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Inhibition of Calmodulin-Dependent Protein Kinase II by Cyclic and Linear Peptide Alkaloids from *Zizyphus* Species

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The effects of sedative peptide alkaloids from *Zizyphus* species on calmodulin- dependent protein kinase II were investigated. Protein kinase II activity was assayed on the basis of its ability to activate tryptophan 5-monooxygenase as its substrate in the presence of calmodulin. All thirteen alkaloids tested were stronger inhibitors than chlorpromazine (IC_{50}, 98 μM) on calmodulin-dependent protein kinase II. Among them, the most potent inhibitor was daechuine S27 (IC_{50}, 2.95 μM), which was stronger than pimozide (IC_{50}, 15.0 μM).

Key words: Zizyphus alkaloids, Calmodulin-dependent protein kinase II, Peptide alkaloid, Calmodulin, Rhamnaceae

INTRODUCTION

We isolated several sedative cyclic peptide alkaloids (CPAs) and peptide alkaloids from the seeds of *Zizyphus vulgaris* Lamark var. *spinosus* Bunge (Rhamnaceae, "sanjoin" in Korean), and the fruits (Rhamnaceae, "daechu" in Korean) and the stem barks of *Z. jujube* Miller var. *inermis* Rehder (Han *et al.*, 1985, 1987, 1990, 1993a).

Sanjoinine A (frangufoline), a major CPA in sanjoin, has Ca^{2+} and Mg^{2+} ionophore activity (Park *et al.*, 1991). Sanjoinine A binds to calmodulin in a Ca^{2+}-dependent manner at two sets of its binding sites; the mole binding ratio to calmodulin was calculated as 2 at the high affinity sites (Kd, 1.1 μM) and 4 at the low affinity sites (Kd, 3.1 μM) (Han *et al.*, 1993b).

Calmodulin is an ubiquitous calciumbinding protein that regulates the activity of several calcium-dependent enzymes such as adenylyl cyclase, protein kinase II, Ca^{2+}-ATPase, phosphodiesterase, *etc* (Klee *et al.*, 1980). We demonstrated that sanjoinine A and its analogues inhibited the activation of Ca^{2+}-ATPase, and that sanjoinine D and daechuine S10 were strong inhibitors against calmodulin-dependent phosphodiesterase (Hwang *et al.*, 2001).

Calmodulin-dependent protein kinase II has been demonstrated to phosphorylate not only tryptophan 5-monooxygenase, tyrosine 3-monooxygenase, tubulin, and microtubule-associated protein 2 (MAP2) as its physiological substrates, but also casein and myosin light chain, presumably as its nonphysiological substrates. A prominent feature of calmodulin-dependent protein kinase II is its broad substrate specificity.Furthermore, this enzyme occurs most abundantly in neuronal tissues such as the cerebral cortex, brainstem, and cerebellum, suggesting that calmodulin-dependent protein kinase II may play a number of roles in the functioning of the nervous system (Fujisawa, 1984).

In this paper, we investigated the effects of cyclic and linear peptide alkaloids from *Zizyphus* species on calmodulin-dependent protein kinase II, using tryptophan 5-monooxygenase as the substrate.

MATERIALS AND METHODS

CPAs and peptide alkaloids were obtained by the methods described in the previous papers (Han *et al.*, 1985, 1987, 1989, 1990). PMSF(phenylmethylsulfonyl fluoride) and $MePteH_4$(2-amino-4-hydroxy-6-methyl tetrahydropteridine) were purchased from Aldrich Chem. Co.(Saint Louis, MO, USA) and another reagents were from Sigma Chem. Co.(Saint Louis, MO, USA); Tris [tris(hydroxylmethyl)aminomethane)], DTT (dithiothreitol), Trp (L-tryptophan), Fe^{2+}-[$Fe(NH_4)_2(SO_4)_2$], Hepes [*N*-(2-hydroxyethyl)piperazine-*N*'-(2-ethane sulfonic acid)], cata-

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lase, ATP, EGTA (ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetra acetic acid), calmodulin (from bovine brain), and 5-HTP (5-hydroxyl-L-tryptophan).

