0026-895X/94/030423-08$03.00/0

MOLECULAR PHARMACOLOGY, 46:423–430, 1994.

Occurrence and Activation of Ca^{2+}/Calmodulin-Dependent Protein Kinase II and Its Endogenous Substrates in Bovine Adrenal Medullary Cells

NOBUYUKI YANAGIHARA, YUMIKO TOYOHIRA, HIDEYUKI YAMAMOTO, YASUTAKA OHTA, MASATO TSUTSUI, EISHICHI MIYAMOTO, and FUTOSHI IZUMI

Department of Pharmacology, University of Occupational and Environmental Health, School of Medicine, Kitakyushu 807, Japan (N.Y., Y.T., M.T., F.I.), and Department of Pharmacology, Kumamoto University School of Medicine, Kumamoto 860, Japan (H.Y., Y.O., E.M.)

Received January 31, 1994; Accepted June 14, 1994

SUMMARY

We investigated the presence of and the endogenous substrates for Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) in cultured bovine adrenal medullary cells. By a series of chromatographic steps using DEAE-cellulose, calmodulin affinity, and Sephacryl S-300 columns, we partially purified two CaM kinases (peaks I and III) and one calmodulin-binding protein (peak II). Both of the kinases (peaks I and III) showed broad substrate specificities. Peak I, but not peak III, was immunoprecipitated with an antibody against rat brain CaM kinase II, suggesting that peak I is CaM kinase II or a closely associated CaM kinase. Although the anticaldesmon antibody recognized a 77-kDa protein (low molecular mass caldesmon) in crude preparations from the cells, the protein in peak II was not immunoblotted with the antibody. The peak II protein was phosphorylated by the CaM kinase in peak I but not by the CaM kinase in peak III. Peak I kinase also phosphorylated purified tyrosine hydroxylase and several proteins from chromaffin granule membranes. Stimulation of cultured bovine adrenal medullary cells with 56 mM K^+ evoked rapid increases in $^{45}Ca^{2+}$ influx and autonomous CaM kinase II activity, both of which were attenuated by the addition of 20 mM $MgSO_4$, an inhibitor of voltage-dependent Ca^{2+} channels. These results suggest that an isozyme of CaM kinase II exists in adrenal medullary cells and is activated by cell depolarization. Furthermore, the peak II protein is apparently a novel endogenous substrate for CaM kinase II.

Calcium ion is widely recognized as an important intracellular messenger in stimulus-secretion coupling (1) and in the regulation of the synthesis of neurotransmitters and hormones (2). One of the primary intracellular receptors of Ca^{2+} is CaM, a ubiquitous Ca^{2+}-binding protein (3, 4) that interacts with many intracellular proteins. Among these targets for CaM are several distinct CaM kinases, such as phosphorylase kinase, myosin light chain kinase, and CaM kinase I, II, III, and IV (5). CaM kinase II is a multifunctional protein kinase that has been studied extensively in the brain and other tissues (for reviews, see Refs. 5–7). The enzyme has broad substrate specificities and is implicated in the regulation of numerous Ca^{2+}-mediated cellular functions in response to extracellular signals.

Adrenal medullary cells are paraneurons of neural crest origin and share many physiological and pharmacological properties with postganglionic sympathetic neurons. Stimulation of acetylcholine receptors in adrenal medullary cells causes an increase in secretion (1, 8) and synthesis (2, 8) of catecholamines in a Ca^{2+}-dependent manner. The enhancement of catecholamine synthesis is associated with the activation of tyrosine hydroxylase, which catalyzes the rate-limiting step in the biosynthesis of catecholamines (9). Furthermore, in rat pheochromocytoma PC-12 cells there is a direct relationship between activation and phosphorylation of tyrosine hydroxylase caused by 56 mM K^+-evoked depolarization (10). Several studies have demonstrated that the tryptic peptide maps of tyrosine hydroxylase phosphorylated in rat pheochromocytoma PC-12 cells after treatment with carbachol or 56 mM K^+ are similar to those obtained after the enzyme is phosphorylated by CaM kinase II *in vitro* (11, 12). Therefore, CaM kinase II is considered to be involved in the Ca^{2+}-mediated phosphorylation of tyrosine hydroxylase in intact cells (12). However, the existence of CaM kinase II has not been well documented in adrenal medulla.

In the present study, we have partially purified and characterized three CaM-binding proteins, including an apparent

This research was supported in part by grants-in-aid (03670115 and 05680688) from the Ministry of Education, Science, and Culture of Japan.

