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INTERACTIONS BETWEEN CALMODULIN AND IMMOBILIZED PHENOTHIAZINES

APPLICATION TO THE PURIFICATION OF CALMODULIN FROM VARIOUS TISSUES BY AFFINITY CHROMATOGRAPHY*

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SUMMARY

The ability of a number of antipsychotic drugs such as phenothiazines to bind to calmodulin with high affinity in a calcium-dependent manner was applied to the study of the nature of their interactions with calmodulin. Thus, a series of phenothiazine derivatives and analogues were immobilized on agarose and examined for their binding characteristics to calmodulin. The binding of calmodulin to fluphenazine, perphenazine and 7-aminotriflupromazine involved on the one hand non-specific electrostatic interactions which are abolished by increasing the eluent salt concentration, and on the other hand, Ca^{2+}-dependent interactions which are reversed by EGTA addition. However, the Ca^{2+}-dependent binding of calmodulin was less specific with phenothiazine structural analogues (Neutral Red, diphenylamine) and was suppressed with other phenothiazine derivatives (thionine, Azure C, Toluidine Blue) or analogues (Brilliant Cresyl Blue). It is suggested that the calcium-dependent interactions between calmodulin and drugs involve a charge transfer π–π interaction which may be modulated by the electron donor–acceptor properties of the substituents of the aromatic ring. Affinity chromatography using immobilized fluphenazine was also used as the basis for the purification of calmodulin from a number of tissues in a rapid one-step procedure.

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Fig. 1. Phenothiazine derivatives and structural analogues.

INTRODUCTION

The role of calcium ions in regulating the response of eukaryotic cells to external stimuli is now well established[1]. Calcium appears to exert its biological effects through binding to calcium-dependent proteins[2,3]. Recent studies have shown that an ubiquitous calcium-binding protein, calmodulin, exists in eukaryotic cells and regulates a number of enzymes, including 3',5' cyclic nucleotide phosphodiesterase[4], adenyl cyclase[5], myosin light chain kinase[6,7], membrane ATPase[8], phosphorylase kinase[9] and a nicotinamide adenine dinucleotide kinase[10].

Calmodulin is an acidic low-molecular-weight protein, composed of 148 amino acids. It lacks cysteine and tryptophan and contains one mole of the unusual amino acid N^{ϵ}-trimethyllysine[11]. Chemical and physical studies have shown that calmodulin binds 4 moles of calcium[12]. This binding produces large changes in calmodulin conformation[13–16] and makes the calmodulin–Ca^{2+} complex capable of activating the calmodulin-dependent systems. Another property ascribed to calmodulin is its ability to bind a class of neuroleptic drugs, the phenothiazines[17], in a calcium-dependent manner through a still unknown mechanism. In the present study, this interesting property was used as the basis (i) for improving the understanding of the nature of the interactions between phenothiazines and calmodulin and (ii) for designing an efficient support for affinity chromatography in order to purify calmodulin from various tissue extracts in a rapid one-step procedure. Thus, a series of phenothiazine derivatives and structural analogues were selected and tested for their ability to inhibit calmodulin activity. Then, after immobilization on a matrix, the characteristics of their binding to calmodulin were examined.

MATERIAL AND METHODS

Materials

Fluphenazine, perphenazine and 7-aminotriflupromazine were kindly provided by E. R. Squibb and Sons (Princeton, NJ, U.S.A.). Toluidine Blue, Azure C, Thionine, Brilliant Cresyl Blue, Neutral Red and diphenylamine were obtained from Aldrich-Europe (Beerse, Belgium). [8-^{3}H]cyclic GMP (38.7 Ci/mmole) was purchased from New England Nuclear Corporation (Boston, MA, U.S.A.). Agarose beads (Ultrogel A-6) were obtained from Reactifs-IBF, France, Acriflavin–Sephadex G-25 was prepared according to the previously described technique[18]. The scintillation mixture used for the radioactivity countings (Monophase 40) was from Packard Instruments International SA (Rungis, France) and ampholines from LKB (Bromma, Sweden). The Ram testis calmodulin used in this study was very generously provided by Dr. J. G. Demaille[19].

Preparation of gels for affinity chromatography

Phenothiazine derivatives and structural analogues (see Fig. 1) were immobilized on 6% agarose beads (Ultrogel A-6) according to the epichlorohydrin method of Sundberg and Porath[18,20]. The final conjugates thus obtained contained 1.5–10 μmoles of drug per ml of gel[21] by HCl titration. In particular, the fluphenazine Ultrogel conjugate used for the purification of calmodulin contained 3.7 μmoles of fluphenazine per ml of gel and had a binding capacity of 4 mg of calmodulin per ml of resin.

