Journal of Chromatography, 368 (1986) 462–467
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 18 903

Note

Use of perphenazine as a ligand for calmodulin affinity chromatography

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(First received March 27th, 1986; revised manuscript received June 26th, 1986)

Most of the stimulatory actions of calcium ions on enzymes are mediated by calmodulin, a low-molecular-weight protein which acts as an intracellular calcium receptor[1,2]. Several kinds of compounds, including phenothiazine-derived drugs, can inhibit calmodulin-stimulated enzymes[3,4]. These drugs bind to calmodulin in the presence of calcium, and this property has been utilized to develop affinity chromatography systems for calmodulin purification[5–7].

In the present report we describe the purification of bovine brain calmodulin by affinity chromatography using immobilized perphenazine, a drug that has not been previously reported as a calmodulin inhibitor. The isolated protein was characterized by physicochemical and biological methods.

MATERIALS AND METHODS

Coupling of perphenazine to sepharose 4B

Coupling was achieved by the bisoxirane method described by Sundberg and Porath[8]. A 50-g amount of vacuum-filtered Sepharose 4B (Pharmacia) was washed under vacuum filtration with 3 l of deionized water. A 60-ml volume of 0.6 *M* sodium hydroxide containing 3 mg/ml sodium borohydride and 60 ml of butene-1,4-diol diglycidyl ether (bisoxirane, Sigma) were added to the suction-dried gel. This mixture was incubated for 8 h at room temperature in a rotatory mixer. The gel was then vacuum filtered and washed with 8 l of deionized water. To this epoxi-activated Sepharose 4B, a mixture was added consisting of 450 mg perphenazine (courtesy of Essex, Spain), in 130 ml dioxane and 150 ml of 0.1 *M* sodium carbonate. The resulting mixture was incubated for 48 h at 70°C in a water-bath with continuous agitation (100 oscillations per min).

Care was taken to prevent exposure of all the solutions containing perphenazine to light. After coupling, the mixture was vacuum filtered and successively washed with 500 ml dioxane, 750 ml acetone, 500 ml ethanol, 3 l deionized water and 1 l of 10 m*M* Tris–HCl (pH 7.0) containing 0.02% sodium azide, and was kept in this buffer at 4°C until used.

0021-9673/86/$03.50

The effectiveness of the coupling procedure was studied by measuring the perphenazine content of the coupled Sepharose 4B, using the method described by Taha *et al.*[9] for phenothiazines. Briefly, standards (0–200 μg of perphenazine) and samples (0–100 μg of coupled Sepharose) were treated with 1 ml of chilled 75% sulphuric acid (final concentration 50%). The amount of Sepharose 4B was kept constant in both samples and standards by adding appropriate amounts (up to 100 μg) of uncoupled Sepharose 4B. After 2 h at room temperature, 500 μl of N-bromosuccinimide (35 mg in 100 ml distilled water) were added and after 30 min the absorbance was measured at 530 nm.

Calmodulin purification

A 100-g amount of bovine cerebrum was homogenized in 300 ml Tris–HCl containing 1 m*M* EGTA (pH 7.0). The homogenate was centrifuged at 25 000 *g* at 4°C for 1 h. Solid calcium chloride was added to the supernatant to a final concentration of 2 m*M*. This supernatant was then placed in a bath of boiling water for 3 min. After rapid cooling in an ice-bath, the mixture was centrifuged at 25 000 *g* for 30 min. The supernatant obtained was then passed through the perphenazine-Sepharose column (25 cm × 1.5 cm) at a rate of 18 ml/h. The column was successively washed with 200 ml of 10 m*M* Tris–HCl containing 1 m*M* calcium chloride (pH 7.0) and 5000 ml of the same buffer containing 0.5 *M* sodium chloride. The retained protein was eluted with 500 ml of 50 m*M* Tris–HCl, 10 m*M* EGTA (pH 8). Fractions of 2.8 ml were collected and assayed for calmodulin. The column was regenerated by successive washing with 1 l of 50 m*M* Tris–HCl, 0.5 *M* sodium chloride, 10 m*M* EGTA (pH 8) and with 1 l of 10 m*M* Tris–HCl, 1 m*M* EGTA, 0.02% sodium azide (pH 7). All the manipulations were done at 4°C.

