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Changes of the expression of protein substrates of Ca^{2+}/calmodulin-dependent protein kinase II in neonate and adult rats

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Abstract

We studied the expression of whole protein substrates of Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) in the forebrain of neonate and adult rats. Protein substrates were determined by phosphorylation of the soluble and particulate fractions by CaM kinase II with [γ-^{33}P]ATP. Phosphorylated proteins were analyzed by SDS-PAGE and two-dimensional gel electrophoresis. More than 50 endogenous proteins were found to be phosphorylated by CaM kinase II in both soluble and particulate fractions. The expression of about 15 protein substrates increased in the particulate fraction from neonate to adult rats, and that of several proteins also changed in the soluble fraction. These findings suggest that the expression of protein substrates was regulated during development as well as that of CaM kinase II itself.

Key words: Ca^{2+}/calmodulin-dependent protein kinase II; Protein substrate; Brain development; Two-dimensional gel electrophoresis; [γ-^{33}P]ATP

1. Introduction

Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) is one of the most abundant protein kinases in the mammalian brain [1–3]. CaM kinase II is implicated in a variety of cellular events that utilize Ca^{2+} as a second messenger due to its self-regulatory property, subcellular localization at postsynaptic densities, and broad substrate specificity (for reviews, see [4–6]). The native form of CaM kinase II consists of a distinct homopolymer of either α or β polypeptide [7]. The composition of α and β isoforms of CaM kinase II depends on the region of the brain and on the stage of development [5,6]. The concentration of α and β polypeptides varied markedly in different brain regions at increasing postnatal ages [8]. The enzyme increased most rapidly during the third postnatal week, and changed from being mostly cytosolic in the immature brain to being primarily membrane bound in the adult [9,10]. However, these studies have not determined the changes of the expression of endogenous protein substrates. It is not known how many proteins are phosphorylated by CaM kinase II, although about 15 brain proteins were reported as putative substrates for CaM kinase II [6]. To understand the physiological function of CaM kinase II in neuronal tissue, it is important to analyze the whole protein substrates of this enzyme in the brain.

In the present study, we investigated the expression of protein substrates of CaM kinase II using [γ-^{33}P]ATP in neonate and adult rats. Protein substrates were analyzed by one or two dimensional gel electrophoresis followed by autoradiography and counting radioactivity. The findings indicated that the expression of many protein substrates was increased from neonates to adults according to the increase in the level of CaM kinase II.

2. Materials and methods

2.1. Materials

[γ-^{33}P]ATP was purchased from New England Nuclear. Pharmalyte was from Pharmacia LKB Biotechnology Inc. CaM kinase II was purified from rat forebrain as described previously [1]. The Ca^{2+}-independent form of the kinase was generated by autophosphorylation as described previously [11].

2.2. Preparation of endogenous protein substrates

The forebrain of Wistar rats was homogenized with 5 volumes of 40 mM Tris-HCl buffer, pH 7.6, containing 1 mM dithiothreitol, 1 mM EGTA, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml each of protease inhibitors antipain, leupeptin and pepstatin A (Buffer A). Soluble and particulate fractions were prepared from the homogenate as described previously [8]. To remove the endogenous CaM kinase II activity, the soluble and particulate fractions were treated as follows. The soluble fraction was incubated with 0.1 M acetate buffer, pH 5.0 for 30 min at 0°C, then ethanol was added to make a final concentration of 15% and stood for 15 min at 0 °C. This fraction was used as endogenous protein substrates of the soluble frac-

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Abbreviations: CaM kinase II, Ca^{2+}/calmodulin-dependent protein kinase II; EGTA, ethylene glycol bis (β-amonoethylether)-*N,N,N',N'*-tetra acetic acid.

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tion. The particulate fraction was incubated with 3 M urea for 20 min at 20°C, then centrifuged at 18,500 × *g* for 30 min. The resulting precipitate was suspended with 40 mM Tris buffer, pH 7.6, containing 1 mM dithiothreitol, 50 mM NaCl, 10% ethylene glycol, 0.05% Tween 40, 10 μg/ml of each of the protease inhibitors antipain, leupeptin and pepstatin A, and used as endogenous protein substrates of the particulate fraction.

About 80% of endogenous CaM kinase II activity was removed by these treatments in both soluble and particulate fractions (data not shown).

