ISOLATION AND PARTIAL CHARACTERIZATION OF AN ACIDIC CALCIUM-BINDING PROTEIN FROM SYNAPTIC PLASMA MEMBRANES OF RAT BRAIN

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1. Introduction

For elucidating the role of calcium in the process of neural transmission, a number of calcium-binding proteins have been isolated from brain and other neurosecretory tissues. S-100 protein is localized in glia cells [1]. Calmodulin (modulator protein or calcium-dependent regulator protein) which regulates the activity of cyclic nucleotide phosphodiesterase was discovered [2,3] and subsequently isolated from a wide variety of tissues.

Here we report a simple procedure for purifying an acidic calcium-binding protein from rat brain synaptic plasma membranes to apparent homogeneity. The procedure takes advantage of the observations that the synaptic plasma membranes are easily solubilized with LIS and that the step of phenol extraction removes the bulk of contaminating proteins. The acidic protein isolated here shows a remarkable resemblance in its chemical and physical properties to calmodulin but differs significantly in several respects.

2. Materials and methods

Synaptic plasma membranes were prepared by a modification of the method in [4]. Cerebral cortex from the brains of Donryu rats were homogenized in a glass–Teflon homogenizer in 9 vol. 0.32 M sucrose. The homogenate was fractionated by differential centrifugation to yield a crude mitochondrial pellet. The pellet was subjected to osmotic shock, then centrifuged at 6000 × *g* for 30 min. The pellet containing disrupted nerve endings was layered on gradient tubes containing 6 ml each of the following sucrose solutions in a discontinuous gradient: 1.2 M, 1.0 M, 0.8 M, 0.6 M and 0.4 M. The gradients were centrifuged in a Hitachi 55P-2 centrifuge with a SW-27 rotor for 75 min at 4°C and 88 000 × *g*. Purified plasma membrane fraction was collected at the 0.8 M and 1.0 M sucrose interface. In agreement with [5], the specific activities of acetylcholine esterase and Na^+, K^+-ATPase, markers for synaptic membranes, were enriched ~3-fold and 5-fold, respectively, in the purified synaptic membrane fraction relative to those of the crude mitochondrial pellet.

Calmodulin from bovine brain was the kind gift of Drs Hisataka Kasai and Tsuneo Okuyama of Tokyo Metropolitan University. Polyacrylamide gel electrophoresis was performed as in [6] using 12.9% acrylamide and 0.1% SDS. The isoelectric point was measured by analytical isoelectric focusing in polyacrylamide gels as in [7]. Amino acids were determined on a Hitachi 835 amino acid analyzer after hydrolysis in 6 N HCl at 110°C for 22 h. Trimethyllysine was identified as a distinct peak before lysine by comigration with the hydrolysate of bovine brain calmodulin and quantitated using a color constant of 0.89 × the color constant of lysine. The trimethyllysine content of calmodulin thus measured was in excellent agreement with the value in [8]. The NH_2-terminal residue of the isolated protein was measured by the method of dansylation as in [9]. Calcium binding was measured by equilibrium dialysis as in [10].

Abbreviation: LIS, lithium diiodosalycylate; SDS, sodium dodecyl sulfate

3. Results

When synaptic plasma membrane fraction was treated with 0.3 M LIS, >90% of the protein of all membranes was solubilized as determined by measuring proteins in the supernatant after centrifugation at 100 000 × *g* for 1 h. The solubilized material was extracted with 50% phenol and the aqueous layer was lyophilized after extensive dialysis against water. The lyophilized material contained one major and several minor bands stainable with Coomassie blue as analyzed by SDS gel electrophoresis (gel A, fig.1). This fraction was further subjected to gel filtration on Sepharose 6-B (fig.1). Peak I in the void volume was composed largely of a high molecular weight material which migrated at the top of SDS gels. The material from peak II contained several bands stainable with both Coomassie blue and periodate-Schiff reagent on the gels (result not shown). Peak III, which represents ~15% of the recovered material, contained a single Coomassie blue staining band with mol. wt 23 000 on the gels (gel B, fig.1). The yield of this protein from the synaptic plasma membrane fraction was ~3%. It is to be noted that the 23 000 mol. wt protein was not found either in the soluble or mitochondrial fraction of rat brain or in rat liver homogenate (results not shown).

