Nicotinic Acetylcholine Receptor Transmembrane Mutations Convert Ivermectin from a Positive to a Negative Allosteric Modulator

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ABSTRACT

Ivermectin is a macrocyclic lactone that acts as a positive allosteric modulator of $\alpha 7$ nicotinic acetylcholine receptors (nAChRs) but has no modulatory activity on 5-hydroxytryptamine (5-HT) type 3 (5-HT$_3$) receptors. By examining the influence of ivermectin on subunit chimeras containing domains from the nAChR $\alpha 7$ subunit and the 5-HT3A subunit, we have concluded that the transmembrane domains play a critical role in influencing allosteric modulation by ivermectin. A series of mutations located within the α-helical transmembrane domains of the $\alpha 7$ subunit were examined, and seven were found to have significant effects on allosteric modulation by ivermectin. Four mutations (A225D, Q272V, T456Y, and C459Y) caused a significant reduction in the potency of ivermectin as an allosteric potentiator. Compared with wild-type $\alpha 7$ nAChRs, potentiation by ivermectin was reduced dramatically (by 89–97%) by these mutations. Somewhat unexpectedly, three mutations (S222M, M253L, and S276V located in TM1, TM2, and TM3) converted ivermectin from a positive allosteric modulator into an antagonist. Levels of inhibition of 56, 84, and 89% were observed on M253L, S276V, and S222M, respectively. Antagonism by ivermectin was insurmountable and had no effect on EC$_{50}$ of acetylcholine, indicating that it is acting noncompetitively. The seven mutations that influence allosteric modulation by ivermectin are located near a predicted intrasubunit transmembrane cavity. Computer docking simulations provide support for the hypothesis that ivermectin binds in close proximity to this cavity. We conclude that transmembrane mutations in $\alpha 7$ nAChRs are able to convert ivermectin from a positive to a negative allosteric modulator.

Ivermectin is a large macrocyclic lactone (Fig. 1A). It is a synthetic derivative of a naturally occurring compound isolated by fermentation of *Streptomyces avermitilis* and is used commercially as an antiparasitic agent in both human and veterinary medicine (Burkhart, 2000). The antiparasitic activity of ivermectin is thought to be due to its action as an agonist of invertebrate glutamate-gated chloride channels (Arena et al., 1994; Raymond and Sattelle, 2002). In addition, ivermectin has been shown to modulate several other ligand-gated ion channels. For example, it is an agonist of invertebrate histamine-gated chloride channels (Zheng et al., 2002) and a positive allosteric modulator of vertebrate $\alpha 7$ nicotinic acetylcholine receptors (nAChRs) (Krause et al., 1998), GABA$_A$ receptors (Sigel and Baur, 1987; Krůsek and Zemková, 1994), glycine receptors (Shan et al., 2001) and ATP

(P2X) receptors (Khakh et al., 1999). Ivermectin has also been reported to act as an agonist of GABA$_A$ and glycine receptors (Sigel and Baur, 1987; Adelsberger et al., 2000; Shan et al., 2001).

There has been considerable interest in positive allosteric modulators of $\alpha 7$ nAChRs (for review, see Bertrand and Gopalakrishnan, 2007; Moaddel et al., 2007). In part, this interest has been stimulated by suggestions that such compounds may have therapeutic potential as cognitive enhancers in connection with disorders such as Alzheimer's disease and schizophrenia (Hurst et al., 2005; Bertrand and Gopalakrishnan, 2007; Moaddel et al., 2007). A wide variety of compounds in addition to ivermectin have been reported to act as positive allosteric modulators of $\alpha 7$ nAChRs. These include proteins such as serum albumins (Conroy et al., 2003) and SLURP-1 (Chimienti et al., 2003), as well as small molecules such as galanthamine (Samochocki et al., 2003), 5-hydroxyindole (Zwart et al., 2002), [2-(4-fluoro-phenylamino)-4-methyl-thiazol-5-yl]-thiophen-3-yl-methanone (LY-2087101) (Broad et al., 2006),

ABBREVIATIONS: nAChR, nicotinic acetylcholine receptor; LY-2087101, [2-(4-fluoro-phenylamino)-4-methyl-thiazol-5-yl]-thiophen-3-yl-methanone; NS1738, 1-(5-chloro-2-hydroxy-phenyl)-3-(2-chloro-5-trifluoromethyl-phenyl)-urea; PNU-120596, 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea; MLA, methyllycaconitine; CPBG, 1-(3-chlorophenyl)biguanide hydrochloride.

