DEMONSTRATION OF CALMODULIN-BINDING PROTEINS IN RAT BRAIN

TERUO ABE and HIDEO SAISU

Department of Neurochemistry, Brain Research Institute, Niigata University, Asahimachi, Niigata 951, Japan

ABSTRACT

The soluble proteins of rat brain retained on calmodulin-Sepharose were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and two-dimensional electrophoresis. Calmodulin-binding proteins were then detected by overlaying the electrophoretic gels with calmodulin labeled with ^{125}I . About 22 calmodulin-binding proteins were identified by autoradiography, those of M_r 220 K, 71 K, 60 K, 59 K and 49 K being the main components.

Calmodulin (CaM) is now thought to be an intracellular receptor for Ca^{2+} . It regulates the activities of various enzymes with shift of the Ca^{2+} concentration in a physiological range. CaM is also known to bind specifically with several proteins of unknown function in a Ca^{2+} -dependent manner. Since CaMs present in various tissues are very similar to each other, if not identical, differences in the responses of various tissues to increase in the intracellular concentration of Ca^{2+} cannot be due to differences in CaM, but to differences in the molecules whose activities are regulated by CaM.

The brain is among the richest sources of CaM and also of CaM-binding proteins (CBPs). It is thus important to determine the CBPs in brain for understanding the mechanisms underlying the complex and dynamic responses of neuronal cells to increased influx of Ca^{2+} . Several brain CBPs have been identified (1, 3, 5, 7, 9, 10, 14, 18, 19), but the brain probably contains many others. In the present study we examined the CBPs in the soluble fraction of the brain by autoradiography of electrophoretic gel overlaid with CaM labeled with ^{125}I (^{125}I -CaM).

Abbreviations: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; DTT, dithiothreitol; EGTA, ethyleneglycol-bis(β -aminoethyl ether)*N,N'*-tetraacetic acid; FP, fluphenazine; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

MATERIALS AND METHODS

All procedures were carried out at 0–4°C unless otherwise stated.

CaM and Phosphodiesterase

CaM was purified from bovine brain by the method of Watterson *et al.* (22). Cyclic nucleotide phosphodiesterase was prepared from bovine brain as described by Klee and Krinks (11).

Soluble Fraction of Rat Brain

Three rat brains were homogenized in a Polytron in 10 volumes of 10 mM Hepes-NaOH-0.1 mM DTT-2 mM EGTA, pH 7.5, for 1 min. After further addition of 0.11 volume of the same buffer containing 5 M NaCl, homogenization was repeated for 1 min. The homogenate was then centrifuged at 100,000 *g* for 1.5 h, and CaCl_2 was added to the supernatant to a final concentration of 4 mM.

FP-Sepharose Column

The supernatant was then applied to a column (2.2 $\times$ 8 cm) of FP-Sepharose 4B (2) equilibrated with Solution A (20 mM Tris-HCl-0.5 M NaCl-0.1 mM DTT-0.3 mM CaCl_2 , pH 7.5). Then

the column was washed thoroughly with Solution A, and the flow-through fraction was collected (Fig. 2). The material retained on the column was eluted with Solution B (20 mM Tris-HCl-0.5 M NaCl-0.1 mM DTT-4 mM EGTA, pH 7.5).

CaM-Sepharose Column

The flow-through fraction from the FP-Sepharose column was subjected to affinity chromatography on CaM-Sepharose 4B prepared as described previously (14). The column (2.2 × 4.5 cm) was equilibrated with Solution A and thoroughly washed with Solution A to remove unbound material before elution of CBPs with Solution B.