Tryptophan 5-monooxygenase assay

The enzyme was prepared by the following methods used by Nakada and Fujisawa (1982): All procedures were carried out at 0~4 °C. After adult Sprague-Dawley rats were sacrificed by decapitation, the brain-stems (approximately 70 g wet weight) were immediately removed and homogenized in three volumes of 50 mM Tris acetate buffer, pH 7.6 containing 2 mM DTT and 2 mM PMSF. The homogenate was centrifuged at 39,000 × g for 35 min. The supernatant was used for the enzyme source.

The activity of tryptophan 5-monooxygenase was assayed fluorimetrically by measuring the formation of 5-HTP by the method used by Friedman *et al.* (1972). The standard assay system contained 400 μM Trp, 200 μM $MePteH_4$, 2 mM DTT, 40 μM Fe^{2+}, 120 mM Hepes buffer, pH 7.6, 100 μg catalase, and a suitable amount of the enzyme in a final volume of 250 μL. The reaction was started by the addition of $MePteH_4$ and carried out at 30 °C for 20 min with shaking. The reaction was stopped by the addition of 25 μL of 40% perchloric acid. After precipitated protein had been removed by centrifugation, an aliquot (200 μL) of the supernatant was taken and added to 300 μL of 5M HCl. The fluorescence of the solution was measured at an excitation wavelength of 295 nm and an emission wavelength of 530 nm. One unit of tryptophan 5-monooxygenase is defined as the amount which produces 1 nmol 5-HTP/min at 30 °C.

Protein kinase II assay

The enzyme was prepared by the method used by Yamauchi and Fujisawa (1983) as follows: The cerebral cortices (45 g) of adult Sprague-Dawley rats were homogenized in five volumes of 50 mM Tris·HCl buffer, pH 7.6, containing 1 mM DTT, 1 mM EGTA and 1 mM PMSF. The homogenate was centrifuged at 39,000 × g for 30 min, and the supernatant was used for the enzyme source.

The activity of protein kinase II was assayed on the basis of its ability to activate tryptophan 5-monooxygenase. The standard incubation mixture contained 50 mM Hepes buffer, 0.4 mM Trp, 2 mM DTT, 0.12 mM $CaCl_2$, 10 mM NaF, 0.1 mM EGTA, 0.03 unit tryptophan 5-monooxygenase, 50 μg catalase, 0.05 mM Fe^{2+}, 0.5 mM ATP, 5 mM $Mg(Ac)_2$, 100 nM calmodulin, 0.3 mM $MePteH_4$, and a suitable amount of protein kinase II in a final volume of 0.4 mL. The reaction was started by the addition of $MePteH_4$ and carried out at 30°C for 20 min with shaking. The reaction was stopped by the addition of 40 μL of 60% perchloric acid. After the resulting precipitate was removed by centrifugation, 5-HTP was determined fluorimetrically. One unit of the activity of protein kinase II is defined as one incremental nmol 5-HTP produced under standard conditions in the presence of protein kinase II over controls without protein kinase II or calmodulin.

Protein determination

Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine albumin as a standard.

RESULTS AND DISCUSSION

Effects of CPAs and peptide alkaloids on calmodulin-dependent protein kinase II

Calmodulin-dependent protein kinase II was assayed on the basis of its ability to activate tryptophan 5-monooxygenase (Yamauchi and Fujisawa,1983). The preparations of tryptophan 5-monooxygenase and calmodulin-dependent protein kinase II to be used for assay were the isotonic soluble fractions from the rat brainstems and cerebral cortices, respectively. It was confirmed that the activation of tryptophan 5-monooxygenase showed a linear dependence from 0.1 to 0.5 units of calmodulin-dependent kinase II until the activation reached twofold from 0.025 to 0.05 units of the monooxygenase enzyme. Subsequently, the effects of cyclic peptide alkaloids (CPAs) and peptide alkaloids on calmodulin-dependent kinase II were investigated and compared to those of the known antipsychotics such as pimozide, fluphenazine, and chlorpromazine (Fig. 1). The IC_{50} values of the three known antipsychotics, used as positive controls, were similar to those of other researchers (Kuhn, *et al.*, 1980) : 15/28 μM for pimozide, 26/30 μM for fluphenzazine and 98/90 μM for chlorpromazine. All thirteen alkaloids exhibited stronger inhibition on calmodulin-dependent protein kinase II than chlorpromazine (IC_{50}, 98 μM).