1-(5-chloro-2-hydroxy-phenyl)-3-(2-chloro-5-trifluoro-methyl-phenyl)-urea (NS1738) (Timmermann et al., 2007), and 1-(5-chloro-2,4-dimethoxy-phenyl)-3-(5-methyl-isoxazol-3-yl)-urea (PNU-120596) (Hurst et al., 2005). All of these compounds potentiate peak agonist-induced responses but have been classified as either "type I" or "type II" positive allosteric modulators based on their effect on receptor desensitization (Bertrand and Gopalakrishnan, 2007). Type I compounds (such as ivermectin) have little or no effect on receptor desensitization, whereas type II compounds (such as PNU-120596) reduce levels of desensitization seen during prolonged agonist exposure (Krause et al., 1998; Hurst et al., 2005).
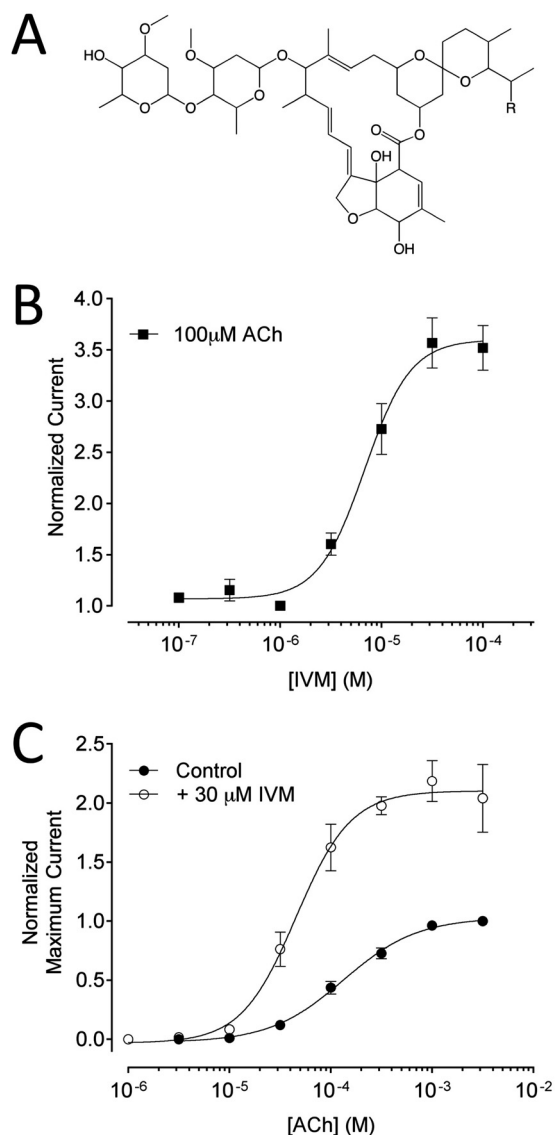

Fig. 1. Positive allosteric modulation of α7 nAChRs by ivermectin. A, the chemical structure of ivermectin. The preparation of ivermectin used in this study (Sigma) is composed primarily of ivermectin B_{1a} (where $R = CH_2CH_3$) with ivermectin B_{1b} (where $R = CH_3$) being a minor component. B, dose-response curve for ivermectin on rat α7 nAChR expressed in *X. laevis* oocytes illustrating potentiation of responses to an EC_{50} concentration of acetylcholine (100 μM) by ivermectin. Data points are means of three to four responses and are normalized to the responses in the absence of ivermectin. C, acetylcholine dose-response curve obtained in the presence (open circles) and absence (closed circles) of 30 μM ivermectin. Data points are means of four to five responses and are normalized to the maximum response in the absence of ivermectin.

For some of these allosteric potentiators (e.g., galanthamine), the location of the binding site has been proposed to be on the nAChR extracellular domain (Hansen and Taylor, 2007). In contrast, for allosteric potentiators such as LY-2087101 and PNU-120596, a transmembrane binding site has been proposed (Young et al., 2008). It has been suggested, on the strength of computer docking simulations, that ivermectin may also interact with nAChRs via a transmembrane binding site (Sattelle et al., 2009). In the present study, we examined the ability of ivermectin to act as an allosteric modulator on a series of mutated and chimeric α7 nAChR subunits. We conclude that the nAChR transmembrane domains play a critical role in influencing allosteric modulation by ivermectin. In addition, we have demonstrated that single point mutations located within the α7 nAChR TM1, TM2, and TM3 transmembrane domains are able to convert ivermectin from a positive to a negative allosteric modulator.

Materials and Methods

Materials. All chemicals were obtained from Sigma (Poole, UK) with the exception of 1-(3-chlorophenyl)biguanide hydrochloride (CPBG) and methyllycaconitine (MLA), which were obtained from Tocris Bioscience (Bristol, UK).

Subunit cDNAs, Subunit Chimeras, and Site-Directed Mutagenesis. Rat nAChR α7 subunit cDNA was obtained from Jim Patrick (Baylor College of Medicine, Houston, TX). Mouse 5-HT3A subunit cDNA was obtained from David Julius (University of California, San Francisco, San Francisco, CA). Subunit chimeras $α7^{4TM-5HT3A}$ and $α7^{V201-5HT3A}$, containing domains derived from the rat α7 subunit and mouse 5-HT3A subunit in plasmid pZeoSV2 have been described previously (Cooper and Millar, 1998; Gee et al., 2007), as has a plasmid expression construct (pcDNA3-hRIC-3) containing the human RIC-3 cDNA (Lansdell et al., 2005). Construction of α7 nAChRs containing site-directed mutations has also been described previously (Young et al., 2008).

