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Binding of Sanjoinine-A (Frangufoline) to Calmodulin

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A binding protein of radio-labeled sanjoinine-A (frangufoline) in rat brain cytoplasm was investigated, using an equilibrium dialysis technique. The labeled agent was bound to the cytosol fraction with two distinctly different types of sets in a calcium ion-dependent manner. The bound protein was identified as calmodulin by gel filtration of the sanjoinine-A bound cytosol fraction on a Sephadex G-75 column. Calmodulin was bound to sanjoinine-A at two sets of binding sites in the calcium ion-dependent manner. The mole binding ratio of sanjoinine-A to calmodulin were calculated as two at high affinity sites (Kd=1.1 μM) and four at low affinity sites (Kd=3.1 μM).

Key words: Cyclopeptide alkaloid, Frangufoline, *Zizyphus vulgaris* var. *spinosus*, Calmodulin binding.

INTRODUCTION

"Sanjoin", the seeds of *Zizyphus vulgaris* var. *spinosus* Bunge (Rhamnaceae),has been used as an important sedative and nervine herbal medicine in the Orient.In the earlier studies on its pharmacological aspect, hypnotic (Kawaguchi *et al.*, 1940), tranquilizing (Kim, 1971), sedative (Watanabe *et al.*, 1973; Shibata *et al.*, 1975; Woo *et al.*, 1980; Shin *et al.*, 1981), analgesic (Watanabe *et al.*, 1973), antiinflammatory (Watanabe *et al.*, 1973), antiarrhythmic (Cho *et al.*, 1976), and hypotensive (Ahn *et al.*, 1982) activities have been described.

We reported that alkaloids were the sedative principles of sanjoin (Han *et al.*, 1987a), and we isolated four aporphine alkaloids (APAs) and nine cyclopeptide alkaloids(CPAs) from the seeds (Han *et al.*, 1987a, 1989, 1990) and the fruits and stem bark of *Zizyphus jujuba* Miller var. *inermis* Rehder (Han *et al.*, 1987b, 1989a). Evaluation of the sedative activity (prolongation of sleeping time induced by hexobarbital) of the APAs and the CPAs resulted in the conclusion that nuciferine (APA) and sanjoinine-A (CPA) were major components of native sanjoin (Han *et al.*, 1993). On heat treatment, nuciferine was converted to less effective lysicamine (APA), while sanjoinine-A was in equilibrium with its epimeric artifact, sanjoinine-Ah1, which showed enhanced sedative activity (Han *et al.*, 1987c, 1993). Sanjoinine-A was identified as frangufoline, a known 14-membered CPA (Han *et al.*, 1987c).

The reports of the isolation and structure elucidation of more than 70 CPAs have appeared since 1966 (Tschesche and Kaussmann, 1975). No definitive pharmacological activity has been demonstrated for this class of natural product by other workers, although Legaris *et al.* (1978) reported that ceanothine-β and its synthetic model belonging to 14-membered CPA bound to monovalent (Li^+) and divalent (Ca^{2+}, Mg^{2+}) cations. The binding properties of them were determined by circular dichroism in acetonitrile.

The above informations prompted us to study the binding of radio-labeled sanjoinine-A to rat tissue homogenate, and we found that sanjoinine-A specifically binds to calmodulin, which is well known to be a calcium-binding protein in eukaryotic cells (Cheung, 1980).

MATERIALS AND METHODS

Materials

Sanjoinine-A was isolated from sanjoin by the method described in the previous paper (Han *et al.*, 1990). Tritium labelled sanjoinine-A was prepared by heating sanjoinine-A in CH_3OH/T_2O at 210℃ for 15 min, and was purified by preparative TLC on a silica gel plate, using $CHCl_3$/MeOH (20 : 1) as a developing solvent (Han *et al.*, 1987c, 1993). [^{3}H]Sanjoinine-A (5.68 mg) was dissolved in MeOH (0.5 ml) and 0.1 N HCl (3 ml), and then the mixture was adjusted to pH 7.0 with 0.1 M tris and water to make 0.85 mM [^{3}H] sanjoinine-A suspension (radioactivity: 12,000 cpm/0.01

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ml).The suspension was diluted 10-fold before use with 67 mM phosphate buffer (Na_2HPO_4, KH_2PO_4, pH 7.0) containing 5 mM $MgSO_4$, 0.05 mM $CaCl_2$ and 0.02% NaN_3.

