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The antiparasitic isoxazoline A1443 is a potent blocker of insect ligand-gated chloride channels

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ABSTRACT

A structurally unique isoxazoline class compound, A1443, exhibits antiparasitic activity against cat fleas and dog ticks comparable to that of the commercial ectoparasiticide fipronil. This isoxazoline compound inhibits specific binding of the γ-aminobutyric acid (GABA) receptor channel blocker [^{3}H]4′-ethynyl-4-*n*-propylbicycloorthobenzoate (EBOB) to housefly-head membranes, with an IC_{50} value of 455 pM. In contrast, the IC_{50} value in rat-brain membranes is >10 μM. To study the mode of action of this isoxazoline, we utilized MdGBCl and MdGluCl cDNAs, which encode the subunits of housefly GABA- and glutamate-gated chloride channels, respectively. Two-electrode voltage clamp electrophysiology was used to confirm that A1443 blocks GABA- and glutamate-induced chloride currents in *Xenopus* oocytes expressing MdGBCl or MdGluCl channels, with IC_{50} values of 5.32 and 79.9 nM, respectively. Blockade by A1443 was observed in A2′S-MdGBCl and S2′A-MdGluCl mutant channels at levels similar to those of the respective wild-types, and houseflies expressing A2′S-MdGBCl channels were as susceptible to A1443 as standard houseflies. These findings indicate that A1443 is a novel and specific blocker of insect ligand-gated chloride channels.

Introduction

Neurotransmitter receptors are membrane proteins that are directly involved in transmembrane signaling in both neurons and muscle cells. They are important not only in the function and regulation of the nervous system but also as common targets of drugs, insecticides, and parasiticides [1]. Neonicotinoid insecticides act as agonists of nicotinic acetylcholine receptors (nAChRs) [2]. Phenylpyrazoles such as fipronil and ethiprole, and macrolides such as avermectins and milbemycins, are commercially available insecticides and parasiticides that target γ-aminobutyric acid (GABA) receptors and inhibitory glutamate receptors in invertebrates [3].

The ionotropic GABA receptors comprise a family of "Cys-loop receptors", together with nAChRs, glycine receptors, and 5-hydroxytryptamine type 3 receptors (5-HT_3Rs). The receptors that belong to this family are composed of five subunits that form integral ion channels. nAChRs and 5-HT_3Rs form cation channels at the center of the pentamers, and the other receptors similarly form anion (chloride) channels. Because each neurotransmitter gates its specific ion channels, these receptors are termed ligand (neurotransmitter)-gated ion channels. Ligand-gated cation channels mediate fast excitatory synaptic transmission by allowing the influx of cations into neurons or muscle cells, whereas ligand-gated chloride channels (LGCCs) mediate fast inhibitory synaptic transmission by enhancing chloride ion permeability through the postsynaptic membrane.

Glutamate receptors exist as both excitatory cation channels and inhibitory anion channels. However, inhibitory glutamate receptors are found only in invertebrates. It is interesting that invertebrates uniquely express not only glutamate- but also biogenic amine-gated chloride channels, whereas most of the vertebrate biogenic amine receptors are G protein-coupled receptors [1,4–6]. The specific expression of these LGCCs in invertebrates encouraged us to explore whether they might serve as insecticide and parasiticide targets.

GABA receptors were first shown to be a target of the organochlorine insecticides lindane and dieldrin [7,8], both of which were banned because of its environmental persistence on account of their chemical structures. In the past three decades, structurally diverse insecticidal compounds have been reported to act as noncompetitive antagonists or blockers for GABA and inhibitory glutamate receptors [3]. However, no further development of practical insecticides or parasiticides has been reported since the discovery of the phenylpyrazole insecticide two decades ago. We have recently found that a novel isoxazoline class compound, A1443 (Fig 1A), which is under investigation as an ectoparasiticide, acts as a potent blocker of housefly GABA- and glutamate-gated chloride channels (named MdGBCls and MdGluCls, respectively).

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We provide here evidence for the unique blocking actions of A1443 on invertebrate LGCCs.

