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Neuropharmacological Mechanism of Action of PF1022A, an Antinematode Anthelmintic with a New Structure of Cyclic Depsipeptide, on *Angiostrongylus cantonensis* and Isolated Frog Rectus*

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Abstract

Mechanism of action of PF1022A was studied neuropharmacologically. Against *Angiostrongylus cantonensis,* PF1022A inhibited the motility at such a low concentration as 10^{-13} g/ml, and paralyzed the worm at 10^{-12} – 10^{-6} g/ml. The paralysis by the drug at 10^{-12} g/ml was partially antagonized by gabergic antagonists like picrotoxin and bicuculline, and completely reversed when N-methylcytisine (N-MC) was added with gabergic antagonists. On the other hand, in the preparations paralyzed by PF1022A (10^{-10} g/ml), the spasmogenic effects of N-MC and eserine were kept inhibited even with gabergic antagonists, while those of pyrantel were not inhibited. Paralysis by PF1022A (10^{-12} g/ml) was antagonized by Ca^{2+} combined with gabergic antagonists. The reversed motility by Ca^{2+} was again paralyzed by the addition of PF1022A (10^{-10} g/ml). The guanidine (2.5×10^{-3} M)-induced twitch response in the isolated frog rectus with or without N-MC was inhibited by PF1022A (10^{-6} g/ml), while contraction by pyrantel was not inhibited in the paralyzed preparation. From these results, it is suggested that PF1022A affects neuropharmacologically the nematode and the frog rectus. And in *A. cantonensis,* the inhibition is produced synergistically by stimulating the gabergic mechanism and inhibiting the cholinergic mechanism. As the drug is extremely less toxic against host animals, it is quite likely that PF1022A becomes available as a superior antinematode drug.

Key words: PF1022A, a new antinematode anthelmintic, neuropharmacological mechanism, *Angiostrongylus cantonensis,* isolated frog rectus

Introduction

PF1022A is one of a group of substances produced in fermentation broth of a strain PF1022 belonging to the order Agonomycetales (Mycelia Sterilia). The strain was newly isolated from the microflora on the leaf of plant, *Camellia japonica,* collected at Ibaragi Prefecture in Japan. This substance has a structure of cyclic depsipeptide consisting of four L-N-methyl leucins, two D-phenyl lactic acids and two D-lactic acids (Fig. 1) and a formula of $C_{52}H_{76}N_4O_{12}$ (Takagi *et al.*, 1991). It was reported that PF1022A had superior antinematode effects (Fukashe *et al.*, 1990; Takagi *et al.*, 1991). By a visual observation method it was observed that *in vitro* motility of intestinal nematodes such as *Heterakis spumosa* was inhibited completely 2 hrs after treatment with 10^{-7} g/ml. Rapid effects of the fermentation product were also observed *in vivo.* When chickens infected with *Ascaridia galli* were treated orally with 4.0 mg/kg, worms were expelled such quickly as several hours after treatment. Against dogs infected with *Toxocara canis* and *T. cati,* the compound orally given was effective at 0.2 mg/kg and greater, and worms were also expelled from the host intestines on the first day after treatment.

These results suggest that PF1022A acts on neuropharmacological mechanisms rather than on energy metabolism in worms because of its

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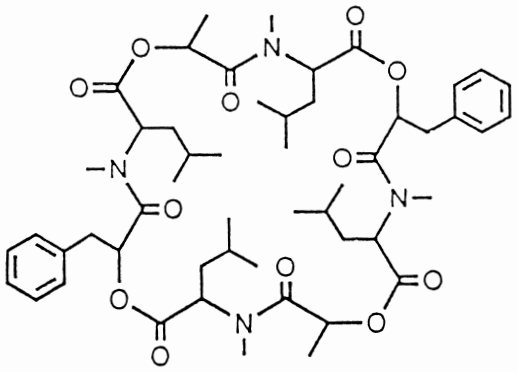

Fig. 1 Chemical structure of PF1022A.

prompt onset of action. Thus, in the present study, mechanism of action of PF1022A was examined neuropharmacologically using *Angiostrongylus cantonensis,* our worm model for studying effects of antinematode anthelmintics (Terada *et al.*, 1982, 1984). The isolated frog rectus preparation was also used because the preparation having only the cholinergic mechanism was demonstrated to be favorable for studying effects of anthelmintic actions on this mechanism (Terada *et al.*, 1992).

