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Inhibition of Calmodulin-Dependent Calcium-ATPase and Phosphodiesterase by Various Cyclopeptides and Peptide Alkaloids from the Zizyphus Species

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The effects of various sedative cyclopeptides and peptide alkaloids from the Zizyphus species on calmodulin-dependent Ca^{2+}-ATPase and phosphodiesterase were investigated. Calmodulin-induced activation of Ca^{2+}-ATPase was strongly inhibited by sanjoinine-A dialdehyde (IC_{50}, 2.3 μM), -Ah1 (IC_{50}, 4.0 μM), -A (IC_{50}, 4.6 μM), and -G2 (IC_{50}, 7.2 μM), while calmodulin-induced activation of phosphodiesterase was strongly inhibited by both deachuine-S10 (IC_{50}, 4.9 μM) and sanjoinine-D (IC_{50}, 9.0 μM). The inhibitory activity of the various cyclopeptides and peptide alkaloids on Ca^{2+}-ATPase was found to correlate well with their sedative activity.

Key words: *Zizyphus vulgaris* var. *spinosus*, *Zizyphus jujuba* var. *inermis*, Cyclopeptide alkaloid, Ca^{2+}-ATPase, Phosphodiesterase, Calmodulin

INTRODUCTION

Cyclopeptides and peptide alkaloids were isolated from the seeds of *Zizyphus vulgaris* Lamark var. *spinosus* Bunge (Rhamnaceae, "Sanjoin" in Korean). This herb, which is used as a sedative and nerve tonic in the Oriental medicine, is reputed to be an important herbal remedy insomnia (Han and Park, 1987a; Han *et al.*, 1990). Various kinds of cyclopeptide alkaloids were also isolated from Zizyphi fructus ("Daechu" in Korean), the fruits of *Zizyphus jujuba* Miller var. *inermis* Rehder and from the stem barks (Han and Park, 1987b; Han *et al.*, 1989).

Sanjoinine-A with a 14-membered ring was identified as frangufoline (Han *et al.*, 1987c). It was demonstrated to be a major sedative component of sanjoin (Han *et al.*, 1993a). Sanjoinine-A exhibited the ion binding activity to calcium and magnesium ions (Park *et al.*, 1991), and specifically bound to calmodulin at two sets of binding sites. The sanjoinine-A to calmodulin mole binding ratio was reported to be two at the high affinity sites and four at the low affinity sites (Han *et al.*, 1993b).

Calmodulin is an ubiquitous, calcium-binding protein that regulates the activity of several calcium-dependent enzymes, including phosphodiesterase, Ca^{2+}-ATPase, protein kinase II, and adenylate cyclase, *etc* (Klee *et al.*, 1980). The alteration of calmodulin activity is known to have profound biological consequences (Weiss and Wallace, 1980). Antipsychotic and antidepressant agents that interfere with calmodulin's action were reported to have the ability to inhibit the calmodulin-induced activation of phosphodiesterase, Ca^{2+}-ATPase, and adenylate cyclase (Weiss and Wallace, 1980; Roufogalis, 1982).

The aim of this study was to investigate the effects respective that various cyclopeptides and peptide alkaloids from the Zizyphus species have on calmodulin-dependent Ca^{2+}-ATPase and phosphodiesterase. It was found that sanjoinine-A and its analogs were strong inhibitors of Ca^{2+}-ATPase, which correlated with their sedative activity.

MATERIALS AND METHODS

Materials

Cyclopeptides and peptide alkaloids were isolated from sanjoin and daechu by the methods described previously (Han *et al.*, 1985, 1987, 1989, 1990). Calmodulin (from bovine brain tissue, p-2277), phosphodiesterase (calmodulin-deficient, p-9529), snake venom (*Ophiophagus hannah*), AG1-X2 resin and ATP were purchased from

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Sigma Chem. Co. (USA). ^{3}H-cAMP was a product of Dupont NEN (USA).