Affinity chromatography of calmodulin

Tissues (liver, heart, pancreas, etc.) were homogenized in three volumes of buffer A (20 m*M* Tris–HCl buffer pH 7.0, 1 m*M* $CaCl_2$, 1 m*M* 2-mercaptoethanol) and the homogenate was centrifuged at 100,000 *g* for 60 min. The supernatant was heat-treated at 90°C for 1 min. The resulting suspension was then recentrifuged for 20 min at 24,000 *g* and applied to a column (3 × 1.14 cm) of immobilized phenothiazine derivative or analogue which had been equilibrated with buffer A.

After the sample was applied, the column was washed with ten column volumes of buffer A, and then with the same buffer containing 0.3 *M* NaCl until the A_{280} of the eluent dropped to its baseline level. Calmodulin was then eluted by a buffer containing 20 m*M* Tris–HCl pH 7.0, 1 m*M* 2-mercaptoethanol, 0.3 *M* NaCl and 10 m*M* ethyleneglycol bis(β-aminoethyl ether)-N,N′-tetraacetic acid (EGTA). Fractions (3 ml) were collected at a flow-rate of 12 ml/h and aliquots (50 μl) were tested for their ability to activate the calmodulin-dependent phosphodiesterase. The active fractions were pooled, adjusted to 10 m*M* $CaCl_2$, extensively dialysed against buffer A and lyophilized. All operations were performed at 4°C.

Phosphodiesterase assay for calmodulin

Calmodulin was assayed by its ability to activate a standard quantity (5 μg) of a calmodulin-dependent phosphodiesterase from rat pancreas prepared according to Kakiuchi *et al.*[22].

The standard reaction mixture contained in a final volume of 0.1 ml: 50 m*M* Tris–HCl buffer (pH 7.5), 30 μ*M* Ca^{2+}, 200 m*M* NH_4Cl, 1 μ*M* [^{3}H]cyclic GMP ($1 \cdot 10^5$ cpm per 0.1 nmol), 5 μg phosphodiesterase and appropriate amounts of calmodulin. The reaction was initiated by addition of the substrate and carried out at 30°C for 10 min, then stopped by boiling for 1 min. The entire contents of the assay tube were applied onto acriflavin–Sephadex G-25 gel packed in 5-cm-high pasteur pipettes equilibrated in 0.2 *M* ethylmorpholine buffer (pH 7.0) containing 0.3 *M* NaCl. The 5′-GMP formed was eluted with the same buffer as previously described[23]. Results were calculated as nmoles of 5′-GMP formed per 10 min and per mg of enzyme.

Other methods

Sodium dodecyl sulphate (SDS) (0.1%), 10–20% linear gradient polyacrylamide gel electrophoresis was performed in slab gels with the buffer system described by Maizel[24]. Isoelectrofocusing (pH 3.6–5.2) was performed in 5% polyacrylamide gel slabs according to Basset *et al.*[25].

RESULTS

A number of antipsychotic phenothiazine drugs are capable of inhibiting several calmodulin-activated systems[17,26,27]. As shown in Fig. 2, phenothiazine derivatives such as fluphenazine, perphenazine and 7-aminotriflupromazine (see Fig. 1) antagonized the calmodulin-stimulated cyclic nucleotide phosphodiesterase. Concentrations as low as 10 μ*M* were sufficient to produce a 50% decrease in the activation of phosphodiesterase by calmodulin. However, other phenothiazine derivatives including Toluidine Blue, Azure C and thionine were without effect on phosphodiesterase

