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ISOLATION OF A NEW CALMODULIN-BINDING PROTEIN FROM RAT BRAIN

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SUMMARY. A new calmodulin-binding protein was isolated from rat brain by chromatographies on DEAE-Sephadex and hydroxyapatite followed by affinity chromatography on calmodulin-Sepharose. This protein, which constituted over 10% of the total amount of calmodulin-binding proteins in the supernatant from rat brain, gave one band of molecular weight 50K on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Although bound to calmodulin-Sepharose even in the presence of 6 M urea, the protein was quickly released on removal of calcium. Rapid postmortem decrease of the protein was observed.

Calmodulin, originally discovered as a Ca^{2+}-dependent regulator of mammalian phosphodiesterase (PDE) (1-3), is now believed to be ubiquitous in eukaryotic cells (4-6). This small, acidic, Ca^{2+}-binding protein is known to activate not only PDE but also various other enzymes. Calmodulin has also been shown to be associated with microtubular protein and to affect its assembly and disassembly. The Ca^{2+}-dependent multifunctional roles and properties of calmodulin have recently been reviewed (7-9).

The growing list of calmodulin-binding proteins (CBPs) includes several proteins whose biological functions remain to be elucidated. A heat-labile protein of molecular weight (M_r) 80K consisting of two subunits, 60K and 18K, has been purified (10,11). This protein was recently named calcineurin on the basis of its localization to the nervous system and the Ca^{2+}-binding property of its smaller subunit (12). Another calmodulin-binding protein has been isolated from bovine brain by a method utilizing its heat stability and shown to have M_r of 70K (13). Grand and Perry (14) showed that the myelin basic protein (M_r 18.4K) binds calmodulin.

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Since the brain is particulary rich in calmodulin and CBPs, it is very likely that calmodulin is involved in a variety of cellular processes in the brain. The isolation and characterization of CBPs from the brain should thus provide useful information on the neural mechanisms related to Ca^{2+}. In this communication we report the isolation of a new CBP from rat brain.

MATERIALS AND METHODS

Calmodulin and PDE: Calmodulin and PDE were purified from bovine brain by the methods of Teo et al. (15) and Klee and Krinks (11), respectively. Calmodulin was coupled to CNBr-activated Sepharose 4B (Pharmacia) as described in (11); about 1 mg of calmodulin was linked to 1 ml of Sepharose. PDE activity was determined by the method of Butcher and Sutherland (16) using *C. atrox* venom as a source of 5'-nucleotidase.

Supernatant from Rat Brain: Rat brains were homogenized in a Polytron for 30 sec in 10 vol of 5 mM 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES)-KOH-1 mM ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA)-1 mM dithiothreitol (DTT), pH 7.5. After the addition of 0.1 vol of 0.2 M Tris-Cl-10 mM $MgCl_2$-1.0 M NaCl, pH 7.5, the mixture was further homogenized for 30 sec and then centrifuged at 100,000g for 60 min and the supernatant was obtained. All these procedures were carried out at 0-4°C. In some cases, rat brains were kept at 37°C for 20 min before homogenization to investigate the postmortem change of CBPs.

Stepwise Elution of CBPs from Calmodulin-Sepharose: A column of calmodulin-Sepharose 4B (1.6 x 18 cm) equilibrated with Solution A (40 mM Tris-Cl-3 mM $MgCl_2$-0.1 mM DTT-0.2 M NaCl-0.2 mM $CaCl_2$, pH 7.5) was loaded with the supernatant containing 1.2 mM $CaCl_2$ and washed with Solution A. CBPs were then eluted stepwise with the following solutions: (1) Solution A containing 1 M NaCl, (2) Solution A containing 3 M urea, (3) Solution A containing 6 M urea, (4) Solution B (40 mM Tris-Cl-1 mM $MgCl_2$-0.1 mM DTT-0.2 M NaCl-2 mM EGTA, pH 7.5).