Xenopus laevis **Oocyte Electrophysiology.** *X. laevis* oocytes were isolated and defolliculated as described previously (Young et al., 2007). Heterologous expression was achieved by injection of plasmid cDNA constructs (10–30 ng) into oocyte nuclei. In the case of the α7 cDNA containing the A225D mutation (Young et al., 2008), expression of functional receptors was inefficient. For this reason, the A225D mutation was coexpressed with the molecular chaperone RIC-3, which has been found previously to enhance nAChR maturation (Lansdell et al., 2005; Millar, 2008). Oocytes were injected in a volume of 18.4 nl using a variable-volume microinjector (Drummond Scientific, Broomall, PA). Two-electrode voltage-clamp recordings were performed essentially as described previously (Young et al., 2007) using an Axon Geneclamp 500B amplifier, Axon Digidata 1200, and pClamp software (Molecular Devices, Sunnyvale, CA). Rapid solution exchange was achieved using a computer-controlled eight-way solenoid valve perfusion system (BPS-8; ALA Scientific Instruments, Westbury, NY). Data were analyzed using Prism software (GraphPad Software, San Diego, CA) using the Hill equation. In all cases, data were best fit with a single-site model. Statistical significance was determined by analysis of variance.

Computer Docking Simulations. Computational molecular docking was performed with AutoDock 4 (http://autodock.scripps.edu/) (Morris et al., 1998) using a homology model of the human α7 nAChR transmembrane region (Cheng et al., 2006), as described previously (Young et al., 2008). To avoid bias, a "blind docking" approach was used in which no assumptions were made concerning where within the transmembrane region ivermectin might be expected to bind. Flexibility of rotatable bonds in ivermectin was permitted during the docking simulation. Predicted Gibbs free

energy of binding (ΔG) was calculated as described previously (Morris et al., 1998), as was the predicted equilibrium constant for binding (K_{eq}), using the equation $K_{eq} = e^{-\Delta G/R \cdot T}$ (where R is the gas constant, and T is absolute temperature).

Results

Ivermectin displayed no agonist activity on rat $\alpha 7$ nAChRs expressed in *X. laevus* oocytes and caused little or no potentiation of agonist-induced responses when it was coapplied with acetylcholine (in the absence of preapplication). Strong potentiation, however, was observed when ivermectin was preapplied (for 60 s) in addition to being coapplied with acetylcholine (Fig. 1, B and C). Consequently, this application protocol (a combination of pre- and coapplication of ivermectin) was used for all experiments described in the present study. These findings, conducted with rat $\alpha 7$ nAChRs, are in agreement with previous studies conducted with human and chick $\alpha 7$ nAChRs (Krause et al., 1998; Raymond et al., 2000) and are consistent with the classification of ivermectin as a positive allosteric modulator of $\alpha 7$ nAChRs.

Initial studies, performed with rat $\alpha 7$ nAChRs, were aimed at determining the influence of ivermectin on responses evoked by an EC_{50} concentration of acetylcholine (100 μM). Ivermectin potentiated acetylcholine responses (with an EC_{50} of 6.8 $\pm$ 1.2 μM) and maximal levels of potentiation were obtained with 30 μM ivermectin (Fig. 1B). The effect of this maximal concentration of ivermectin was then examined on a range of concentrations of acetylcholine. In the absence of ivermectin, acetylcholine activated $\alpha 7$ nAChRs with an EC_{50} of 141 $\pm$ 23 μM and a Hill coefficient of 1.2 $\pm$ 0.1. In the presence of ivermectin, the acetylcholine dose-response curve was shifted to the left (EC_{50} = 50 $\pm$ 3 μM) and had a steeper slope (Hill coefficient = 1.6 $\pm$ 0.4). Both of these are effects that have been observed consistently with nAChR potentiators and are consistent with an allosteric mechanism of action (Bertrand and Gopalakrishnan, 2007). Responses to a maximal concentration of acetylcholine (3 mM) were potentiated 2.0 $\pm$ 0.3 fold ($n = 4$) by ivermectin, whereas higher levels of potentiation were observed with submaximal concentrations of acetylcholine (Fig. 1C). Responses to an EC_{50} concentration of acetylcholine (100 μM) were potentiated 3.6 $\pm$ 0.2-fold ($n = 4$), and responses to an EC_{20} concentration (50 μM) were potentiated 4.4 $\pm$ 0.6-fold ($n = 8$). In contrast, 30 μM ivermectin caused no significant potentiation of agonist-evoked responses with the 5-HT3A subunit expressed in *X. laevis* oocytes (Fig. 2). The influence of ivermectin was also examined on two artificial subunit chimeras ($\alpha 7^{4TM-5HT3A}$ and $\alpha 7^{V201-5HT3A}$), both of which contain the $\alpha 7$ nAChR extracellular domain fused to the 5-HT3A subunit transmembrane domain (Fig. 2). In contrast to what was observed with the $\alpha 7$ and 5-HT3A, ivermectin caused a significant inhibition of both of the chimeras (Fig. 2 and Table 1). Ivermectin (30 μM) caused responses evoked by an EC_{50} concentration of acetylcholine (50 μM) to be inhibited by 44.9 $\pm$ 8.8% ($n = 5$) with $\alpha 7^{4TM-5HT3A}$ and by 26.6 $\pm$ 3.4% with $\alpha 7^{V201-5HT3A}$.