Electrophoreses

SDS-PAGE was carried out by the method of Laemmli (13) on 7.5–15% acrylamide gradient gels. Two-dimensional electrophoresis was performed by the method of O'Farrell (17) with some modifications. The first dimension (isoelectric focusing) was carried out on cylindrical gels (2.5 × 60 mm) containing ampholine pH 3.5–10 (LKB), 9 M urea and 2% Triton X-100. Samples (50 µg) were applied as solutions containing 2.5% 2-mercaptoethanol, SDS and Triton X-100 (protein: SDS : Triton X-100 = 1:4:32, w/w). SDS and Triton X-100 were added to avoid possible formation of aggregates. The second dimension (SDS-PAGE) was carried out on 7 cm wide slab gels. Electrophoresis was stopped when the marker dye (bromophenol blue) had migrated to 5 cm from the top to the running gel.

Iodination of CaM

CaM was radioiodinated by the method of Graf *et al.* (6). The reaction mixture (0.4 ml) contained 100 mM imidazole-HCl, pH 6.9, 50 µM CaCl₂, 10 µM phenylmethylsulfonyl fluoride, 280 µM KI, 2 mCi Na¹²⁵I (15.6 mCi/µg of iodine, Amersham), 0.76 mM glucose, 0.065 µM lactoperoxidase (Boehringer), 0.23 µM glucose oxidase (Sigma) and 100 µg CaM purified from bovine brain. The mixture was incubated at 30°C for 10 min, and then the reaction was stopped by adding 5 µl of 2-mercaptoethanol. The reaction mixture was applied to a column of Sephacryl S-200 (Pharmacia) equilibrated with 50 mM Tris-HCl-0.2 M NaCl, pH 7.6. The column was operated at 0–4°C and the peak frac-

tions containing CaM were pooled. The specific activity of the preparation was about 2.5×10^6 cpm/µg CaM. The ¹²⁵I-CaM was indistinguishable from native CaM in activating CaM-deficient cyclic nucleotide phosphodiesterase.

Binding of ¹²⁵I-CaM to Proteins on the Gel

This was done essentially as described by Carlin *et al.* (1). The procedures were carried out at room temperature. After SDS-PAGE, each lane was cut out of the gel. These strips as well as slabs used for two-dimensional electrophoresis were treated as described below. They were shaken overnight in 25% isopropanol-10% acetic acid solution and then washed with 10% acetic acid for 2 h. They were then washed for 8 h with 4 changes of Solution C (50 mM Tris-HCl-0.2 M NaCl-1 mM CaCl₂, pH 7.6) or Solution D (50 mM Tris-HCl-0.2 M NaCl-2 mM EGTA, pH 7.6). Gel strips and slabs were then kept in the respective solutions containing bovine serum albumin (1 mg/ml) for 2 h, and subsequently incubated in the respective solutions containing ¹²⁵I-CaM (0.2–0.4 µg/ml) for 14 h. In some cases FP (final concentration 0.1 mM) or cold CaM (final 100 µg/ml) was added to Solution C. Unbound ¹²⁵I-CaM was removed by washing the gel strips or slabs for 1 day with at least 5 changes of the respective solutions. Then they were stained with 0.1% Coomassie blue in 25% isopropanol-10% acetic acid for 2 h. Destained gel strips and slabs were dried on filter paper on which Ultrofilm (LKB) was placed. The film was exposed for 0.5–2 days at –80°C.

RESULTS AND DISCUSSION

The procedures described by Graf *et al.* (6) gave ¹²⁵I-CaM suitable for our experiments. More than 95% of the total radioactivity applied comigrated with native CaM on PAGE in Davis' system (4), as seen in Fig. 1, and SDS-PAGE of ¹²⁵I-CaM showed that essentially all the radioactivity comigrated with native CaM.