ABBREVIATIONS: CaM, calmodulin; CaM kinase, Ca^{2+}/calmodulin-dependent protein kinase; PAGE, polyacrylamide gel electrophoresis; SDS, sodium dodecyl sulfate; PKC, protein kinase C; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid.

isoform of CaM kinase II, from cultured bovine adrenal medullary cells. Furthermore, we have searched for endogenous substrates for CaM kinase II and have examined the effect of high-K^+ depolarization on the activation of CaM kinase II in the cells.

Experimental Procedures

Materials. CaM (bovine brain) was purchased from Calbiochem-Behring (La Jolla, CA); DEAE-cellulose, CaM-agarose, and Sephacryl S-300 from Whatman Bio Systems (Maidstone, Kent, UK), Sigma Chemical Co. (St. Louis, MO), and Pharmacia (Uppsala, Sweden), respectively; [γ-^{32}P]ATP (3000 Ci/mmol) from New England Nuclear (Boston, MA); $^{45}CaCl_2$ (0.5–2 Ci/mmol) and ^{125}I Protein A (2–10 μCi/μg) from Amersham International (Amersham, UK); CaM kinase II substrate peptide, syntide 2, and synthetic peptide inhibitors of cAMP-dependent protein kinase (PKI-tide) and PKC (PKC_{19-36}) from Bachem Feinchemkalien AG (Bubendorf, Switzerland); and poly(vinylidene fluoride) membrane (GVH membrane) from Millipore (Bedford, MA). Rat brain CaM kinase II was purified according to the method of Fukunaga *et al.* (13). Tyrosine hydroxylase purified from rat pheochromocytoma PC-12 cells and polyclonal anticaldesmon antibody were kindly provided by Dr. E. Tachikawa (Department of Pharmacology, Iwate Medical School, Morioka, Japan) and Dr. K. Sobue (Department of Neurochemistry and Neuropharmacology, Biomedical Research Center, Osaka University Medical School, Osaka, Japan), respectively. Other chemicals used were of analytical grade from Nacalai Tesque (Kyoto, Japan).

Preparation of cultured adrenal medullary cells. Bovine adrenal medullary cells were isolated by collagenase digestion, as described previously (14). The isolated cells were purified by a selective plating method (15). In brief, cells were suspended in Eagle's minimum essential medium containing 10% calf serum, 10 μM cytosine arabinoside, and antibiotics and were plated on tissue culture dishes (100-mm Falcon) for 3 hr. The nonattached cells (adrenal medullary cells) were decanted away from the attached cells and cultured for 2–4 days in bacterial (non-tissue culture) plastic dishes (150-mm Falcon) under 5% CO_2/95% air. We checked the purity of cultured cells and found that the final cell preparation contained at least 90% adrenal medullary cells.

Partial purification of CaM kinases and a CaM-binding protein from cultured adrenal medullary cells. CaM kinases (peaks I and III) and a CaM-binding protein (peak II) were partially purified from the cultured bovine adrenal medullary cells, essentially according to the method described by Fukunaga *et al.* (13), with a slight modification. Cultured cells (1.5×10^9) were homogenized with 10 volumes of 0.1 M NaCl, 20 mM Tris·HCl buffer, pH 7.5, 2 mM EDTA, 4 mM EGTA, 10 mM 2-mercaptoethanol, 0.5% Triton X-100, 0.43 mM phenylmethylsulfonyl fluoride, 0.05 mM leupeptin, 50 mg/liter trypsin inhibitor. The homogenate was centrifuged at 105,000 × *g* for 1 hr. The resultant supernatant was applied to a DEAE-cellulose column (4 × 8 cm) that had been equilibrated with 20 mM Tris·HCl buffer, pH 7.5, 0.1 mM EGTA, 10 mM 2-mercaptoethanol, 10% glycerol (buffer A), containing 20 mM NaCl. The protein fraction was eluted with 200 ml of buffer A containing 200 mM NaCl. 200 ml of 100% ammonium sulfate solution, pH 7.4, was added to the eluate. After 30 min of stirring, the precipitate was collected by centrifugation and dissolved in buffer A containing 20 mM NaCl. The solution was diluted with 2 volumes of dilution buffer (40 mM Tris·HCl, pH 7.5, 1 mM $CaCl_2$, 10 mM 2-mercaptoethanol) and applied to a CaM-agarose column (1.2 × 4.4 cm). The column was washed with 100 ml of buffer (40 mM Tris·HCl, pH 7.5, 2 M NaCl, 0.2 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol). Then, the fraction of CaM-binding proteins was eluted with 60 ml of buffer (40 mM Tris·HCl, pH 7.5, 0.2 M NaCl, 1 mM EGTA, 1 mM $MgCl_2$, 10 mM 2-mercaptoethanol). The fraction was concentrated by ultrafiltration with an Amicon PM-10 membrane. The concentrate was applied to a Sephacryl S-300 column (2.2 × 60 cm) and the proteins were eluted with buffer (20 mM Tris·HCl, pH 7.5, 0.2 M NaCl, 0.1 mM EGTA, 10 mM 2-mercaptoethanol). The protein fractions (peaks I, II, and III) were each pooled and concentrated by ultrafiltration.