Materials and methods

Chemicals. GABA and fipronil were purchased from Wako Pure Chemicals Industries (Osaka, Japan). Sodium L-(+)-glutamate monohydrate was obtained from Sigma–Aldrich (St. Louis, MO). [^{3}H]EBOB (47.5 Ci/mmol) was purchased from PerkinElmer (Waltham, MA). Isoxazoline A1443 was synthesized according to the procedure described in a patent [9]. As A1443 is a racemate, the enantiomers, (*S*)-A1443 (A664) and (*R*)-A1443 (A663), were separated using HPLC equipped with a CHIRALPAK® AD-H column (2.0 × 25 cm, 1/1 hexane/EtOH) (Daicel Chemical Industries, Osaka, Japan). (*S*)-A1443 (A664): mp 173.0–175.0 °C, $[\alpha]_D^{23.1}$ +61.96 (c 1.10, ethanol), 99% ee. (*R*)-A1443 (A663): amorphous, $[\alpha]_D^{23.1}$ −58.95 (c 1.15, ethanol), 99% ee. The enantiomer excess was determined using CHIRALPAK® AD-H (4.6 × 250 mm, 1/1 hexane/EtOH). The absolute configuration of A664 was determined by single crystal X-ray analysis (data not shown).

Specimens. The dieldrin-resistant (OCR) and susceptible (SRS) strains of houseflies (*Musca domestica*) were kindly supplied by Professor Jeffrey G. Scott (Cornell University, Ithaca, NY). OCR houseflies exhibit a homozygous A299S mutation [10]. Mature female African clawed frogs (*Xenopus laevis*) were purchased from Japan SLC (Shizuoka, Japan). Rats (male Wister, 5 weeks old) were purchased from Shimizu Laboratory Supplies (Kyoto, Japan). Cat fleas (*Ctenocephalides felis*) and American dog ticks (*Dermacentor variabilis*) were obtained from Stillmeadow (Sugar Land, TX).

Antiparasitic assays. Acetone solutions of several relevant compounds were uniformly coated onto the inner surface of 5.3-cm TPX petri dishes (Nalgene Nunc International, Tokyo, Japan) at 10 μl/cm^2 and then dried to produce a film of the test compounds. Twelve flea adults or tick nymphs were released in each petri dish and kept at 25 °C without food. LD_{95} values were determined from the mean 4-d mortality in three (*D. variabilis*) or five (*C. felis*) replications.

Insecticidal assays. Acetone solutions (1 μl) of compounds were topically applied on the notum of adult 3- to 5-day-old female OCR and SRS houseflies, using an Arnold hand microapplicator (Burkard Scientific, Rickmansworth, England). Thirty flies were used for each dosage. Treated flies were kept at 25 °C with sugar and water. LD_{50} values were calculated from the mean 24-h mortality data across three replications using standard probit analysis.

[^{3}H]EBOB binding assays. Binding assays were performed using housefly (SRS)-head membranes and rat-brain membranes as previously described [11]. Nonspecific binding was determined in the presence of 1 μM α-endosulfan (Wako). Experiments were replicated three times.

cDNAs encoding the wild type and the 2′ mutant of MdGBCl and MdGluCl subunits. In previous studies [12,13], we have described how the expression vector pcDNA3 can be inserted with cDNAs that encode wild-type MdGBCl (MdRdl) and MdGluCl subunits and a 2′ (S278A) mutant MdGluCl subunit. The cDNA that encodes a 2′ (A299S) mutant MdGBCl subunit was prepared from the cDNA of the wild type, using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). Oligonucleotide primers used for the mutagenesis were MdGBCl-A299S-f (CCAGCCCGTGTATCTTTAGGT GTCAC) and MdGBCl-A299S-r (GTGACACCTAAAGATACACGGGCT GG).

Preparation of cRNA. Templates for *in vitro* transcription were obtained by amplifying the inserts of pcDNA3-MdGluCl and pcDNA3-MdGBCl plasmids by PCR using KOD-Plus polymerase (Toyobo, Osaka, Japan) and the primers pcDNA3-F (CTCTCTGGCTA ACTAGAGAACC) and pcDNA3-R (CTAGAAGGCACAGTCGAGGCTG). The capped RNA transcripts were synthesized using T7 polymerase (mMESSAGE mMACHINE® T7 Ultra Kit, Ambion, Austin, TX). cRNA samples were stored at −80 °C until use.

