EVIDENCE FOR THREE DISTINCT FORMS OF CALMODULIN-DEPENDENT PROTEIN KINASES FROM RAT BRAIN

TAKASHI YAMAUCHI and HITOSHI FUJISAWA
Department of Biochemistry, Asahikawa Medical College, Asahikawa 078-11, Japan

Received 4 June 1980

1. Introduction

Calcium ions, which are known to play important roles in the release and biosynthesis of neurotransmitters and other physiological functions of the nervous system [1], regulate the level of phosphorylation of a number of endogenous proteins in nerve terminals apparently through activation of a Ca^{2+}-sensitive protein kinase [2]. The involvement of a heat-stable Ca^{2+}-sensitive regulator protein (calmodulin) in the Ca^{2+}-sensitive protein phosphorylation in the nervous system has been suggested [3–6]. These studies have demonstrated that at least three distinct calmodulin-dependent protein kinases with different substrate specificities, one of which is involved in the regulation of tryptophan 5-monooxygenase, are present in rat brain cytosol.

2. Materials and methods

[γ-^{32}P]ATP (3000 Ci/mmol) was obtained from the Radiochemical Centre, Amersham. Phosphorylase *b* from rabbit muscle, obtained from Boehringer Mannheim, was further purified by gel filtration on Ultrogel AcA 34 in order to remove contaminating calmodulin. Rat brain calmodulin was prepared as in [7]. The light chain from chicken gizzard myosin was prepared essentially as in [8]. The light chain preparation was free from calmodulin. Phosphorylase kinase from rabbit skeletal muscle was prepared as in [9].

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether) *N,N'*-tetraacetic acid; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate; DTT, dithiothreitol

Tryptophan 5-monooxygenase was purified ~7-fold from rat brain-stem extracts by ammonium sulfate precipitation (55% saturation), gel filtration on Sepharose CL-6B and chromatography on calmodulin-substituted Sepharose 4B. The last step was required to remove calmodulin-dependent protein kinase from this preparation. Endogenous substrates of calmodulin-dependent protein kinases were prepared from rat cerebral cortex by ammonium sulfate fractionation and calmodulin-substituted Sepharose 4B chromatography. Calmodulin-substituted Sepharose 4B was prepared as in [10].

Calmodulin-dependent protein kinases were prepared from rat cerebral cortex. The cerebral cortex (30 g wet wt) of Wistar rats was homogenized in 120 ml 50 mM Tris–HCl (pH 7.6) containing 2 mM DTT and 1 mM EGTA and centrifuged at 39 000 × *g* for 30 min. To the supernatant a saturated ammonium sulfate solution (pH 7.6) was added to a final saturation of 55%. The precipitate was collected by centrifugation and dissolved in 50 ml buffer A (40 mM Tris–HCl (pH 7.6), containing 50 mM NaCl, 3 mM $Mg(CH_3COO)_2$ and 1 mM DTT) containing 0.2 mM $CaCl_2$ and dialyzed against the same buffer. The dialyzed solution was applied to a column of calmodulin–Sepharose 4B (1.6 × 12 cm) pre-equilibrated with Buffer A containing 0.2 mM $CaCl_2$. The column was washed with the same buffer containing 1 M NaCl. Calmodulin-dependent protein kinases were eluted with buffer A containing 1 mM EGTA, concentrated by ammonium sulfate precipitation, and then applied to a column of Sepharose CL-6B (1.4 × 60 cm) pre-equilibrated with buffer A and eluted with the same buffer.

Assays for calmodulin-dependent protein kinase activity were done essentially as in [4]. Endogenous

substrates, chicken gizzard myosin light chain, or casein were used as protein substrates. Activation of tryptophan 5-monooxygenase was done as in [3]. Phosphorylase kinase was assayed as in [11] with a slight modification. Calmodulin was assayed on the basis of its ability to activate the Ca^{2+}-activatable phosphodiesterase [7].

SDS–polyacrylamide slab gel electrophoresis and autoradiography were done as in [4]. Protein was determined as in [12].

