Isolation of Inhibitors of Adenylate Cyclase from Dan-shen, the Root of *Salvia miltiorrhiza*

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The effect of dan-shen extract, the root of *Salvia miltiorrhiza*, on adenylate cyclase was investigated in both rat brain and rat erythrocytes. The ETOAc fraction of the MeOH extract was proved to have significant inhibitory activity. Potent inhibitory principles in the ETOAc fraction were isolated and identified as 4 polyphenolic acids, rosemarinic acid, lithospermic acid, and their methyl ester derivatives.

Keywords dan-shen; *Salvia miltiorrhiza*; adenylate cyclase; rosemarinic acid; lithospermic acid

Introduction

Folk medicines and crude drugs contain many potentially useful substances and medicinal materials. We have looked for activators and inhibitors of adenylate cyclase in medicinal plants and crude drugs. Adenylate cyclase produces cyclic adenosine monophosphate (cyclic-AMP), which is known to mediate the actions of hormones and neurotransmitters on their target cells.21 The results of preliminary screening have already been reported.11 Forskolin, a diterpene extracted from the roots of *Coleus forskohlii* Briq. has been demonstrated to be a unique activator of adenylate cyclase in the brain and various tissues.23 To date, only the *C. forskohlii* species growing in India is known to produce forskolin.4

In a series of investigations, the MeOH extract of dan-shen was found to inhibit this enzyme system. Dan-shen, the dried roots of *Salvia miltiorrhiza* Bunge, is an important Chinese drug in the treatment of heart disease.53 Many chemists have studied the physiologically active constituents of this crude drug and have isolated many pigments, abietanoids.5

This paper describes the isolation of active components of dan-shen by monitoring its inhibitory activity against adenylate cyclase.

Materials and Methods

Preparation of Membrane Fraction from Rat Erythrocyte

All procedures were carried out at 4°C. Male rats of the Wistar strain (150–200 g) were killed under ether anesthesia and whole blood was collected into a tube containing saline with 10 mm ethylenediaminetetraacetic acid (EDTA). The erythrocytes were sedimented by centrifugation at 500 × g for 10 min and washed 3 times with saline. Then the erythrocytes were lysed hypotonically by addition of 10 volumes of 5 mm Tris-HCl/1 mm EDTA (pH 7.4). The lysate was centrifuged at 20000 × g, and the pellet was washed and suspended 3 times with the same buffer as above. The final pellet was suspended in 50 mm Tris-HCl buffer (pH 7.4), and was used as the membrane preparation in the following experiments.

Preparation of Membrane Fraction from Rat Brain Synaptosomal membranes were prepared from brains of male Wistar rats (150–200 g). The brains were homogenized in 10 volumes of 0.3 m sucrose solution containing 5 mm Tris-HCl and 1 mm EDTA (pH 7.4) with a Potter-Elvehjem homogenizer for 3 min. The homogenate was centrifuged at 500 × g for 10 min, and the supernatant was centrifuged at 20000 × g for 30 min. The pellet was resuspended in 20 volumes of 5 mm Tris-HCl buffer (pH 7.4). After standing for 30 min, the suspension was centrifuged at 20000 × g for 30 min. The final pellet was suspended in 0.25 m sucrose–50 mm Tris-HCl buffer (pH 7.4). The membrane suspension was stocked at −20°C.

Measurements of Adenylate Cyclase Activity and Protein

Adenylate cyclase activity was measured according to the method of Salomon et al.27 with some modifications.26 The assay system consisted of 50 mm Tris-HCl (pH 8.0), 10 mm MgCl₂, 8 mm theophylline, 12.0 mm phosphocreatine, 35 units/ml creatine phosphokinase, 0.01 mm guanosine triphosphate (GTP), 0.1 mm adenosine triphosphate (ATP), 1 µCi of [³²P]ATP (4 × 10⁶ cpml as a substrate and sample solution in a final volume of 200 µl. After incubation at 30°C for 30 min (brain preparation; for 15 min), the reaction was terminated by the addition of 200 µl of 10% sodium dodecyl sulfate/10 mm EDTA solution. The cyclic AMP produced was separated by successive chromatographies on Dowex 50W × 4 and neutral alumina and the radioactivity was measured in a liquid scintillation spectrometer. Protein was determined by the method of Lowry et al.28 using bovine serum albumin as the standard. The presence of 5% methanol in the final assay mixture did not affect the basal adenylate cyclase activity.

Materials Forskolin was purchased from Calbiochem, ATP, creatine phosphate, and creatine phosphokinase were purchased from Sigma Chemical Co., [³²P]ATP from Amerham International, Ltd., and GTP from Yamasa Co. All other drugs and chemicals were of reagent grade from commercial sources.

