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Emamectin is a non-selective allosteric activator of nicotinic acetylcholine receptors and GABA_{A/C} receptors

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Abstract

Avermectins are a group of compounds isolated from a soil-dwelling bacterium. They have been widely used as parasitocides and insecticides, acting by relatively irreversible activation of invertebrate chloride channels. Emamectin is a soluble derivative of an avermectin. It is an insecticide, which persistently activates glutamate-gated chloride channels. However, its effects on mammalian ligand-gated ion channels are unknown. To this end, we tested the effect of emamectin on two cation selective nicotinic receptors and two GABA-gated chloride channels expressed in *Xenopus* oocytes using two-electrode voltage clamp. Our results demonstrate that emamectin could directly activate $\alpha 7$ nAChR, $\alpha 4\beta 2$ nAChR, $\alpha 1\beta 2\gamma 2$ GABA_A receptor and $\rho 1$ GABA_C receptor concentration dependently, with similar potencies for each channel. However, the potencies for it to activate these channels were at least two orders of magnitude lower than its potency of activating invertebrate glutamate-gated chloride channel. In contrast, ivermectin only activated the $\alpha 1\beta 2\gamma 2$ GABA_A receptor.

Keywords

Emamectin; ivermectin; nicotinic receptor; GABA_{A/C} receptor; allosteric activation

1. Introduction

Ligand-gated ion channels are a group of ion channels that are activated by extracellular chemical messengers, mainly neurotransmitters. There are three superfamilies of ligand-gated ion channels, each differs from the others by number of subunits required to form a

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functional channel. Channels in the pentameric ligand-gated ion channel superfamily have five subunits per channel. The vertebrate channels in this family include cation selective nicotinic receptors, serotonin receptor type 3, and zinc activated cation channels, and anion selective GABA_{A/C} receptors, and glycine receptors. Their invertebrate counterparts include GABA-gated cation channel, glutamate-gated chloride channel, serotonin-gated chloride channel, and histamine-gated chloride channel. More recently, bacterial pentameric ligand-gated ion channels also joined this superfamily [1]. These ligand-gated ion channels play essential roles in fast synaptic transmission between neurons. Many of them, such as nicotinic receptors [2] and GABA_A receptors [3], are also found to be expressed in peripheral cells, such as immune cells, and potentially play other important roles, such as anti-inflammation in the body. They are important targets of many therapeutic drugs in vertebrate animals, including humans [1, 4]. Similarly, the pentameric ligand-gated ion channels in invertebrate animals are important targets for insecticides and parasitocides [5].

The Nobel Prize winning discovery of avermectins signifies the importance of their application in medicine as anti-parasite agents [6]. Avermectins are produced by fermentation of a soil bacterium *Streptomyces avermitilis* [7]. Emamectin is a relatively new synthetic insecticide [8]. It is a derivative of abamectin, a member of the avermectin family. Structurally, it is also similar to ivermectin, a widely used anti-parasite agent. Emamectin exerts its action by irreversibly activating chloride channels in insects [9]. However, less is known about its effect on mammalian channels in the pentameric ligand-gated ion channel family. In this study, we investigated emamectin's effect on mammalian GABA-gated chloride channels (GABA_{A/C} receptors) and cation selective nicotinic receptors.

2. Materials and methods

2.1. cRNA Preparation

The cDNAs encoding human wild type $\alpha 7$, $\alpha 4$, $\beta 2$ nicotinic receptor subunits (in the pGEMHE vector), rat $\alpha 1$, $\beta 2$ and $\gamma 2S$ (in the pGEM-T vector) and human $\rho 1$ (in the pAlter-1 vector) GABA_{A/C} receptor subunits were used as the DNA template for cRNA synthesis. The cRNA was transcribed by standard *in vitro* transcription using T7 RNA polymerase. The cRNAs were purified and resuspended in diethyl pyrocarbonate (DEPC)-treated water. cRNA yield and integrity were examined with an Eppendorf BioPhotometer and a 1% agarose gel.

