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Comparative pharmacology and computational modelling yield insights into allosteric modulation of human $\alpha 7$ nicotinic acetylcholine receptors

David B. Sattelle^{a*}, Steven D. Buckingham^a, Miki Akamatsu^b, Kazuhiko Matsuda^c, Ilse Pienaar^a, Andrew K. Jones^a, Benedict M. Sattelle^d, Andrew Almond^d and Charles D. Blundell^e

^aMRC Functional Genomics Unit, Department of Physiology, Anatomy and Genetics, University of Oxford, South Parks Road, Oxford, Oxford OX1 3QX, UK. david.sattelle@dpag.ox.ac.uk, steven.buckingham@dpag.ox.ac.uk, ilse.pienaar@dpag.ox.ac.uk, andrew.jones@dpag.ox.ac.uk

^bGraduate School of Agriculture, Kyoto University, Kita-Shirakawa, Sakyo-ku, Kyoto, 606-8502, Japan. akamatsu@kais.kyoto-u.ac.jp

^cDepartment of Applied Biological Chemistry, Faculty of Agriculture, Kinki University, 3327-204 Nakamachi, Nara 631-8505, Japa kmatsuda@nara.kindai.ac.jp

^dManchester Interdisciplinary Biocentre, 131 Princess Street, Manchester M1 7DN, UK. ben.sattelle@manchester.ac.uk, andrew.almond@manchester.ac.uk

^eConformetrix Ltd., Core Technology Facility, 46 Grafton Street, Manchester, M13 9NT, UK. charles.blundell@conformetrix.com

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*Corresponding author and presenting author Tel: 44 (0)-1865-272145; Fax: 44(0)-1865-285862; Email: david.sattelle@dpag.ox.ac.uk

Abstract

The human $\alpha 7$ nicotinic acetylcholine receptor (nAChR) subunit and its *Caenorhabditis elegans* homolog, ACR-16, can generate functional recombinant homomeric receptors when expressed in *Xenopus laevis* oocytes. Both nAChRs express robustly in the presence of the co-injected chaperone RIC-3 and show striking differences in the actions of a type I positive allosteric modulator (PAM), ivermectin (IVM). Type I PAMs are characterised by an increase in amplitude only of the response to acetylcholine (ACh), whereas type II PAMs exhibit, in addition, changes in time-course / desensitization of the ACh response. The type I PAMs, ivermectin, 5-hydroxyindole (5-HI), NS-1738 and genistein and the type II PAM, PNU-120596, are all active on human $\alpha 7$ but are without PAM activity on ACR-16, where they attenuate the amplitude of the ACh response. We used the published structure of avermectin B1a to generate a model of IVM, which was then docked into the candidate transmembrane allosteric binding site on $\alpha 7$ and ACR-16 in attempt to gain insights into the observed differences in IVM actions. The new pharmacological findings and computational approaches being developed may inform the design of novel PAM drugs targeting major neurological disorders.

Keywords: computational modeling, electrophysiology, ivermectin, nicotinic acetylcholine receptor, positive allosteric modulators, ion channels

1. Introduction

Nicotinic acetylcholine receptors (nAChRs) containing the $\alpha 7$ subunit are widely expressed in human brain regions associated with cognitive functions [1-3]. This subtype of nAChR has major pre-synaptic roles in the brain [4], which include regulating the release of dopamine from nerve terminals in the dopaminergic reward pathway [5, 6]. The activation of $\alpha 7$ nAChRs is neuroprotective against toxicity resulting from exposure to the neurotoxic β -amyloid peptide, A β 1-42, important in Alzheimer's disease and other toxins [7]. The $\alpha 7$ nAChRs participate in auditory sensory gating, defects in which contribute to the attention and cognitive problems associated with schizophrenia [8, 9]. They are also involved in the mechanism of nicotine addiction [10]. The $\alpha 7$ nAChR subunit, which forms robust functional homomeric receptors, has been proposed as a candidate target for new drugs designed to ameliorate symptoms of Alzheimer's disease and schizophrenia as well as to control pain [11].

A number of $\alpha 7$ -specific agonists have been designed with therapeutic goals in mind but this strategy raises concerns relating to long-term drug activation and / or rapid desensitization of nAChRs, which may compromise the utility of such ligands. In attempts to circumvent such problems, positive allosteric modulators of the $\alpha 7$ nAChR have been generated, which act by enhancing the actions of the neurotransmitter acetylcholine (ACh) [11]. Such drugs offer a therapeutic approach to cognitive and attention deficits, which, importantly, preserve the integrity of cholinergic neurotransmission [12]. Two classes of positive allosteric modulators (PAMs) can be recognised in terms of their actions on $\alpha 7$ nAChRs [11, 13]. Type I PAMs enhance the amplitude of the response to ACh with little or no effect on the time-course of the response, including desensitization. Examples include ivermectin (IVM), genistein, 5-hydroxyindole (5-HI), compound-6 and NS-1738. Type II PAMs, such as PNU-120596, and the

tetrahydroquinoline, 4-naphthalene-1-yl-3a,4,5,9b-tetrahydro-3-H-cyclopenta[c]quinoline-8-sulfonic acid amide (TQS). In addition to enhancing the amplitude of ACh responses, Type II PAMs also greatly prolong the time-course of $\alpha 7$ -mediated ACh responses [13].