To investigate in greater detail the influence of the $\alpha 7$ transmembrane domain upon allosteric modulation by ivermectin, a series of mutations within the four α-helical transmembrane regions (TM1–TM4) were examined (Fig. 3). As in a previous study that examined allosteric modulation of $\alpha 7$ nAChRs (Young et al., 2008), the rationale that we have used

for mutagenesis is to alter amino acids in the $\alpha 7$ subunit to the corresponding amino acid in 5-HT3A. In all cases, the influence of 30 μM ivermectin was examined on an EC_{20} concentration of acetylcholine. Of 11 transmembrane mutations examined, four (G221V, L230Y, M260L, and F455A) had no significant effect on allosteric modulation by ivermectin, whereas seven mutations had significant effects (Fig. 3
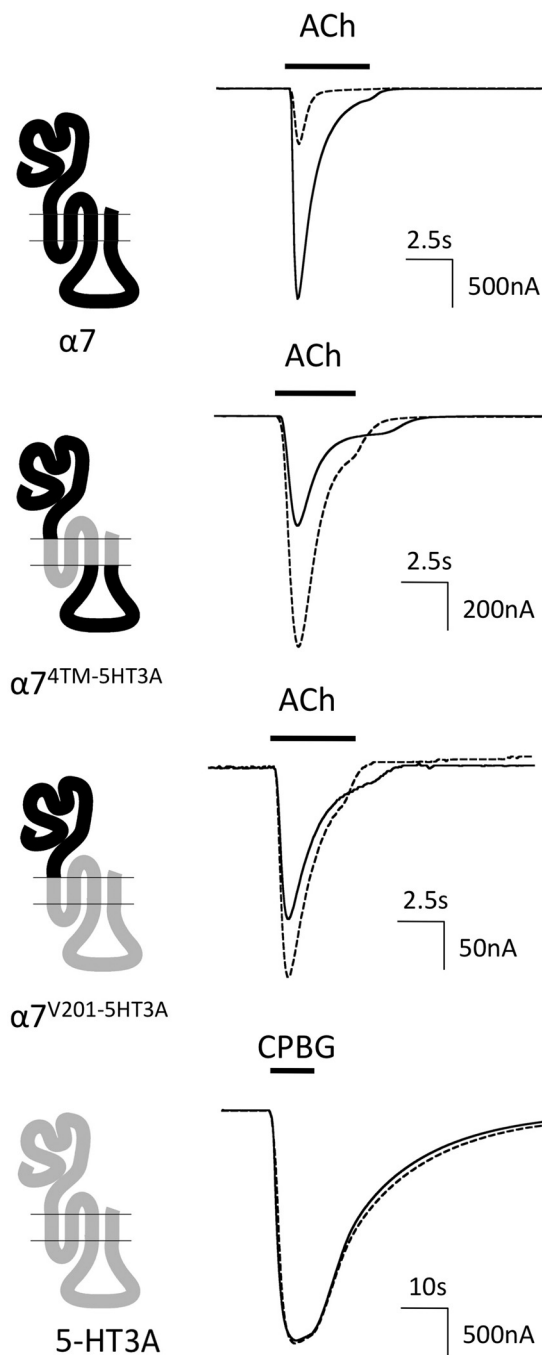

Fig. 2. Representative two-electrode voltage-clamp recordings from *X. laevis* oocytes illustrating the effects of ivermectin on agonist-evoked responses. Agonist-evoked responses are shown for rat $\alpha 7$ subunit, $\alpha 7^{4TM-5HT3A}$ chimera, $\alpha 7^{V201-5HT3A}$ chimera, and mouse 5-HT3A subunit. Control EC_{50} agonist responses elicited with either acetylcholine (in the case of $\alpha 7$, $\alpha 7^{4TM-5HT3A}$, and $\alpha 7^{V201-5HT3A}$) or CPBG (5-HT3A) are shown (black dashed lines). Also shown are responses evoked by EC_{50} concentration of agonists after coapplication and 60-s preapplication of 30 μM ivermectin (solid lines).

and Table 2). Four mutations caused a significant reduction in potentiation by ivermectin (A225D, Q272V, T456V, and C459Y). These mutations are located in TM1 (A225D), TM3 (Q272V), and TM4 (T456V and C259Y). Compared with the level of potentiation observed in wild-type $\alpha 7$ nAChRs in response to an EC_{20} concentration of acetylcholine (4.6 ± 0.6-fold), ivermectin caused potentiation of only 1.1- to 1.4-fold ($n = 3$–7) in $\alpha 7$ receptors containing one of these four mutations (Table 2). Three mutations (S222M, M253L, and S276V located in TM1, TM2, and TM3, respectively) had a particularly dramatic and somewhat unexpected effect. All of these mutations caused ivermectin to act as an inhibitor, rather than as a potentiator. Ivermectin caused agonist-evoked responses to be inhibited by 89.1 ± 6.6% ($n = 4$) in S222M, 55.6 ± 8.8% ($n = 3$) in M253L, and 84.2 ± 3.5% ($n = 4$) in S276V (Fig. 3 and Table 2).