2.2. Oocyte preparation and injection

Oocytes were harvested from female *Xenopus laevis* (*Xenopus* I, Ann Arbor, MI, USA), using the IACUC-approved protocol as previously described [10]. Micropipettes for injection were pulled from borosilicate glass on a Sutter P87 horizontal puller, and the tips were cut with forceps to $\approx 40 \mu\text{m}$ in diameter. The cRNA was drawn up into the micropipette and injected into oocytes with a Nanoject micro-injection system (Drummond, Broomall, PA, USA) at a total volume of 20~60 nl.

2.3. Two-electrode voltage-clamp

Two to 5 days after injection, the oocytes expressing each of these receptor types were placed in a custom made small volume chamber with continuous perfusion with Oocyte Ringer's solution (92.5 mM NaCl, 2.5 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, and 5 mM HEPES, pH 7.5). The chamber was grounded through an agar KCl bridge to prevent ligand-induced junction potential change. The oocytes were voltage-clamped at -70 mV to measure agonist-induced currents using an AxoClamp 900A amplifier (Molecular Devices, Sunnyvale, CA, USA). The current signal was filtered at 50 Hz with a built-in 4 pole low-pass Bessel filter in the AxoClamp 900A and digitized at 100 Hz with pClamp 10.3 and Digidata1440A (Molecular Devices).

2.4. Drug Preparation

Emamectin was from Dr. Ehrenstorfer GmbH (Augsburg, Germany). Ivermectin, GABA, acetylcholine, and picrotoxin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Mecamylamine hydrochloride was purchased from R&D systems (Minneapolis, MN, USA). The stock solutions were prepared from the solid materials and stored at -20°C in aliquots. Working concentrations of these drugs were prepared from stock solutions immediately before use.

2.5. Data analysis

The ligand-induced currents were measured using Clampfit 10.3 (Molecular Devices). The concentration-response relationship of the agonist-induced current was least-squares fit to a Hill equation with GraphPad Prism 6.0 (GraphPad Software, Inc, La Jolla, CA) to derive the EC₅₀ value and Hill coefficient, as well as the maximum current, which was then used to normalize the concentration-response curve from individual oocytes. The averages of the normalized currents or changes for each agonist concentration were used to plot the data. All the data were presented as mean ± SEM (standard error). Statistical comparisons for logEC₅₀s, were performed with two-sided grouped or paired (if two measurements were performed in the same oocytes) t-tests for two group comparison.

3. Results and Discussion

3.1. Direct activation of $\alpha 7$ nAChR by emamectin

Emamectin is known to activate invertebrate glutamate gated chloride channels [9]. However, it is unknown whether it can activate cation selective channels. To test its effect on human $\alpha 7$ nAChR, we expressed the receptor in *Xenopus* oocytes, and directly perfused emamectin to the oocytes. Figure 1A shows that 100 μ M emamectin directly activated human $\alpha 7$ nAChR with slow deactivation kinetics. The activation is not through other channels in the *Xenopus* oocytes, because an even higher concentration of emamectin did not induce any measurable current in un-injected oocytes (Figure 1A inset). The current was relatively stable after termination of drug application, suggesting the activation is relatively irreversible, similar to the activation of glutamate-gated chloride channels. Thus, in addition to activating chloride channels, emamectin also directly activates cation selective channels. In contrast, the same concentration of its analog, ivermectin, could not activate the channel.