Expression of channels in Xenopus laevis oocytes. *Xenopus laevis* was anesthetized by immersion in 0.1% tricaine (Sigma–Aldrich)

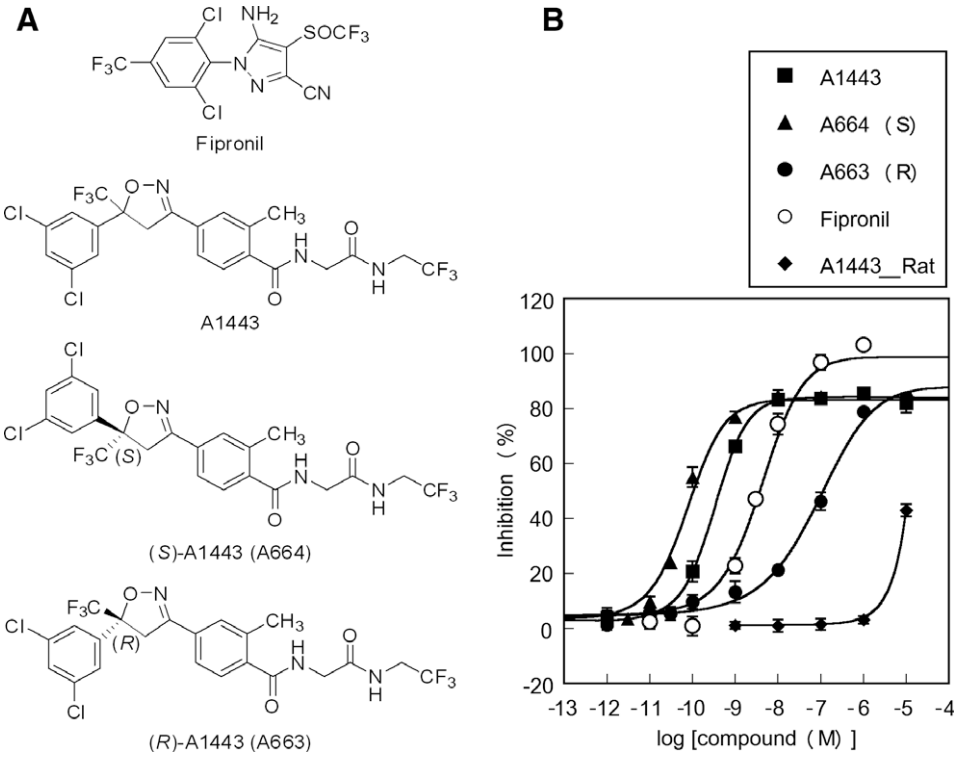

Fig. 1. Inhibition of [^{3}H]EBOB binding to housefly-head and rat-brain membranes by the isoxazolines A1443, A663, and A664, and fipronil. (A) Structures of the compounds tested. (B) Concentration-inhibition curves. Each point represents the mean ± SE of at least three experiments.

for 30 min. Ovary lobes were treated with collagenase type 1A (Sigma–Aldrich; 2 mg/ml) in Ca^{2+}-free standard oocyte saline (SOS) (100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6) for 90 min at 25 °C. Each oocyte (stage V-VI) was injected with 2–10 ng of either MdGBCl or MdGluCl cRNA dissolved in nuclease-free water, and then the oocytes were incubated at 16 °C in a sterile SOS medium (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.6) containing gentamycin (Sigma–Aldrich; 50 μg/ml), penicillin–streptomycin (Sigma–Aldrich; 100 U/ml, 100 μg/ml), 2.5 mM sodium pyruvate (Wako), and 4% horse serum (Life Technologies, Carlsbad, CA). Oocytes incubated at 16 °C for 2–6 days after cRNA injection were used for electrophysiological recordings.