2.3. Phosphorylation and analysis of endogenous protein substrates

Endogenous protein substrates were phosphorylated by CaM kinase II as described previously [1] with some modifications. The reaction mixture contained 50 μM [γ-^{33}P]ATP (2–4 × 10^6 cpm), 8 mM $Mg(CH_3COO)_2$, 0.15 mM $CaCl_2$, 0.8 μM calmodulin, 10 μg/ml each of protease inhibitors antipain, leupeptin and pepstatin A, 1 μM okadaic acid, 100 μM orthovanadate, 5 mM NaF, 50 mM HEPES buffer, pH 8.0, and a suitable amount of CaM kinase II and protein substrates. Control experiments were carried out in the reaction mixture contained 1 μM EGTA in place of $CaCl_2$.

After phosphorylation at 30°C for 15 min, an aliquot was subjected to SDS-PAGE or two dimensional gel electrophoresis. After electrophoresis, gels were dried and phosphorylated proteins were visualized by autoradiography at −80 °C overnight. In some experiments, radioactive spots were excised, and placed in a toluene scintillation solution, then counted in a liquid scintillation counter.

2.4. Gel electrophoresis

SDS-PAGE was carried out with a 10% polyacrylamide gel containing 0.1% SDS by the method of Laemmli [12]. Two-dimensional gel electrophoresis was carried out as described previously [13]. The first dimension consisted of isoelectric focussing on a 1% agarose gel in Pharmalyte, pH 3–10. Isoelectric focussing was carried out at 200 V for 1 h, then at 400 V for 4 h at 4°C. The second dimension was SDS-PAGE on a 7–15% polyacrylamide gradient gel at 6 mA for 1 h, then 17 mA for 1.5 h.

3. Results and discussion

Whole protein substrates were phosphorylated in the soluble and particulate fractions by purified CaM kinase II with [γ-^{33}P]ATP, after removal of endogenous CaM kinase II activity as described in section 2. The change of phosphorylated proteins during development was analyzed by SDS-PAGE. As shown in Fig. 1, many phosphorylated protein bands were observed from day 1 to 60 in both soluble and particulate fractions. Each band of proteins phosphorylated with [γ-^{33}P]ATP became much more sharp as compared with those phosphorylated with [γ-^{32}P]ATP (data not shown). In the particulate fraction, the phosphorylation level of at least 8 bands (~330, ~250, 88, 80, 46, 24, 21, and 19 kDa) increased and that of at least 5 bands (~280, 170, 140, 110, and 31 kDa) decreased during development. In the soluble fraction, the level of phosphorylation of at least 3 bands (~270, 85, and 78 kDa) increased and that of at least one band (110 kDa) decreased. Microtubule associated protein 2 (~270 kDa band in Fig. 1B), a good substrate for CaM kinase II [14], greatly increased, although other proteins were not identified in these experiments.

To characterize protein substrates which were changed in their phosphorylation level during development, phosphorylated proteins were analyzed by two-dimensional gel electrophoresis. In the particulate fraction, about 50 radioactive spots were observed in both neonate and adult rats as shown in Fig. 2. Since the level of phosphorylation of CaM kinase II was high as shown by the arrow in Fig. 2D, phosphorylation of tubulin and tau, good substrates of CaM kinase II, could not be dissolved under the experimental conditions. However, we can see that about 20 proteins were changed in the phosphorylation level from day 1 to day 60. Fig. 2D shows a schematic drawing of protein substrates and typical spots were numbered from 1 to 16. Table 1 shows the molecular size and isoelectric point of these proteins. Spot numbers 1, 13, and 16 were expanded in a wide range of pH. Several spots did not correspond to bands of SDS-PAGE, because of the difference of the dissolution of gel electrophoresis. We cannot identify most spots of protein substrates at present. In the soluble fraction, several spots were also changed by two-dimensional gel electrophoresis, and microtubule-associated protein 2 was greatly increased, consistent with the observation of SDS-PAGE (data not shown).

Since phosphorylation of protein substrates was thought to be correlated to the content of protein substrates, we tried to analyze these proteins quantitatively. Radioactive spots were excised and counted as described in section 2. The change of the content of each protein substrate was determined by measuring radioactivity and summarized in Table 1. Although the content of spot numbers 1, 5, 6, and 7 was decreased by about 43, 29, 62, and 20%, respectively, other spots were increased by

Table 1
Change of the content of protein substrates of CaM kinase II in the particulate fraction on day 1 and 60

Spot no.	Molecular size	Isoelectric point	Change of protein substrates
	kDa	pH	Ratio on day 60/day 1
1	~300	5.8 (6.7–5.0)	0.57
2	260	4.5	1.9
3	70	~9.0	4.2
4	75	8.5	4.0
5	66	6.7	0.71
6	51	8.3	0.38
7	55	4.8	0.80
8	51	4.8	4.6
9	45	4.9	2.0
10	20	~9.0	14
11	20	8.6	15
12	20	8.0	17
13	20	6.3 (7.5–5.2)	20
14	~17	8.9	36
15	~17	8.0	27
16	~17	6.1 (7.1–5.0)	31

Protein substrates which changed from day 1 to day 60 are numbered from 1 to 16 in Fig. 2. The molecular size and isoelectric point were calculated from the data of Fig. 2. Change of protein substrates was calculated from the measured radioactivity of each spot which was excised from the gels as described in section 2.