Materials and Methods

Angiostrongylus cantonensis (Hawaii strain) was obtained from rats (Wistar strain) experimentally infected in our laboratory. The female worms (2.5–3.0 cm) were used in this study. The rectus (2.0–2.5 cm) was isolated from *Rana nigromaculata*. Effects of drugs were examined by the isotonic transducer method described by Terada *et al.* (1984) using Tyrode's solution for *A. cantonensis* and Ringer's frog solution for the isolated rectus.

PF1022A was kindly supplied by Meiji Seika Ltd., and other drugs used were obtained from following sources; eserine salicylate, bicuculline (Sigma), strychnine sulfate (Nakarai), picrotoxin (Tokyokasei) and pyrantel tartrate (Pfizer Taito). The PF1022A and bicuculline were dissolved in methanol and dimethylsulfoxide, respectively, and added to the organ bath. The final concentration of the organic solvent was 0.5% or less, which had little effect on the motility of the preparations used. Other drugs were dissolved in a 0.9% NaCl solution and the concentrations refer to the weight of the salts.

Results

Effects of PF1022A and some neuropharmacological agents on the motility of Angiostrongylus cantonensis

PF1022A exerted inhibitory effects on the motility of *A. cantonensis* at a concentration as low as 10^{-13} g/ml (1.05×10^{-13} M), and paralysis was caused at concentrations of 10^{-12} to 10^{-6} g/ml (Fig. 2A, B). Although amplitude of relaxation was not always dependent on the concentration of PF1022A, the time required to cause a complete paralysis depended on the concentration of the drug, and the time varied from 78.8 ± 7.7 min at 10^{-12} g/ml to 4.2 ± 1.1 min at 10^{-9} g/ml (Fig. 3).

The paralysis caused by PF1022A (10^{-12} g/ml) was not reversed after washing with Tyrode's solution. The paralysis was, however, partially reversed by the addition of gabergic antagonists such as bicuculline (3×10^{-5} M) and picrotoxin (5×10^{-5} M) (Fig. 4A). Spasmogenic effects of N-methylcytisine (N-MC, 2×10^{-5} M), a stimulant of the release of acetylcholine (ACh) from the nerve endings of the nematode (Terada *et al.*, 1982) were inhibited when it was given to the preparation paralyzed by PF1022A (10^{-12} g/ml). The spasmogenic effect was reversed when gabergic antagonists were accompanied with N-MC, and picrotoxin was more remarkable than bicuculline in the antagonistic effect (Fig. 4B). On the preparation contracted by N-MC (2×10^{-5} M) or eserine (10^{-6} M), an inhibitor of acetylcholinesterase, PF1022A (10^{-10} g/ml) caused paralysis. In the paralyzed preparations, the spasmogenic effects of N-MC and eserine newly added were kept inhibited even with gabergic antagonists. In the presence of gabergic antagonists, spasmogenic effects of pyrantel which contracts nematodes by stimulating a nicotinic ACh receptors (Aubry *et al.*, 1970; Terada *et al.*, 1983) were, however, not inhibited in the preparations paralyzed by PF1022A