Calmodulin(from bovine brain),ethyleneglycol-bis(β-aminoethylether)N,N,N',N'-tetraacetic acid (EGTA), Sephadex G-75, cellulose dialysis membrane and bovine serum albumin (BSA) were purchased from Sigma Chem Co. (St. Louis, USA).

Preperation of rat brain homogenate

Brains (5 g) obtained from male Sprague-Dawley rats weighing 180-200 g were disintegrated in a Waring blendor for one min at 4℃ with 10 ml of the phosphate buffer. The resultant mixture was centrifuged at 3,000×g for 5 min at 4℃. The supernatant was used as H fraction, which was subjected to recentrifuge at 45,000×g for 10 min at 4℃. The sediment was suspended into 10 ml of buffer to make a synaptosome (S) fraction, and the supernatant was used as a cytosol (C) fraction. Protein contents in the H,C and S fractions were 4.2, 3.3 and 7.1 mg BSA/ml, respectively, when measured by the method of Lowry *et al.* (1951).

Equilibrium dialysis

The experiment was performed at 37℃ in a dialysis cell (3 ml volume), in which cellulose dialysis membrane having a molecular weight cut off of 12,000 seperated one ml of 85 μM [^{3}H]sanjoinine-A (45.6 μg) suspension from one ml of brain homogenate or calmodulin dissolved in the phosphate buffer. A portion (100 μl) of the contents of each half-cell was counted in a liquid scintillation spectrometer (Hewlet Packard Co.), using a toluene cocktail.

RESULTS

Binding of labeled sanjoinine-A to rat brain homogenate

Fig. 1 shows time course of equilibrium between [^{3}H]sanjoinine-A and rat brain homogenate in the presence of calcium and magnesium ions. The brain homogenate reached equilibrium by about 8 hr and 48 hr, indicating that sanjoinine-A bound to it at two distinctly different sites. Similar results were obtained from other rat organs, such as liver,lung and heart (data not shown), suggesting that sanjoinine-A binds to protein(s) universally occuring in rat cells.

The brain homogenate was sub-fractionated into cytosol and synaptosomal fractions by centrifugation at 45,000×g, and then both the fractions were equilibrated with sanjoinine-A (Fig. 1). The former fraction reached about two times earler than the latter one at the high affinity site, indicating that the binding of sanjoinine-A to the cytosol fraction is more rapid in equilibrium than that to the synaptosomal one.