Assay for CaM kinase activity *in vitro*. The standard incubation medium (final volume, 100 μl) contained 25 mM Tris·HCl buffer, pH 7.5, 10 mM $MgCl_2$, 0.1 mM $CaCl_2$, 20 μM [γ-^{32}P]ATP ($3–5 \times 10^5$ cpm), 40 μg of chicken gizzard myosin light chain as substrate, and the enzyme, with or without 0.5 μg of CaM. Incubation was carried out at 30° for 10 min. The reaction was terminated, and the amount of phosphate incorporated into myosin light chain was measured as described previously (16). The enzyme activity was expressed in picomoles or nanomoles of phosphate incorporated per minute per fraction or milligram of protein. Protein was determined according to the procedure of Bradford (17).

Subcellular localization of CaM kinase activity. Cultured cells (3×10^7) were washed three times with Dulbecco's phosphate-buffered saline (Nissui Pharmaceutical, Tokyo, Japan). The cell pellet was homogenized with 10 volumes of 0.29 M sucrose, 20 mM Tris·HCl buffer, pH 7.5, 2 mM EDTA, 2 mM EGTA, 10 mM 2-mercaptoethanol, 0.43 mM phenylmethylsulfonyl fluoride, 0.05 mM leupeptin, 50 mg/liter trypsin inhibitor. The homogenate was centrifuged at 850 × *g* for 10 min to remove cell debris, and the supernatant was centrifuged at 105,000 × *g* for 1 hr. The pellet (particulate fraction) was resuspended in the homogenizing buffer with 0.1% Triton X-100. The CaM kinase activity in the supernatant and particulate fractions was measured as described for the assay for CaM kinase activity *in vitro*, except for the concentrations of 0.1 mM [γ-^{32}P]ATP (3.5×10^6 cpm) and 0.2 mM $CaCl_2$.

Immunoprecipitation of CaM kinase with the anti-CaM kinase II antibody. Affinity-purified polyclonal antibody against rat brain CaM kinase II was prepared as described previously (18, 19). The enzyme fraction (2.6 μg of peak I or peak III) or brain CaM kinase II (0.5 μg) was incubated at 4° for 90 min with or without the anti-CaM kinase II antibody (10 μl of IgG), in a buffer containing 150 mM NaCl and 20 mM Tris·HCl, pH 7.4. After incubation, the antibody was immobilized by incubation at 0° for 15 min with 50 μl of a solution containing 50% (v/v) Protein A-Sepharose gel (Pharmacia), 20 mM Tris·HCl buffer, pH 7.4, and 150 mM NaCl. Immunoprecipitate-Protein A-Sepharose gel complex was washed and incubated at 30° for 20 min with 40 μM [γ-^{32}P]ATP (8.8×10^6 cpm) in a phosphorylation medium (100 μl) containing 10 mM $MgCl_2$, 1 mM $CaCl_2$, 2 μg of CaM, and 40 μg of chicken gizzard myosin light chain. The phosphorylation reaction was stopped by addition of SDS stop solution (20), containing 60 mM Tris·HCl buffer, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, and 0.001% bromphenol blue, and boiling for 3 min. After centrifugation, aliquots of the supernatant were analyzed by SDS-PAGE in 13.5% acrylamide gels (20).

Immunoblotting of peak II with the anticaldesmon antibody. Each sample was separated by SDS-PAGE (9% acrylamide gels). After electrophoresis, proteins were transferred to GVH membrane (21). After the membrane was incubated for 1 hr at room temperature with 2.5% (w/v) bovine serum albumin in 100 mM Tris·HCl buffer, pH 7.5, 0.9% (w/v) NaCl, it was incubated with the polyclonal anticaldesmon antibody at 4° overnight and the bound antibody was treated with ^{125}I Protein A. The radioactivity was analyzed by autoradiography.