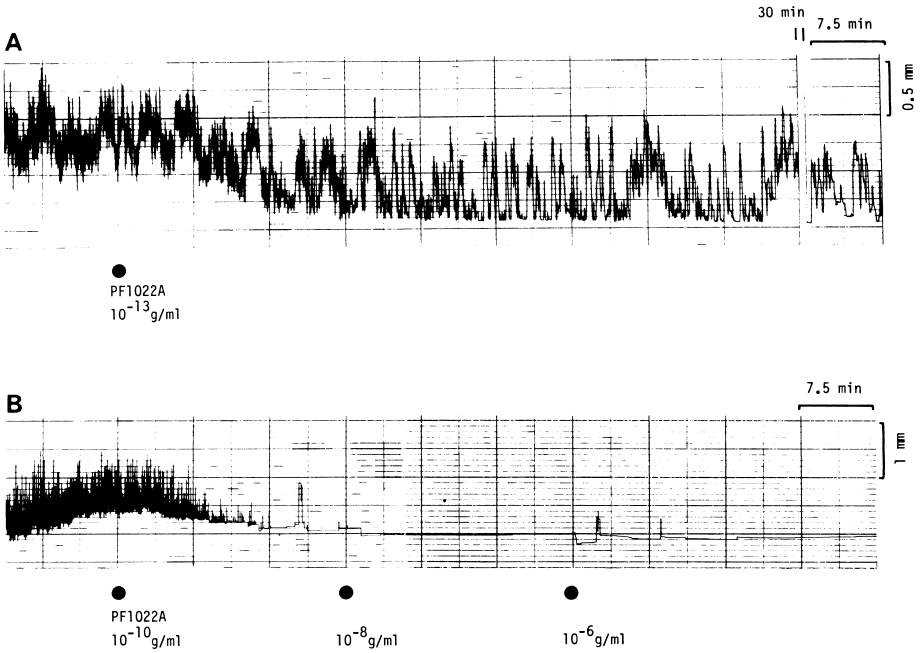

Fig. 2 Effects of different concentrations of PF1022A on the motility of *Angiostrongylus cantonensis*. The female worm was used with a tension of 0.7–0.8 g. In figures 2 and 4–6, drugs in a single or cumulative dose were given successively at the points shown by the symbols. Each figure shows the representative of 3 to 5 similar tracings.

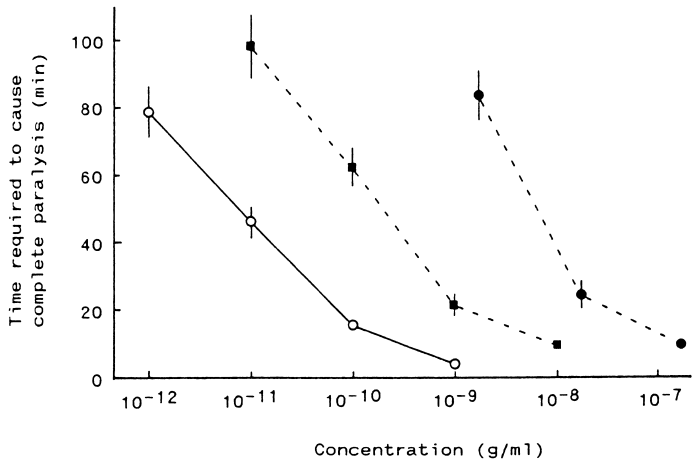

Fig. 3 Effects of PF1022A on the motility of *A. cantonensis*: Concentration-response curve. The result on PF1022A (○———○) is shown as mean ± SE (n = 6–12). The results on milbemycin D (■------■, n = 6–9) and ivermectin (●------●, n = 5–8) were cited from Terada *et al.* (1984, 1986).