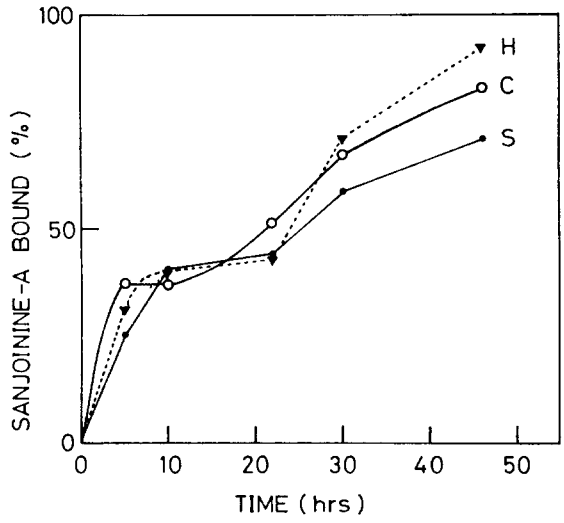

Fig. 1. Binding of sanjoinine-A to rat brain homogenate. Equilibrium dialysis was performed at 37℃ in a dialysis cell (3 ml volume), in which cellulose dialysis membrane having a molecular weight cut off of 12,000 separated one ml of 85 μM [^{3}H]sanjoinine-A (45.6 μg) suspension from one ml of brain homogenate. Each preparation was made in 67 mM phosphate buffer, pH 7.0, containing 5 mM $MgSO_4$, 50 μM $CaCl_2$ and 0.02% NaN_3. A portion (0.1 ml) of the contents of each half-cell was counted in a liquid scintillation spectrometer, using a toluene cocktail. H, 3,000×g supernatant of rat brain homogenate; C, cytosol fraction (45,000×g supernatant); S, synaptosome fraction (the suspension of 45,000×g precipitate of H).

Displacement of labeled sanjoinine-A from the cytosol fraction by unlabeled one

In order to investigate whether the binding of sanjoinine-A to the cytosol fraction of rat brain is a specific reaction or not, equilibrium dialyses were performed in the absence and the presence of cold sanjoinine-A, of which the concentration was equal to that of hot one. Fig. 2 shows that approximately half of the bound sanjoinine-A was displaced from the cytosol fraction by unlabeled sanjoinine-A. The results indicate that the binding is very specific and non-specfic binding occupies a few percent part of total binding.

Calcium dependence of the binding of sanjoinine-A to the cytosol fraction

Equilibrium dialyses were carried out in the absence and the presence of EGTA to study the effect of calcium ion on the specific binding of sanjoinine-A to the cytosol fraction. Fig. 3 shows that the binding ability of sanjoinine-A to the fraction completely was lost. It was also found that the non-specific binding was about five percent of the total binding.

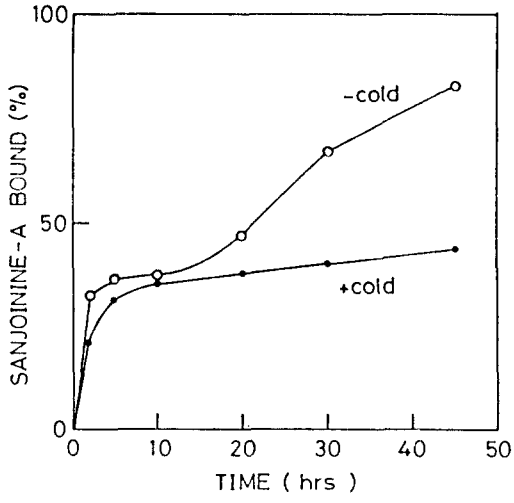

Fig. 2. Displacement of [^{3}H]sanjoinine-A from the cytosol fraction by cold sanjoinine-A. Equilibrium dialysis was carried out in the absence and presence of cold sanjoinine-A (45 μg) as described in the legend of Fig. 1.

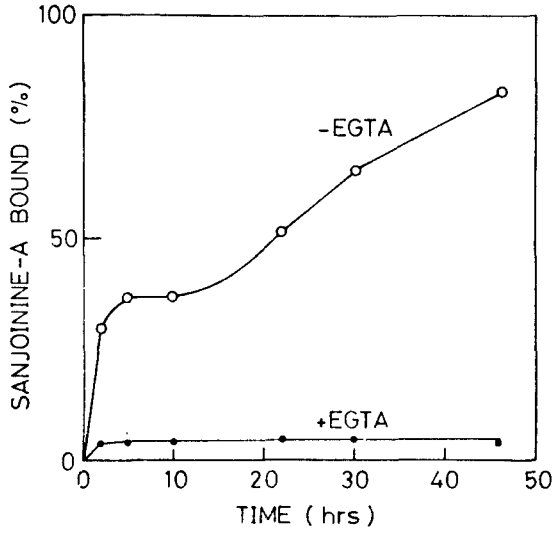

Fig. 3. Effect of calcium ion on the binding of sanjoinine-A to the cytosol fraction. Equilibrium dialysis was performed in the presence of either calcium ion (50 μM) or EGTA (150 μM) as described in the legend of Fig. 1.