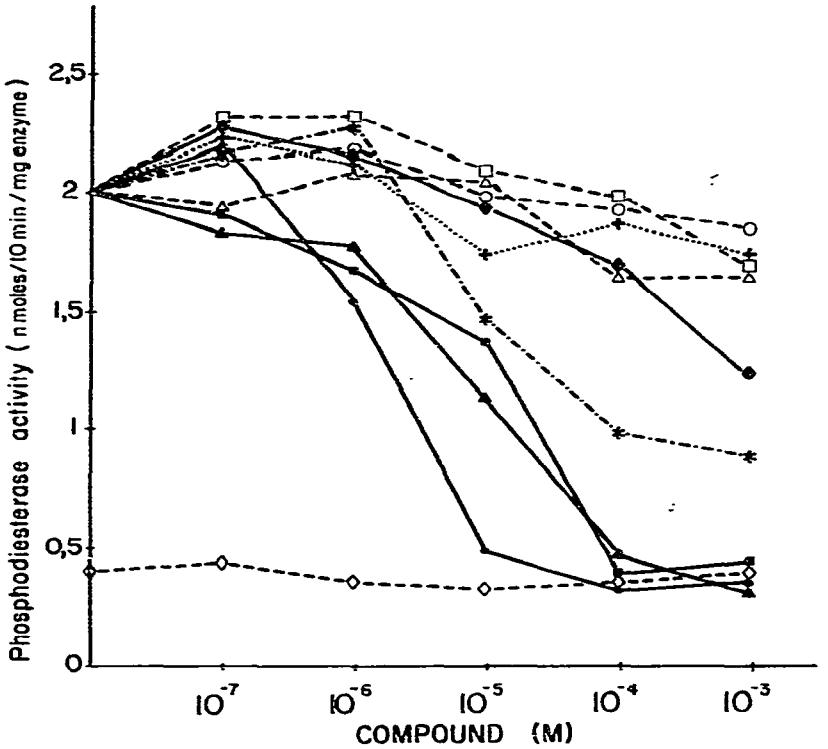

Fig. 2. Effects of phenothiazine derivatives and structural analogues on cyclic GMP phosphodiesterase in the presence of calmodulin. The assay medium contained 5 μg cGMP phosphodiesterase from rat pancreas, and 0.1 μg ram testis calmodulin. ◇—◇, Basal activity in the absence of calmodulin; ●—●, fluphenazine; ■—■, perphenazine; ▲—▲, 7-aminotriflupromazine; ○ — ○, Toluidine Blue; □ — □, Azure C; △ — △, thionine; + ·· +, Brilliant Cresyl Blue; ✳—·—✳, Neutral Red; ◆—◆, diphenylamine. Each point is the mean from three experiments.

activity even at concentrations as high as 1 m*M*. In addition, structural analogues such as Brilliant Cresyl Blue and diphenylamine were also uneffective, whereas Neutral Red inhibited calmodulin activity by 50% at about 50 μM. None of the phenothiazine derivatives or analogues affected the phosphodiesterase activity in the absence of calcium (results not shown).

According to these results the phenothiazine derivatives and structural analogues mentioned above were also tested for their calmodulin-binding capacity. They were immobilized on 4% agarose beads using the epichlorhydrin method, which prevents secondary electrostatic interactions, in contrast to the cyanogen bromide method[28].

Affinity chromatography of calmodulin on immobilized phenothiazine derivatives

To compare the binding of calmodulin to phenothiazine derivatives, extracts prepared from rat liver were applied to immobilized phenothiazines in the presence of $CaCl_2$. Fig. 3A illustrates a typical elution profile for the chromatography of rat liver calmodulin on fluphenazine–agarose. With extensive washing of the column in the presence of Ca^{2+}, most of the applied protein was eluted and the absorbance of the

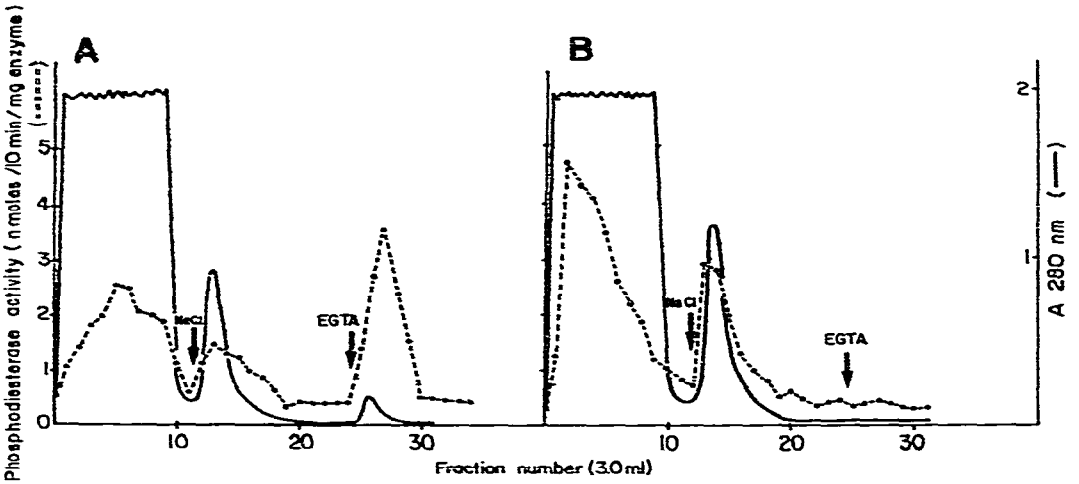

Fig. 3. Phenothiazine–agarose chromatography of rat liver calmodulin. A heat-treated extract of rat liver was applied on a phenothiazine column as described in Materials and methods. The column was washed with the equilibration buffer (buffer A) followed by the same buffer containing 0.3 *M* NaCl and then eluted by replacement of calcium by 10 m*M* EGTA in the buffer. Column: 3 × 1.14 cm. Flow-rate: 12 ml/h. 3-ml fractions were collected. Chromatography was followed spectrophotometrically at 280 nm. Aliquots (50 µl) were tested for their ability to activate the calmodulin-dependent phosphodiesterase. A, Fluphenazine–agarose; B, Azure C–agarose.