Purification of the M_r 50K Protein: The supernatant from about 100g of rat brain prepared as described above was applied to a DEAE-Sephadex A-50 column (4.0 x 45 cm) equilibrated with 20 mM Tris-Cl-1 mM $MgCl_2$-0.1 mM EGTA-0.1 mM DTT-0.1 M NaCl, pH 7.5 (Solution C). The column was washed with Solution C containing 0.17 M NaCl, and then material was eluted with Solution C containing 0.28 M NaCl. Fractions of 20 ml were collected. Appropriate fractions were combined and loaded on a column of hydroxyapatite (2.5 x 13 cm) equilibrated with 10 mM potassium phosphate buffer-1 mM $MgCl_2$-0.1 mM DTT-0.1 M NaCl, pH 6.8. The column was washed first with 0.05 M potassium phosphate buffer-50 mM NaCl-1 mM $MgCl_2$-0.1 mM DTT, pH 6.8 and then with 0.25 M potassium phosphate buffer-1 mM $MgCl_2$-0.1 mM DTT, pH 6.8. Then material was eluted with 0.4 M potassium phosphate buffer-1 mM $MgCl_2$-0.1 mM DTT, pH 6.8. The fraction size was 7 ml. Appropriate fractions were combined and applied to a column of calmodulin-Sepharose 4B (1.5 x 5.5 cm) equilibrated with Solution A. CBPs were eluted stepwise as described above. The eluate with Solution B was the final preparation.

Electrophoresis: Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS) was performed as described by Laemmli (17) using a

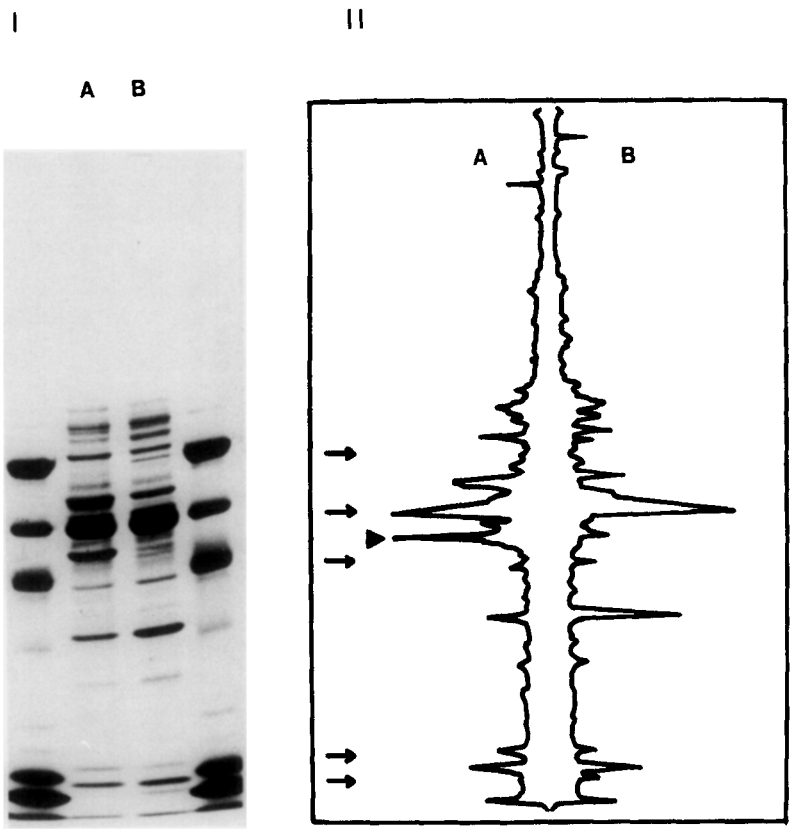

Fig. 1. SDS-polyacrylamide gel electrophoretic patterns of CBPs (I) and their densitograms (II). After addition of $CaCl_2$ at a final concentration of 1.2 mM, the 100,000g supernatant was applied to a column of calmodulin-Sepharose 4B (1.5 x 5.5 cm) equilibrated with Solution A. The column was thoroughly washed with Solution A, and then CBPs were eluted with Solution B. Rat brains were kept at 0°C (A) or 37°C (B) for 20 min before homogenization. In both (A) and (B) 23 µg protein was applied. The lanes at both ends in I are M_r marker proteins, the positions of which are shown by arrows in II. From top to bottom bovine serum albumin (68000), tubulin (55000), ovalbumin (43000), soy bean trypsin inhibitor (21500), myoglobin (17800). The arrowhead in II indicates the M_r 50K protein.

gradient of 7.5-15% acrylamide. Gels were stained and destained as in (18) and scanned with a Joyce Loebl densitometer.

Protein Determination: Protein concentration was determined by the method of Bradford (19) using bovine serum albumin (Sigma) as a standard.