Identification of sanjoinine-A binding protein(s)

In order to identify substance(s) binding to sanjoinine-A, gel filtration of bound and unbound [^{3}H]sanjoinine-A was conducted on a column of Sephadex G-75. After the labeled compound was equilibrated with the cytosol fraction for 46 hr, an aliquot (0.5 ml) of the contents of each half-cell was applied to the column. Fig. 4a shows the two peaks having radio-activity were found at the tube numbers of 17 and 43. The latter was absolutely the major peak. Fig. 4b shows the elution profile of unbound sanjoinine-A

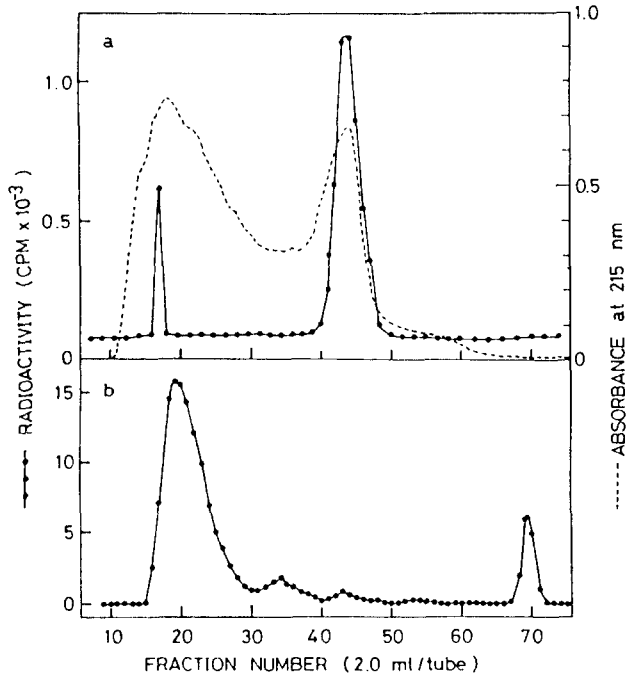

Fig. 4. Gel filtration of bound and unbound sanjoinine-A on a column of Sephadex G-75. After equilibrium dialysis of the cytosol fraction to [^{3}H]sanjoinine-A for 46 hr, one-half ml of the contents of each half-cell was applied to the column (size, 1×88 cm) and eluted with the phosphate buffer at 4°C. Detection of proteins was carried out by measuring absorbance at 215 nm. a, bound; b, unbound [^{3}H]sanjoinine-A.

with two peaks at the tube numbers of 19 and 69. It must be certain that the former is an aggregate of [^{3}H]sanjoinine-A and the latter is its free form, because the compound is very insoluble in the buffer used. It is remarkable that no free form of [^{3}H]sanjoinine-A was found in the elution profile shown in Fig. 4a.

Fig. 5a shows the elution profile of the cytosol fraction before and after equilibrium dialysis with cold sanjoinine-A. Only one peak bound to sanjoinine-A was found in tube no. 42. Its molecular weight was determined as about 17 KD by SDS-polyacrylamide gel electrophoresis (data not shown). Thus, it was estimated that the protein bound to sanjoinine-A was calmodulin. Fig. 5b shows the elution profile of the mixture of BSA and calmodulin on the column. The peak of the protein bound to sanjoinine-A (Fig. 5a) was superimposed on that of calmodulin (Fig. 5b). The minor peak having radio-activity shown in Fig.4a may be ascribed to rat serum albumin in the cytosol fraction.