Of the three mutations that caused ivermectin to act as an inhibitor, one (M253L) was selected for more detailed inves-

TABLE 1
The influence of ivermectin on agonist-evoked responses was examined on the $\alpha 7$ nAChR, the 5-HT$_3$R 3A subunit (5-HT3A) and two chimeras containing domains from the $\alpha 7$ and 5-HT3A subunits ($\alpha 7^{4TM-5HT3A}$ and $\alpha 7^{V201-5HT3A}$)

In each case, the influence of a maximal concentration of ivermectin (30 μM) was examined on an EC_{50} concentration of acetylcholine. The effect of ivermectin is expressed either as potentiation or inhibition compared with the magnitude of responses obtained with an EC_{50} concentration of acetylcholine in the absence of ivermectin.

Subunit	EC_{50} Agonist	Potentiation	Inhibition	n
	μM	*fold*	*%*	
$\alpha 7$	100 (ACh)	3.6 ± 0.2		4
$\alpha 7^{4TM-5HT3A}$	50 (ACh)		44.9 ± 8.8***	5
$\alpha 7^{V201-5HT3A}$	50 (ACh)		26.6 ± 3.4***	5
5-HT3A	1 (CPBG)	1.0 ± 0.02***		4

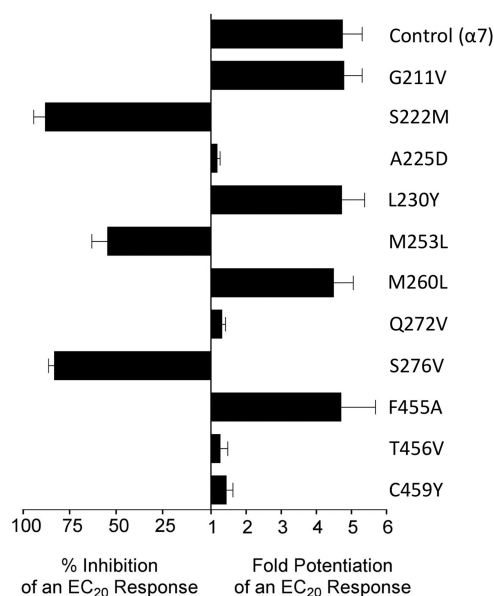*** $P < 0.001$, significantly different from the $\alpha 7$ subunit.

Fig. 3. The influence of $\alpha 7$ transmembrane mutations on allosteric modulation by ivermectin. A bar graph illustrates the influence of single point mutations on allosteric modulation by ivermectin. The effect of ivermectin is expressed either as potentiation (fold effect; right axis) or inhibition (percentage change; left axis), compared with the magnitude of responses obtained with an EC_{20} concentration of acetylcholine in the absence of ivermectin. Levels of potentiation and inhibition are summarized in Table 2. Data are means ± S.E.M. ($n = 3$–8).

tigation (because it was found to generate functional receptors most reliably). The influence of a range of ivermectin concentrations was examined on responses evoked by 100 μM acetylcholine (an EC_{50} concentration) on $\alpha 7$ nAChRs containing the M253L mutation (Fig. 4, A and B). The IC_{50} value for inhibition by ivermectin on receptors containing the M253L mutation was 1.4 ± 0.2 μM ($n = 3$; Fig. 4C), similar to the EC_{50} value for potentiation by ivermectin of agonist (100 μM acetylcholine)-evoked responses on wild-type $\alpha 7$ nAChRs (6.7 ± 1.2 μM; $n = 3$; Fig. 1B). The influence of ivermectin on $\alpha 7$ nAChRs containing the M253L mutation was also examined on a range of acetylcholine concentrations (Fig. 4C). As would be expected of a noncompetitive antagonist, ivermectin (3 μM) caused an insurmountable reduction in the maximal agonist response with no significant effect on the EC_{50} value for acetylcholine. In the absence of ivermectin, the EC_{50} value for acetylcholine was 110 ± 27 μM (with a Hill coefficient of 1.4 ± 0.2; $n = 3$) and in the presence of ivermectin (3 μM), EC_{50} value was 115 ± 24 μM (with a Hill coefficient of 1.6 ± 0.3; $n = 3$). However, the maximum agonist-evoked response in the presence of 3 μM ivermectin was only 42 ± 3% ($n = 3$) of that detected in the absence of ivermectin (Fig. 4.C). In contrast, inhibition of nAChRs M253L by the competitive antagonist MLA was typical of what would be expected of a competitive antagonist. MLA (3 nM) caused a significant ($P < 0.001$) rightward shift of the agonist dose-response curve (the EC_{50} for acetylcholine was 570 ± 86 μM with a Hill coefficient of 1.2 ± 0.2; $n = 3$ in the presence of MLA) and the antagonism was completely surmountable by high concentrations of acetylcholine (Fig. 4C).

Computational docking simulations (Morris et al., 1998) were performed with a nAChR $\alpha 7$ subunit homology model (Cheng et al., 2006) based on the 4Å structure of the *Torpedo marmorata* nAChR (Unwin, 2005), as described previously for docking studies with PNU-120596 (Young et al., 2008). Our aim was to examine whether the $\alpha 7$ transmembrane region was a plausible binding site for ivermectin. The most favorable docked position of ivermectin (that of lowest predicted binding free energy and, hence, highest predicted af-

TABLE 2
The influence of ivermectin on agonist-evoked responses was examined on wild-type $\alpha 7$ nAChRs and a series of $\alpha 7$ nAChRs containing single point mutations

In each case, the influence of a maximal concentration of ivermectin (30 μM) was examined on an EC_{20} concentration of acetylcholine. The effect of ivermectin is expressed either as potentiation or inhibition compared with the magnitude of responses obtained with an EC_{20} concentration of acetylcholine in the absence of ivermectin.