Other procedures

The biological activity of calmodulin was assessed by the activation of calmodulin-free bovine brain phosphodiesterase obtained by the procedure of Cheung and Lin[10], using a radiometric assay of the phosphodiesterase (PDE) activity, based on the conversion of cyclic AMP into 5′-AMP, as previously described[11]. cAMP was obtained from Sigma and 8-(^{3}H) cyclic AMP (1036 GBq/mmol) was from Amersham.

Inhibition of PDE activity was used to compare the inhibitory power of different phenothiazines by incubating the supernatant (50 000 *g*, 30 min) of a rat brain homogenate in the presence of different amounts of phenothiazines. The PDE activity was measured as reported previously[11].

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed in 11% acrylamide[12].

Ultrogel Ac-44 chromatography was done in a 40 cm × 0.75 cm column equilibrated with 0.01 *M* sodium phosphate, 0.15 *M* sodium chloride (pH 7.5). Proteins were measured using the Bradford reagent (Bio-Rad).

RESULTS AND DISCUSSION

Rat brain phosphodiesterase was inhibited by three different phenothiazines: trifluoperazine, fluphenazine and perphenazine (Table I). Trifluoperazine lacks func-

TABLE I

RAT BRAIN PHOSPHODIESTERASE ACTIVITY IN THE PRESENCE OF DIFFERENT AMOUNTS OF CALMODULIN INHIBITORS

Data are expressed as percentages of the phosphodiesterase activity in the absence of inhibitors. Each value represents the mean of triplicate determinations ± the standard deviation.

Inhibitor concentration M	*Trifluoperazine*	*Fluphenazine*	*Perphenazine*
$1 \cdot 10^{-4}$	30.29 ± 0.92	21.92 ± 0.67	25.54 ± 1.53
$5 \cdot 10^{-5}$	32.83 ± 2.79	30.80 ± 0.66	33.45 ± 2.32
$1 \cdot 10^{-5}$	50.78 ± 1.07	57.59 ± 2.42	64.64 ± 2.75
$5 \cdot 10^{-6}$	94.68 ± 2.52	91.01 ± 3.09	73.17 ± 2.76

tional groups that may be bound covalently to Sepharose, and therefore it is not used in affinity chromatography. Fluphenazine has a primary alcohol, as does perphenazine, and has been used for the mentioned purposes. Nevertheless, perphenazine has a slightly higher inhibitory power than the other drugs. To our knowledge this is the first time that the inhibition of phosphodiesterase by this drug has been reported.

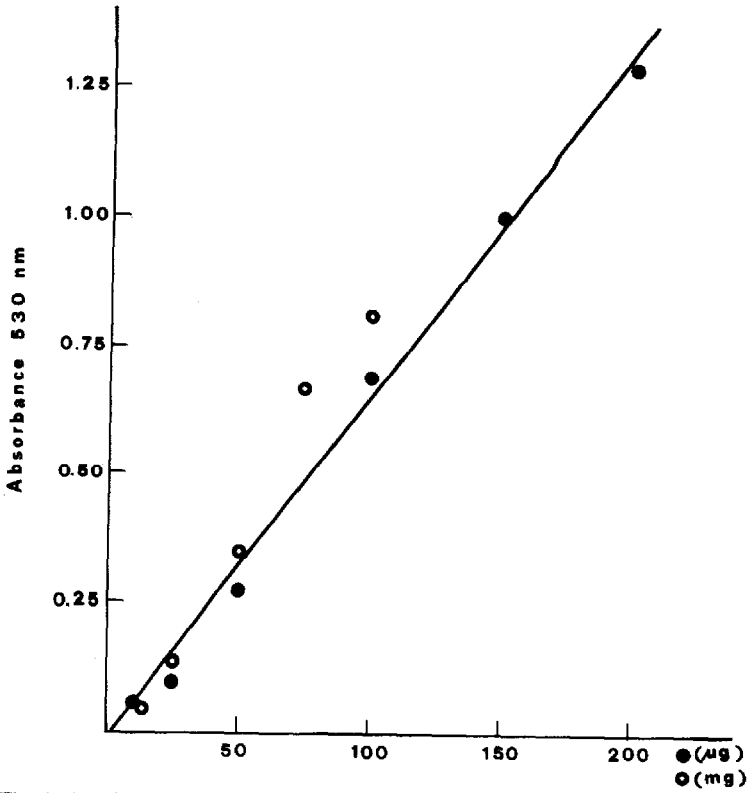

Fig. 1. Perphenazine (●) and perphenazine-Sepharose 4B conjugate (○) measured as indicated in Materials and methods. Data are the means of duplicate determinations.