Binding of sanjoinine-A to calmodulin

Calmodulin and sanjoinine-A were equilibrated under the same condition as that performed with the cytosol fraction. Sanjoinine-A was specifically bound

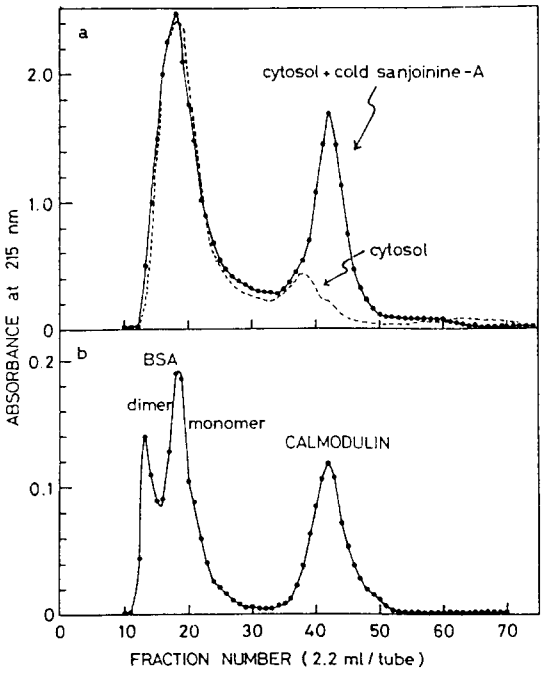

Fig. 5. Gel filtration of the cytosol fractions before and after equilibrium dialysis with cold sanjoinine-A, and of standard proteins on Sephadex G-75 column. (a) Cytosol fraction (one ml) before (---) and after equilibrium dialysis with cold sanjoinine-A (one mg in one ml suspension) for 46 hr. (b) Standard proteins (each 0.1 mg in 0.1 ml phosphate buffer): bovine serum albumin (BSA) and calmodulin. Gel filtration was carried out at 4℃ as described in the legend of Fig. 4.

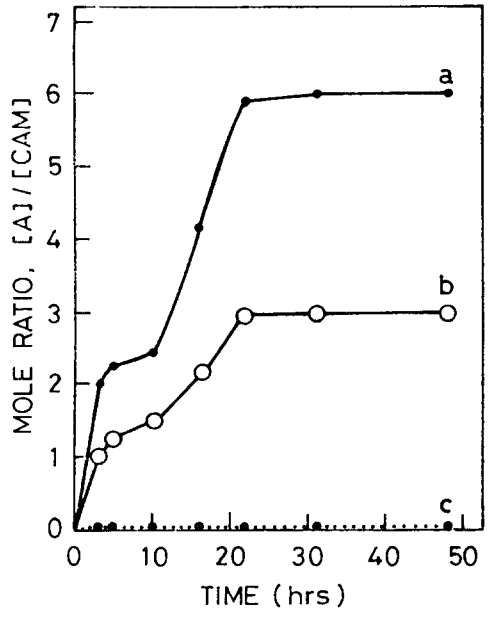

Fig. 6. Binding of [^{3}H]sanjoinine-A to calmodulin (a), displacement of [^{3}H]sanjoinine-A from calmodulin by cold one (b), and effect of EGTA on the binding (c). One ml of 9 μM calmodulin was dialyzed at 37℃ to one ml of 85 μM [^{3}H]sanjoinine-A (a), or to one ml containing each 85 μM concentration of labeled and unlabeled sanjoinine-A (b). Equilibrium dialysis was also conducted in the presence of EGTA (150 μM)(c). The radioactivity of a 100 μl aliquot was determined, and the concentration of [^{3}H]sanjoinine-A and its mole binding to calmodulin were calculated. A, sanjoinine-A; CAM, calmodulin.

to calmodulin at two sets of binding sites as shown in Fig. 6 (curve **a**). The mole binding ratio of sanjoinine-A to calmodulin was calculated as two and four at the high and the low affinity sites, respectively. The bound sanjoinine-A was displaced from calmodulin by the cold ligand (curve **b**). Moreover, the bindings at both the affinity sites were dependent on the presence of calcium ion (curve **c**). In order to measure dissociation constant (Kd), a given concentration (1.5 μM) of calmodulin was equilibrated with sanjoinine-A using a wide range of concentrations. As shown in Fig. 7, total binding of the ligand to calmodulin did not exceed six mole even at the higest concentration of the ligand. Thus, Kd values were directly determined, instead of Scatchard (1949) analysis, from the free ligand concentrations at 50% protein saturation of both the affinity sites (Hulme and Birdsall, 1992) as following; Kd=1.1 μM at the high affinity sites (N=2) and Kd=3.1 μM at the low affinity sites (N=4).