Subunit	Location	EC_{20} ACh	Potentiation	Inhibition	n
		μM	*fold*	*%*	
$\alpha 7$		50	4.6 ± 0.6		8
G211V	TM1	50	4.7 ± 0.5		6
S222M	TM1	50		89.1 ± 6.6***	4
A225D	TM1	50	1.1 ± 0.1**		4
L230Y	TM1	50	4.7 ± 0.5		5
M253L	TM2	50		55.6 ± 8.8***	3
M260L	TM2	25	4.4 ± 0.6		5
Q272V	TM3	60	1.2 ± 0.1*		3
S276V	TM3	50		84.2 ± 3.5***	4
F445A	TM4	50	4.6 ± 1.0		6
T456V	TM4	50	1.2 ± 0.2***		7
C459Y	TM4	50	1.4 ± 0.2***		7

* $P < 0.05$.
** $P < 0.01$.
*** $P < 0.001$, significantly different from wild-type $\alpha 7$ nAChRs, as determined by ANOVA and Tukey's multiple-comparison test.

finity) was toward the top of a cavity located between the four α-helical transmembrane domains (Fig. 5). Two equally favorable conformations of ivermectin were identified in these studies (illustrated in yellow and green in Fig. 5). For both conformations, the predicted binding free energy (ΔG) of ivermectin within the α7 homology model was −8.39 kcal/mol, equivalent to a predicted binding affinity of 0.7 μM. Of the seven amino acids identified as influencing allosteric modulation in this study, both of the docked positions of ivermectin

Fig. 4. Ivermectin acts as a noncompetitive antagonist (negative allosteric modulator) of α7 nAChRs containing the M253L mutation. A, dose-dependent inhibition by ivermectin of agonist-evoked responses with α7^{M253L}. Data ($n = 3$) are normalized to responses obtained with an EC$_{50}$ concentration of acetylcholine (100 μM) in the absence of ivermectin. B, representative traces illustrating reposes to a range of concentrations acetylcholine in the absence (top panel) and presence (bottom) of ivermectin (3 μM). C, acetylcholine dose-response curve for α7^{M253L} nAChRs (filled circles). Also shown are acetylcholine dose-response curve for α7^{M253L} nAChRs determined in the presence of 3 μM ivermectin (open triangles) and 3 nM methyllycaconitine (open squares). Data ($n = 3$) are normalized to responses obtained with a maximum concentration of acetylcholine in the absence of antagonist.

with the lowest energy were in closest proximity to S276V, a mutation that had no significant effect on the type II allosteric modulator PNU-120596 (Young et al., 2008). PNU-120596 is a much smaller molecule than ivermectin and is predicted to bind at a lower position within the intrasubunit cavity (Young et al., 2008).

Discussion

There has been a considerable amount of interest in understanding the mechanism of action of allosteric modulators of nAChRs. In part, this has been prompted by evidence that such modulators may have useful therapeutic applications as cognitive enhancers (Hurst et al., 2005; Bertrand and Gopalakrishnan, 2007; Moaddel et al., 2007). We have previously identified an intrasubunit transmembrane cavity as a plausible binding for small positive allosteric modulators of α7 nAChRs such as PNU-120596 (Young et al., 2008). The work presented here extends these studies and provides evidence that mutations located in the transmembrane region can influence allosteric modulation of agonist-evoked responses by much larger compounds such as ivermectin.

Mutagenesis of amino acids located within the four transmembrane helices of the nAChR α7 subunit lead us to conclude that the transmembrane region is critical in determining the allosteric modulatory activity of ivermectin. Studies conducted with ivermectin on P2X receptors have suggested that it acts as a positive allosteric modulator of the P2X receptors via interaction with transmembrane regions (Silberberg et al., 2007; Jelínkova et al., 2008). This may therefore be a common mechanism by which ivermectin is able to modulate a variety of diverse ion channels. The finding that ivermectin acts as an inhibitor of α7/5-HT3A subunit chimeras was surprising, given that it acts as a potentiator of α7 nAChRs and has no significant effect on 5-HT$_3$ receptors. This finding, however, is consistent with previous studies conducted with other α7/5-HT3A chimeras (Bertrand et al., 2008), although the inhibitory action of ivermectin described previously was less pronounced than in the present study. Given the inhibitory action of ivermectin on α7/5-HT3A chimeras, the lack of any significant effect of ivermectin on wild-type 5-HT$_3$ receptors requires some explanation. It could be argued that, in constructing the α7/5-HT3A chimeras, a novel ivermectin binding site has been created. A more plausible explanation may be that the conformation of the extracellular domain of the 5-HT3A subunit blocks access of ivermectin to a potential transmembrane binding site and that access by ivermectin is possible only when replaced with the extracellular domain of the nAChR α7 subunit (for example, in the two α7/5-HT3A chimeras). This possibility would be consistent with our findings that mutagenesis of transmembrane amino acids in the nAChR α7 subunit into the analogous amino acids in 5-HT3A converts ivermectin into a negative allosteric modulator.