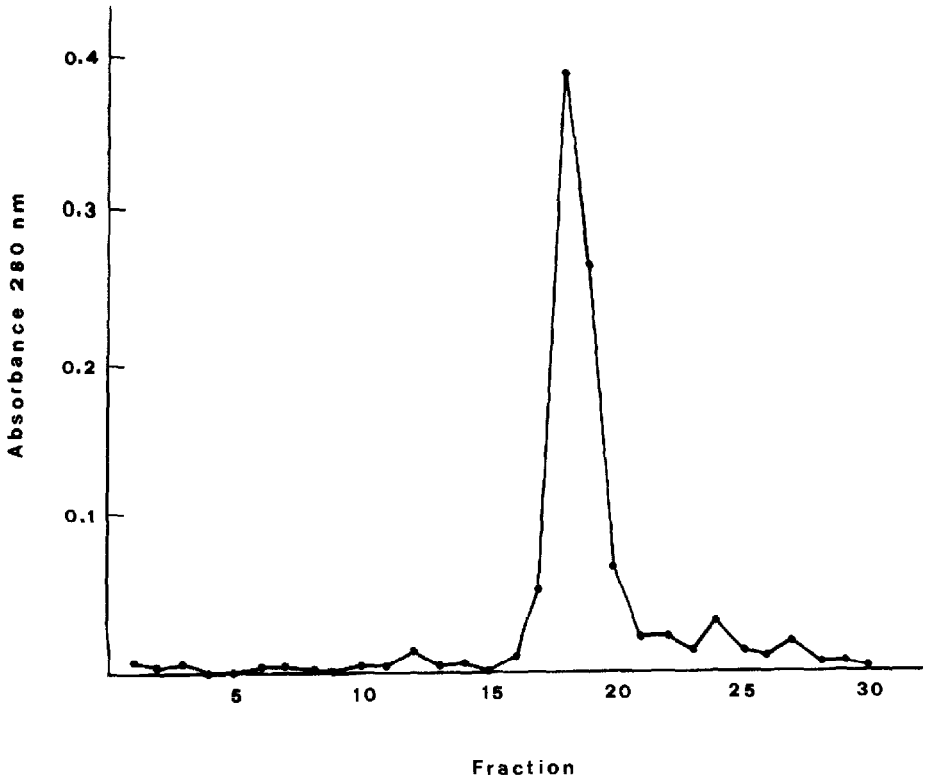

Fig. 2. Elution profile of the retained protein in perphenazine-Sepharose 4B affinity chromatography. Elution was started at the origin with 50 m*M* Tris, 10 m*M* EGTA.

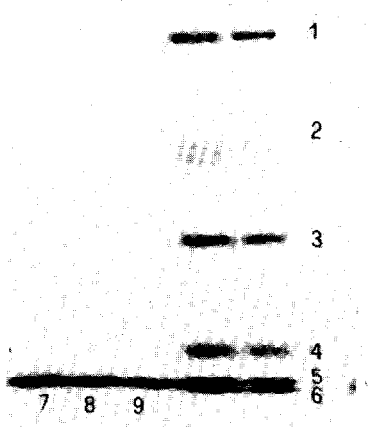

Fig. 3. SDS-PAGE of the protein obtained in perphenazine Sepharose 4B affinity chromatography. Standards of known molecular weight: 1 = bovine serum albumin (66 000); 2 = ovalbumin (45 000); 3 = carbonic anhydrase (29 000); 4 = trypsinogen (24 000); 5 = trypsin inhibitor (20 100); 6 = lactalbumin (14 200). Samples: purified calmodulin (7 and 8) and commercial calmodulin (9). The calculated molecular weight for purified and commercial calmodulin was 19 000.