The discovery that NS-1738 and PNU-120596 are selective for $\alpha 7$, yet show quite different PAM actions suggests that different sites of action on the receptor may exist [14]. Regions important in the actions of these drugs have been explored using chimeras of the $\alpha 7$ and the 5-HT_{3A} cys-loop receptor subunits, both of which exhibit homomer-forming capacity. Based on studies of PNU-120596 and NS-1738, Bertrand and colleagues conclude that there may be different binding sites for different allosteric modulators, each with a distinct profile of drug sensitivities [14]. They also highlight an important role in the actions of these allosteric modulators [14] of a short sequence (AEIMPATSDS) in human $\alpha 7$ at the M2 / M2-M3 loop interface, equivalent to the region (SDTLPATAG) of the 5-HT_{3A} receptor. In a separate study, Millar and colleagues have suggested that there may be a common site of action for two PAMs PNU-120596 and LY-2087101 [15]. These authors also used $\alpha 7$ / 5-HT₃ chimeras, identifying the transmembrane (TM) regions as critical in drug enhancement of agonist evoked responses. In particular, 5 residues (2 in TM1, 1 in TM2 and 2 in TM4) strongly influence the actions of PAMs. Although mutating all these residues in the $\alpha 7$ TM sequence to their 5HT_{3A} equivalents strongly reduced the allosteric actions of these drugs, such activity could not be restored by incorporating the $\alpha 7$ residues into the equivalent site of the 5-HT_{3A} receptor subunit.

Experiments on chimeras have been extremely fruitful but are not without their problems. For example, some are poorly expressed, suggesting that they may not be correctly folded, thereby

complicating interpretation of the results. In addition, the functional organization of receptor domains may not be perfectly modular, so that the effects of substituting a domain may include indirect effects on other, unchanged domains. This may be a particular concern when interspecies chimaeras are used. A complementary approach is to explore the comparative pharmacology of evolutionarily remote members of the same superfamily of cys-loop ligand-gated ion channels. This strategy has already proved instructive in accelerating the identification of drug targets. For example, the hitherto elusive steroid site on the GABA receptor was identified in a comprehensive comparison between *Drosophila melanogaster* GABA-gated chloride channels and human GABA_A type receptors [16]. We have previously shown that the anthelmintic compound, IVM, enhanced ACh-induced $\alpha 7$ amplitude responses, confirming the earlier finding of Bertrand and colleagues [17], while slightly attenuating responses of ACR-16 [18], a *Caenorhabditis elegans* $\alpha 7$ homologue [19, 20]. ACR-16 and $\alpha 7$ nAChR subunits share considerable amino acid sequence identity of 47% but show important differences in residues considered important in allosteric drug actions (Fig. 1). For example, of the 5 transmembrane residues identified by Millar and colleagues in rat $\alpha 7$ (S222, A225, M253, F455 and C459 using mature peptide numbering) only F455 is conserved in *C. elegans* ACR-16 (F458 in ACR-16, Fig.1) [15]. Here, we report comparison of the actions of several other PAMs on the human $\alpha 7$ nAChR and ACR-16. Having established that these two receptors differ greatly in their sensitivity to PAMs, we then modeled the docking of IVM to the transmembrane regions of both receptors in an initial attempt to develop a molecular interpretation of the observed differences in pharmacology.

2. Materials and Methods

2.1 Electrophysiological studies

The vectors pcDNA3.1/Zeo+ (Invitrogen) encoding human $\alpha 7$ or *C. elegans ric-3* as well as pGEM-T Easy (Promega) encoding *acr-16* were used. The vectors were linearised to be used as template for cRNA synthesis with the T7 (pcDNA3.1) or SP6 (pGEM-T Easy) mMACHINE kits (Ambion). Mature stage IV or V *Xenopus laevis* oocytes were manually defolliculated after incubation for 1h in 2mg/ml collagenase (Sigma type IV) in calcium-free oocyte saline (OS) (containing (in mM) NaCl, 100; KCl, 2; CaCl₂, 1.8; MgCl₂, 1; HEPES, 5 adjusted to pH 7.6 with 5 M NaOH). Following defolliculation, the cytoplasm of each oocyte was injected with 50nl 1ng/ml containing a 5:1 ratio of receptor cRNA: RIC-3 cRNA in RNAase-free dH₂O. Oocytes were incubated for 2-7 days in filter-sterilized culture medium consisting of OS supplemented with 100 units/ml of penicillin, 100 μ g/ml of streptomycin and 50 μ g/ml gentamycin sulphate and 2.5 mM sodium pyruvate, the medium being changed daily. After 2-7 days incubation, injected oocytes were transferred to a recording chamber (Warner type RC-3Z) for electrophysiological analysis. Oocytes were impaled with two glass microelectrodes (3M KCl, resistance 0.5-1 M Ω in OS) and held under voltage clamp at -100 mV. Standard two-electrode voltage clamp was achieved using an Axon Axoclamp 900A amplifier under the control of a laboratory PC. Signals were acquired at 1 kHz.

Saline and drugs were applied through a gravity-fed perfusion system (Valvelink 8.2) using computer-controlled pinch valves. With relatively high perfusion rates (5 ml/min), low saline levels in the chamber and a short distance between the perfusion manifold and the chamber, it was possible to achieve consistent amplitude, rapid onset responses in oocytes expressing the rapidly desensitizing $\alpha 7$ and ACR-16 nAChRs. Agonists were applied for 10s, and the effects of allosteric modulators were assessed by applying the drug for 30s immediately prior to a 10s test

application of agonist in the continued presence of modulator. Agonist challenges were at 3 min intervals to minimize any effects of desensitization. Oocytes were only used in experiments if the amplitudes of responses to successive challenges of agonist differed by 10% or less. All drugs were obtained from Sigma-Aldrich UK except for IVM, PNU-120596 and NS-1738 which were purchased from Tocris (Ellisville, Missouri USA) and acetylcholine chloride which was purchased from Alfa Aesar (Karlsruhe, Germany).

2.2 Computational protein modelling and Ivermectin docking

The model of IVM used in this study was constructed using the atom co-ordinates previously determined for avermectin B1a [21]. IVM and avermectin B1a are very similar molecules, differing only at the C22-C23 bond, which is saturated in IVM but unsaturated in avermectin B1a. All IVM atoms conserved in avermectin B1a were therefore assigned the avermectin B1a co-ordinates, leaving only the saturated C22-C23 bond to be modeled. This was readily achieved by simple atom building and local energy minimization with Discovery Studio Visualizer (Accelrys). Figures 2 and 4 were produced using PyMOL (DeLano Scientific).