RESULTS AND DISCUSSION

CBPs bound to the calmodulin-Sepharose column were eluted with EGTA. As shown in Fig. 1 (IA and IIA), many proteins were detected on SDS-gel electro-

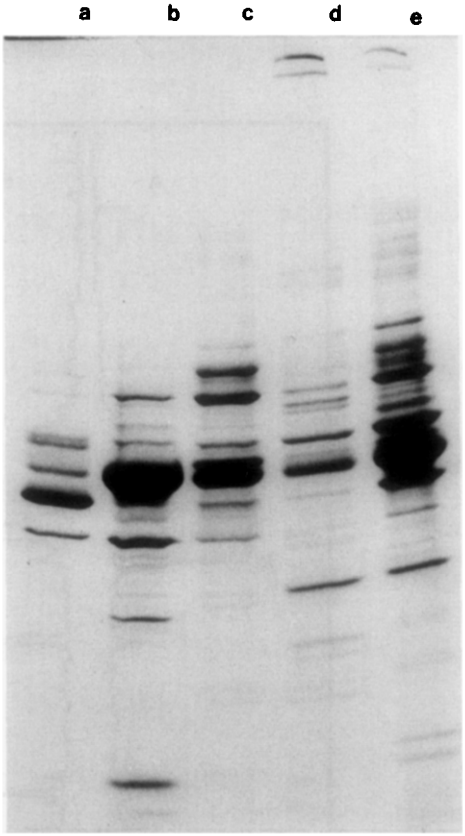

Fig. 2. SDS-polyacrylamide gel electrophoresis of CBPs obtained by stepwise elution of a calmodulin-Sepharose 4B column. For details see MATERIALS AND METHODS. Eluates with Solution A + 1 M NaCl (b), Solution A + 3 M urea (c), Solution A + 6 M urea (d), Solution B (e). Lane a shows CBPs obtained by direct elution with Solution B and is the same as in Fig. 1-I-A.

phoresis including three major proteins with M_r of 60K, 55K and 50K. For measurement of the affinities of various CBPs from rat brain to calmodulin, CBPs bound to a calmodulin-Sepharose column were eluted stepwise as described in the MATERIALS AND METHODS. The results are presented in Fig. 2. Although many CBPs were eluted before or with 6 M urea, several CBPs remained attached to the column and were then released with EGTA. Among the latter, the M_r 50K protein was prominent, indicating that this protein binds firmly to calmodulin-Sepharose.

The M_r 50K protein was purified taking advantage of its strong binding to calmodulin-Sepharose. The results of purification are given in Fig. 3. Before

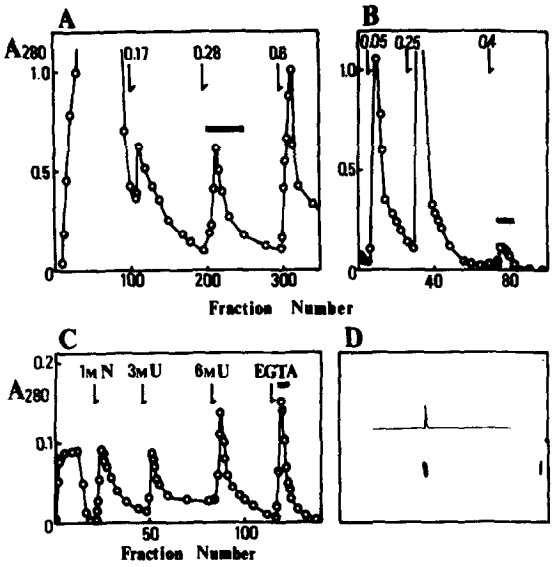

Fig. 3. Purification of the M_r 50K protein. In A, B and C horizontal bars indicate fractions pooled. A; DEAE-Sephadex A-50 chromatography of the supernatant. Figures above the arrows indicate molar concentrations of NaCl. B; hydroxyapatite chromatography of the 0.28 M NaCl eluate in A. Figures above the arrows indicate molar concentrations of phosphate buffer. C; affinity chromatography of the 0.4 M phosphate eluate in B on calmodulin-Sepharose 4B. Fractions of 0.7 ml were collected except for those in tubes 1-20 which were 10 ml. N and U indicate Solution A containing NaCl and urea, respectively. EGTA stands for Solution B. Details are given in the MATERIALS AND METHODS. D; SDS-electrophoresis of the EGTA eluate in C and its densitogram. The stain at the right is an artifact.