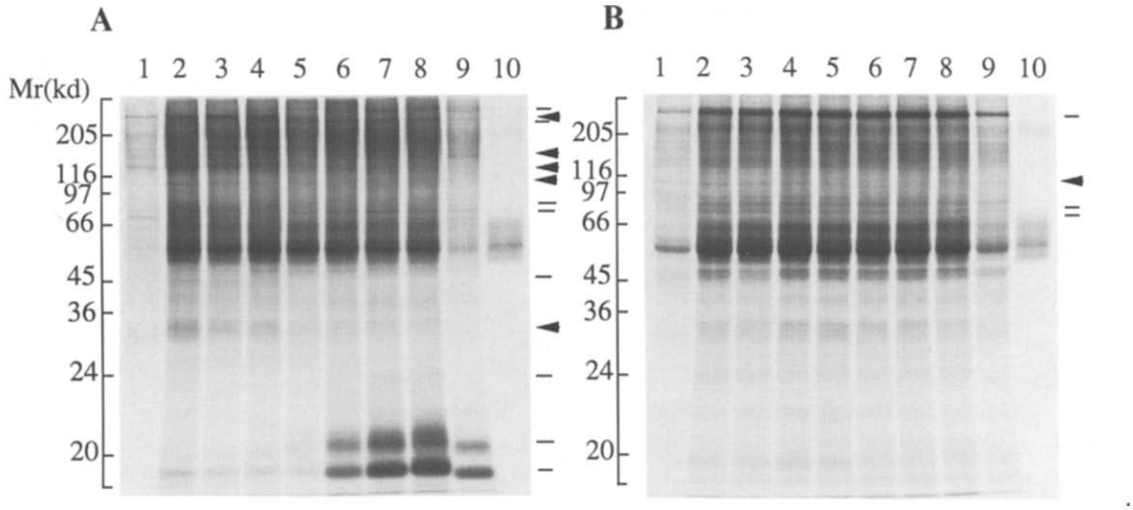

Fig. 1. Developmental change of protein substrates of CaM kinase II analyzed by SDS-PAGE. Endogenous protein substrates (10 μg) were phosphorylated by CaM kinase II (0.2 μg) with [γ-^{33}P]ATP, and then subjected to SDS-PAGE as described in section 2. (A) The particulate fraction. (B) The soluble fraction. Column 1 and 9, phosphorylated without added CaM kinase II on day 1 and 60, respectively; column 2 to 8, phosphorylated by CaM kinase II on day 1, 5, 10, 14, 20, 30, and 60, respectively; column 10, CaM kinase II alone. Bands increased and decreased during development are shown by bar and arrowhead, respectively, at the right. Molecular markers are shown at the left.