3. Results and discussion

The elution patterns of the rat brain cytosol calmodulin-dependent protein kinase activity on Sepharose CL-6B were examined with using several protein substrates as shown in fig.1. Phosphorylase kinase activity was eluted as two peaks (fig.1A), one at the void volume and the other (kinase I) at a position corresponding to mol. wt $\sim 1 \times 10^6$ (fig.2). The phosphorylase kinase activity was stimulated by the addition of calmodulin in the presence of Ca^{2+}. The activating activity of tryptophan 5-monooxygenase was eluted as two peaks (fig.1B), one at the void volume and the other (kinase II) at a position corresponding to mol. wt $\sim 5 \times 10^5$ (fig.2). The activating activity of tryptophan 5-monooxygenase absolutely required the presence of ATP, Mg^{2+}, Ca^{2+} and calmodulin. When casein was used as the substrate, the calmodulin-dependent protein kinase activity was eluted as 3 peaks (fig.1C), one at the void volume, the second at the position of kinase II, and the third (kinase III) at a position corresponding to mol. wt $\sim 1 \times 10^5$ (fig.2). The phosphorylation activity of casein by both kinase II and kinase III absolutely required the presence of both Ca^{2+} and calmodulin. When myosin light chain was used as the substrate, the kinase activity was also eluted as 3 peaks (fig.1D), each elution position of which corresponded to that of each peak of the calmodulin-dependent casein kinase. However, the ratio of myosin light chain kinase activity to calmodulin-dependent casein kinase activity of kinase III was twice as high as that of kinase II. Thus, myosin light chain served as a substrate more efficiently for kinase III than for kinase II, compared with casein. The phosphorylation activity of myosin light chain was absolutely dependent on the presence of Ca^{2+} and calmodulin. Since the kinase activity (kinase IV) eluted near the void volume was active with any of the substrates tested, it appeared to be a heterogeneous mixture of large molecular weight aggregates.

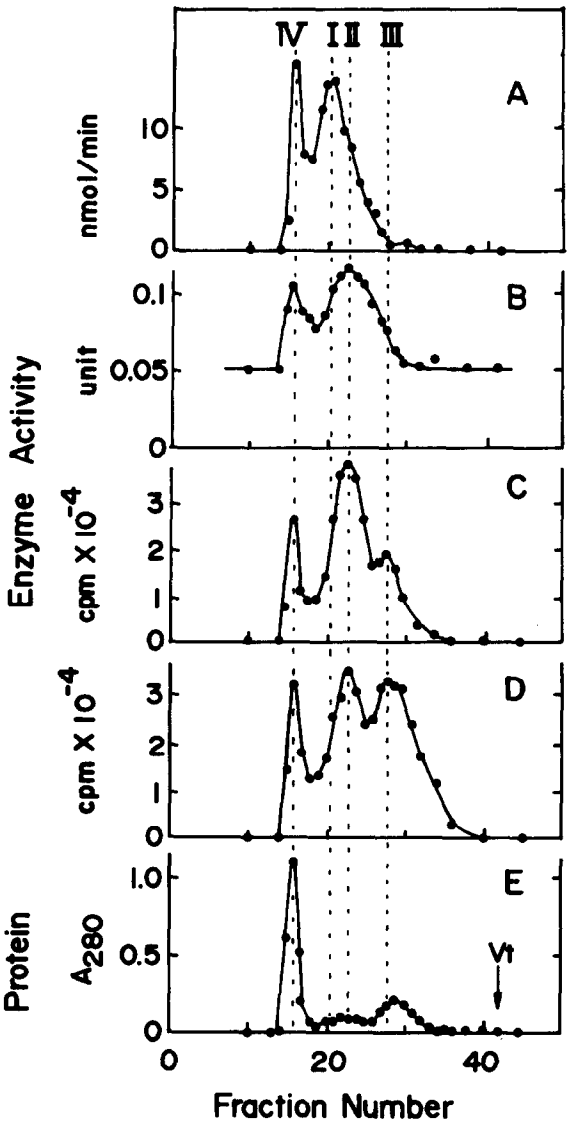

Fig.1. Gel filtration of calmodulin-dependent protein kinases on Sepharose CL-6B column. The column (1.4 × 60 cm) was equilibrated with buffer A and eluted with the same buffer. (A) A 10 μl aliquot of each fraction was assayed for phosphorylase kinase; (B) a 30 μl aliquot was assayed for the activation of tryptophan 5-monooxygenase; (C) a 10 μl aliquot was assayed for calmodulin-dependent phosphorylation of casein; (D) a 5 μl aliquot was assayed for calmodulin-dependent phosphorylation of myosin light chain; (E) protein was determined.