eluent dropped to zero. As shown by the phosphodiesterase test (Fig. 3A) and by SDS–polyacrylamide gel electrophoresis (Fig. 4), some calmodulin eluted during the salt wash. When buffer containing 10 m*M* EGTA was applied to the column, a small peak was eluted. Fractions from this peak were shown by gel electrophoretic analysis to contain a single low-molecular-weight polypeptide (Fig. 4) which stimulated the calmodulin-dependent phosphodiesterase preparation (Fig. 3A).

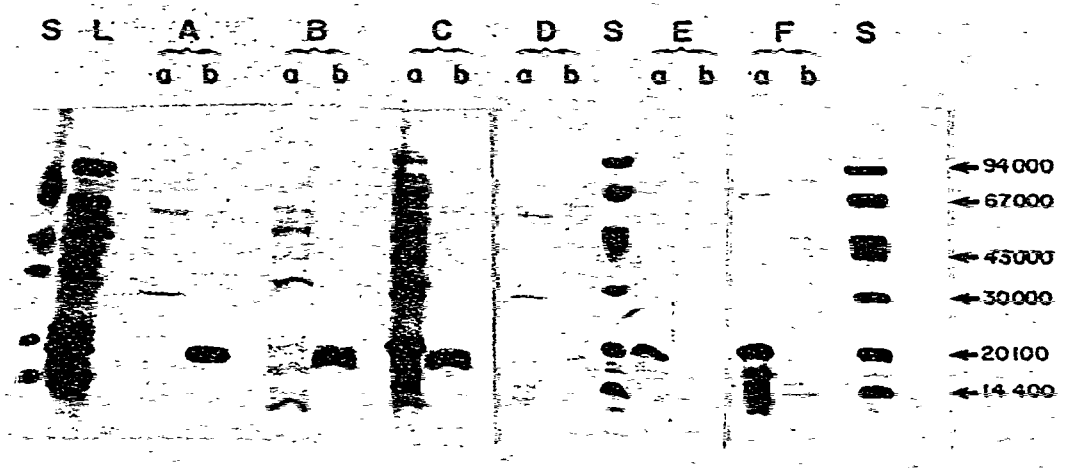

Fig. 4. 0.1% SDS, 10–20% polyacrylamide linear gradient gel electrophoresis of NaCl (a) and EGTA (b) peaks eluted from immobilized phenothiazine derivatives. S = Standards; L = rat liver extract; A = fluphenazine–agarose; B, perphenazine–agarose; C = 7-aminotriflupromazine–agarose; D = Toluidine Blue–agarose; E = Azure C–agarose; F = thionine–agarose.

Similar chromatographic profiles were obtained with immobilized perphenazine and 7-aminotrifluprómazine, these compounds differing from fluphenazine by the halogen in position 2 for the former, and the N(10) lateral chain for the latter. As shown by electrophoresis on SDS–polyacrylamide gels (Fig. 4), some calmodulin was eluted during the salt wash, but most was recovered as a single protein in the EGTA elution peak.

By contrast, with other immobilized phenothiazine derivatives such as Toluidine Blue, Azure C and thionine which lack halogen of the 2 position and lateral chain at N(10), the elution profiles showed a different pattern (Fig. 3B). Some calmodulin was retained on the column without calcium dependence and was eluted during the salt wash, simultaneously with other components, but none was recovered in the EGTA fractions (Fig. 4).

Affinity chromatography of calmodulin on immobilized phenothiazine structural analogues

Phenothiazine analogues such as Brilliant Cresyl Blue and Neutral Red differing from phenothiazines by the substituents on the aromatic ring were also immobilized on agarose beads. As shown in Fig. 5, with Brilliant Cresyl Blue, some calmodulin was retained on the column without calcium dependence and eluted during the salt wash, but none was recovered in the EGTA fractions. However, with im-