The muscle (1 g) from a rat hind thigh was thoroughly homogenized with 9 ml of 0.66 M KCl (pH 6.8) and the homogenate was then centrifuged at 10,000 *g* for 30 min. The supernatant was diluted with 11 volumes of 0.3 mM $NaHCO_3$ and centrifuged at 10,000 *g* for 20 min. The pellet obtained was resuspended into 1.375 volumes of 1 M KCl (pH 6.8) and again centrifuged at 10,000 *g* for 20 min. The supernatant was used as an actomyosin source for Ca^{2+}-ATPase activity measurements. Experiments using this actomyosin were accomplished within 2~3 days, as the calcium and calmodulin-sensitivity often decreased and disappeared in preparations stored for 5~6 days.

Measurement of Ca^{2+}-ATPase activity

The Ca^{2+}-ATPase activity of rat hind thigh actomyosin was measured using the method reported by Sobieszek and Small (1976). The reaction mixture (2 ml) contained 20 mM Tris-maleic acid buffer (pH 6.8), 5 mM $MgCl_2$, 50 μM $CaCl_2$, 30 mM KCl, 400 μg actomyosin and 1 mM ATP, in the absence or presence of calmodulin (100 ng). The assays were pre-equilibrated at 25°C and the reaction was initiated by adding ATP (Na) while mixing gently on a vortex mixer. Incubation was carried out for 20 min at 25 °C and the assay was terminated by adding 1 ml of 10 % trichloroacetic acid. An aliquot (0.5 ml) was taken for inorganic phosphate (Pi) analysis, which was determined using the method described by Martin and Dotty (1949).

Assay of phosphodiesterase activity

The enzyme activity was determined by the method reported by Thomson and Strada (1984). The reaction mixture (0.4 ml) contained 50 mM TrisHCl (pH 8.0), 5 mM $MgCl_2$, 50 μM $CaCl_2$, 0.4 μM phosphodiesterase, 2.02 μM ^{3}H-cAMP, and 100 ng calmodulin. The mixture was incubated for 10 min at 30°C, and the assay was terminated by boiling for 1 min. 5'-AMP was converted to adenosine and inorganic phosphate by incubation with 100 μg (100 μl) snake venom for 10 min at 30°C. The adenosine was separated from the unreacted cAMP by adding the incubation mixture (0.5 ml) to an AGI-X2 column (1 ml of 25% resin slurry). The reaction mixture was eluted into a scintillation vial, and 1 ml methanol was added to the column. The radioactivity of the combined eluate was determined after the addition of an 8 ml toluene cocktail, using a liquid scintillation spectrometer (Hewlet Packard Co.).

Protein determination

The protein concentration was determined using the method reported by Lowry *et al.* (1951) with bovine serum albumin as a standard.

RESULTS AND DISCUSSION

The Ca^{2+}-ATPase activity of the actomyosin prepared from the rat thigh muscle was assessed in either the presence or absence of calmodulin. The basal Ca^{2+}-ATPase activity of actomyosin freshly prepared and that stored for three days was enhanced 240 and 213%, respectively in the presence of 100 ng calmodulin (Table I). It is known that calmodulin addition results in a three- or four-fold concentration-dependent increase in the Ca^{2+}-ATPase activity (Larsen and Vincenzi, 1979). Therefore, the experimental conditions were adequate to the purpose of this study.

The effects that the various several cyclopeptides and peptide alkaloids have on Ca^{2+}-ATPase activity were investigated, with the results shown in Table II. The IC_{50} values of the three known antipsychotics, used as positive controls, were similar to those of other researchers: 6.3/4.5 μM for pimozide (Gietzen *et al.*, 1980); 4.9/10 μM for fluphenazine (Gietzen *et al.*, 1980); chlorpromazine 27/22, 5 and 75 μM (Gietzen *et al.*, 1980; Kobayashi *et al.*, 1979; Raess and Vincenzi, 1980). Among the alkaloids tested (Table II), Sanjoinine-A, -Ah1, -dialdehyde, -F and -G2 proved to be strong Ca^{2+}-ATPase inhibitors. The other alkaloids moderately inhibited enzyme activity.