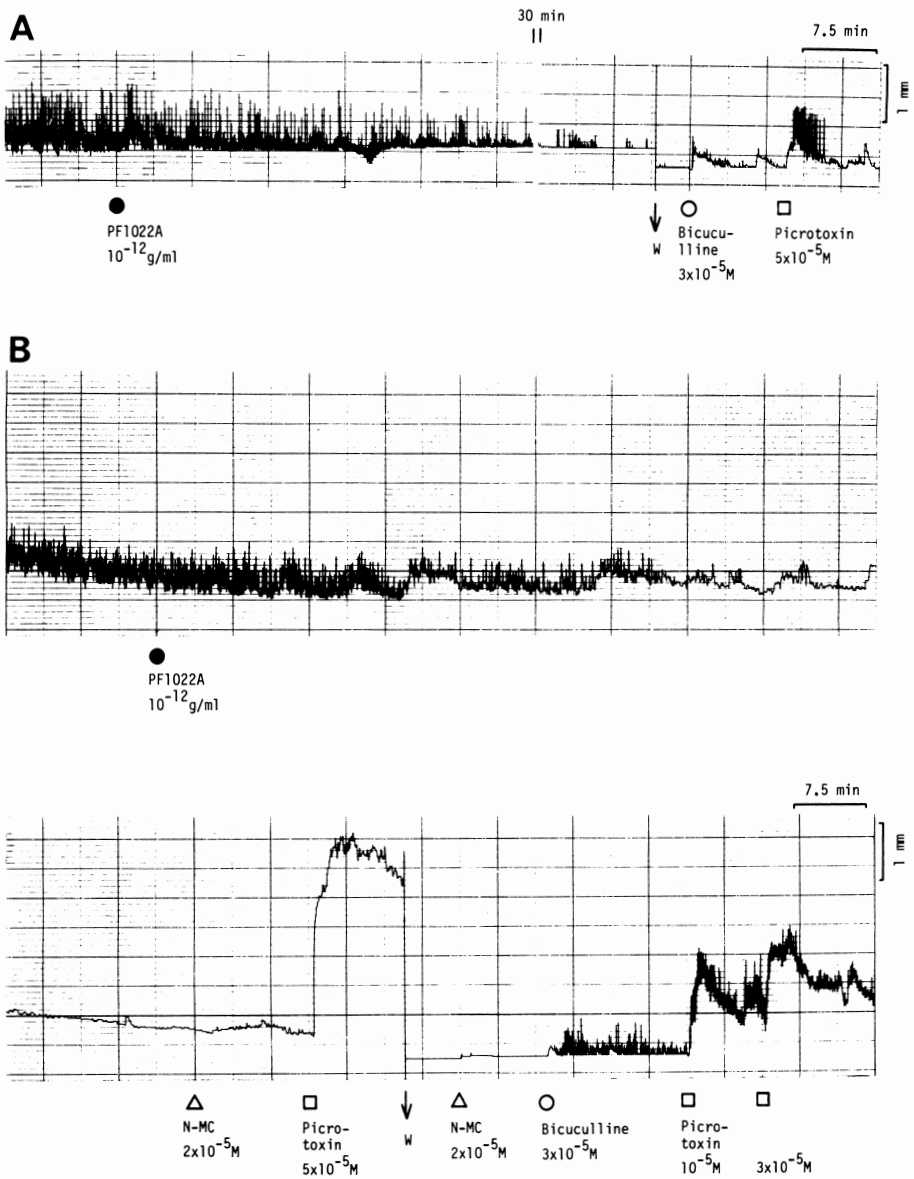

Fig. 4 Inhibitory effects of PF1022A at 10^{-12} g/ml on the motility of *A. cantonensis* and antagonistic effects of gabergic antagonists like picrotoxin and bicuculline, and N-methylcytisine (N-MC), a stimulant of the release of acetylcholine from the cholinergic nerve endings. In figures 4–6, preparations were washed with Tyrode's solution for about 30 min at times shown by point W.

(10^{-10} g/ml). In this experiment the antagonistic effect of picrotoxin was also stronger than that of bicuculline (Fig. 5A, B).

Strychnine (3×10^{-6} M), an inhibitor of the release of ACh from the nerve endings in the worm (Terada *et al.*, 1984), paralyzed the worm preparation, but the paralysis was antagonized by the addition of Ca^{2+} (2×10^{-2} M). The reversed motility was again paralyzed by the further addition of PF1022A (10^{-10} g/ml) and

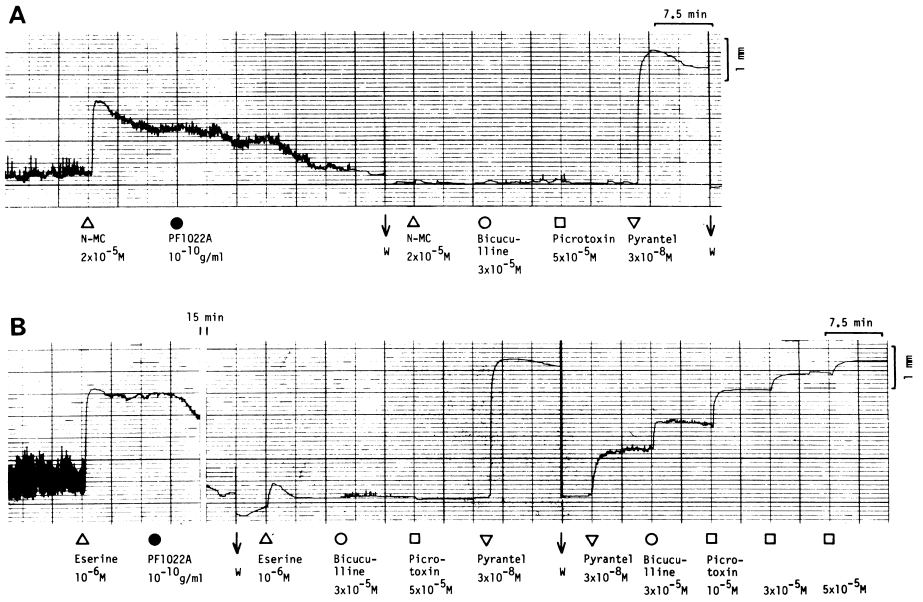

Fig. 5 Effects of PF1022A at 10^{-10} g/ml on the contracted preparations by N-MC (A) and eserine (B), an inhibitor of acetycholinesterase in *A. cantonensis*. Antagonistic effects of picrotoxin and bicuculline were also examined.