Electrophysiology. Electrophysiological experiments were performed at a holding potential of −80 mV, using a two-electrode voltage-clamp setup (TEV 200A, Dagan Corporation, Minneapolis, MN). Micropipettes were prepared from glass capillaries (1.2 mm × 90 mm, A-M Systems, Sequim, WA), using a pipette puller (PE-2, Narishige, Tokyo, Japan), and filled with 2 M KCl to give a resistance of 0.2–5 MΩ in the medium. The current signals were digitized by a PowerLab 4/26 unit (ADInstruments, Colorado Springs, CO). Experiments were carried out at 25 °C. Oocytes were placed in a recording chamber perfused with a SOS solution by gravity flow at 8–10 ml/min. GABA and glutamate dissolved in a SOS medium were applied to oocytes for 20 and 10 s, respectively, at intervals of 2–3 min. Dose–response curves were obtained by sequential applications of increasing concentrations of GABA and glutamate to MdGBCl and MdGluCl channels expressed in oocytes, respectively. Test compounds were first dissolved in dimethyl sulfoxide (DMSO), and diluted with a SOS medium to a final DMSO concentration of 0.1%, which failed to produce any response in oocytes. The test compound solution was added to the perfusate after three successive control applications of GABA or glutamate, and then applied consecutively for the remainder of the experiments. GABA (100 μM) or glutamate (100 μM) was repeatedly applied with a test compound for 20 or 10 s at 2-min intervals during the perfusion of the test compound. The minimum response was regarded as the extent of inhibition (∼5 min after the first application of a test compound). IC_{50} values were determined from the mean of three replications using standard probit analysis.

Results

Effects of isoxazolines on cat fleas and dog ticks

The antiparasitic activity of isoxazolines against cat fleas and dog ticks was investigated using a dry film method. The LD_{95} values of A1443 against cat fleas and dog ticks were slightly greater than those of fipronil (Table 1). As A1443 is a racemic compound, the *R*- and *S*-enantiomers [designated A663 and A664, respectively (Fig. 1A)] were separated and examined for their antiparasitic activity. The LD_{95} values of A664 for cat fleas and dog ticks were not significantly different from those of A1443, whereas A663 was inactive at the tested highest dose for both ectoparasites. This finding indicates that the *S*-enantiomer is the active component of A1443.

Effects of isoxazolines against dieldrin-resistant houseflies

The insecticidal activity of A1443 against the OCR and SRS strains of houseflies was determined in order to investigate the cross-resistance of OCR houseflies to A1443. The OCR strain is resistant to dieldrin, a GABA receptor channel blocker and a classical chlorinated insecticide [10]. The LD_{50} value of A1443 in OCR houseflies (1.01 ± 0.11 (SE) ng/mg) was not significantly different from that in dieldrin-susceptible SRS houseflies (0.853 ± 0.056 ng/mg).

Effects of isoxazolines on [^{3}H]EBOB binding to housefly-head and rat-brain membranes

A binding assay using the radiolabeled GABA receptor channel blocker [^{3}H]4′-ethynyl-*n*-propylbicycloorthobenzoate (EBOB) as a ligand has been established as an excellent means by which to detect inhibition and allosteric modulation of GABA receptors caused by various chemicals [14,15]. The binding of [^{3}H]EBOB to GABA receptors is affected by the binding of not only noncompetitive antagonists (channel blockers), but also agonists, competitive antagonists, and activators such as ivermectin. Thus, we first utilized this ligand to examine whether A1443 acts directly on GABA receptors. Fig. 1B shows the effects of isoxazolines and fipronil on specific [^{3}H]EBOB binding to membranes prepared from housefly heads and rat brains. A1443 inhibited [^{3}H]EBOB binding to housefly-head membranes in a concentration-dependent fashion. In contrast to fipronil, which fully inhibited radioligand binding, the inhibition by A1443 was partial, reaching a maximum at ∼80%. The IC_{50} value of A1443 was estimated to be 455 pM, which is ∼7-fold smaller than that of fipronil (Table 1). We then examined the inhibitory potency of the enantiomers. Fig. 1B shows that the *S*-enantiomer A664 has ∼1300-fold higher potency than the antipode A663. The inhibitory activity of A1443 in rat-brain membranes was also examined (Fig. 1B). A1443 at a 10 μM concentration inhibited specific [^{3}H]EBOB binding to rat-brain membranes by only 43%, indicating that housefly receptors are >2000-fold more sensitive to A1443 than rat receptors.