Results and Discussion

Preparation of Membrane Fraction from Rat Brain Synaptosomal membranes were prepared from brains of male Wistar rats (150–200 g). The brains were homogenized in 10 volumes of 0.3 m sucrose solution containing 5 mm Tris-HCl and 1 mm EDTA (pH 7.4) with a Potter-Elvehjem homogenizer for 3 min. The homogenate was centrifuged at 500 × g for 10 min, and the supernatant was centrifuged at 20000 × g for 30 min. The pellet was resuspended in 20 volumes of 5 mm Tris-HCl buffer (pH 7.4). After standing for 30 min, the suspension was centrifuged at 20000 × g for 30 min. The final pellet was suspended in 0.25 m sucrose–50 mm Tris-HCl buffer (pH 7.4). The membrane suspension was stocked at −20°C.

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Results and Discussion

Figure 1 shows the effect of each fraction separated according to Chart 1 on forskolin-activated and basal adenylate cyclase activities in both rat erythrocyte ghost and rat brain preparation. As shown in Fig. 1, the ETOAc fraction proved to have significant inhibitory activity. Inhibitory activities of the ETOAc fraction against basal and 5 µm forskolin-activated adenylate cyclase activities in rat brain preparation were stronger than those in the case of rat erythrocyte ghosts.

Figure 2 shows the concentration–response curve of ETOAc against basal and 5 µm forskolin-activated adenylate cyclase activities in both rat erythrocyte ghost and rat brain preparation. Figure 3 shows the concentration–response curve for forskolin in the presence or absence of the active ETOAc fraction. The ETOAc fraction has no effect on EC₅₀ of forskolin in both rat erythrocyte and rat brain adenylate cyclase systems. These
data demonstrate that the two adenylate cyclase systems are inhibited by EtOAc fraction in a similar manner. Therefore, the difference in the effect of the extract on the erythrocytes and on the brain in Fig. 1 may be due to the lipophilic nature of the latter, since the active principles in the EtOAc extract were expected to be lipophilic. As shown in Fig. 4, inhibitory activity of the EtOAc fraction against basal and 5 μM forskolin-activated adenylate cyclase activities in rat brain preparation was located in the 12th and 13th fractions. Figure 5 shows the inhibitory activity of compounds 1, 2, 3 and 4 separated by HPLC. Compounds 1, 2, 3, and 4 all showed strong inhibitory activity against basal and 5 μM forskolin-activated adenylate cyclase activities in rat brain preparation.

Compounds 1, 2, 3 and 4 all showed a positive reaction to iron chloride. The carbon-13 nuclear magnetic resonance (13C-NMR) spectra of the four compounds (1–4) isolated from dan-shen suggested similar chemical structures (Chart 2). The proton magnetic resonance (1H-NMR) and 13C-NMR spectra of 1 are strikingly similar to those of 3. The 13C-NMR spectra of 1 and 3 in CD3OD showed a unit of caffeic acid.10) The 1H-NMR spectra of 1 and 3 in CD3OD showed trans-olefin proton signals at δ 6.27 and δ 7.55 (each d, J = 15.8 Hz). The 13C-NMR spectrum of 1 showed 2 carboxyl groups (δ 168.4 ppm, δ 173.4 ppm), 1 methylene (δ 37.9 ppm), and 1 methine linked to oxygen (δ 74.6 ppm). By the 13C- and 1H-NMR analyses, 1 was identified as rosemarinic acid.10)

The 13C- and 1H-NMR spectra of 3 showed one more methyl ester group (δ 52.7, δ 3.70 ppm) than 1. Eventually, 3 The 1H- and 13C-NMR spectra of 2 closely resembled those of 4. In the 13C-NMR spectrum of 2, ten more carbon signals compared with 1 were observed, and among them one carbon signal was found to be due to a carbomethoxyl group (δ 53.2 ppm). Moreover, the other 27 carbon signals
of 2 corresponded closely with those reported for lithospermic acid.\(^{10}\) and the 3-(3,4-dihydroxyphenyl)lactic acid moiety was essentially the same as that of 1.\(^ {10}\) Therefore, the carbomethoxyl group of 2 should be located on the dihydrobenzofuran ring. These results indicated that 2 is the monomethylester of lithospermic acid.

In the \(^1\)H- and \(^13\)C-NMR spectra of 4, two carbomethoxyl groups were indicated. Therefore, 4 was concluded to be the dimethylester of lithospermic acid.

Rosmarinic acid was first isolated from \textit{Rosmarinus officinalis}, and has been reported to occur in several species of the family Labiatae.\(^ {13}\) This compound has been reported to be an anti-inflammatory constituent of \textit{Symphytum officinale}\(^ {12}\) and \textit{Ehretia microphylla}.\(^ {13}\) Though the isolation of lithospermic acid was first reported from

Lithospermum ruderale,\(^ {14}\) the structure reported was not correct. In 1975, Carmack \textit{et al.} undertook a study of the structure and antagonodotropic activity in animals.\(^ {15}\) This was the first demonstration that these polyphenolic acids have a strong inhibitory effect against adenylate cyclase. Further studies of the inhibitory effects of polyphenolic acids on adenylate cyclase as well as the relationship between this effect and the reported antagonodotropic effect are in progress.

References