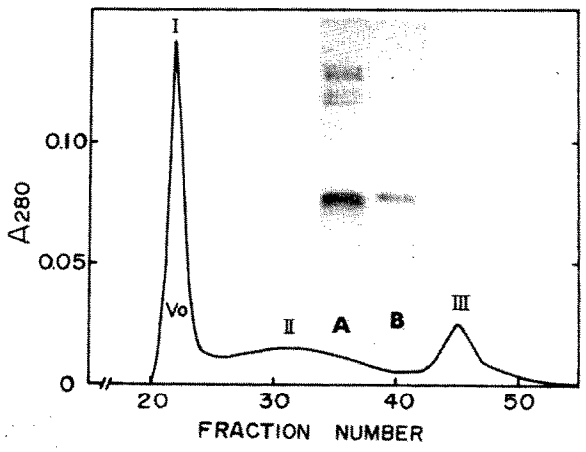

Fig.1. Gel filtration of the proteins solubilized from synaptic plasma membranes with 0.3 M LIS and extracted with phenol. The proteins were applied to a 2 × 94 cm column of Sepharose 6-B equilibrated with a solution containing 24 mM NaCl and 5 mM phosphate buffer (pH 8.5) and eluted with the same buffer. V_0 is the void volume. The inset shows the electrophoretic patterns of the crude acidic protein fraction before gel filtration (A) and the purified acidic protein from peak III(B). The electrophoretic conditions were described in the text.

Table 1
Amino acid composition of acidic calcium-binding proteins (mol %)

Amino acid	Present study	Bovine brain calmodulin[a]
Lys	4.1	5.0
His	0.6	0.6
Trimethyl-lysine	1.5	0.6
Arg	2.6	4.4
Asp	15.5	15.1
Thr	6.6	7.5
Ser	4.3	3.1
Glu	18.9	18.2
Pro	2.6	1.3
Gly	9.8	7.5
Ala	7.3	7.5
Cys	0	0
Val	5.9	5.0
Met	5.0	6.3
Ile	4.7	5.0
Leu	5.2	6.3
Tyr	1.0	1.3
Phe	4.4	5.0
Trp	0	0

[a] Calculated from [8]

The amino acid composition of the isolated protein is remarkably similar to that of calmodulin from bovine brain (table 1). The only significant differences were the presence of a greater proportion of proline and trimethyllysine and less arginine in the isolated protein than in calmodulin. Neither glucosamine nor galactosamine was detected. The NH_2-terminal residue of the isolated protein, like calmodulin, was masked as examined by the method of dansylation. On isoelectric focusing in polyacrylamide gels, the isolated protein migrated as a single band. The isoelectric point was determined to be 4.1, which is in close agreement with the pI value for calmodulin of 4.1–4.3. Fig.2 shows a reciprocal plot of calcium binding by the isolated protein. The intercept on the ordinate indicates that there are 3.3 binding sites/molecule of the protein. An app. K_d of 3.46×10^{-5} M was calculated from these data.

In order to distinguish between the isolated protein and calmodulin, these two proteins were coelectrophoresed on a slab gel (fig.3). The isolated protein migrated towards the anode with a mobility signifi-

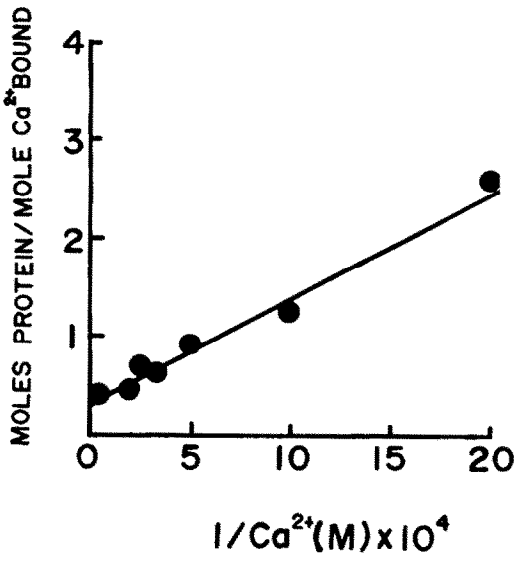

Fig.2. Reciprocal plot for binding of calcium by the purified acidic protein. The measurements were made by the method of equilibrium dialysis in the presence of 5 mM Tris–HCl (pH 7.5) [9].