DISCUSSION

It is known that several antipsychotic agents including phenothiazines, butyrophenones and diphenylbutylpiperidines (Levin and Weiss, 1977, 1979; Weiss and Wallace, 1980), and smooth muscle relaxants including naphthalenesulfonamides (Hidaka *et al.*, 1980, 1981) all have a high degree of calcium-specific binding to calmodulin. The number of calcium-specific binding sites for these drugs on calmodulin appears to vary between one and three at the high-affinity, low-capacity sites. For example, a Scatchard analysis of the binding of several agents to calmodulin revealed two calcium-specific binding sites on calmodulin for trifluoperazine (Kd=1.5 μM), three for chloropromazine (Kd=5 μM), two for haloperidol (Kd=9 μM), one for pimozide (Kd=0.8 μM), and two sites for W-7 (Kd=11 μM). These agents except haloperidol and pimozide also bind to the low-affinity, high-capacity sites on calmodulin. For example, the number of the low affinity binding sites on calmodulin was determined as 24 for trifluoperazine (Kd=5 mM) (Levin and Weiss, 1977), 7 for chloropromazine (Kd=0.13 mM) (Levin and Weiss, 1979) and 7 sites for W-7 (Kd=0.2 mM) (Hidaka *et al.*, 1981). However, all these bindings at the low-affinity, high-capacity sites on calmodulin are independent on the presence of calcium ion. A similar distribution of binding sites was demonstrated by us for sanjoinine-A(N=2 and 4; Kd=1.1 and 3.1 μM) (Fig.

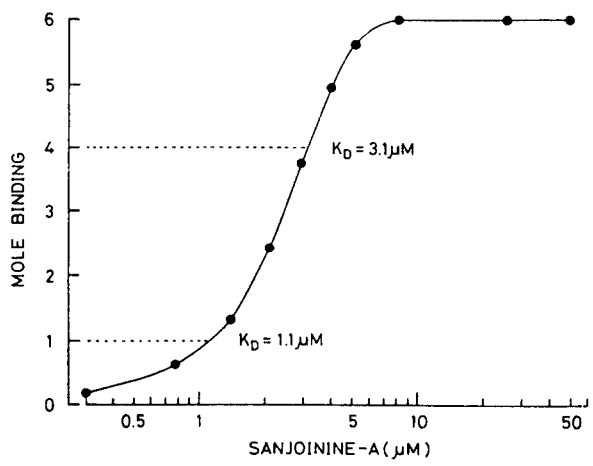

Fig. 7. Mole binding of sanjoinine-A to calmodulin. One ml of 1.5 μM calmodulin and one ml of [^{3}H]sanjoinine-A ranging 0.57 μM to 57 μM were equilibrated at 37℃ for 48 hr as described in the legend of Fig.1. The abscissa represents the concentrations of free sanjoinine-A at equilibrium.

7). However, the low affinity sites of sanjoinine-A on calmodulin were dependent on calcium ion (Fig. 6).

Calmodulin is known to activate various enzymes and to interact with cytoskeleton proteins (Klee *et al.*, 1980). Four moles of calcium ion binding to calmodulin are necessary for the enzyme activation. It has been shown the high affinity sites of calcium ion are located in the C-terminal half-region (C-domain), and the calcium ion binding to the N-terminal half-region (N-domain) is thought to be important for the enzyme activation (Yazawa *et al.*, 1987). It is anticipated that two moles of sanjoinine-A bind to C-domain and four moles to N-domain under the presence of calcium ion.

Calmodulin is widely distributed in eukaryotes (Klee *et al.*, 1980). The subcellular distribution of calmodulin has been examined in various cells and tissues. In rat brain 42% of the total calmodulin was particulate in the presence of calcium ion (Kakiuchi *et al.*, 1978). The binding of sanjoinine-A to the synaptosomal fraction is thought to be due to the particulate calmodulin (Fig. 1).

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