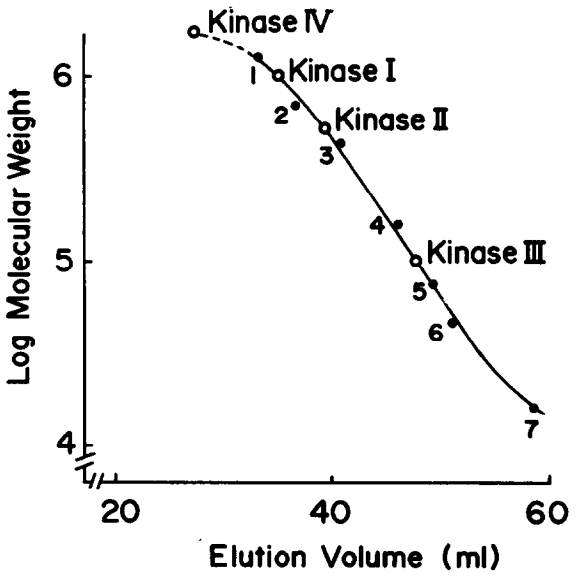

Fig.2. Determination of molecular weights of calmodulin-dependent protein kinases by gel filtration on Sepharose CL-6B column. Gel filtration was carried out as in fig.1. The standard proteins were: (1) skeletal muscle phosphorylase kinase; (2) thyroglobulin; (3) ferritin; (4) γ-globulin; (5) transferrin; (6) ovalbumin; (7) myoglobin.

An autoradiograph illustrating the incorporation of ^{32}P into cytosol proteins by the action of each fraction from Sepharose CL-6B column is shown in fig.3. The pattern of phosphorylation of the preparation precipitating at 55% saturated ammonium sulfate (line 1) was very similar to that of the phosphorylation of endogenous substrates by the kinase preparation from the purification step of calmodulin affinity chromatography (line 3). Since the former preparation contained almost all the calmodulin-dependent protein kinases as well as their endogenous protein substrates [4], the results suggested that almost all of the calmodulin-dependent protein kinases in brain cytosol were recovered in the latter preparation. Many protein bands including the proteins of mol. wt 81 000, 52 000, 47 000, 43 000, 37 000 and 17 000 were phosphorylated by fraction 23 (kinase II) (line 7), indicating that kinase II either had a broad substrate specificity or was a mixture of several different enzymes. The pattern of the phosphorylation by fraction 21 (kinase I) (line 6) was similar to that by fraction 23 (kinase II) (line 7), presumably due to contamination with kinase II in fraction 21. The phosphorylation of two protein bands of mol. wt 63 000 and 15 000 were selectively stimulated by fraction 29 (kinase III) (line 10), indicating that kinase III had a high degree of substrate specificity. Thus, kinase II differed from kinase III not only in size but also in substrate specificity.

These results demonstrate that at least 3 distinct calmodulin-dependent protein kinases, kinase I, kinase II and kinase III are present in rat brain cytosol. Kinase I with mol. wt ~1 × 10^6 which could be assayed with phosphorylase *b* as substrate may be identical with the kinase reported to be phosphorylase kinase in brain tissue [13,14], although its physiological protein substrates remain unknown. Kinase III with mol. wt ~1 × 10^5 which could be assayed with casein or myosin light chain as substrates may be identical with the kinase reported to be myosin light chain kinase [15], since kinase III showed a relatively high activity with myosin light chains. However, kinase III appears to differ in substrate specificity from myosin light chain kinase from muscle [16] or