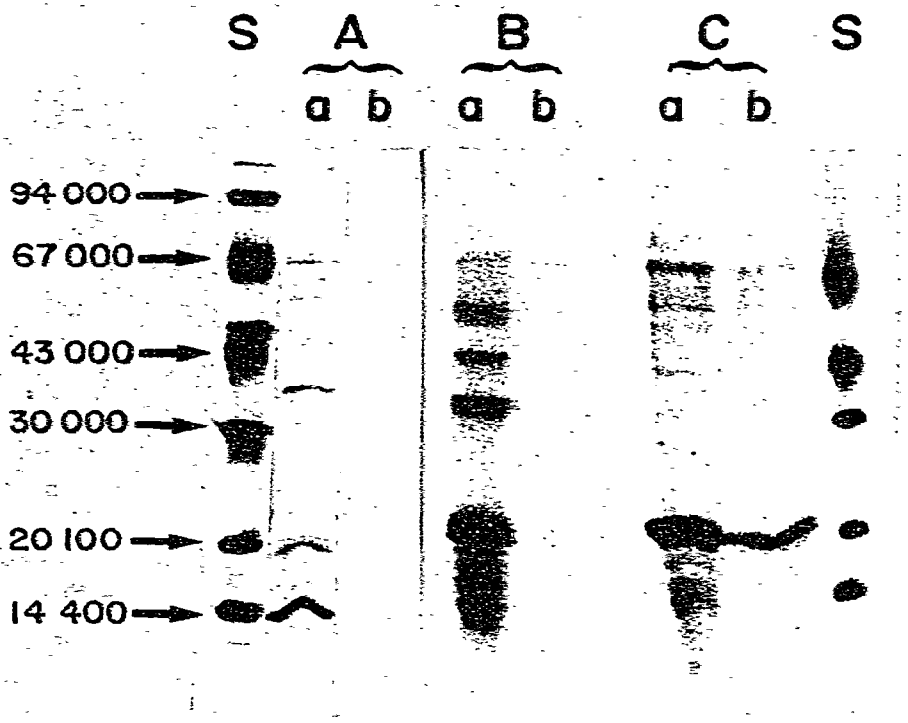

Fig. 5. 0.1% SDS, 10–20% polyacrylamide gradient gel electrophoresis of NaCl (a) and EGTA (b) peaks eluted from immobilized phenothiazine structural analogues. S = Standards; A = Brilliant Cresyl Blue–agarose; B = Neutral Red–agarose; C = diphenylamine–agarose.

mobilized Neutral Red and diphenylamine some calmodulin was retained on the column and eluted with EGTA, but several other components coeluted with calmodulin (Fig. 5).

Properties of calmodulins purified by affinity chromatography on fluphenazine–agarose

According to the above results, immobilized fluphenazine was used as the basis for the purification of calmodulin from a number of tissues by affinity chromatography. By this method, homogeneous calmodulin was obtained from bovine brain, rat liver, rat pancreas or rat heart. As isolated, in the presence of 10 m*M* EGTA, all calmodulins migrated with the same mobility as standard ram testis calmodulin, showing a single Coomassie Blue staining band in polyacrylamide gels, corresponding to an apparent molecular weight of approximately 19,500 (Fig. 6). In addition, as previously reported by Burgess *et al.*[29] and by Autric *et al.*[19], these calmodulins showed a different migration rate when electrophoresis was carried out in the presence of calcium. When subjected to isoelectrofocusing in 5% polyacrylamide gels, all calmodulins migrated as single bands with a p*I* of about 4.2 (Fig. 7).

A widely used functional test for calmodulin is its ability to activate preparations of calmodulin-dependent cyclic nucleotide phosphodiesterase in a calcium-dependent manner. Fig. 6 shows that the activation curves for the various calmodulins obtained by affinity chromatography are very similar to that obtained for standard ram testis calmodulin. The calmodulins increased the activity of the phosphodiesterase preparation 4–5-fold. No stimulation occurred in the absence of calcium (data not shown).