The 14-membered cyclopeptide alkaloids described in both Table II and Fig. 1 belong to a frangulanine type, which is characterized by an aryl ether bridge resulting from *p*-hydroxystyrylamine and β-hydroxyleucine (Tschesche and Kaussmann, 1975). Sanjoinine-A contains leucine as a ring bond amino acid and N,N-dimethylphenylalanine as a basic amino acid. Sanjoinine-A and its analogs were proven to inhibit calmodulin-dependent Ca^{2+}-ATPase (Fig. 1). Sanjoinine-A dialdehyde and sanjoinine G2, which are products obtained through the cleavage of the olefinic bond on the sanjoinine-A ring unit, exhibited a similar inhibitory activity. This suggests that the 14-membered ring structure is not important for inhibiting enzyme activity. The configuration of the basic amino acid, N,N-dimethylphenylalanine did not greatly alter the inhibitory activity, since the IC_{50} value (4.0 μM) of sanjoinine-Ah1, an epimer (R-form) of sanjoinine-A (S-form)

Table I. Activation of Ca^{2+}-ATPase of actomyosin from the rat thigh flesh muscle of rat hind thigh in the presence of calmodulin

Actomyosin Preparation	Ca^{2+}-ATPase activity (μ mol Pi/mg/min)		Activation (%)
	-Calmodulin	+Calmodulin[1)]	
Fresh Preparation	0.720[2)]	1.725	240
Stocked 2 days	0.662	1.412	213

[1)]The presence of 100 ng calmodulin was contained in the reaction mixture (2 ml).
[2)]Data from duplicate experiments

Table II. The IC_{50} values of the cyclopeptides and peptide alkaloids and some known antipsychotics on Ca^{2+}-ATPase in the presence of calmodulin

Compounds	IC_{50} (μM)	
	Ours[a]	Reference
A. Cyclopeptide alkaloids (CPAs)		
14-Membered CPAs (frangulanine type)		
Sanjoinine-A	4.6	-
Sanjoinine-Ah1	4.0	-
Dihydrosanjoinine-A	23.7	-
Sanjoinine-C	14.9	-
Sanjoinine-D	23.0	-
Sanjoinine-F	7.9	-
Sanjoinine-G1	14.6	-
Sanjoinenine	11.0	-
Daechuine-S4	19.6	-
13-Membered CPAs (zizyphine-A type)		
Daechuine-S10	29.0	-
Daechuine-S27	27.0	-
B. Peptide alkaloids		
Sanjoinine-A dialdehyde	2.3	-
Sanjoinine-G2	7.2	-
C. Known antipsychotics		
Pimozide	6.3	4.5[b] (Gietzen *et al.*, 1980)
Fluphenazine	4.9	10[b] (Gietzen *et al*, 1980)
Chlorpromazine	27.0	22[b] (Gietzen *et al.*, 1980)

[a]Rat muscle Ca^{2+}-ATPase. Data from 3 or 4 experiments in the presence of 100 ng calmodulin. Drugs were dissolved in dimethyl sulfoxide and diluted with 20 mM Tris·maleic buffer (pH 6.8) before using.
[b]Human erythrocyte Ca^{2+}-ATPase.