Direct activation of nicotinic receptor by emamectin has not been reported before. This is different from the effects of emamectin and ivermectin on invertebrate glutamate-gated chloride channels in that both of them can directly activate those channels. The emamectin activation of the $\alpha 7$ nAChR could be blocked by the nicotinic receptor noncompetitive antagonist mecamylamine (Figure 1B), suggesting the activated current was through the $\alpha 7$ nAChR. Figure 1 C&D show that activation of $\alpha 7$ nAChR by emamectin was concentration dependent. The activation was not through the orthosteric binding site, because the non-functional Y93C or C191Y binding site mutants of the $\alpha 7$ nAChR [10] could be activated by (100 μ M or 207 μ M) emamectin (Y93C: Current=2815 $\pm$ 186 nA, N=11; C191Y: Current=2518 $\pm$ 152 nA, N=9, data not shown). This is consistent with the conclusion that emamectin activates the channel through an allosteric site. 4BP-TQS is an allosteric agonist of the $\alpha 7$ nAChR with binding to an allosteric site in the channel lining domain. The mutation (M254S) that knocks out the 4BP-TQS activation[11] did not influence activation by (100 μ M) emamectin (current=2396 $\pm$ 208 nA, N=8, data not shown), suggesting that activation by emamectin is through a distinct mechanism. The detailed mechanism for emamectin activation of the $\alpha 7$ nAChR is unknown. However, it is highly likely that it is similar to the mechanism of ivermectin activation of the glutamate-gated chloride channel by binding to the subunit interface in the transmembrane domain [12].

3.2. Emamectin activation of $\alpha 4\beta 2$ nAChR

To see whether the effect of emamectin is more widespread, we tested its effect on another major subtype of nicotinic receptors, $\alpha 4\beta 2$. Figure 2A shows that, like $\alpha 7$ nAChR, the $\alpha 4\beta 2$ nAChR is also sensitive to direct activation by emamectin. The emamectin-induced current was sensitive to mecamylamine antagonism, suggesting the current was through the expressed receptors. Similarly, the $\alpha 4\beta 2$ nAChR was not sensitive to ivermectin (Figure 2B). The mechanism of the differential sensitivity of these two types of receptor to emamectin and ivermectin is unknown. The detailed structural differences of the two compounds that influence their interactions with different receptors are worth further investigation. Figure 2C&D show the concentration response relationship of the emamectin action on the $\alpha 4\beta 2$ nAChR. The EC₅₀ value of emamectin on $\alpha 4\beta 2$ nAChR derived from fitting was slightly higher than that on the $\alpha 7$ nAChR. However, there is an unusually steep Hill slope (Hill coefficient) for the concentration dependent activation of both subtypes of nicotinic receptor. We speculate that the activation may require bindings of more than 5 ligands to the same receptor (two molecules in each subunit interface) to activate the channel. In addition, there may be a critical point of lipid solubility for this relatively hydrophilic compound to get access to the membrane hydrophobic environment to bind the receptor.

3.3. Emamectin activation of the $\alpha 1\beta 2\gamma 2$ GABA_A receptor

The GABA_A receptors are also chloride channels. Their subunit sequences are homologous to the glutamate-gated chloride channels in invertebrates. Thus, we expect that emamectin also activates mammalian GABA_A receptors. Figure 3A shows that emamectin directly activated the $\alpha 1\beta 2\gamma 2$ GABA_A receptor. The current could be antagonized by the GABA_A receptor channel blocker, picrotoxin, further confirming the activated current was through this receptor. However, the current could not be antagonized by the GABA_A receptor competitive antagonist, bicuculline (data not shown), suggesting that emamectin activates

the receptor through an allosteric site rather than orthosteric site. In comparison, ivermectin also activated the GABA_A receptor (Figure 3B), similar to a recently reported result [13]. Thus, the $\alpha 1\beta 2\gamma 2$ GABA_A receptor was sensitive to both emamectin and ivermectin. The concentration dependence of emamectin action on the GABA_A receptor is shown in Figure 3C&D. We expected to see a higher potency of this compound on the GABA_A receptor when compared to those on the nicotinic receptors. However, the results suggest that its potency on the GABA_A receptor was comparable to those on the nAChRs. The major difference is that the Hill slope was not as steep as in the nAChR activation.