Modeling of nAChRs was carried out using the molecular modeling software package Sybyl, version 7.3 (Sybyl Molecular Modeling Software, Tripos Associates, Inc., St Louis, Missouri, U.S.A.) and the homology modeling software PDFAMS pro, version 2.0 (Protein Discovery Full Automatic Modeling System, In-Silico Sciences, Inc., Tokyo, Japan), originally developed by Ogata and Umeyama [22]. The three dimensional structures of the nAChRs were constructed based on the sequence and coordinates of the standard protein, AChR (2BG9 [23]) by the simulated annealing method [24] according to Taly et al, 2006 [25]. The receptor model was energy-minimized for 1,000 iterations of conjugated gradients using the MMFF94 force field and

MMFF94 partial charges [26, 27]. The coordinates of backbone atoms were fixed during the minimization. The flexible ligand/receptor docking was performed with the FlexiDock, a genetic algorithm-based flexible docking software in Sybyl. IVM was placed manually into the cavity of the ligand binding site. The conformation of IVM was fixed during FlexiDock, except for the torsion angles of the 3-methoxy groups and the sec-butyl group. The docked receptor / ligand models were energy-minimized for 1,000 iterations of conjugated gradients using the MMFF94 force field and MMFF94 partial charges [26, 27] with co-ordinates of the backbone atoms fixed.

3. Results

3.1 Functional studies

Responses mediated by $\alpha 7$ receptors expressed robustly in oocytes in the presence of RIC-3 were reversibly enhanced by both type I and type II allosteric modulators (Fig. 3). These findings resemble and extend those obtained previously in the absence of RIC-3 [11, 14, 17]. The type I modulators, IVM (30 μM), 5-hydroxyindole (1mM), NS-1738 (10 μM) and genistein (10 μM), enhanced $\alpha 7$ responses to $123 \pm 7\%$ (n=5), $300 \pm 91\%$, (n=3), $1740 \pm 1358\%$ (n=3) and $376 \pm 77\%$, (n=3) respectively, while the type II modulator, PNU-120596 (3 μM) enhanced $\alpha 7$ responses by $1867 \pm 871\%$ (n=4). In accordance with previous reports, the type II modulator, PNU-120596 greatly enhanced the duration of the response, while the type I modulators investigated exerted a variable effect upon the time course of the responses, but much less markedly so than type II modulators. The similarity of our findings with type I and type II allosteric modulators and those previously reported [11, 14, 17] also suggests that RIC-3, which

enhances the robustness of $\alpha 7$ expression does not affect allosteric responses of the drugs tested in this study.

In contrast to the findings on human $\alpha 7$ -mediated responses, ACR-16 showed no positive allosteric modulation of ACh actions by any of the compounds we tested. Rather, all type I PAMs investigated significantly ($P < 0.05$, 1-sample t -test) attenuated the amplitude of ACR-16-mediated ACh response (Fig 3). The results with IVM are in accord with earlier experiments conducted on ACR-16 in the absence of RIC-3 [18]. In addition, there was no obvious effect with the type II modulator, PNU120596, on the time-course of ACh-evoked responses. Reducing or increasing the concentration of the modulators 10-fold did not result in enhancement (data not shown).

3.2 Computational modelling studies

The structure of IVM used in this study is based on the crystal structure of Avermectin B_{1a} [21]. A stick representation of the molecule, based on the published crystal co-ordinates [21] is shown (Fig 2). Receptor models for the putative transmembrane region were generated and the IVM molecule docked. IVM is so large and rigid that when manually inserted only a limited complex structure was allowed for docking. Also the Flexidock software yielded only one solution as an initial IVM-docked structure. Whatever method was used to obtain the initial complex structure, the resulting complex structures were always very similar. Of particular interest was the finding that the interaction energies for the $\alpha 7$ /IVM complex was less than that for ACR16, suggesting that the $\alpha 7$ -IVM complex is much more energetically favourable than the ACR-16-IVM complex.

4. Discussion

The cys-loop superfamily of ligand-gated ion channel receptors mediate the fast actions of the neurotransmitters ACh, GABA, glycine and 5-HT in humans. These important transmembrane proteins are the targets of major drugs for the treatment of disorders such as anxiety, nicotine addiction, pain, Alzheimer's disease and schizophrenia. Allosteric drug binding sites on these proteins are of growing importance as targets for new drugs, yet their identity remains to be fully elucidated. Here we begin to explore the combination of comparative pharmacology and computational modelling to better understand the human $\alpha 7$ allosteric drug binding site(s).

4.1 Major differences in amino acid sequence and the interactions of allosteric ligands between human $\alpha 7$ and *C. elegans* ACR-16 nAChRs

We show that drugs exhibiting type I (IVM, 5-HI, genistein, NS-1738) and type II (PNU-1205096) PAM activity on human $\alpha 7$ receptor are all without PAM activity on ACR-16, instead displaying varying degrees of attenuation of the amplitude of the ACh response. Genistein and 5-HI result in particularly strong attenuation in the amplitude of ACh responses (Fig. 3). In line with this, comparisons reveal that despite an overall sequence identity of 47% [20], human $\alpha 7$ and *C. elegans* ACR-16 subunits differ at residues known in nAChRs and in other cys-loop receptors to influence the actions of allosteric modulators. For example, ACR-16 differs in 4 of 5 key transmembrane residues, which in rat $\alpha 7$ receptors (these residues are identical in human $\alpha 7$) are known to be important in the actions of the PAMs PNU-120596 and LY-2087101 [15] (Fig. 5). Also, the short region at the extracellular end of TM2 and part of the inter TM2-TM3 loop region of $\alpha 7$, which was shown to be involved in NS-1738 potentiation [14], differs by eight amino acid residues when compared to ACR-16.