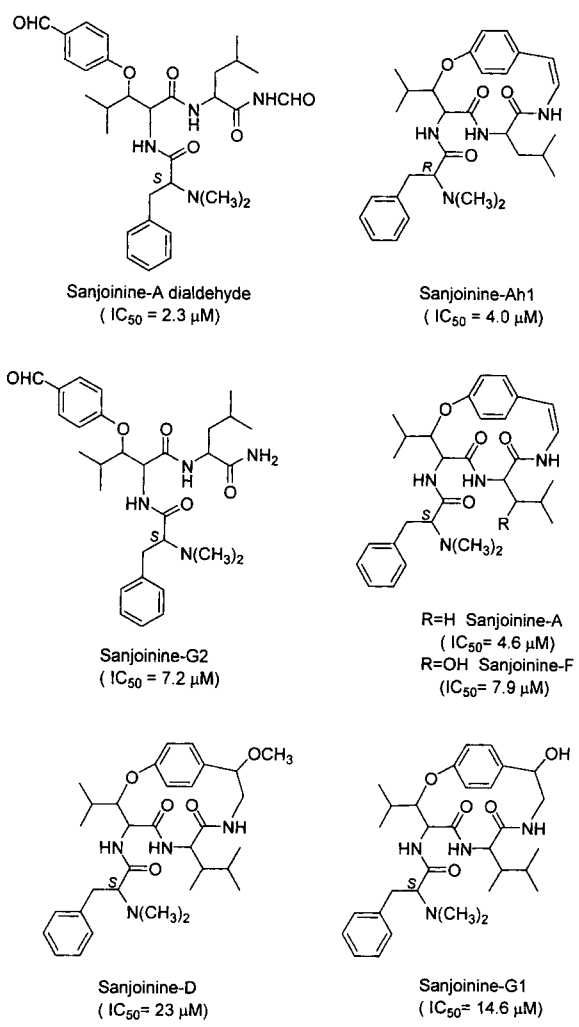


Fig. 1. The chemical structures of sanjoinine-A, its analogs, and their respective IC_{50} values on calmodulin-dependent Ca^{2+}-ATPase

was similar to that of the S-form (Fig. 1). Oxidation of the olefinic double bond results in significant loss in activity similar to that observed with sanjoinine-D and -G1.

Calcium-dependent phosphodiesterase is one of the many forms of cyclic 3',5'-nucleotide phosphodiesterase. Calcium-dependent phosphodiesterase requires Mg^{2+} for catalytic activity and Ca^{2+} for its activation by calmodulin. The simultaneous presence of Ca^{2+} and calmodulin is necessary to activate the enzyme, and one agent increases the sensitivity of the enzyme to the other (Lin *et al.*, 1974). The Ca^{2+} and Mg^{2+} concentrations were fixed at 50 μM and 5 mM, respectively, because sanjoinine-A has ionophore activity to Ca^{2+} and Mg^{2+}. Enzyme activity was greatly enhanced at calmodulin levels of between 100 ng to 400 ng, and the half-maximum stimulation of the enzyme appears to occur at 100 ng of calmodulin(Table III).

In the presence of 100 ng of calmodulin, the effects of several cyclopeptides and peptide alkaloids on phosphodiesterase were investigated (Table IV). Sanjoinine-D and daechuine-S10 strongly inhibited phosphodiesterase activity.

Table III. Enhancement of phosphodiesterase activity in the presence of calmodulin

Calmodulin(ng) in 0.4 ml of reaction mixture	Activity of phosphodiesterase* (AMP nmol/mg/min)	Activation (%)
0	28.6**	100
50	49.8	174.1
100	107.1	374.5
200	111.4	389.5
400	138.2	483.2

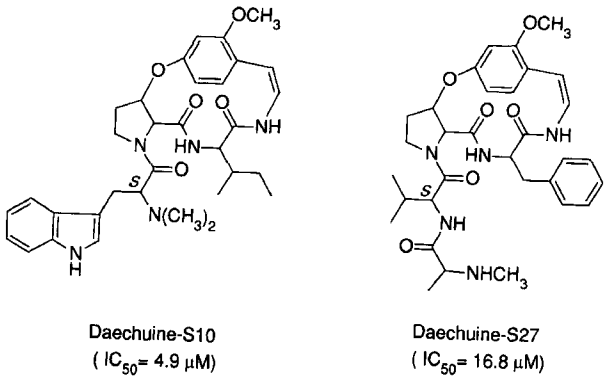
*Bovine brain, **Data from duplicate experiments

Daechuine-S10 and -S27 (Fig. 2) belong to a zizyphine-A type, which contains a 13-membered ring with a 5-hydroxy-2-methoxystyrylamine unit and trans 3-hydro-

Table IV. IC_{50} Values of the cyclopeptides and peptide alkaloids and some known antipsychotics on phosphodiesterase in the presence of calmodulin