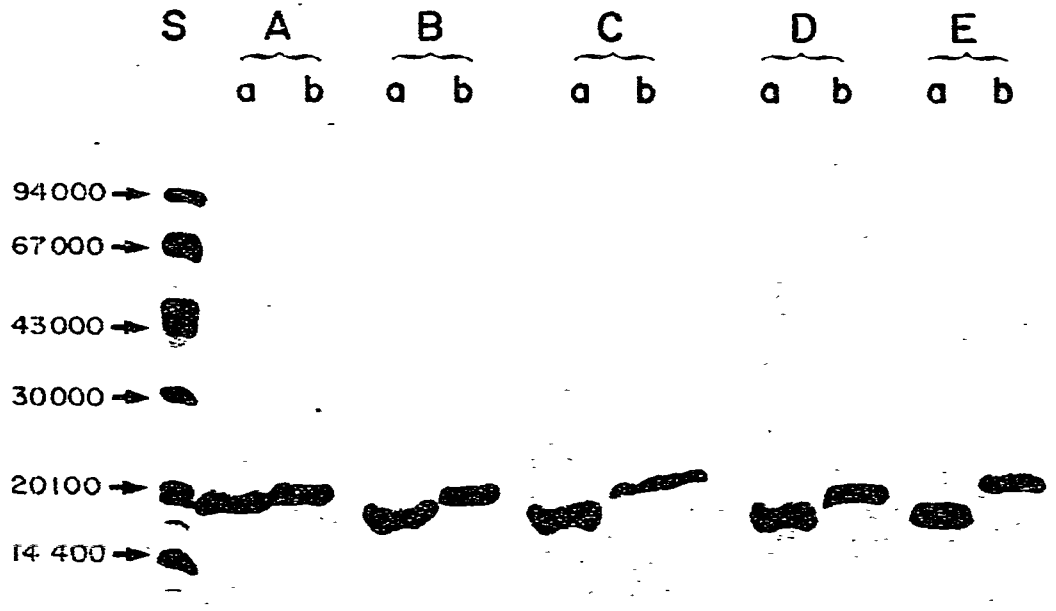

Fig. 6. 0.1% SDS, 10–20% polyacrylamide linear gradient gel electrophoresis of purified calmodulins (5 μg) in the presence of 10 m*M* $CaCl_2$ (a) or 10 m*M* EGTA (b). S = Standards; A = ram testis calmodulin; B = bovine brain calmodulin; C = rat liver calmodulin; D = rat pancreas calmodulin; E = rat heart calmodulin.

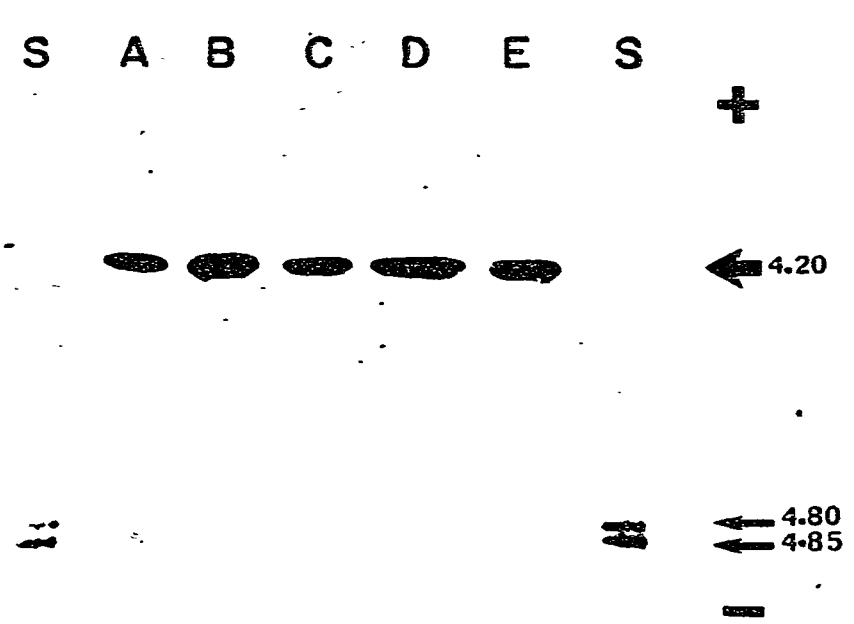

Fig. 7. Isoelectrofocusing (pH 3.6–5.2) of purified calmodulins (10 μg) on 5% polyacrylamide gels. S = Standard albumin; A–E as in Fig. 6.

DISCUSSION

The present report describes the use of affinity chromatography for studying the interactions between phenothiazines or analogues and calmodulin. By this method, immobilized phenothiazine derivatives substituted by an halogen in position 2 and a lateral chain at N(10) (fluphenazine, perphenazine, 7-aminotrifluprom azine) were found to have a similar chromatographic behaviour and to bind calmodulin with high affinity and specificity. This binding involves, on the one hand, non-specific electrostatic interactions which are abolished by increasing the eluent salt concentration, and on the other hand, Ca^{2+}-dependent interactions which are reversed by EGTA addition. Although various non-specifically bound proteins were eluted simultaneously with calmodulin by increasing the salt concentration, calmodulin obtained by EGTA elution was homogeneous.