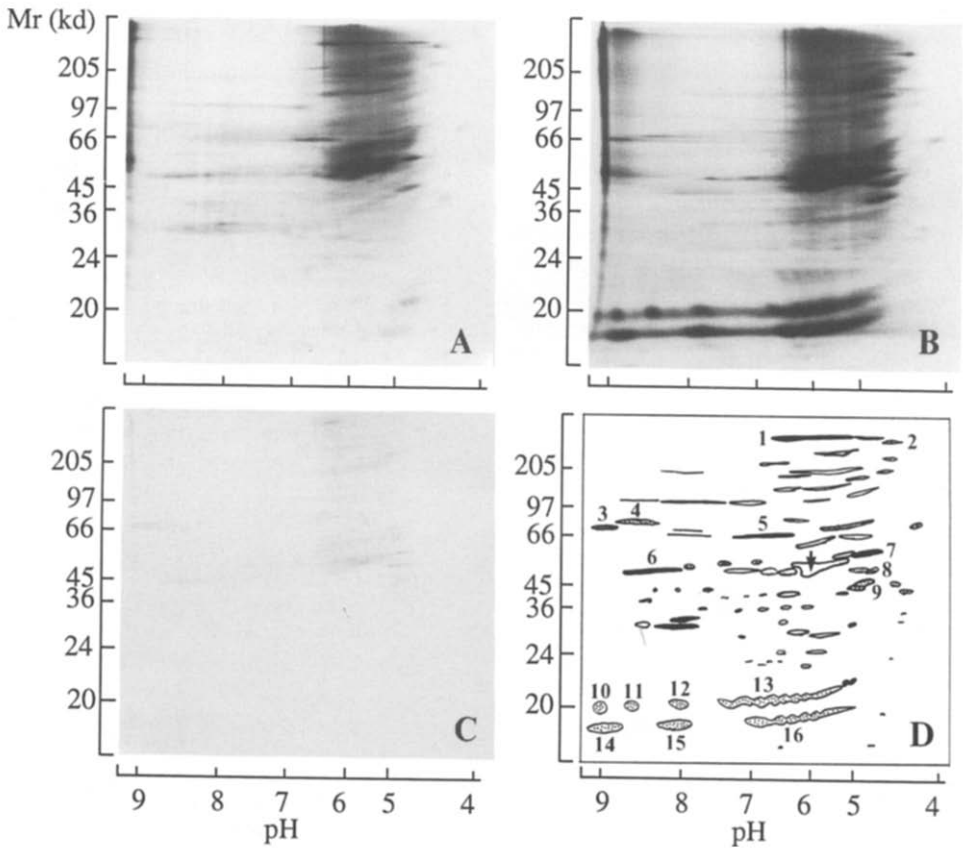

Fig. 2. Comparison of protein substrates of CaM kinase II on day 1 and 60 by two-dimensional gel electrophoresis. Endogenous protein substrates (18 μg) of the particulate fraction were phosphorylated by CaM kinase II (0.4 μg) with [γ-^{33}P]ATP, and then subjected to two-dimensional gel electrophoresis as described in section 2. (A) and (B), on day 1 and 60, respectively; (C), on day 60 without added CaM kinase II; (D) schematic representation of protein substrates. ○, almost the same on day 1 and 60; ◎, increased from day 1 to 60; ●, decreased from day 1 to 60, *arrow*, spot of CaM kinase II. Typical spots were numbered from 1 to 16. Molecular markers are shown at the left. A pH gradient of the isoelectric focusing gel is shown at the bottom.

about 2- to 35-fold from neonates to adults. The content of spot numbers 13, 14, 15, and 16 were greatly increased. These findings suggest that the expression of protein substrates of CaM kinase II was regulated during development, as well as CaM kinase II itself.

The present study demonstrates by two-dimensional gel electrophoresis that more than 50 protein substrates were phosphorylated by CaM kinase II in the brain. By using [γ-^{33}P]ATP which was recently made commercially available, we could obtain clearer radioactive spots on autoradiography than with [γ-^{32}P]ATP, generally used for the phosphorylation reaction. [^{33}P]phosphate is a β-emitter and the energy of radioactive decay is lower than that of [^{32}P]phosphate.

To remove the endogenous CaM kinase II activity, the soluble fraction was treated with ethanol and the particulate fraction was treated with urea. These treatments did not change the total phosphorylation level of endogenous protein substrates (data not shown), indicating that these substrates were almost native and natural substrates of CaM kinase II. Although about 15 brain proteins have been reported to be phosphorylated by CaM kinase II in vitro [6], analysis of whole protein substrates in the brain has not been carried out. The present studies showed that more than 50 brain proteins were phosphorylated by CaM kinase II. In the particulate fraction, spot numbers 3 and 4 may have corresponded to synapsin Ib and Ia, respectively, although their molecular weight and isoelectric point were somewhat different from the previous report [15]. Synapsin I is known as a good substrate for CaM kinase II [2]. In the soluble fraction, the level of microtubule-associated protein 2 was found to increase from day 1 to 60. These findings were consistent with the previous observation that synapsin I and microtubule-associated protein 2 are developmentally regulated [16,17].

To assess the role of CaM kinase II in regulating the enzymatic activity of physiological process the molecular components of which have not been identified, the present study will be an unique approach. The increase in CaM kinase II near the end of the most active period of synaptogenesis may coincide with synaptic maturation [8,10]. The expression of protein substrates of CaM kinase II was found to be correlated to the expression of CaM kinase II during synaptogenesis. Although many protein substrates could not be correlated to known substrates in the present study, identification of these substrates and their regulation by phosphorylation should be elucidated to understand the full aspect of CaM kinase II function.

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