3.4. Emamectin activation of the $\rho 1$ GABA receptor

The $\rho 1$ GABA receptor is also a GABA-gated chloride channel. It has different pharmacological properties than $\alpha\beta\gamma$ GABA receptors [14]. Pharmacologically, it is classified as a GABA_C receptor [15], but structurally it is classified as a GABA_A receptor [16]. Thus, it is interesting to see whether it has distinct properties in response to emamectin and ivermectin. Figure 4A shows that emamectin also directly induced a current in the oocytes expressing $\rho 1$ GABA receptor. The current could be blocked by picrotoxin, further confirming that activation was through the GABA receptor. Since the $\rho 1$ GABA receptor is also a chloride channel, we expect that it might also be sensitive to direct activation by ivermectin, similar to the $\alpha 1\beta 2\gamma 2$ GABA_A receptor. Surprisingly, this GABA-gated chloride channel was not sensitive to ivermectin (Figure 4B). It would be interesting to determine the structural mechanism for this difference, given that the sequence of the $\rho 1$ GABA receptor subunit is highly homologous to those of the $\alpha 1$, $\beta 2$, and $\gamma 2$ GABA receptor subunits, especially in the transmembrane domains. The emamectin concentration dependent activation of the $\rho 1$ GABA receptor is shown in Figure 4C&D. The EC₅₀ is similar to those in nAChR and GABA_A receptor shown above.

In summary, we have demonstrated the direct activation of multiple pentameric ligand-gated ion channels by emamectin, including two cation-selective nicotinic receptors and two GABA-gated chloride channels. The activation of each channel was long lasting and relatively irreversible. In contrast, ivermectin only activated the $\alpha 1\beta 2\gamma 2$ GABA_A receptors. The major structural differences between emamectin and ivermectin are that there is a double bond between C22 and C23 in emamectin but a single bond in ivermectin, and that emamectin has a methylamino group connecting C4'', to replace the hydroxyl group in ivermectin, which increases its water solubility. One possible explanation for the differences in activating these 4 channels by emamectin and ivermectin is that emamectin has higher water solubility, so that it can achieve higher concentrations in solution. In contrast, ivermectin has lower water solubility and may not be able to reach a concentration higher than 100 μ M in solution. However, this cannot explain the result that the same concentration of ivermectin could only activate the GABA_A receptor, but not other receptors. In comparison, emamectin activated all four receptors with similar potency. Thus, these structural differences between the two analogs must influence their means of interaction with the different receptors. However, the persistent current induced by emamectin is kinetically similar to that activated by ivermectin. Thus, the mechanisms of action for both compounds are likely similar. That is, emamectin would also bind to the subunit interface in the transmembrane domains of receptors to activate the channels as ivermectin does [12, 17,

18]. Regardless of the detailed molecular mechanism, our results have provided new information on the differential response of four different channels to emamectin and ivermectin, as well as on the potency of emamectin activation of these channels. The activation by emamectin of all four mammalian pentameric ligand-gated ion channels we tested suggests that it may have higher potential toxicities to mammals than ivermectin. However, its higher water solubility could make it more difficult to pass the blood brain barrier. In addition, The EC₅₀ values of the direct activation of the mammalian nicotinic receptors and GABA receptors were more than 100 fold higher than the EC₅₀s of its activating invertebrate chloride channels, suggesting that its influence on mammals is minimal, unless a large amount is accidentally ingested. Thus, the results derived from this study will provide novel information for the molecular toxicology of this insecticide. In addition, we have also identified a new compound for studying the mechanism of allosteric activation of the pentameric ligand-gated ion channels.

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Abbreviations

EB	emamectin benzoate
nAChR	nicotinic acetylcholine receptor
GABA	γ-aminobutyric acid
IVM	ivermectin
PTX	picrotoxin
MCA	mecamylamine
I_{max}	maximal current
EC₅₀	half maximal effective concentration

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Highlights

- Emamectin directly activated cation selective nicotinic receptors, as well as anion selective GABA_{A/C} receptors, whereas its analog, ivermectin, only activated the GABA_A receptor.
- The emamectin activation could be blocked by non-competitive antagonists.
- The EC₅₀s of the activation were similar among the tested receptors, ranging from 97-136 μ M.
- We have identified a non-selective allosteric activator for the pentameric ligand-gated ion channels.
- The non-specific activation of multiple ligand-gated ion channels in vertebrate suggests that emamectin is potentially toxic to vertebrate animals.