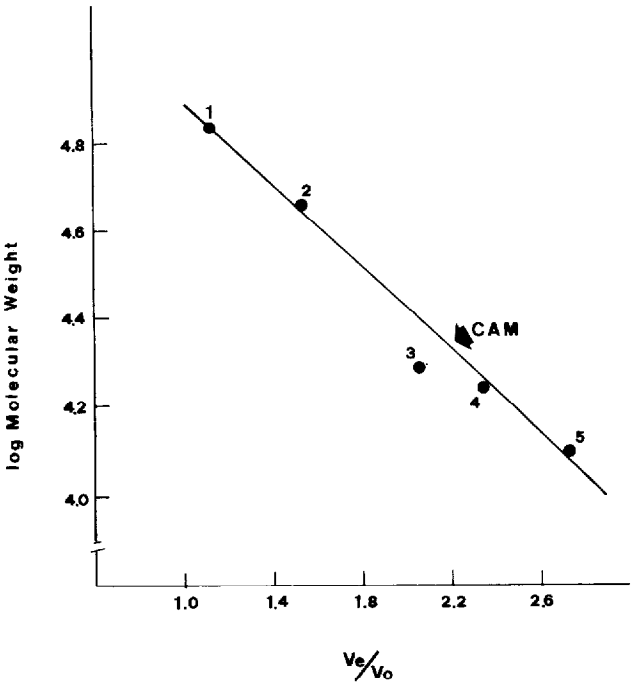

Fig. 4. Molecular weight determination of the protein (CAM) obtained in perphenazine Sepharose 4B affinity chromatography by gel permeation in Ultrogel Ac-44. The outer volume, V_0, was determined with blue dextran; V_e = elution volume. Standards of known molecular weight: 1 = human serum albumin (65 600); 2 = ovalbumin (45 000); 3 = myoglobin (18 800); 4 = lactalbumin (14 200); 5 = cytochrome *c* (12 700).

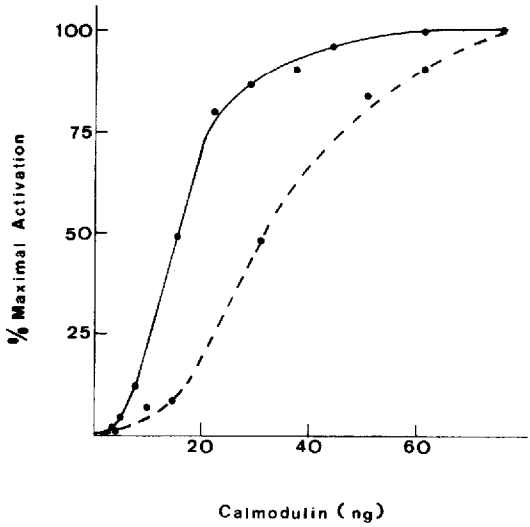

Fig. 5. Activation of calmodulin-deficient bovine brain phosphodiesterase by the protein obtained in perphenazine-Sepharose 4B affinity chromatography (continuous line) and by commercial calmodulin (dashed line). Data are expressed as the means of duplicate determinations.

The extent of coupling of perphenazine to Sepharose 4B was estimated to be 2.6 mg per g of vacuum-dried gel, representing 14.4% coupling. Fig. 1 shows that there is good agreement between the amount of the standard and the perphenazine-Sepharose 4B conjugate, indicating that the technique used[9] provides a good method for estimating coupling. It should be noted that the stated concentration of sulphuric acid is critical, and that it must be chilled to avoid caramelization.

Calmodulin was eluted after EGTA was passed through the column (Fig. 2). Approximately 5 mg of protein were recovered. Polyacrylamide gel electrophoresis of this peak showed only a band of molecular weight 19 000. The same R_F value was obtained with commercial calmodulin (Fig. 3). Ultrogel filtration gave a molecular weight of 19 500, with the same mobility as that of the reference preparation (Fig. 4). These data agree with those found in the literature. The purified protein activates calmodulin-free phosphodiesterase in a dose-related manner, 16 ng of the protein being equivalent to 1 unit of calmodulin, *i..e.*, the amount producing 50% of the maximum phosphodiesterase activation. Commercial calmodulin showed about one half of this activity (Fig. 5).

The results presented here show that perphenazine-Sepharose 4B is an excellent matrix for calmodulin purification by affinity chromatography. The protein seems to be very pure and shows a specific activity about twice that reported by purification using other ligands[6,7,10,13,14].

ACKNOWLEDGEMENTS

The authors are grateful to Essex, Spain, for the supply of perphenazine, and wish to thank Professor García Vargas, Department of Analytical Chemistry, Facultad de Ciencias, Universidad de Cádiz for his invaluable help during the course of this work, and Ms. Paloma Peers for editorial assistance. This work was carried out with grant No. 1180 from the Comisión Asesora de Investigación Cientifica y Técnica.

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