The supernatant was first applied to an FP-Sepharose column to remove endogenous CaM, which may inhibit the binding of CBPs to CaM-Sepharose. The EGTA eluate from FP-Sepharose (Fig. 2, F2) contained only CaM as shown by SDS-PAGE, indicating its selective removal from the supernatant (data not shown). The flow-through fraction from the FP-Sepharose column (Fig. 2, F1) was subjected to affinity chromatography on CaM-Sepharose to obtain CBPs. As shown in Fig. 3A the CBP

fraction (Fig. 2, FII) gave many bands on SDS-PAGE, M_r 49 K, 55 K, 59 K and 81 K being the main components. Fig. 3B shows the binding of ^{125}I -CaM to the proteins on the gel shown in Fig. 3A. This binding was completely abolished in the absence of Ca^{2+} (Fig. 3C) or in the presence of FP (D) or excess cold CaM (E), indicating the specificity of ^{125}I -CaM-binding in the present study. For detailed analysis of CBPs, the proteins of the CBP fraction were subjected to two-dimensional electrophoresis and then exposed to ^{125}I -CaM. The results are shown in Fig. 4. As in the case of SDS-PAGE, the binding of ^{125}I -CaM to the proteins developed by two-dimensional electrophoresis was not observed in the absence of Ca^{2+} or in the presence of FP or excess cold CaM (data not shown). As can be seen from Figs. 3 and 4, some of the proteins retained on CaM-Sepharose did not bind ^{125}I -CaM, possibly owing to irreversible denaturation of CBPs in SDS resulting in the loss of CaM-binding activity, or to the existence of some proteins in the CBP fraction bound to CaM-Sepharose through non-specific or indirect interactions; for example, if a protein that itself is not a CBP binds to CBP, it may be retained on CaM-Sepharose by binding to CBP bound to CaM-Sepharose. Table 1 summarizes findings on the CBPs in the rat brain soluble fraction based on the results in Figs. 3 and 4.

Fig. 1 PAGE of ^{125}I -CaM in Davis' system. The acrylamide concentration was 12.5%. All solutions used contained 2 mM EGTA. A: native CaM (5 μg), Coomassie blue stain; B: native CaM (5 μg) + ^{125}I -CaM (30 ng), Coomassie blue stain; C: autoradiograph of lane B

Fig. 2 Preparation of CBP fraction. a: FP-Sepharose column chromatography of the supernatant. b: CaM-Sepharose column chromatography of the flow-through fraction of a (F1). In both a and b, vertical arrows indicate the start of elution with Solution B. The fraction size was 7 ml before the arrows and 3 ml after the arrows.

Fig. 3 Binding of ^{125}I -CaM to proteins on the gel. The CBP fraction was subjected to SDS-PAGE and the gel strips were overlaid with ^{125}I -CaM under various conditions. A: Coomassie blue stain; B-E: autoradiographs. B: Ca^{2+} (+); C: Ca^{2+} (-), EGTA (+); D: Ca^{2+} (+), FP (+); E: Ca^{2+} (+), cold CaM (+)

Fig. 4 Binding of ^{125}I -CaM to proteins on two-dimensional electrophoretic gel. After two-dimensional electrophoresis of the CBP fraction, the gel was overlaid with ^{125}I -CaM. A: Coomassie blue stain; B: autoradiograph after ^{125}I -CaM overlay in the presence of Ca^{2+} . Spot numbers correspond to the band numbers shown in Fig. 3.

Table 1 Soluble Calmodulin-Binding Proteins of Rat Brain*

Spot or band No.	M_r	^{125}I -CaM bound
1a	240 K	+++
1b	220	+++
2a	156	++
5**	76	+
6	71	++
7a	60	+++
7b	60	+++
7d	60	+
7e	60	++
8a	59	+++
8b	59	+++
10	52	+
11a	50	++
11b	50	+++
12	49	+++
13	44	++
14b	42	++
15	39	+
16	37	+
17**	35	+
18**	33	+
20**	28	+

* Based on the data shown in Figs. 3 and 4.