The most potent alkaloid was daechuine S27 (IC_{50}, 2.95 μM), which was shown to be a stronger inhibitor than pimozide (IC_{50}, 15.0 μM). Sanjoinine F (IC_{50}, 18.2 μM) and G2 (IC_{50}, 19.0 μM) were stronger inhibitors than fluphenazine (IC_{50}, 26.0 μM). Daechuine S4 (IC_{50}, 86.0 μM) and S10 (IC_{50}, 57.0 μM) were weaker inhibitors than fluphenazine, while the alkaloids not mentioned above gave a similar activity to fluphenazine.

Structure-activity relationships (SAR)

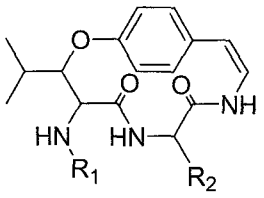
Cyclic peptide alkaloids (CPAs) are defined as basic compounds embodying an ansa structure, in which a 10- or 12-membered peptide-type bridge spans the 1,3 or 1,4 positions of a benzene ring. Compounds that fit this definition have so far been isolated from Rhamnaceae, Sterculiaceae, Pandaceae, Rubiaceae, Urticaceae,

Hymenocardiaceae, and Celastraceae. Expanding this definition, "linear peptide alkaloids" are defined as those compounds that can be derived from CPAs by scission of the bridge in an elimination reaction (Tschesche and Kaussmann, 1975).

The peptide alkalolids we isolated from *Zizyphus* species (Rhamnaceae) belong to the following classification :

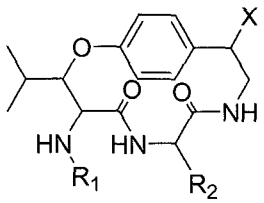
- Frangulanine type: a *p*-ansa compounds with a 14-membered ring incorporating a 10-membered bridge with a β-hydroxystyrylamine unit and β-hydroxyleucine (e.g., [1] and [2], Fig. 1).
- Zizyphine type: an *m*-ansa compound with a 13-membered ring incorporating a 10-membered bridge with a β-(2-methoxy-5-hydroxyphenyl)vinylamine unit and β-hydroxyproline (e.g., [4], Fig. 1).
- Linear peptide alkaloids: a compound with a free aldehyde group derived from CPA of the frangulanine type by scission of the bridge in an unusual enamide cleavage (Suh *et al.*, 1996) (e.g., [3], Fig. 1).

The SAR study of the peptide alkaloids on calmodulin-dependent protein kinase II revealed that : 1. Daechuine S27, the peptide alkaloid having the aromatic amino acid as the ring bond amino acid (R_2 group) and the methoxy group on the bridge benzene was shown to be the most potent inhibitor among all the drugs tested. Daechuine S27 (IC_{50}, 2.95 μM) was five-fold stronger than pimozide (IC_{50}, 15.0 μM); 2. Daechuine S4 (IC_{50}, 86.0 μM), the peptide alkaloid having a non-aromatic amino acid as the basic end amino acid (R_1, group) was far weaker than those having an aromatic amino acid (e.g., sanjoinine A, AhI, B, D, F, G1 in the range of IC_{50}, 23~35 μM); 3. Among the 14-membered CPAs having aromatic amino acid as



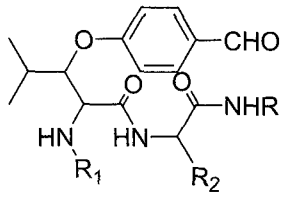
[1]

14-CPAs [1]	R_1	R_2	IC_{50} (μM)
Sanjoinine A	$N(Me)_2Phe(S)$-	$(CH_3)_2CHCH_2$-	24.1
Sanjoinine AhI	$N(Me)_2Phe(R)$-	$(CH_3)_2CHCH_2$-	27.2
Sanjoinine B	N(Me)Phe-	$(CH_3)_2CHCH_2$-	23.5
Sanjoinine F	$N(Me)_2Phe$-	$(CH_3)_2CHCH(OH)$-	18.2
Dachuine S4	$N(Me)_2Leu$-	$(CH_3)_2CHCH_2$-	86.0
Sanjoinenine	*t*-cinnamoyl-	$(CH_3)_2CHCH_2$-	35.0