Interestingly, the 4 key transmembrane residues highlighted by Young et al [15] (S222 and A225 in TM1, M253 in TM2 and C459 in TM4 after the number in [15]), which are identical in human and rat $\alpha 7$ but differ in *C. elegans* ACR-16 and are known to influence PAM activity, are in close proximity to (and in some cases overlap with) residues known to influence the actions of other allosteric ligands in a variety of cys-loop receptors. These molecules include anaesthetics [28, 29], alcohols [30], endogenous neurosteroids [16] and androgenic steroids [31] (Fig. 5). Three residues in TM1-3, which have been well-characterised in GABA receptors, form a binding pocket with which general anaesthetics can interact [16, 28]. Residues in TM1 and TM4 play a major role in the binding of neurosteroids. Residues already identified as determinants of sensitivity to PNU-120596 [15], one of the allosteric modulators employed in the present study are among those that differ between ACR-16 and $\alpha 7$.

Computational modelling suggests differences in interaction energies (Energy (Complex)-Energy (Receptor)-Energy (IVM)) between IVM complexes with $\alpha 7$ and ACR-16 nAChRs, with the $\alpha 7$ -IVM complex being much more stable than the ACR-16-IVM complex (Fig. 4). Indeed energy considerations suggest that IVM-ACR-16 interactions may be severely limited. However, the small attenuation of the ACh response argues that there must be some interaction between IVM and ACR-16. The sequence differences between $\alpha 7$ and ACR16 invite systematic site-directed mutagenesis studies aimed at elucidating all key residues determining PAM activity and such studies are in progress in our laboratory. Indeed, the valuable insights already gained by comparing $\alpha 7$ nAChRs with a $\alpha 7$ / 5HT3R chimeras [15] encourages the hope that additional insights may be achieved by comparing $\alpha 7$ with ACR16.

4.2 Ivermectin can have allosteric and agonist effects on ligand-gated ion channels

IVM acts upon a range of diverse ligand-gated ion channels of both vertebrates and invertebrates, where it can act as an agonist, an allosteric modulator, or both (Table 1). In addition to nematode acetylcholine receptors, these targets also include P2X receptors of *Schistosoma* [32] and of mouse ([33]) and histamine-gated chloride channels of *Drosophila* [34], as well as nematode [35-38] and insect [39, 40] glutamate-gated chloride channels, GABA receptors from nematodes to vertebrates [41-44] and human glycine receptors [45]. There is also evidence for an action of IVM on an intracellular ligand-gated ion channel, the ryanodine receptor [46]. In most cases examined so far, the action of IVM is through an allosteric stabilization of the open state of the channel, but in the case of recombinant nematode glutamate-gated chloride channels and rat GABA $\alpha_1\beta_1\gamma_2$ receptors, IVM can also act as an agonist [43]. Residues in both transmembrane regions of P2X receptors that affect IVM action have been identified using scanning mutagenesis [32, 47]. Thus IVM exerts a range of actions on diverse members of the cys-loop receptor family and some other ligand-gated ion channels, providing ample scope for identifying the roles of specific amino acid residues in determining IVM action.

4.3 Prospects for further understanding of allosteric drug actions and the development of novel allosteric ligands

The use of $\alpha 7 / 5$ -HT3A chimeras has begun to elucidate the site of action of PAMs [14, 15]. Site-directed mutagenesis informed by comparisons of evolutionarily remote members of the $\alpha 7$ -like nAChRs (and other members of the cys-loop family of receptors) combined with pharmacological studies and computational modelling, along with access to resolved structures of relatively rigid molecules such as IVM, will complement the work on such chimeras [14, 15].

Questions of interest include: in the light of its diverse actions (Table 1), does IVM bind to an equivalent site on several types of cys-loop receptor? Can occupation of the transmembrane binding pocket and/or the resultant coupling to channel activation in the different cys-loop receptors account for the observed diversity of IVM actions? It will also be of interest to explore the extent to which computational studies on a complex molecule such as IVM can also add to our understanding of the actions of small molecule allosteric modulators. Mutagenesis and molecular modelling studies in progress on $\alpha 7$ and ACR-16 are aimed at enhancing our understanding of allosteric drug - nAChR interactions and accelerating the development of new allosteric drug candidates.

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Receptor		Species	Ivermectin Actions	Reference
P2X	P2X ₄ R	Mouse	Enhances	[33]
	P2X ₇ R	"	Enhances	[48]
	P2X ₁ R	"	Prolongs response	[49]
	P2X ₂ R	"	Enhances	[50]
	P2X ₄ R	"	Binds open conformation	[47]
GluCIR		D. melanogaster		[39]
		C. elegans	Gates GluCIR, Resistance involves avr14+avr15+glc1	[35], [37], [38]
		Locusta migratoria	Activates	[40]
		Haemonchus contortus Cylicocyclus nassatus Cooperia oncophora		[51]
GluCIR+RDL(GABAR)		D. melanogaster	Activates	[52]
		D. melanogaster	Binds	[53]
nAChRs	ACR-16 $\alpha 7$	C. elegans	No PAM action (slight inhibition)	[18]
		Human	PAM action	[17]
GABAR		Haemonchus contortus	PAM	[41]
		Apis mellifera	Reduces learning via GABARs	[42]
		Rat	Agonist of $\alpha_1\beta_1\gamma_2s$	[43]
		Rat	Binds $\alpha 1\beta_3,2$	[44]
GlyR		Human	PAM and activates	[45]
RyR		Rabbit	Activates	[46]
Histamine R		D. melanogaster	Mutants in HisClB alter sensitivity to IVM	[54]

Figure captions

Fig. 1. Alignment of the amino acid sequences of the human $\alpha 7$ and *C. elegans* ACR-16 homomer - forming nAChR subunits. The signal sequences (brown box) , acetylcholine binding loops A-F

(purple boxes) and transmembrane regions TM1-4 (blue boxes) are indicated. The alignment and feature annotation were performed using the SWISSPROT database. Although the signal sequence is illustrated, the numbering of residues shown is that of the mature peptide.

