observed after a 10 minute wash. C. Spinosad showed agonist action when applied alone. D. Co-
399 application of 60 μM spinosad with 3 μM acetylcholine led to a response similar to 3 μM
400 acetylcholine alone, however the channel remained open.

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