Effects of isoxazolines on agonist-induced currents in housefly GABA and inhibitory glutamate receptors

While EBOB is an excellent probe for GABA receptors, it has been reported not to be highly specific to GABA receptors [16].

Table 1
Antiparasitic activity and potency of isoxazolines and fipronil in the inhibition of [^{3}H]EBOB binding and the blockade of GABA- and glutamate-induced currents.

Compound	Antiparasitic activity (LC_{95})[a]		[^{3}H]EBOB binding inhibition[b]			Current blockade[b]			
	Flea	Tick	Housefly		Rat	MdGBCl		MdGluCl	
			IC_{50}	95% CI	IC_{50}	IC_{50}	95% CI	IC_{50}	95% CI
A1443	21 ± 15	1.3 ± 0.7	0.455	0.320 –0.680	>10,000	5.32	3.10 –8.78	79.9	52.7 –121.2
A663	>10000	>10000	110	70 –174	—	6600	3750 –13160	15300	9200 –29800
A664	16 ± 9	1.0 ± 0.3	0.0831	0.0636 –0.129	—	6.54	3.78 –10.94	61.2	40.7 –94.2
Fipronil	4.3 ± 1.6	1.0 ± 0.6	3.40	2.64 –4.42	—	24.5	16.2 –36.9	165	112 –245

[a] Units (mean ± SE) are ng/cm^2.
[b] Units are nM.

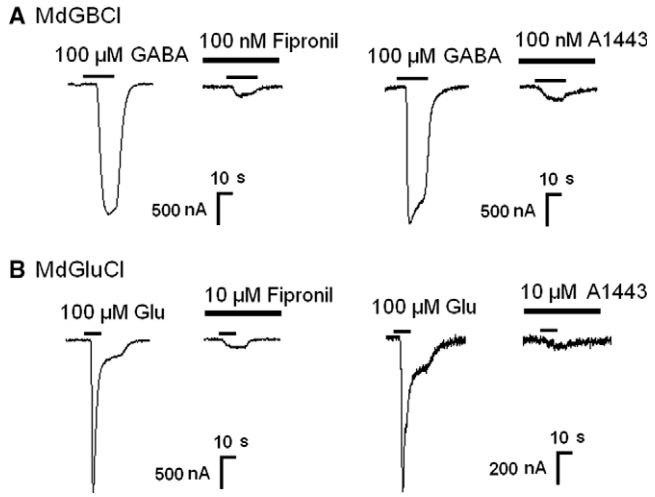

Fig. 2. Blockade of GABA- and glutamate-induced currents by A1443 and fipronil. (A) Blockade of the response to 100 μM GABA by 100 nM A1443 or fipronil in MdGBCl channels. (B) Blockade of the response to 100 μM glutamate by 10 μM A1443 or fipronil in MdGluCl channels. GABA and glutamate were added for 20 s and 10 s, respectively, at intervals of 2 min. A1443 or fipronil was bath-applied continuously after three successive control applications of GABA or glutamate.