the paralysis was not reversed by picrotoxin (5×10^{-5} M) and bicuculline (3×10^{-5} M) (Fig. 6A). Ca^{2+} (2×10^{-2} M) with gabergic antagonists also reversed the paralysis elicited by PF1022A (10^{-12} g/ml) and the reversed motility was blocked again by PF1022A (10^{-10} g/ml) (Fig. 6B).

Effects of PF1022A and some neuropharmacological agents on the twitch response in the isolated frog rectus preparation

The guanidine (2.5×10^{-3} M)-induced twitch response was influenced little by PF1022A (10^{-7} g/ml), but 10^{-6} g/ml of the drug prevented the response. The spasmogenic effect of pyrantel (10^{-4} M) was, however seen in the preparation paralyzed by PF1022A (Fig. 7A). N-MC (2×10^{-5} M) stimulated the twitch response, but PF1022A (10^{-6} g/ml) also paralyzed the stimulated preparation (Fig. 7B).

Discussion

It is suggested that the motility of nematodes such as *Ascaris suum* and *A. cantonensis* is regulated by an excitatory cholinergic mechanism and an inhibitory gabergic mechanism (Mellanby, 1955; Norton and De Beer, 1957; Natoff, 1969; Del Castillo and Morales, 1969; Terada *et al.*, 1984). From the neuropharmacological standpoints, if we can find drugs which synergistically affect the nematode worms by stimulating the gabergic mechanism and inhibiting the cholinergic mechanism, they will become ideal anti-nematode anthelmintics paralyzing worms rapidly and strongly.

In the frog rectus preparations isolated from *R. nigromaculata,* guanidine (5×10^{-4} g/ml, 5.3×10^{-3} M) was reported to cause twitch response through stimulating the release of ACh from the cholinergic nerve endings (Otsuka and Endo, 1960). As it was suggested that some gabergic anthelmintics activated the cholinergic mechanism at their higher concentrations (Terada and Sano, 1985, 1986; Lee and Terada, 1992), we examined the influence of gabergic anthelmintics on the guanidine-induced twitch response. Drugs except piperazine all stimulated the