Fig. 2. *Structure of Ivermectin.* The stick model was constructed from the co-ordinates of avermectin B1a [21]. Carbon atoms are coloured grey and oxygen atoms red. Hydrogen atoms have been omitted for clarity.

Fig. 3. *Major differences are observed in the actions of type 1 and type 2 allosteric modulators on human $\alpha 7$ and *C. elegans* receptors.* The actions of PNU120596 (type 2 action characterised by enhanced amplitude and duration of the ACh response) as well as IVM, 5-HI, genistein and NS-1738 (type 1 actions characterised by enhanced amplitude) as positive allosteric modulators of the human $\alpha 7$ nAChR contrast strikingly with their blocking actions on the *C. elegans* ACR-16 nAChR.

Fig. 4. *The docking of Ivermectin to $\alpha 7$ and ACR-16 nAChRs.* Top and side views of the complexes are shown in the upper and lower parts of this figure. IVM was docked into $\alpha 7$ and ACR-16 using the PDFAMS software (see Methods for details). Only one subunit is shown to facilitate observation. IVM is drawn as sticks where carbons and oxygens are colored white and red, respectively, while omitting hydrogens. The transmembrane (TM1) regions are shown as cyan helices, while the TM2-TM3 linker is shown as sticks where carbons, nitrogens and oxygens are colored green, blue and red, respectively. The two models differed in their interaction energies ($\alpha 7$ -IVM complex: -68.4 kcal/mol; ACR16-IVM complex: -19.1 kcal/mol).

Fig. 5. *Sequence alignment of the transmembrane regions of human $\alpha 7$ and *C. elegans* ACR-16 showing residues in transmembrane regions (red boxes, [15]) and segments between transmembrane regions (yellow boxes[14]) that are known to mediate the effects of positive allosteric modulators (PAMs). In addition, equivalent residues in other ligand-gated ion channels that have been implicated in the actions of allosteric modulators are also indicated. These sites include a well-characterized binding cavity to which residues in the extracellular portions of TM1-3 contribute. The close proximity of residues in different receptors mediating different allosteric actions suggests that common binding sites are conserved across different members of the cys-loop family. That $\alpha 7$ and ACR16, despite their overall sequence similarity, should 1) differ at most of these sites, 2) have widely different pharmacologies and 3) robustly form functional homomers underscores the great usefulness of these receptors in exploring the molecular basis of PAM action. Residue numbering is that for the mature peptide.*

Table 1 The diverse actions of ivermectin (IVM) on cys-loop neurotransmitter receptors and other ligand-gated ion channels. In most cases, IVM is a positive allosteric modulator, although there is some evidence that it can also act as an agonist. This diversity of IVM targets includes both excitatory and inhibitory neurotransmitter receptors and includes both vertebrate and invertebrate targets. There is therefore scope for comparative studies on related, but distinct, cys-loop receptor subunits. Abbreviations: CHO: Chinese hamster ovary; GABAR: GABA receptor; GluClR: glutamate-gated chloride channel; GlyR: glycine receptor; HCIB: histamine-gated chloride channel B; nAChRs: nicotinic acetylcholine receptors; PAM: positive allosteric modulator; P2XR: P2X receptor; RDL: resistant to dieldrin; RyR: ryanodine receptor; TM: transmembrane regions.