affinity chromatography, soluble proteins were fractionated first by DEAE-Sephadex and then by hydroxyapatite chromatography. The hydroxyapatite step was found to be effective in removing contaminants, because only about 3% of the applied protein was eluted with 0.4 M phosphate. The final preparation was reasonably pure as shown by the densitogram in Fig. 3. At this step, the yield was about 0.2 mg protein from 100 g of rat brain. About 3% of the total protein in the 100,000g supernatant was recovered as CBPs,and it was calculated from the densitogram in Fig. 1 that the M_r 50K protein amounted to 12% of the total CBPs. From these values about 0.4% of the total protein in the supernatant is M_r 50K protein, but the yield was reduced during removal of closely associated proteins.

The binding of the M_r 50K protein to calmodulin was confirmed by high speed liquid chromatography of a mixture of the two proteins (data not shown).

The two proteins were eluted together in a peak in the void volume (Toyo Soda, G3000SW, exclusion limit: M_r around 300K). Since the M_r 50K protein also appeared in the void volume when applied to the same column without calmodulin, it probably formed an aggregate.

We attempted to purify the M_r 50K protein from bovine brain, but found that bovine brain extract contained much less of this protein than of other CBPs. This fact suggested that this protein decreased rapidly after death, because bovine brain could not be removed from the skull in less than 30 min after death. To examine the postmortem change of this protein, we kept isolated rat brains at 37°C for 20 min before homogenization. Control brain was kept at 0°C. As clearly demonstrated in Fig. 1, several CBPs underwent changes during this period, but decrease in the M_r 50K protein was the most striking change (arrowhead in Fig. 1-II). This indicates that the M_r 50K protein undergoes rapid postmortem decrease, which may explain why it has not previously been found.

The function of the M_r 50K protein is unknown at present. It did not react with antiserum against glial fibrillary acidic protein (GFA). Preliminary results indicate that it has no ATPase, GTPase or PDE activity. The rapid postmortem change and the tight binding of this protein with calmodulin suggest that it has dynamic functions *in vivo* regulated by Ca^{2+}.

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REFERENCES.

1. Kakiuchi, S. and Yamazaki, K. (1970) Biochem. Biophys. Res. Commun. 43, 1104-1110.
2. Cheung, W.Y. (1970) Biochem. Biophys. Res. Commun. 38, 533-538.
3. Cheung, W.Y. (1971) J. Biol. Chem. 246, 2859-2869.
4. Waisman, D., Stevens, F.C. and Wang, J.H. (1975) Biochem. Biophys. Res. Commun. 65, 975-982.
5. Smoake, J.A., Song, S-Y. and Cheung, W.Y. (1974) Biochim. Biophys. Acta 341, 402-411.

6. Kumagai, H., Nishida, E., Ishiguro, K. and Murofushi, H. (1980) J. Biochem. 87, 667-670.
7. Wolff, D.J. and Brostrom, C.O. (1979) Adv. Cyclic Nucleot. Res. 11, 27-88.
8. Cheung, W.Y. (1980) Science 207, 19-27.
9. Means, A.R. and Dedman, J.R. (1980) Nature 285, 73-77.
10. Wang, J.H. and Desai, R. (1977) J. Biol. Chem. 252, 4175-4184.
11. Klee, C.B. and Krinks, M.H. (1978) Biochemistry 17, 120-126.
12. Klee, C.B., Crough, T.H. and Krinks, M.H. (1979) Proc. Nat. Acad. Sci. USA 76, 6270-6273.
13. Sharma, R.K., Desai, R., Thompson, T.R. and Wang, J.H. (1978) Can. J. Biochem. 56, 598-604.
14. Grand, R.J.A. and Perry, S.V. (1979) Biochem. J. 183, 285-295.
15. Teo, T.S., Wang, T.H. and Wang, J.H. (1973) J. Biol. Chem. 248, 588-595.
16. Butcher, R.W. and Sutherland, E.W. (1962) J. Biol. Chem. 237, 1244-1251.
17. Laemmli, U.K. (1970) Nature 227, 680-685.
18. O'Farrell, P.H. (1975) J. Biol. Chem. 250, 4007-4021.
19. Bradford, M. (1976) Anal. Biochem. 72, 248-254.