The use of immobilized phenothiazine derivatives and structural analogues was helpful in the understanding of the nature of the interactions between calmodulin and phenothiazines. When the halogen substituents (electron donors) were suppressed and NH_2, $NHCH_3$ or $N(CH_3)_2$ groups (electron acceptors) were introduced in the molecule, the phenothiazine derivatives thus obtained (Toluidine Blue, Azure C, thionine) did not bind calmodulin in a calcium-dependent manner, since no calmodulin was obtained by EGTA elution. However, they kept their ability to bind calmodulin through non-specific electrostatic interactions. Further suppression of the sulphur in the heterocycle and its replacement by oxygen (as in Brilliant Cresyl Blue) did not alter these electrostatic interactions. However, when the sulphur was replaced by nitrogen in the heterocycle (as in Neutral Red) the calcium-dependent interactions

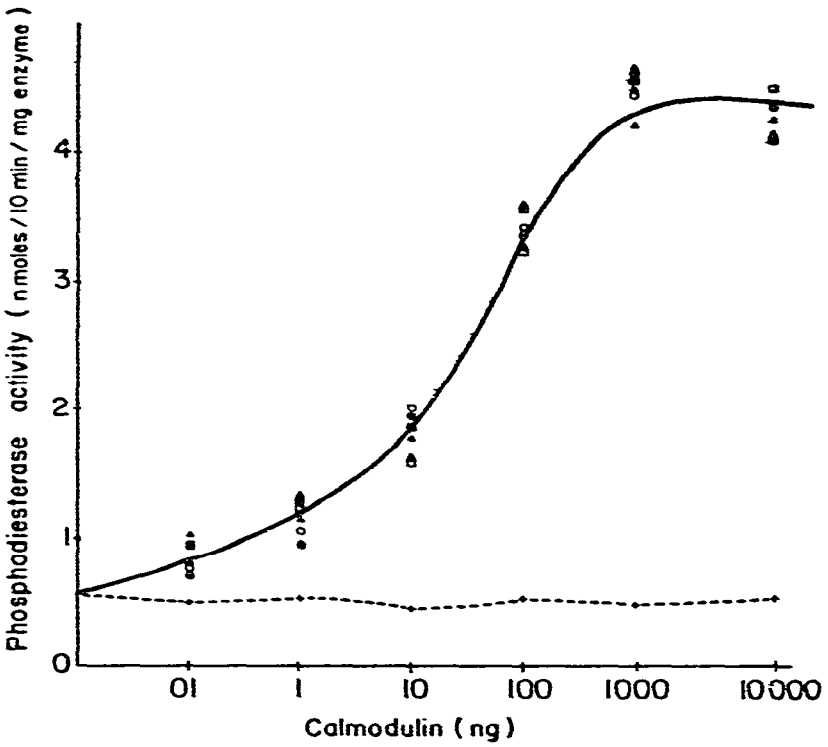

Fig. 8. Activity of rat pancreas cyclic GMP phosphodiesterase in the absence (+--+) and in the presence of various amounts of calmodulin from rat testis (●), bovine brain (▲), rat brain (■), rat liver (○), rat pancreas (△) and rat heart (□). All assays contained 30 μM calcium and 5 μg phosphodiesterase. Each point is the mean from three experiments.

were restored but the affinity and the specificity of the binding remained poor, since contaminants coeluted with calmodulin at the EGTA step. The same phenomenon was observed with diphenylamine which lacks electron acceptor–donor substituents and the sulphur.

From these results, two aromatic rings joined by a nitrogen as in diphenylamine seem necessary for drugs to interact in a calcium-dependent manner with calmodulin. Furthermore, electron donor (halogen) substituents increase the affinity and the specificity of the calcium-dependent binding of calmodulin. By contrast, electron attractor (amino) substituents decrease these properties. Therefore, the calcium-dependent interactions between calmodulin and drugs seem to involve a charge transfer π–π interaction which may be modulated by the electron donor–acceptor properties of the substituents of the aromatic rings. According to these results, the drugs which bind calmodulin in a calcium-dependent manner with the highest affinity and specificity are the phenothiazine derivatives substituted by an halogen (fluphenazine, perphenazine, 7-aminotriflupromazine). Furthermore, these drugs have the highest capacity to antagonize the calmodulin-stimulated systems. These results are in agreement with recent reports[14,16] providing evidence that, when binding to calmodulin, calcium induces conformational transitions affecting a number of aromatic residues (phenylalanine). These conformational changes would be responsible for the

binding of certain aromatic ligands such as phenothiazines[13–16] which then antagonize the interaction of calmodulin with its target enzymes.

Affinity chromatography using immobilized antipsychotic drugs is a valuable tool not only for studying the nature of the interactions between calmodulin and drugs but also for the search for new calmodulin-binding drugs. Furthermore, this procedure can also be used for the purification of calmodulin from various tissues as previously described by Jamieson and Vanaman[30] and by Charbonneau and Cormier[31]. In the present study, with fluphenazine coupled to the agarose matrix by the epichlorhydrin method[18], which avoids the non-specific electrostatic secondary interactions displayed by the cyanogen-bromide coupling method[28,30], homogeneous calmodulin was rapidly obtained from a number of tissue sources in only one step. All preparations of calmodulin prepared by fluphenazine–agarose affinity chromatography showed physical and chemical properties (molecular weight, acidity, heat stability and biological activity) similar to those of previously characterized mammalian calmodulins[32,33], suggesting that this procedure can be employed for the purification of calmodulin from any tissue source.