Compounds	IC_{50} (μM)	
	Ours*	Reference
A. Cyclopeptide alkaloids		
14-Membered CPAs (frangulanine type)		
Sanjoinine-A	82	-
Sanjoinine-Ah1	55	-
Dihydrosanjoinine-A	21	-
Sanjoinine-C	27	-
Sanjoinine-D	9	-
Sanjoinine-F	94	-
Sanjoinine-G1	140	-
Sanjoinenine	25.5	-
Daechuine-S4	21	-
13-Membered CPAs (zizyphine-A type)		
Daechuine-S10	4.9	-
Daechuine-S27	16.8	-
B. Peptide alkaloids		
Sanjoinine-A dialdehyde	34	-
Sanjoinine-G2	125	-
C. Known antipsychotics		
Pimozide	3.5	7 (Levin and Weiss, 1976)
		0.7 (Norman *et al.*, 1979)
Fluphenazine	3.7	11 (Kanno and Sasaki, 1982)
Chlorpromazine	4.6	24 (Kanno and Sasaki, 1982)
		6 (Norman *et al.*, 1979)

*Data from 3 or 4 experiments in the presence of 100 ng calmodulin. Drugs were dissolved in dimethyl sulfoxide and diluted with 50 mM Tris·HCl buffer (pH 8.0) before using.



Daechuine-S10 (IC_{50}= 4.9 μM)

Daechuine-S27 (IC_{50}= 16.8 μM)

Fig. 2. The chemical structures of daechuine-S10 and -S27, and their respective IC_{50} values on calmodulin-dependent phosphodiesterase

xyproline (Tschesche and Kaussmann, 1975). As the basic end amino acid moiety, daechuine-S10 contains the aromatic amino acid, N,N-dimethyltryptophan, whereas daechuine-S27 contains the aliphatic dipeptide, monomethylalanylvaline. Furthermore, daechuine-S10 possesses leucine as the ring bond amino acid moiety, whereas daechuine-S27 contains phenylalanine. Since daechuine-S10 is a stronger calmodulin-dependent phosphodiesterase inhibitor than daechuine S27, it was concluded that the aromatic moiety on the basic end amino acid is important for inhibiting enzyme activity. It is also found that the methoxy group on the styrylamine unit is responsible for inhibiting enzyme activity, since sanjoinine-D (Table VI and Fig. 1) and daechuine-S10 contains this group. Briefly, the presence of the methoxy group on the styryamine unit and aromatic moiety as the basic end amino acid is very important for inhibiting calmodulin-dependent phosphodiesterase activity.

Levin and Weiss (1979) demonstrated that the degree to which drugs bind to calmodulin was directly related to their ability to inhibit Ca^{2+}-dependent phosphodiesterase activity. Moreover, antipsychotic drugs showed the highest degree of binding to calmodulin and the largest inhibitory effect on calmodulin-induced phosphodiesterase activity. In contrast, the antidepressants and anxiolytics showed significantly less binding to calmodulin and lower phosphodiesterase inhibition. As daechuine-S10 and sanjoinine-D strongly inhibited Ca^{2+}-dependent phosphodiesterase to an extent comparable to known antipschotics such as pimozide, fluphenazine and chlorpromazine (Table IV), both the alkaloids may exhibit a high level of calcium-specific binding to calmodulin.

In a previous report (Han *et al.*, 1993a), the sedative activity of several cyclopeptides and peptide alkaloids was assessed by employing a hexobarbital-induced sleeping time method in mice. The results correlated well with the Ca^{2+}-ATPase inhibition observed in this study. The lower the IC_{50} values on the enzyme were, the stronger the sedative activity. Park *et al.* (1996) reported the effects of sanjoinine-A, a major alkaloid in sanjoin, on the central nervous system and in general pharmacology. Sanjoinine-A depresses the spontaneous locomotor activity without motor uncoordination and it has a weak analgesic effect. Those effects are quantitatively similar to those of diazepam. However, it has a lower potency (20 times). Sanjoinine-A slightly depresses the spontaneous or acetylcholine-induced motility of the smooth muscles but the degree of the depressant effect varies according to the various tissues. The pharmacological activities of sanjoinine-A may be attributed to its specific inhibition of calmodulin-dependent Ca^{2+}-ATPase.

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