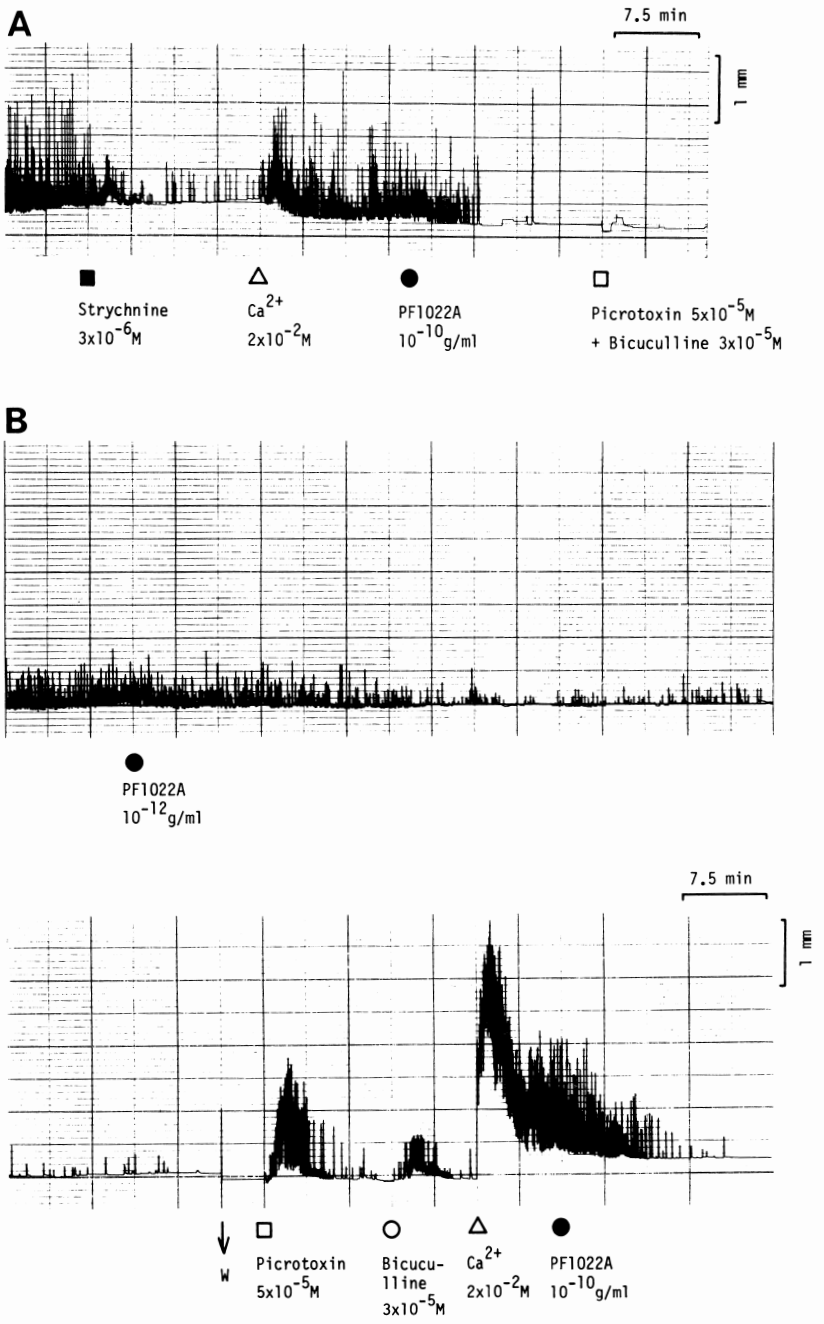

Fig. 6 Effects of Ca^{2+} on the paralyzed preparations by strychnine (A) and PF1022A at 10^{-12} (B) in *A. cantonensis*. Effects of picrotoxin and bicuculline, and PF1022A at 10^{-10} g/ml were also examined.