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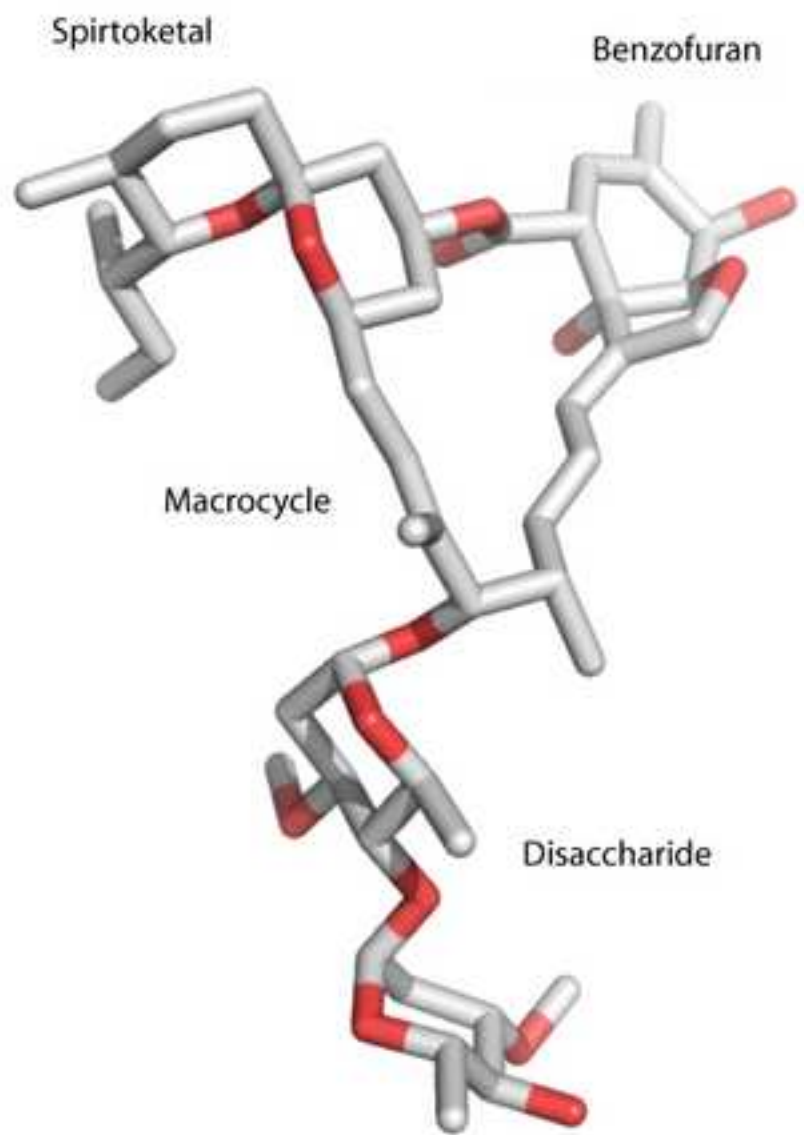
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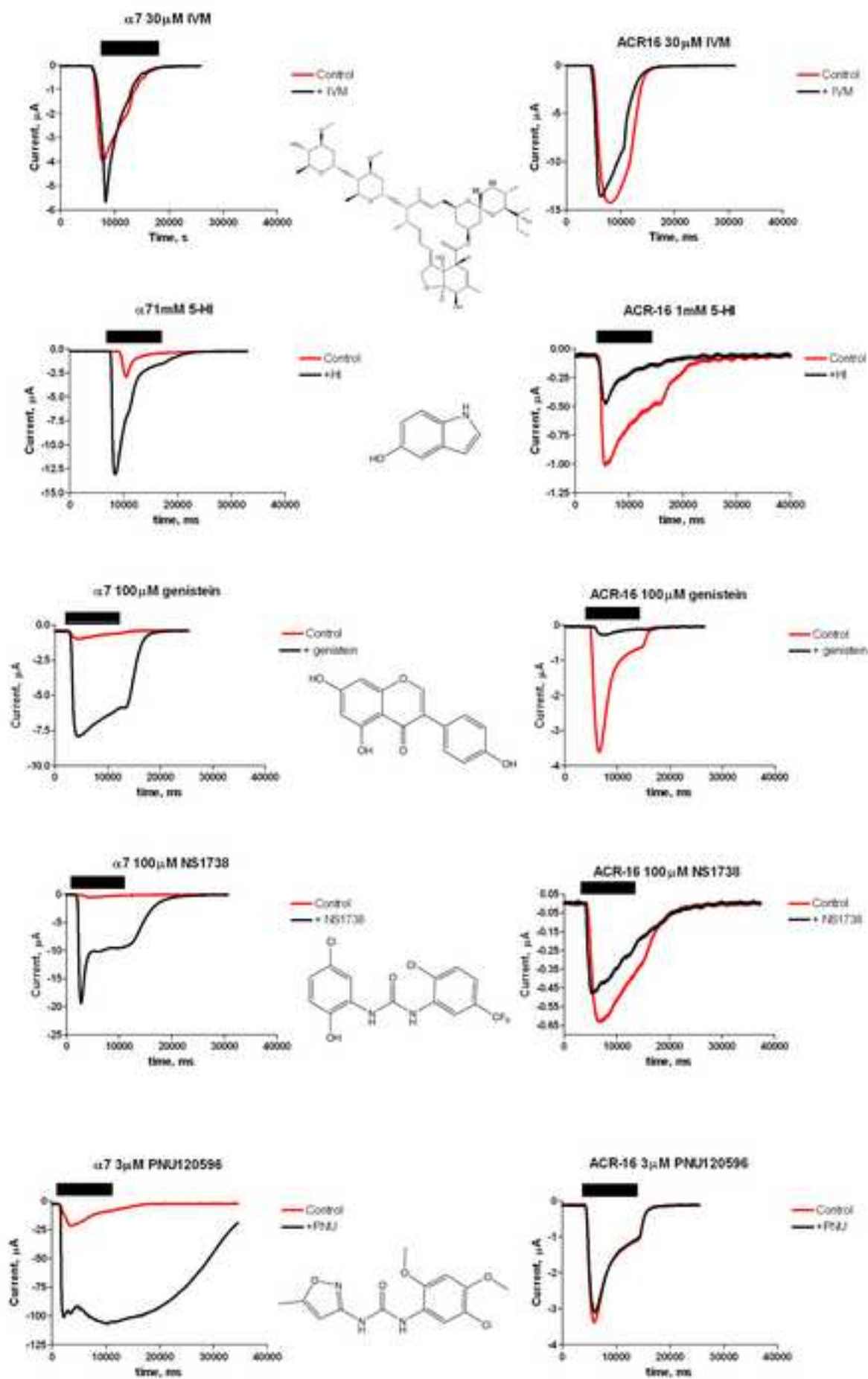
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Figure 2