Several previous studies have demonstrated that ivermectin acts as a positive allosteric modulator of α7 nAChRs (Krause et al., 1998; Raymond et al., 2000; Bertrand et al., 2008; Sattelle et al., 2009). Of some significance to the findings reported in the present study is that, in addition to acting as an allosteric potentiator of α7 nAChRs, ivermectin has been reported to cause a slight attenuation of agonist-evoked responses in ACR-16, a nAChR from the nematode
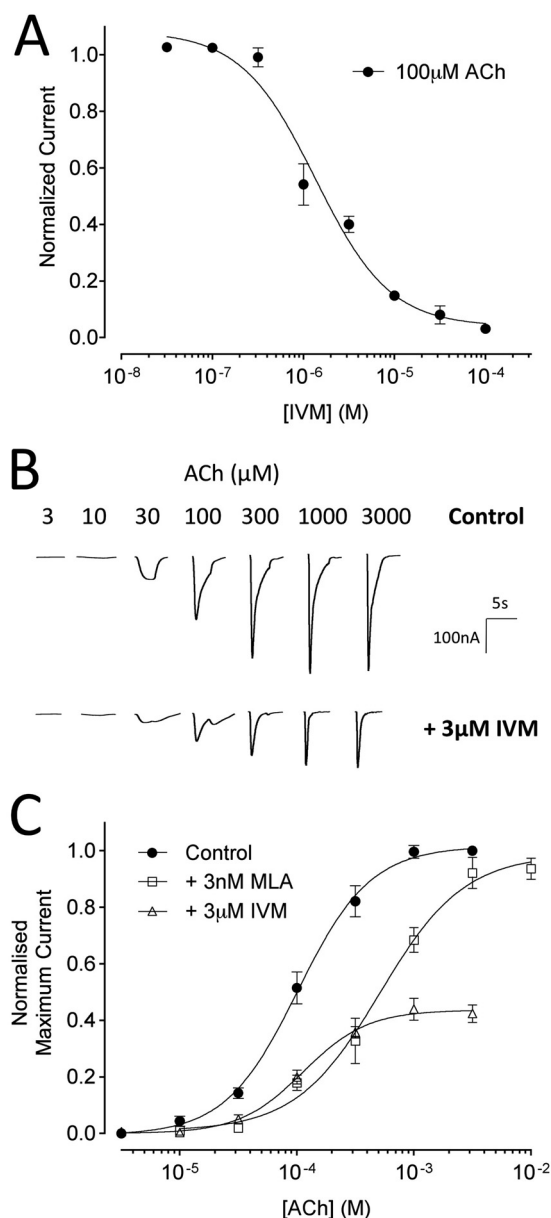
Caenorhabditis elegans (Raymond et al., 2000; Sattelle et al., 2009). Our findings would suggest that the inhibitory effect of ivermectin on ACR-16 might be a consequence of amino acid residues located within the transmembrane domain. It is noteworthy that, of the three mutations identified in this study that convert ivermectin from a potentiator into an inhibitor (S222M, M253L, and S276V), none is conserved between α7 and ACR-16 (the corresponding amino acids in ACR-16 are threonine, leucine, and cysteine, respectively). Indeed, the M253L mutation corresponds exactly to a change into the analogous amino acid in ACR-16. Of the four mutations that had no effect on allosteric modulation by ivermectin in α7 (G211V, L230Y, M260L, and F455A), three of these amino acids (Gly211, Leu230, and Phe455) are conserved between α7 and ACR-16. It seems plausible that amino acid differences at one or more of the positions identified in the present study may explain the different modulatory effect of ivermectin on these two nAChRs.

Inspection of the 4-Å resolution structure of the *T. marmorata* nAChR reveals an intrasubunit cavity located between the four transmembrane α-helices (Miyazawa et al., 2003; Unwin, 2005). The cavity is open at the extracellular side of the helices but closes approximately halfway into the lipid bilayer, and it has been suggested as being water-accessible (Miyazawa et al., 2003; Lobo et al., 2006). In a recent study involving site-directed mutagenesis of amino acids located in the α7 transmembrane regions, we have obtained evidence to suggest that low-molecular-weight allosteric potentiators such as PNU-120596 bind close to the bottom of this transmembrane cavity (Young et al., 2008). Other studies have suggested that allosteric modulators of GABA$_A$ and glycine receptors, such as neurosteroids and volatile anesthetics, may also bind near or within this cavity (Mihic et al., 1997; Ye et al., 1998; Hosie et al., 2006). Thus, this region may correspond to a common site for allosteric modulation of Cys-loop ligand-gated ion channels.