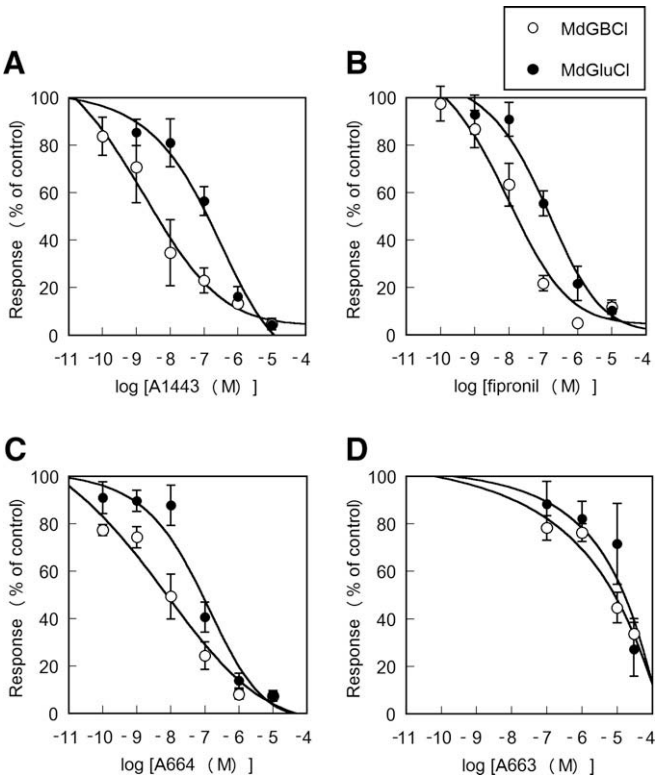

Fig. 3. Dose–response curves of the blockade of MdGBCl and MdGluCl channels by (A) A1443, (B) fipronil, (C) A664, and (D) A663. The percentage agonist response based on the peak inward current is plotted against the logarithm of the concentration of each chemical. Each point represents the mean ± SE of responses in at least three oocytes.

Thus, we examined the potency of isoxazolines on both GABA and inhibitory glutamate receptors cloned from houseflies, i.e., homo-oligomeric MdGBCl and MdGluCl channels transiently expressed in *Xenopus* oocytes. As previously reported [12], respective robust chloride-permeable channels were formed in oocytes after micro-injections of cRNAs that encode the MdGBCl and MdGluCl subunits. The EC_{50} values of GABA and glutamate were estimated in this study to be 160 ± 2 μM (mean ± SE, $n = 5$) and 94.0 ± 24.0 μM ($n = 5$) for MdGBCl and MdGluCl channels, respectively.

Fig. 2A shows that 100 nM A1443 and fipronil blocked GABA (100 μM)-induced currents MdGBCl channels; Fig. 2B shows that these compounds applied at 10 μM also blocked glutamate (100 μM)-induced currents in MdGluCl channels. Fig. 3A shows that the blockade of MdGBCl and MdGluCl channels by A1443 is dose-dependent, with IC_{50} values of 5.32 nM and 79.9 nM, respectively, as determined on the basis of the amplitudes of peak currents (Table 1). The MdGBCl channel is ~15-fold more sensitive to A1443 than the MdGluCl channel. For comparison, the blocking activity of fipronil on both channels was also tested (Fig. 3B). Fipronil was ~7-fold more selective to the MdGBCl channel than to the MdGluCl channel, with IC_{50} values of 24.5 nM and 165 nM, respectively. The potency of A664 was not significantly different from that of A1443 in either MdGBCl or MdGluCl channels, whereas A663 was a ~1200-fold and ~190-fold weaker blocker than A1443 in MdGBCl and MdGluCl channels, respectively (Fig. 3C and D). This finding indicates that the *S*-enantiomer is the active component of A1443.

Effects of isoxazolines on the 2′ mutants of housefly GABA and inhibitory glutamate receptors

The amino acid residue at the so-called 2′ position of LGCC subunits (Fig. 4A) greatly affects the sensitivity of the channels to blockers [13,17]. Therefore, we examined the effects of A1443 on glutamate-induced currents in both the wild type and the 2′ (S278A) mutant of MdGluCl channels. In agreement with our previous report [13], the EC_{50} value of glutamate on the mutant channel was increased to 270 ± 55 (SE) μM ($n = 3$). Fig. 4B shows that the blocking potency of fipronil on glutamate (100 μM)-induced currents was enhanced ~9-fold by the mutation, with IC_{50} values of 165 (112–245) nM and 18.1 (11.2–28.9) nM for the wild-type and the mutant channels, respectively (95% confidence intervals in parentheses). Not only did the mutant channels exhibit increased sensitivity to fipronil, but the currents in the presence of fipronil decayed more slowly than those in the wild-type equivalents (Fig. 4C). In contrast, the potency of A1443 was unchanged by the 2′ mutation [79.9 (52.7–121.2) nM (wild-type) vs. 77.9 (52.0–116.8) nM (mutant)] (Fig. 4D), and no change in the time course of current decay was observed in its presence (Fig. 4C).

We also investigated the blocking actions of A1443 on the 2′ (A299S) mutant of MdGBCl channels. The EC_{50} value of GABA was also increased in this mutant (269 ± 75 (SE) μM, $n = 4$). However, the potencies of A1443 and fipronil were unaffected by this mutation, with IC_{50} values of 2.81 (1.91–4.10) nM and 11.0 (6.5–17.6) nM, respectively (95% confidence intervals in parentheses).