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REFERENCES

1 M. J. Berridge, *Advan. Cyclic Nucl. Res.*, 6 (1975) 1–98.
2 W. Y. Cheung, *Science*, 207 (1980) 19–27.
3 A. R. Means and J. R. Dedman, *Nature (London)*, 285 (1980) 73–77.
4 W. Y. Cheung, *J. Biol. Chem.*, 246 (1971) 2859–2869.
5 C. O. Brostrom, Y. C. Huang, B. M. Cl. Breckenridge and D. J. Wolff, *Proc. Nat. Acad. Sci. U.S.*, 72 (1975) 64–68.
6 R. Dabrowska, J. M. F. Sherry, D. K. Aromatorio and D. J. Hartshorne, *Biochemistry*, 17 (1978) 253–258.
7 M. P. Walsh, B. Vallet, F. Autric and J. G. Demaille, *J. Biol. Chem.*, 254 (1979) 12136–12144.
8 H. W. Jarrett and J. T. Penniston, *J. Biol. Chem.*, 253 (1978) 4676–4682.
9 P. Cohen, A. Burchell, J. G. Foulkes, P. T. W. Cohen, T. C. Vanaman and A. C. Nairn, *FEBS Lett.*, 92 (1978) 287–293.
10 J. M. Anderson, H. Charbonneau, H. P. Jones, R. O. McCann and J. M. Cormier, *Biochemistry*, 19 (1980) 3113–3120.
11 C. B. Klee, *Calcium and Cell Function*, 1 (1980) 59–77.
12 T. M. Crouch and C. B. Klee, *Biochemistry*, 19 (1980) 3692–3698.
13 D. C. Laporte, B. M. Wierman and D. R. Storm, *Biochemistry*, 19 (1980) 3814–3819.
14 K. B. Seamon, *Biochemistry*, 19 (1980) 207–215.
15 T. Tanaka and H. Hidaka, *J. Biol. Chem.*, 255 (1980) 11078–11080.
16 C. B. Klee, *Biochemistry*, 16 (1977) 1017–1024.
17 B. Weiss and R. M. Levin, *Advan. Cyclic Nucl. Res.*, 9 (1978) 285–302.
18 J.-M. Egly and J. Porath, *J. Chromatogr.*, 168 (1979) 35–47.
19 F. Autric, C. Ferraz, M. C. Kilhoffer, J. C. Cavadore and J. G. Demaille, *Biochim. Biophys. Acta*, 631 (1980) 139–147.
20 L. Sundberg and J. Porath, *J. Chromatogr.*, 90 (1974) 87–98.
21 E. Boschetti, unpublished results.

22 S. Kakiuchi, R. Yamazaki, Y. Teshima, K. Uenishi and E. Miyamoto, *Biochem. J.*, 146 (1975) 109–120.
23 C. Rochette-Egiy and J. M. Egly, *J. Cyclic Nucl. Res.*, 6 (1980) 335–345.
24 J. V. Maizel, *Methods Virol.*, 5 (1971) 180.
25 P. Basset, Y. Beuzard, M. C. Garel and J. Rosa, *Blood*, 51 (1978) 971–982.
26 K. Gietzen, A. Mansard and H. Bader, *Biochem. Biophys. Res. Commun.*, 94 (1980) 674–681.
27 B. D. Roufogalis, *Biochem. Biophys. Res. Commun.*, 98 (1981) 607–613.
28 R. Jost, T. Miron and M. Wilchek, *Biochim. Biophys. Acta*, 362 (1974) 75–82.
29 W. H. Burgess, D. K. Jemiolo and R. H. Kretsinger, *Biochim. Biophys. Acta*, 623 (1980) 257–270.
30 G. A. Jamieson and M. J. Vanaman, *Biochem. Biophys. Res. Commun.*, 90 (1979) 1048–1056.
31 H. Charbonneau and M. J. Cormier, *Biochem. Biophys. Res. Commun.*, 90 (1979) 1039–1047.
32 C. B. Klee, T. H. Crouch and P. G. Richman, *Annu. Rev. Biochem.*, 49 (1980) 489–515.
33 D. M. Watterson, P. A. Mendel and T. C. Vanaman, *Biochemistry*, 19 (1980) 2672–2676.