$\alpha 7$

ACR-16

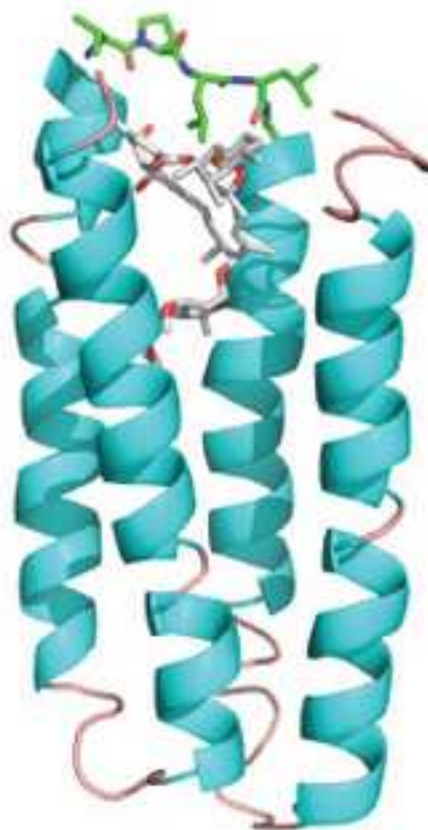
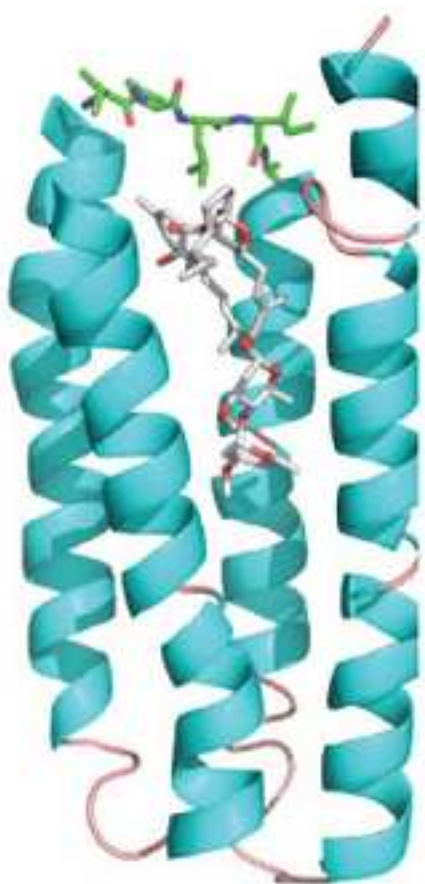
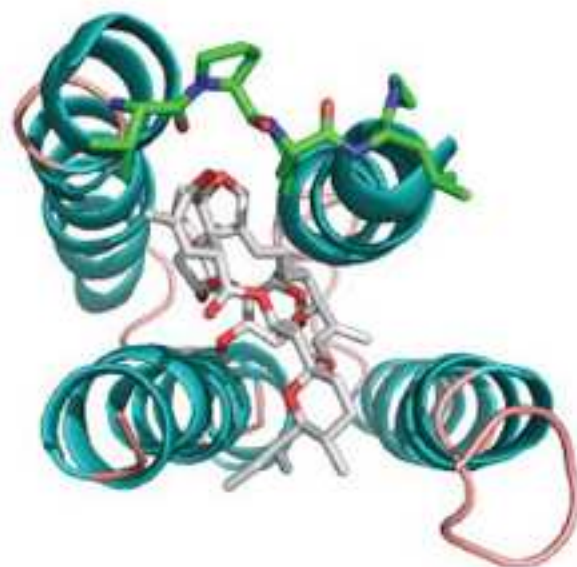
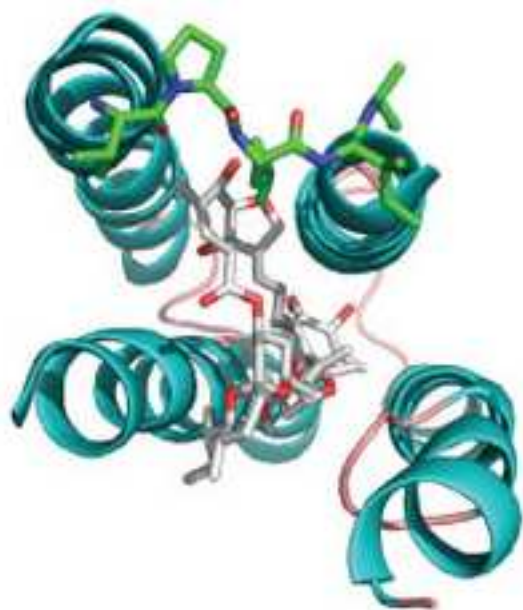
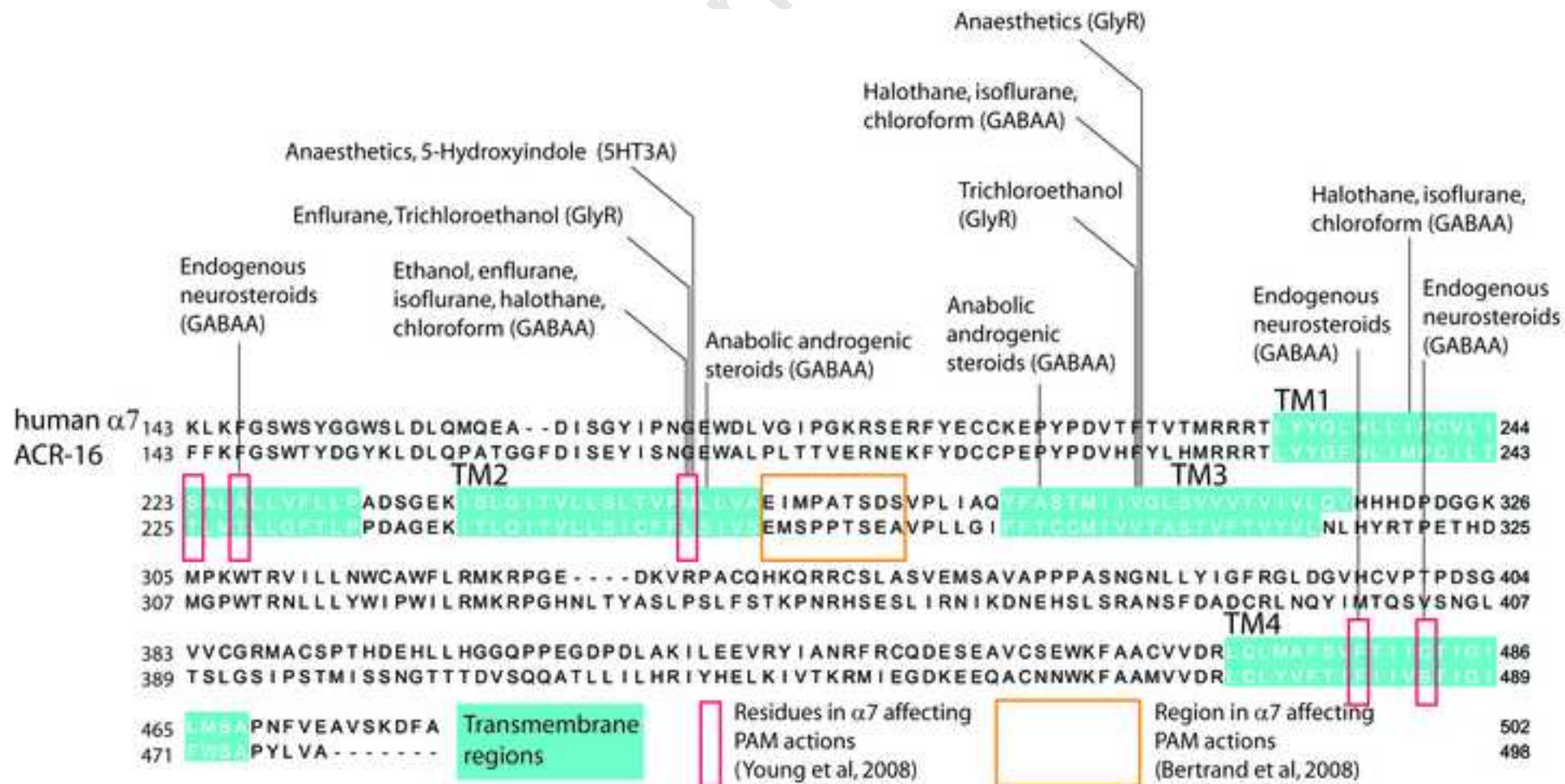
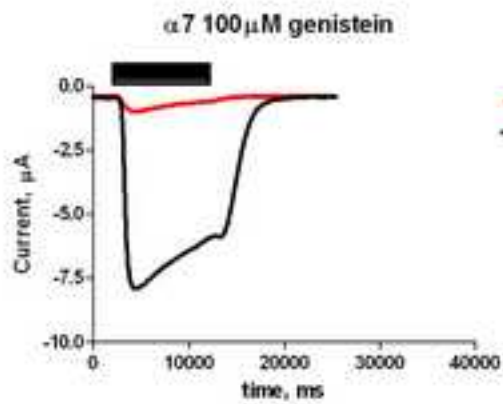