Discussion

In the present study, we provided two lines of evidence that the antiparasitic isoxazoline A1443 is a potent blocker of insect LGCCs. First, A1443 inhibited the binding of [^{3}H]EBOB to housefly-head membranes at subnanomolar concentrations; second, it blocked agonist-induced currents of cloned housefly GABA and inhibitory glutamate receptor channels at nanomolar concentrations. In contrast, A1443 inhibited [^{3}H]EBOB binding to rat-brain membranes by only 43% at 10 μM, indicating that A1443 has only an extremely weak blocking action on rat GABA receptors. In addition to its outstanding potency and selectivity for insect LGCCs, A1443 is structurally unique compared with the other blockers of GABA receptor channels reported to date. Although A1443 belongs to the phenylheterocycle class of compounds, which includes the phenylpyrazole insecticides fipronil and ethiprole, the heterocyclic

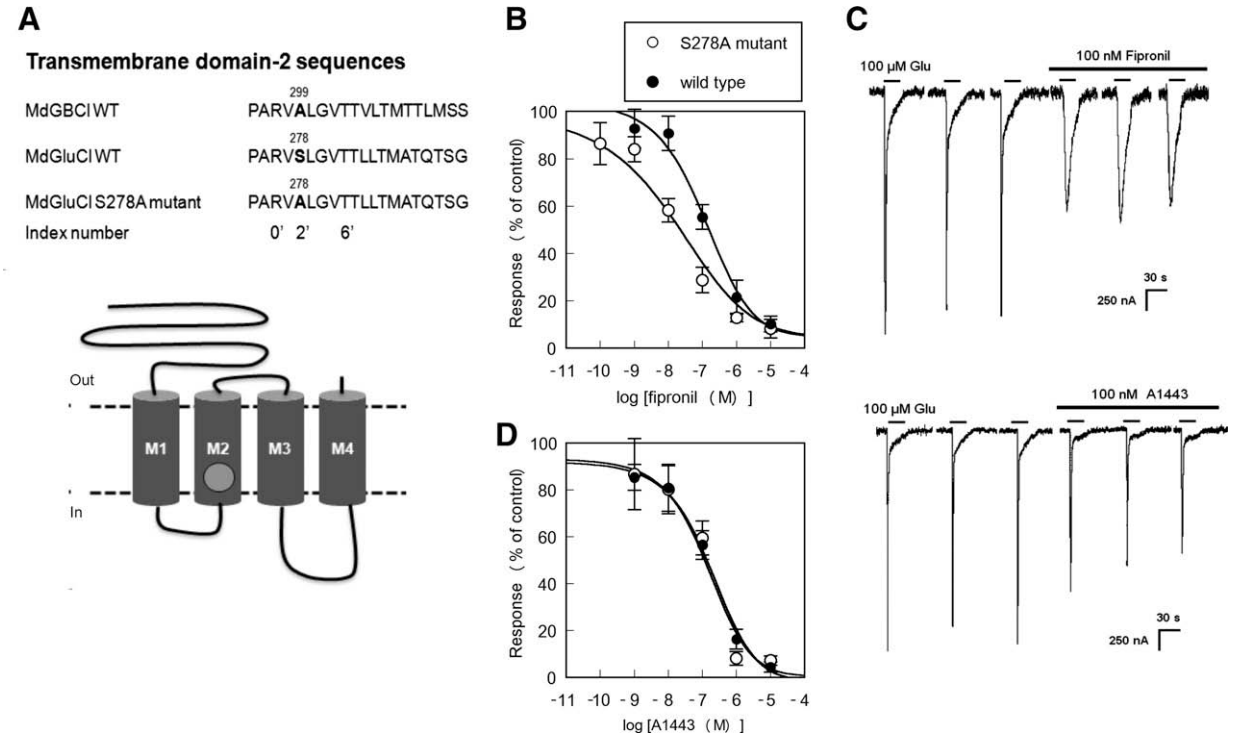

Fig. 4. Effects of A1443 and fipronil on the S278A mutant of MdGluCl channels. (A) Amino acid sequences of transmembrane domain 2 in wild-type MdGBCl and wild-type and mutant MdGluCl subunits. (B) Dose–response relationships of the blockade of glutamate (100 μM)-induced currents by fipronil in wild-type and S278A mutant MdGluCl channels. (C) Typical blockade of glutamate (100 μM)-induced currents by 100 nM A1443 and fipronil in S278A mutant MdGluCl channels. (D) Dose–response relationships of the blockade of glutamate (100 μM)-induced currents by A1443 in wild-type and A278A mutant MdGluCl channels. Each point in (B) and (D) represents the mean ± SE of responses in at least three oocytes.