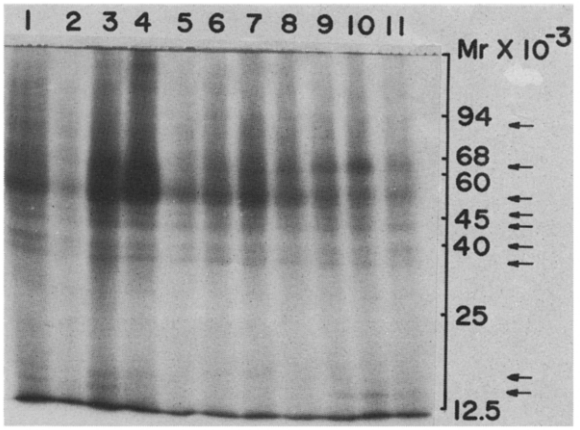

Fig.3. Analysis of phosphorylated cytosol proteins by SDS–polyacrylamide slab gel electrophoresis. The protein fraction precipitating with 55% saturated ammonium sulfate from: (1) cerebral cortex cytosol (120 μg protein); (2) endogenous substrates (140 μg of protein; (3) endogenous substrates (140 μg protein) and calmodulin-dependent protein kinase from the purification step of calmodulin affinity chromatography (9 μg protein); (4) endogenous substrates (140 μg protein) and a 10 μl aliquot of fraction 16, (5) fraction 18, (6) fraction 21, (7) fraction 23, (8) fraction 25, (9) fraction 27, (10) fraction 29, or (11) fraction 31 of Sepharose CL-6B were incubated with 0.1 mM [γ-^{32}P]ATP (1.9 × 10^6 cpm) in a mixture containing 50 mM Hepes buffer (pH 7.0), 5 mM $Mg(CH_3COO)_2$, 0.03 mM Ca^{2+}, 5 mM NaF and 1.4 μg calmodulin in 140 μl total vol. After incubation for 5 min at 30°C, SDS-slab gel electrophoresis and autoradiography were done. Arrows indicate protein bands markedly phosphorylated by calmodulin-dependent protein kinases.

platelet [17] which has been shown not to use casein as substrate. Two proteins with minimal mol. wt 63 000 and 15 000 were specifically phosphorylated by the action of kinase III. The biological function of these proteins remains, however, to be established. Kinase II with mol. wt $\sim 5 \times 10^5$, a new calmodulin-dependent protein kinase, showed a broad substrate specificity, suggesting that kinase II might play a number of roles in the function of nervous system. One of them may be related to regulation of serotonin biosynthesis, since kinase II appeared to be involved in the activation of tryptophan 5-monooxygenase, a rate-limiting enzyme in the biosynthesis of serotonin in the nervous system.

Acknowledgements

The authors thank Professor K. Uchida in this college and Dr H. Onishi in Tokyo Institute of Technology for their advice on the preparation of myosin. This work has been supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science, and Culture of Japan and by a grant from Takeda Science Foundation.

References

[1] Rubin, R. P. (1970) Pharmacol. Rev. 22, 389–428.
[2] Greengard, P. (1978) Science 199, 146–152.
[3] Yamauchi, T. and Fujisawa, H. (1979) Biochem. Biophys. Res. Commun. 90, 28–35.
[4] Yamauchi, T. and Fujisawa, H. (1979) Biochem. Biophys. Res. Commun. 90, 1172–1178.
[5] Schulman, H. and Greengard, P. (1978) Proc. Natl. Acad. Sci. USA 75, 5432–5436.
[6] DeLorenzo, R. J., Freedman, S. D., Yohe, W. B. and Maurer, S. C. (1979) Proc. Natl. Acad. Sci. USA 76, 1838–1842.
[7] Wang, J. H. and Desai, R. (1977) J. Biol. Chem. 252, 4175–4184.
[8] Perrie, W. T. and Perry, S. V. (1970) Biochem. J. 119, 31–38.
[9] Cohen, P. (1973) Eur. J. Biochem. 34, 1–14.
[10] Klee, C. B. and Krinks, M. H. (1978) Biochemistry 17, 120–126.
[11] Yamamura, H., Nishiyama, K., Shimomura, R. and Nishizuka, Y. (1973) Biochemistry 12, 856–862.
[12] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275.
[13] Drummond, G. I. and Bellward, G. (1970) J. Neurochem. 17, 475–482.
[14] Ozawa, E. (1973) J. Neurochem. 20, 1487–1488.
[15] Dabrowska, R. and Hartshorne, D. J. (1978) Biochem. Biophys. Res. Commun. 85, 1352–1359.
[16] Walsh, M. P., Vallet, B., Autric, F. and Demaille, J. G. (1979) J. Biol. Chem. 254, 12136–12144.
[17] Hathaway, D. R. and Adelstein, R. S. (1979) Proc. Natl. Acad. Sci. USA 76, 1653–1657.