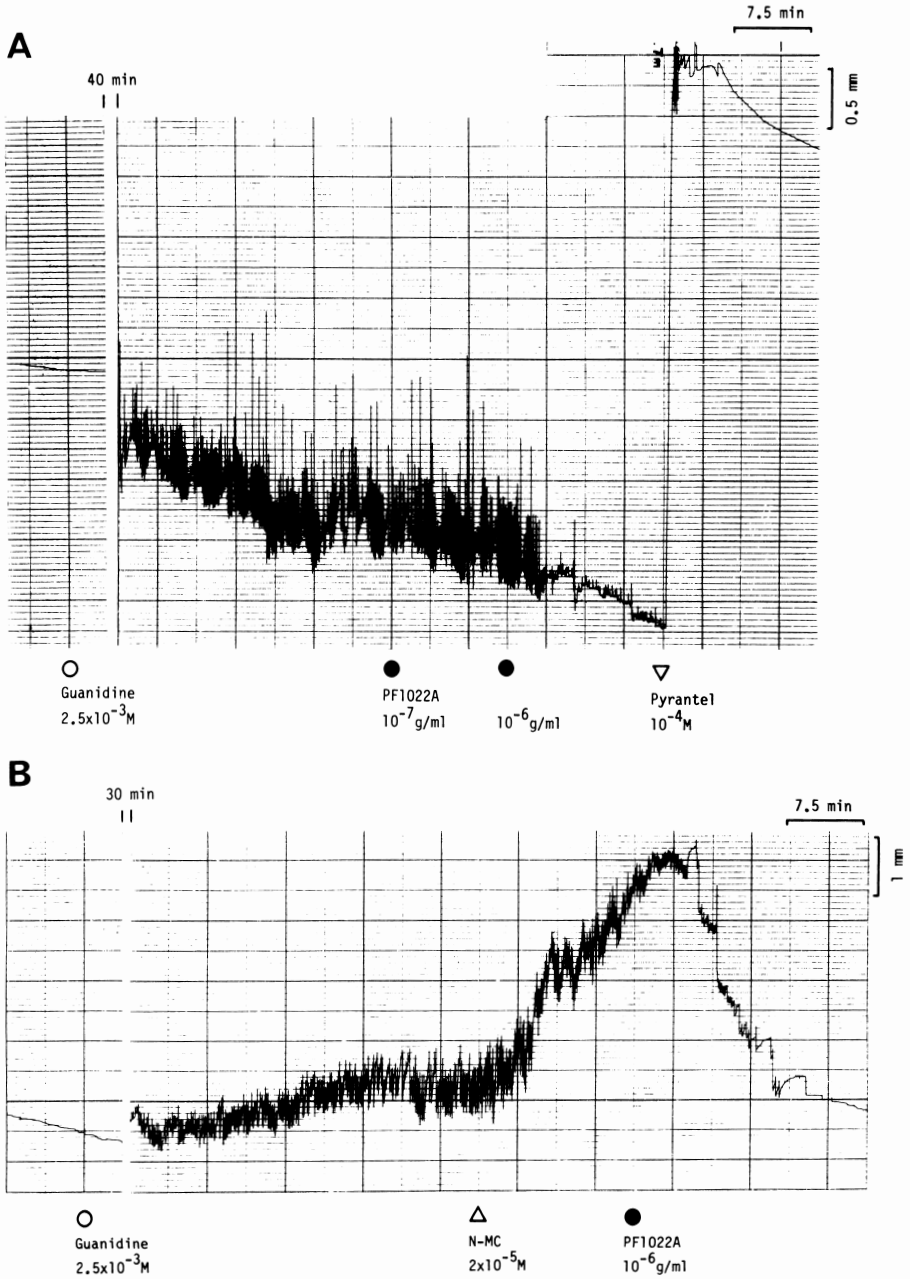

Fig. 7 Effects of PF1022A at 10^{-7}–10^{-6} g/ml on the guanidine (2.5×10^{-3} M)-induced twitch response in isolated frog rectus preparation. Effects of pyrantel and N-MC were also examined. The rectus preparation was suspended in Ringer's frog solution with a tension of 0.8 g.

response at their higher concentrations, whereas only piperazine inhibited the response even at its higher concentrations (Terada *et al.*, 1992). These results suggest that piperazine could paralyze nematodes by affecting synergistically both gabergic and cholinergic mechanisms. The results also show us the reason why this old anthelmintic having a very lower potency as a gabergic anthelmintic has been clinically highly effective.

In the present study, it was demonstrated that PF1022A had the ideal mechanism of action like piperazine and was highly superior to piperazine in its dose-response relationship and its irreversibility of the action. Compared with piperazine which was effective at 1.8×10^{-5} g/ml (10^{-4} M) and greater, PF1022A exerted effects at 10^{-13} g/ml (1.05×10^{-13} M) and greater. In addition, although the inhibitory effects of piperazine were reversed by washing with Tyrode's solution, the effects of PF1022A seemed to be irreversible.

Regarding the inhibitory effects of PF1022A at its lower concentrations such as 10^{-12} g/ml, it is suggested from the antagonistic effects of picrotoxin (an inhibitor of chloride ionophore) and bicuculline (a GABA receptor antagonist) that the drug paralyzes the nematode worms through stimulating the gabergic mechanism. Though PF1022A is superior to milbemycin D and ivermecin with respect to the effective concentrations, the new substance may resemble avermectins rather than milbemycins in relation to the antagonistic action of picrotoxin and bicuculline (Terada *et al.*, 1984, 1986; Lee and Terada, 1992). Therefore, PF1022A seems to affect the nematodes through stimulating the release of GABA and/or enhancing the binding of GABA to its receptors in the worms described for avermectins by Campbell (1985, 1989).