Figure 1. Direct activation of $\alpha 7$ nAChR by emamectin. A, Direct activation of $\alpha 7$ nAChR by 100 μ M EB but not by 100 μ M ivermectin (N=10). Note the long lasting nature of the emamectin-induced current. Inset: 150 μ M EB failed to induced any current in un-injected control oocytes (N=5). B, The emamectin-induced current was antagonized by co-application of 1mM mecamylamine, a non-selective and non-competitive antagonist of nAChRs (N=10). C, Current trace of the concentration dependent activation of $\alpha 7$ nAChR by emamectin. (N=9). D, Normalized and averaged concentration response of $\alpha 7$ nAChR to emamectin. The continuous line is the least squares fit of the data to the Hill equation. The derived values of the fitting parameters were: $I_{\max}$: 4738 ± 214 nA; EC_{50} : 97.4 ± 3.3 μ M; Hill coefficient: 10.5 ± 0.9 .

Figure 2.

Direct activation of $\alpha 4\beta 2$ nAChR by emamectin. A, Direct activation of $\alpha 4\beta 2$ nAChR by 150 μ M EB (N=9). The emamectin-induced current was antagonized by co-application of 1mM mecamylamine (N=9). B, 100 μ M ivermectin did not induce any noticeable current in the $\alpha 4\beta 2$ nAChR expressing oocytes (N=6). C, Current trace of the concentration dependent activation of $\alpha 4\beta 2$ nAChR by emamectin (N=7). D, Normalized and averaged concentration response of $\alpha 4\beta 2$ nAChR to emamectin. The continuous line is the least squares fit of the data to the Hill equation. The derived values of the fitting parameters were: $I_{\max}$: 4029 $\pm$ 146 nA; EC_{50} : 136.4 $\pm$ 4.2 μ M; Hill coefficient: 11.0 $\pm$ 0.7.

Figure 3.

Direct activation of $\alpha 1\beta 2\gamma 2$ GABA_A receptor by emamectin. A, Direct activation of $\alpha 1\beta 2\gamma 2$ GABA_A receptor by $150\mu\text{M}$ EB (N=9). The emamectin-induced current was antagonized by co-application of 1mM picrotoxin (N=9). B, $100\mu\text{M}$ ivermectin induced a current (316 ± 116 nA) in the $\alpha 1\beta 2\gamma 2$ GABA_A receptor expressing oocytes (N=6). C, A representative current trace of the concentration dependent activation of $\alpha 1\beta 2\gamma 2$ GABA_A receptor by emamectin (N=9). D, Normalized and averaged concentration response of $\alpha 1\beta 2\gamma 2$ GABA_A receptor to emamectin. The continuous line is the least squares fit of the data to the Hill equation. The derived values of the fitting parameters for fitting to the single Hill equation were: I_{max} : 5143 ± 277 nA; EC_{50} : $103.7\pm 1.9\mu\text{M}$; Hill coefficient: 5.5 ± 0.3 .

Figure 4.

Direct activation of $\rho 1$ GABA_C receptor by emamectin. A, Direct activation of $\rho 1$ GABA_C receptor by 150 μ M EB (N=9). The emamectin-induced current was antagonized by co-application of 1mM picrotoxin (N=9). B, 100 μ M ivermectin failed to induce any current in the $\rho 1$ GABA_C receptor expressing oocytes (N=6). C, A representative current trace of the concentration dependent activation of $\rho 1$ GABA_C receptor by emamectin (N=8). D, Normalized and averaged concentration response of $\rho 1$ GABA_C receptor to emamectin. The continuous line is the least squares fit of the data to the Hill equation. The derived values of the fitting parameters were: $I_{\max}$: 5539 $\pm$ 332 nA; EC_{50} : 120.1 $\pm$ 3.2 μ M; Hill coefficient: 8.1 $\pm$ 0.7.