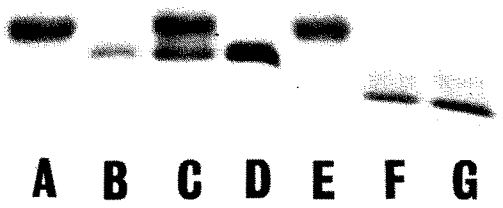

Fig.3. Gel electrophoresis of the purified acidic protein and bovine brain calmodulin. SDS gel electrophoresis were performed as in the text except that 2 mM EDTA (lanes A–D) or 2 mM Ca^{2+} (lanes E–G) was added to the samples [11]. Amounts of proteins applied were 10 μg. Lanes: A,E, the purified acidic protein; B,F, calmodulin; C, mixture of the acidic protein and calmodulin; D,G, calmodulin treated by the LIS–phenol procedure.

cantly less than that of calmodulin even in the presence of either EDTA or Ca^{2+} [11].

4. Discussion

The isolated acidic protein is remarkably similar to calmodulin in that both proteins have similar amino acid compositions, blocked NH_2-terminal residues, almost identical isoelectric points, and high affinity calcium-binding properties. In addition, a preliminary experiment showed that a low concentration (0.5 μg) of the isolated protein stimulated cyclic nucleotide phosphodiesterase activity ~9-fold. However, the isolated protein differs significantly in several respects from calmodulin.

(1) The molecular weight of the isolated protein as determined by SDS gel electrophoresis (23 000) is greater than the value of 15 000 to 20 500 reported for calmodulin from various tissues. In order to exclude the possibility that the electrophoretic mobility of calmodulin associated with synaptic membranes was changed during the isolation procedure, bovine brain calmodulin was treated with 0.3 M LIS, extracted with 50% phenol and electrophoresed. As shown by lanes D,G in fig.3, the electrophoretic mobility of calmodulin was not changed at all by the LIS–phenol treatment. When the molecular weight of the isolated acidic protein was estimated by gel filtration on Sephadex G-100, the protein eluted at a molecular weight corresponding to 52 000, which is also greater than the value of 28 000–32 000 of rat brain calmodulin [12].

(2) The isolated protein contains a greater proportion of proline and trimethyllysine and less arginine than calmodulin.

(3) The acidic protein is detected by the LIS–phenol procedure only in the synaptic plasma membranes but not either in the soluble or mitochondrial fraction of rat brain or in rat liver homogenate.

We conclude from these data that the acidic protein isolated from the synaptic plasma membrane is not identical with calmodulin reported thus far. It will be interesting to examine whether the acidic protein could have any relation to membrane-associated calmodulin which is not removed from synaptic plasma membranes by EDTA treatment [13,14].

References

[1] Moore, B. W. (1965) Biochem. Biophys. Res. Commun. 19, 739–744.

[2] Cheung, W. Y. (1970) Biochem. Biophys. Res. Commun. 38, 533–538.
[3] Kakiuchi, S., Yamazaki, R. and Nakajima, H. (1970) Proc. Jap. Acad. 46, 589–592.
[4] Abood, L. G., Hong, J. S., Takeda, F. and Tometsko, A. M. (1976) Biochim. Biophys. Acta 443, 414–427.
[5] Cotman, C. W. and Matthews, D. A. (1971) Biochim. Biophys. Acta 249, 380–394.
[6] Laemmli, U. K. (1970) Nature 227, 680–685.
[7] Wrigley, C. W. (1968) J. Chromatog. 36, 362–365.
[8] Watterson, D. M., Harrelson, W. G. jr, Keller, P. M., Scarief, F. and Vanaman, T. C. (1978) J. Biol. Chem. 254, 4501–4513.
[9] Gray, W. R. (1972) Methods Enzymol. vol. 25, 121–138.
[10] Maclennan, D. H. and Wong, P. T. S. (1971) Proc. Natl. Acad. Sci. USA 68, 1231–1235.
[11] Grab, D. J., Berzins, K., Cohen, R. S. and Siekevitz, P. (1979) J. Biol. Chem. 254, 8690–8696.
[12] Teshima, Y. and Kakiuchi, S. (1974) Biochem. Biophys. Res. Commun. 56, 489–495.
[13] Kakiuchi, S., Yamazaki, R., Teshima, Y., Uenishi, K., Yasuda, S., Kashiba, A., Sobue, K., Ohshima, M. and Nakajima, T. (1978) Adv. Cyclic Nucl. Res, 4, 219–231.
[14] Gnegy, M. E., Costa, E. and Uzunov, P. (1976) Proc. Natl. Acad. Sci. USA 73, 352–355.