The paralysis caused by PF1022A (10^{-12} g/ml) was reversed by the combined addition of gabergic antagonists with N-MC. On the other hand, in the preparation paralyzed by PF1022A (10^{-10} g/ml), the spasmogenic effects of N-MC and eserine which contract worms through accumulation of endogenous ACh are inhibited even in the presence of gabergic antagonists. The spasmogenic effect of pyrantel with gabergic antagonists was, however, not inhibited in the paralyzed preparation. Thus, it may be probable that PF1022A affects only presynaptic sites of the cholinergic mechanism, and that the inhibition comes from indirectly through the hyperpolarization by the gabergic stimulation. But, there may be a possibility that the inhibition is induced directly by affecting the release mechanism of ACh. It was reported in *A. cantonensis* that verapamil and ethyl p-methyl-transcinnamate, a derivative of cinnamic acid changed the motility through affecting the utilization of Ca^{2+} at presynaptic sites (Sano *et al.*, 1985; 1987). Strychnine is also suggested to paralyze the worm by inhibiting utilization of Ca^{2+} at the sites (Sano *et al.*, 1985). In the present study, the paralyzing action of PF1022A (10^{-12} g/ml) was antagonized by Ca^{2+} (2×10^{-2} M) in the presence of gabergic antagonists, but the restored motility disappeared when PF1022A (10^{-10} g/ml) was given. Thus, PF1022A is likely to cause a very strong inhibition on the utilization of Ca^{2+} and inhibit the release of ACh from the nerve endings. On the other hand, from the relation between the paralyzing effects of PF1022A and the spasmogenic effect of pyrantel on the worm and the frog rectus, this substance seems not to inhibit the utilization of Ca^{2+} at postsynaptic sites.

From these results, it is concluded that PF1022A affects neuropharmacologically the nematode worm and the frog rectus. And in *A. cantonensis,* the inhibition is produced synergistically by stimulating the gabergic mechanism and inhibiting the cholinergic mechanism.

The surprising mechanism of action of PF1022A may be related to the fact that it has an entirely new structure compared with traditional all anthelmintics (Vanden Bossche, 1985; Campbell and Rew, 1986). In addition, PF1022A is completely unique from the standpoints of its source, microflora on the plant leaf and of microbial group, Agonomycetales (Mycelia Sterilia), because all macrocyclic lactons including avermectins and milbemycins are produced through fermentation of Actinomycetes, *Streptomyces* spp. from soil samples (Campbell, 1989; Takiguchi *et al.*, 1981).

To introduce drugs into clinical trials,

although studies on efficacy and mode and mechanism of actions are important, another studies on safety or toxicity of drugs are inevitable. It was reported that PF1022A had little toxicity against animals, and LD_{50} values for p.o. and i.p. administration in male ICR mice were reported to be more than 2,000 mg/kg and more than 1,000 mg/kg, respectively (Takagi *et al.*, 1991). The values are extremely greater than those of ivermectin; 25 mg/kg p.o. and 30 mg/kg i.p. for mice (Campbell, 1989). And the values are also greater than those of milbemycin D; 1,547 and 1,610 mg/kg p.o. for male and female mice and 668 and 772 mg/kg i.p. for male and female mice (Matsunuma *et al.*, 1983). In this study, the low toxicity of PF1022A was also demonstrated with regard to the concentrations causing inhibition on the release of ACh from the cholinergic nerve endings. There was a striking difference of 10^6 in the effective concentrations between *A. cantonensis* and the frog rectus. Regarding selective toxicity of traditional anthelmintics, only a few differences were reported in effective concentrations between helminthic worms and host tissues. For example, only a difference of 30 to 50 was observed between inhibiting concentrations of antimonials against phosphofructokinase activity from *Schistosoma mansoni* and the rat's brain (Mansour and Bueding, 1954). A difference of about 10^2 was reported between contracting concentrations of pyrantel against *A. cantonensis* and the isolated frog rectus (Terada *et al.*, 1983). It is suggested that the striking difference in the effective concentrations of PF1022A against *A. cantonensis* and the frog rectus may be attributable to the differences in roles and mechanisms of utilization of Ca^{2+} between the nematode muscle and host skeletal muscle as reported by us (Sano *et al.*, 1985).

As it was reported that PF1022A could be hardly absorbed from host intestines (Takagi *et al.*, 1991), the fermentation product will undoubtedly become a superior anthelmintic against intestinal nematodes. In addition, it was preliminarily observed that the anthelmintic given intravenously was effective against *Haemonchus contortus* biting into the mucosae of the host intestine (Takagi *et al.*, 1991). Therefore, PF1022A may become available against parasitic diseases caused by tissue nematodes after detailed studies on efficacy and toxicity of the drug by parenteral administration and on its drug delivery system.

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