ring of A1443 differs from that of the phenylpyrazoles in that it is not aromatic. Additionally, A1443 has a longer side chain than the phenylpyrazoles (Fig. 1A). The 2,6-dichloro-4-trifluoromethyl substitution on the phenyl group of the phenylpyrazole class of blockers is essential for their high potency [18], but A1443 has two chlorines at the 3 and 5 positions. Thus, A1443 displays a fairly novel chemistry among the LGCC blockers.

The unique structural features of A1443 prompted us to examine whether A1443 shares a binding site with the classical channel blockers. To this end, we constructed two mutant channels with an amino acid replacement at the so-called 2′ position of each subunit. Insect GABA receptor subunits feature an alanine at this position (Fig. 4A), and replacing this amino acid with a serine or a glycine (the so-called Rdl mutation) confers reduced sensitivity to blockers and resistance to insecticides [17]. This amino acid resides in the second transmembrane domain (M2) of the subunits, which assemble to form a pentameric channel complex (Fig. 4A). The position is on the cytoplasmic side of the channel lumen [3]. It is interesting that inhibitory glutamate receptors have a serine at the equivalent position (Fig. 4A). The fact that wild-type glutamate receptors have the same 2′ amino acid as the GABA receptor Rdl mutants may explain why most blockers have a lower affinity for glutamate receptors than for GABA receptors [12,16]. We recently tested this possibility by replacing the 2′ (278) serine residue of MdGluCl with an alanine [13]. The GABA receptor channel blockers, such as fipronil, lindane, and picrotoxinin, showed higher potencies toward the S278A mutant of MdGluCl channels than toward the wild-type channels when the channels were activated by 30 μM glutamate. Taken together with the findings regarding the GABA receptor mutants [17], these results can be interpreted as indicating that hydrophobic amino acids at the 2′ position are favorable to interactions with classical blockers.

In this study, we compared the effects of A1443 on the 2′ mutants with those of fipronil to examine whether the two compounds share a site of action. If the two compounds bind to a common site, the responses of the mutants to these compounds are assumed to be similar. Consistent with our previous results [13], the blocking potency of fipronil toward 100 μM glutamate-induced currents was enhanced by the S278A mutation (Fig. 4B). By contrast, the potency of A1443 was not significantly changed by the S278A mutation (Fig. 4C). Fipronil also affected the decay rate of glutamate-induced currents in the mutant, whereas A1443 did not. These findings with the MdGluCl mutant suggest that fipronil might interact with the 2′ amino acid in MdGluCl channels whereas this residue might not be involved in the interaction with A1443. The effects of the reverse mutation (A → S) were also tested in MdGBCl channels; the results confirmed that both A1443 and fipronil were as effective at blocking currents induced by 100 μM GABA in the A299S mutant MdGBCl channel as they were in the wild type. This was a somewhat unexpected finding because the homologous *Drosophila* Rdl mutant channels expressed in *Xenopus* oocytes were less sensitive to fipronil than the wild type [19]. However, [^{3}H]EBOB binding assays have shown that head membranes prepared from houseflies with the A299S mutation will bind to fipronil with high affinity, as did membranes from wild-type houseflies [10]. Accordingly, there may be certain functional or structural differences between the *Drosophila* and *Musca* channels. Taken together with the finding that OCR houseflies, which are resistant to known LGCC blockers [10,20], are susceptible to A1443, these results suggest that A1443 might bind to a site at least partly different from the one bound by known blockers in MdGBCl channels. Together, the results of this study demonstrate that the isoxazoline A1443 is a novel and potent LGCC blocker that has considerable potential for antiparasitic use.

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