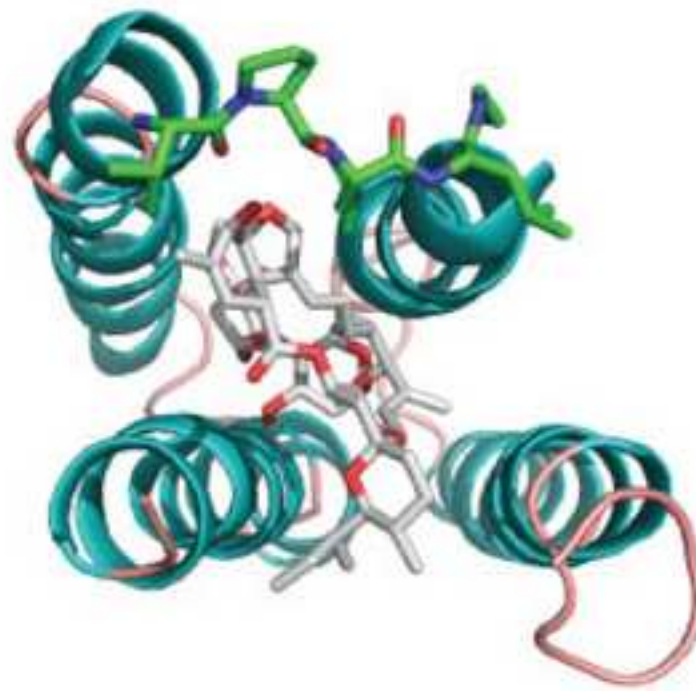
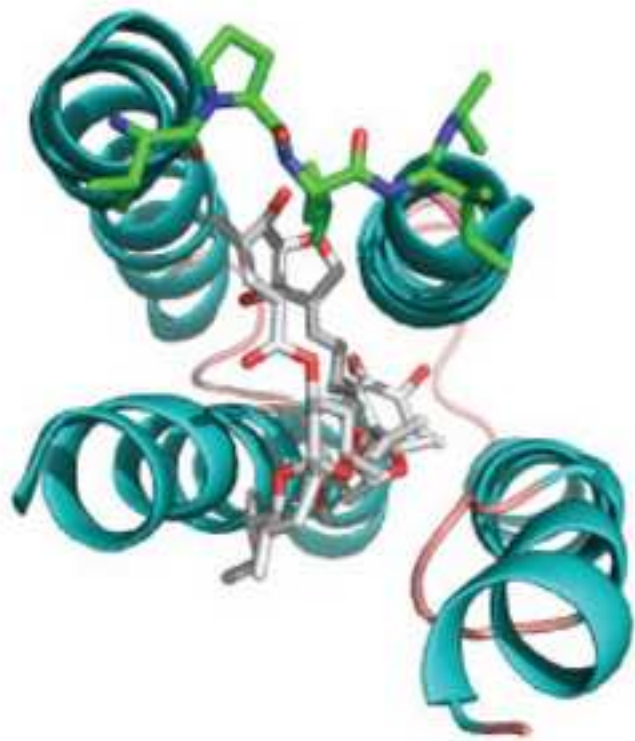


Figure 5

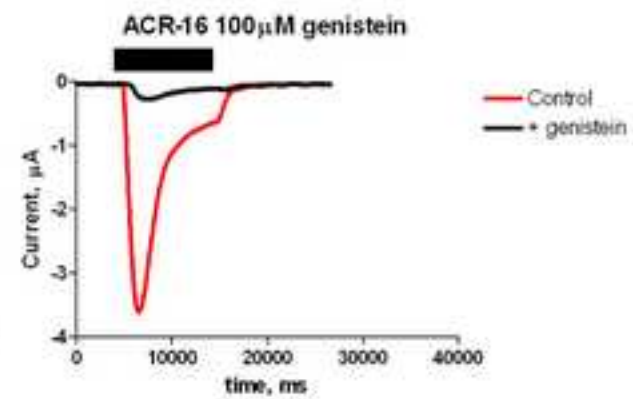
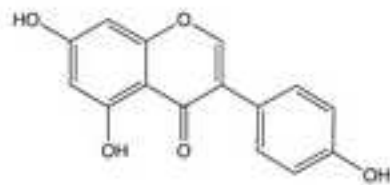


$\alpha 7$

ACR-16



— Control
— + genistein



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