In a previous study of mutations affecting the α7 nAChR positive allosteric modulator PNU-120596, all of the amino acids that were found to influence PNU-120596 were predicted to line the base and sides of the putative intrasubunit

transmembrane cavity and had side chains pointing toward its interior (Young et al., 2008). In addition, those amino acids that had no significant effect upon allosteric modulation by PNU-120596 had side chains that point away from the interior of the cavity or are located toward the ends of the α-helices—either below the cavity or above the central part of it (Young et al., 2008). These findings are consistent with computer docking simulations that predict that the lowest energy docked conformation of PNU-120596 is situated close to the bottom of the cavity. Four of the mutations that we have identified in the present study as influencing allosteric modulation by ivermectin (S222M, A225D, M253L, and C459Y) also reduce potentiation by PNU-120596. In contrast, three mutations (Q272V, S276V, and T456V) have a significant effect on ivermectin but have no effect on PNU-120596. The side chain of Ser276 is predicted to point toward the interior of the cavity (as are all of those found to influence PNU-120596), but it is located much closer to the top of the transmembrane cavity than are the mutations that effect PNU-120596 (Fig. 5). In contrast to all of the above mutations, the side chains of Gln272 and Thr456 are not predicted to point toward the center of the cavity (Fig. 5). Whereas the predicted binding site for PNU-120596 is in very close proximity to all amino acids that, when mutated, exert a significant effect on potentiation (Young et al., 2008), this is not the case for ivermectin. The two docked positions of ivermectin with lowest energy lie in closest proximity to Gln272, which, interestingly, is one of the three amino acids that, when mutated, affect ivermectin but have no significant effect on potentiation by PNU-120596 (Fig. 5). It is tempting to speculate that the positive and negative modulatory activity of ivermectin might be a consequence of its ability to interact with the receptor in different orientations, but we do not wish to over-interpret these computer-docking studies, particularly because they are conducted with a homology model (based on the *T. marmorata* nAChR). Nevertheless, these docking studies provide support for the possibility that ivermectin binds in the nAChR transmembrane domain. At present, we cannot conclude whether the side chains of these mutated amino acids interact directly with bound ivermectin
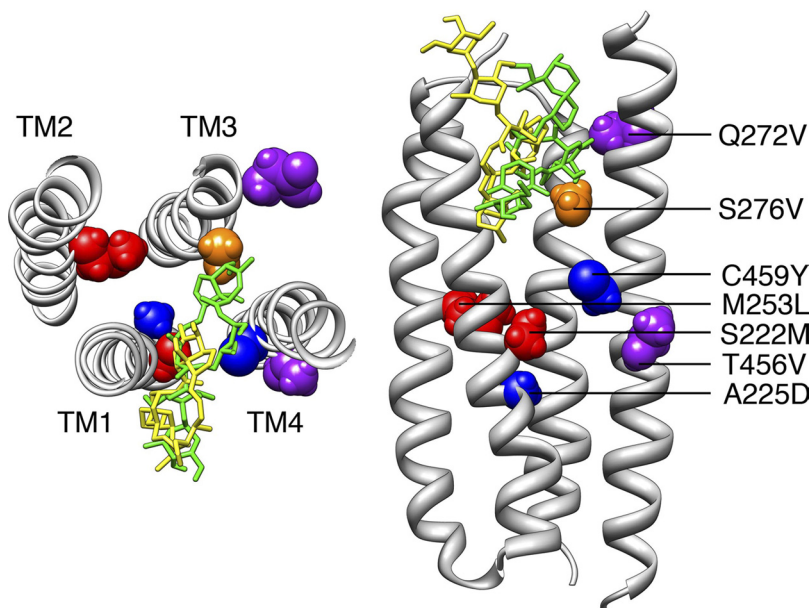
Fig. 5. Computer docking simulation. A homology model of the α7 transmembrane domain, illustrating the location of mutated amino acids. The backbone of the four transmembrane α-helices (TM1–TM4) are colored gray. The model is shown from a side-on view (right) and as viewed from above, looking down from the extracellular face of the lipid membrane (left). Also illustrated are the two lowest energy (highest predicted binding affinity) docked positions of ivermectin within the α7 homology model (represented as a stick model in yellow and green). Side chains of amino acids that, when mutated, had a significant effect on potentiation by ivermectin are indicated by colored spheres. Different colors have been used to indicate differing effects of mutations on allosteric modulation by ivermectin (this study) and PNU-120596 (Young et al., 2008). A225D (TM1) and C459Y (TM4), which caused reduced potentiation of both ivermectin and PNU-120596 are shown in blue. Q272V (TM3) and T456V (TM4), which caused reduced potentiation of ivermectin but had no effect on PNU-120596, are shown in purple. S222M (TM1) and M253L (TM2), which caused ivermectin to act as an inhibitor and caused reduced potentiation by PNU-120596 are shown in red. S276V (TM3), which caused ivermectin to act as an inhibitor but had no effect on PNU-120596, is shown in orange.

or whether these mutations have a longer-range effect on ivermectin as a result of induced conformational changes.

We conclude that point mutations located within the transmembrane domain of the $\alpha 7$ nAChR can convert a positive allosteric modulator into a negative allosteric modulator. These findings are consistent with previous studies that have demonstrated that nAChR mutations can convert agonists binding to the conventional (orthosteric) binding site into competitive antagonists (Hussy et al., 1994; Young et al., 2007). It seems plausible that ivermectin binds to a common site in wild-type and mutated $\alpha 7$ nAChRs and exerts its effect by stabilizing either an open conformation (resulting in positive allosteric modulation) or a closed conformation (resulting in noncompetitive antagonism or negative allosteric modulation).

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