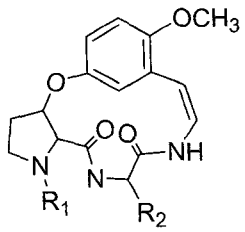
[2]

14-CPAs [2]	X	IC_{50} (μM)
Dihydrosanjoinine A	H	35.0
Sanjoinine D	OCH_3	33.0
Sanjoinine G1	OH	28.5



[3]

Peptide alkaloids [3]	R	IC_{50} (μM)
Sanjoinine G2	H	19.0
Sanjoinine A aldehyde	CHO	40.5



[4]

13-CPAs [4]	R_1	R_2	IC_{50} (μM)
Daechuine S10	$N(Me)_2Try$-	$CH_3CH_2CH(CH_3)$-	57.0
Daechuine S27	NH(Me)Ala-Val-	Benzyl-	2.95

Fig. 1. The chemical structure of cyclic and linear peptide alkaloids isolated from *Zizyphus* species, and their IC_{50} values on calmodulin-dependent protein kinase II. The IC_{50} values of the known antipsychotics pimozide, fluphenazine and chlorpromazine, used as positive controls, were measured to be 15.0, 26.0, and 98.0 μM, respectively.

the basic end amino acid (R_1, group), sanjoinine F (IC_{50},18.2 μM) with the hydroxyl group on the R_2 amino acid was a stronger inhibitor than the CPAs without the hydroxyl group (e.g., sanjoinine A, Ahl, B, D and G1 in the range of IC_{50}, 23~33 μM), and 4 Among the 14-membered CPAs having an aromatic amino acid as the basic end amino acid (R_1 group), the reduced (dehydrosanjoinine A), and the oxidized (sanjoinine D and G1) bridge unit on the olefinic bond of styrylamine did not affect inhibitory activity, as shown in [**2**] (Fig. 1).

Interestingly, CPAs and peptide alkaloids are quite different from each other in their SARs on the calmodulin-dependent enzymes, in the presence of calmodulin. The most potent inhibitors are daechuin S27 (this paper), sanjoinine A dialdehyde (Hwang, *et al.*, 2001) and daechuine S10 (Hwang, *et al.*, 2001) on protein kinase II, Ca^{2+} ATPase and phosphodieaterase, respectively. In a previous report (Han *et al.*, 1993a), the sedative activity of peptide alkaloids well correlated with the Ca^{2+}-ATPase inhibition but did not with the phosphodiesterase inhibition. The lower the IC values on Ca^{2+}-ATPase, the stronger the sedative activity (Han *et al.*, 1993a). In this paper, protein kinase II inhibition was demonstrated to not correlate with the sedative activity of the peptide alkaloids.

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, the antipsychotic drugs such as pimozide, fluphenazine and chlorpromazine showed the highest degree of binding to calmodulin and the largest inhibitory effect on calmodulin-induced phosphodiesterase activity. In contrast, the antidepressants and axiolytics showed significantly less binding to calmodulin and lower phosphodiesterase inhibition. Daechuine S10 and S27 belong to the 13-membered CPAs with the 5-hydroxy-2-methoxystyrylamine unit and *trans*-3-hydroxyproline ([**4**], Fig. 1). As the basic end amino acid moiety (R_1 group), daechuine S10 contains the aromatic amino acid, *N,N*-dimethyltryptophan, whereas daechuine S27 contains the aliphatic dipeptide, monomethyl alanylvaline. Furthermore, daechuine S10 possesses leucine as the ring bond amino acid moiety, whereas daechuine S27 contains phenylalanine. Since daechuine S10 is the stronger calmodulin-dependent phosphodiesterase inhibitor than daechuine S27, and the latter is the more potent calmodulin-dependent protein kinase II, it could be presumed that daechuine S10 and S27 may have antipsychotic and antidepressant properties, respectively. In conclusion, the structure activity relationships of the peptide alkaloids could pave the way for development of new drugs concerning calmodulin-dependent systems in the human body.

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