** Not detected in the autoradiograph after two-dimensional electrophoresis.

Caution must be taken in identifying a protein as a CBP. If a protein is very basic it could bind to CaM, a very acidic protein, in a Ca^{2+} -independent manner (8). Autoradiography of gel after two-dimensional electrophoresis (Fig. 4) showed that most of the proteins bound to ^{125}I -CaM are not basic. Moreover, the binding of ^{125}I -CaM to the proteins on the gel (Fig. 3) is entirely Ca^{2+} -dependent. These findings exclude the possibility that any of the binding of ^{125}I -CaM to proteins observed in the present study was due to non-specific interactions. The contribution of CBPs in the blood must also be taken into consideration. However, when the procedures described above for ^{125}I -CaM-binding were applied to blood proteins, no CBPs with similar M_r values to those in Table 1 were detected. Thus none of the CBPs in brain shown in Table 1 were derived from the blood.

In the very high M_r region, two CaM-binding components (spot 1a and spot 1b in Fig. 4) were detected. The M_r 220 K protein, which showed strong binding of ^{125}I -CaM, formed a

clear spot (spot 1b). It remains to be seen whether the M_r 220 K protein is identical to the protein of M_r 230–240 K recently reported as CBP in brain (1, 3, 9). The nature of the protein of M_r 156 K is unknown at present. The M_r 71 K protein (spot 6) may be identical to the heat-stable CBP reported previously (18). Band 7 (M_r 60 K) was split into 5 spots (7a–7e) on two-dimensional electrophoresis, of which 4 showed CaM-binding activity. Band 8 (M_r 59 K) gave two spots (8a and 8b) on autoradiography, although it was stained only weakly with Coomassie blue. Bands 7 and 8 may contain two CBPs, calcineurin and cyclic nucleotide phosphodiesterase. Calcineurin (10, 19), mainly present in the nervous system, consists of two subunits, calcineurin A (M_r 60–61 K) and calcineurin B (M_r 15–16 K). The former is responsible for CaM-binding. It is not known why the band corresponding to calcineurin B was not observed in our CBP fraction (Fig. 3A). Cyclic nucleotide phosphodiesterase of M_r 58 K on SDS-PAGE has been reported to have an isoelectric point of 4.85 (20). Since the isoelectric point of a protein varies appreciably depending on the conditions employed for its isoelectric focusing, it was not possible to identify the spot of phosphodiesterase on two-dimensional electrophoretic gel. Spots 9a and 9b were identified as tubulin by comparison with authentic tubulin prepared from rat brain. It is known that microtubule assembly-disassembly *in vitro* is regulated by CaM (15, 16). However, these spots did not bind CaM as seen in Fig. 4B, although tubulin is reported to bind CaM (12). Recently Sobue *et al.* (21) reported that tau factor, not tubulin, binds CaM, and our results seem to support theirs. However, it is possible that the CaM-binding activity of tubulin was not recovered on removal of SDS from the gel. Spots 11a and 11b (M_r 50 K) showed strong CaM-binding activity, but their nature remains to be investigated. The M_r 49 K CBP (band 12) is identical to the CBP that shows rapid post-mortem decrease (14). On reinvestigation, the M_r of this protein was found to be 49 K, not the value reported previously (50 K). The M_r 49 K band formed a rather extended spot (spot 12) on two-dimensional electrophoresis (Fig. 4, A and B). It is not clear whether this means that the protein contains several isoforms or whether it aggregates (14). Spot 13 (M_r 44 K) is situated very close to actin, but it is not actin. The M_r 42 K protein (spot 14b) may be formed by degradation of calcineurin A as has been suggested (3). There were also some CBPs with M_r

values of less than 40 K (Fig. 3B), but their nature and function are unknown.

The method of detection of CBPs by the ^{125}I -CaM overlay technique has the limitation that when CBPs are denatured irreversibly by SDS-treatment they do not recover CaM-binding activity. But by coupling affinity chromatography on CaM-Sepharose with ^{125}I -CaM overlay we were able to detect more than 20 CBPs, including several hitherto unknown proteins. This procedure is a simple method for identifying CBPs in various tissues.

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