Phosphoamino acid analysis. Peak I (2 μg) was autophosphorylated by incubation with 20 μM [γ-^{32}P]ATP (8.8×10^6 cpm) in the presence of 0.2 mM $CaCl_2$ and 0.8 μg of CaM. The phosphorylated protein in peak I was separated by SDS-PAGE. It was cut out of the gel and partially hydrolyzed in 6 M HCl at 110° for 1 hr. Phosphoamino acids in the hydrolysate were located by high-voltage paper electrophoresis, as described previously (22).

Assay of the Ca^{2+}-independent activity of CaM kinase II after depolarization of the cells with 56 mM K^+. Cultured adrenal medullary cells (4×10^6/dish, 35-mm Falcon) were washed three times with oxygenated Krebs-Ringer phosphate buffer. It was composed of

154 mM NaCl, 5.6 mM KCl, 1.1 mM $MgSO_4$, 2.2 mM $CaCl_2$, 0.85 mM NaH_2PO_4, 2.15 mM Na_2HPO_4, and 10 mM glucose, adjusted to pH 7.4. In the 56 mM K^+ medium, NaCl was reduced to 103.6 mM from 154 mM. Cells were incubated with or without 56 mM K^+ at 37° for the indicated times and then quickly frozen on dry ice. The frozen cells were scraped and homogenized in 160 μl of solubilization medium, which contained 50 mM HEPES buffer, pH 7.4, 0.1% Triton X-100, 4 mM EGTA, 5 mM EDTA, 15 mM $Na_4P_2O_4$, 100 mM β-glycerophosphate, 25 mM NaF, 0.1 mM leupeptin, and 75 μM pepstatin. After centrifugation at 15,000 × *g* for 5 min, aliquots of the supernatant were assayed for CaM kinase II activity, as described previously (23). The medium (20 μl) contained 50 mM HEPES buffer, pH 7.5, 10 mM $MgCl_2$, 0.1 mM [γ-^{32}P]ATP (3000–5000 cpm/pmol), 1 mg/ml bovine serum albumin, 40 μM syntide 2, 0.1 mM PKI-tide, and 1 μM PKC_{19-36}, with 1 mM EGTA (Ca^{2+}-independent activity) or with 1 mM $CaCl_2$ and 2 μM CaM (total CaM kinase II activity). The reaction was initiated by the addition of 5 μl of the supernatant and was carried out at 30° for 3 min. After incubation, 20 μl of an aliquot of each sample was spotted on a P-cellulose paper square (Whatman) and processed as described by Roskoski (24). The specificity of the assay for CaM kinase II activity was confirmed, as described previously (25). The Ca^{2+}-independent activity was expressed as a percentage of the total CaM kinase II activity.

Statistical analysis. Data are expressed as mean ± standard deviation. Statistical evaluation of the data was performed by analysis of variance. If a significant *F* value was found, Scheffe's test or Dunnett's test for multiple comparisons was carried out to identify differences among groups.

In Fig. 7, a relationship between Ca^{2+}-independent activity of CaM kinase II and $^{45}Ca^{2+}$ influx was assessed by a linear regression analysis (*y*-axis, Ca^{2+}-independent activity of CaM kinase II; *x*-axis, $^{45}Ca^{2+}$ influx).

Results

Separation of two CaM kinases and one major CaM-binding protein by Sephacryl S-300 column chromatography. The supernatant fraction from cultured adrenal medullary cells was subjected to a series of chromatographic steps using DEAE-cellulose, CaM affinity, and Sephacryl S-300 columns. Two peaks of CaM kinase activity (peaks I and III) and one major peak of CaM-binding protein (peak II) were observed on the Sephacryl S-300 column (Fig. 1A). Fractions 34–38, 42–43, and 47–48 were collected and designated as peaks I, II, and III, respectively. Apparent molecular masses of the three peaks (peaks I, II, and III) were estimated to be approximately 650, 300, and 200 kDa, respectively (Fig. 1B, a). Apparent molecular masses of the proteins in peak I and peak II were approximately 50 and 70 kDa, respectively, using SDS-PAGE (Fig. 1B, b). These results are also summarized in Table 1.

The specificities of the protein kinases (peaks I and III) for various exogenous substrates were investigated. Chicken gizzard myosin light chain, myelin basic protein, and β-casein served as good substrates, whereas lysine-rich histone, arginine-rich histone, and whole histone were only slightly phosphorylated by the two kinases. Phosphorylase *b* was not phosphorylated by these kinases (Table 1).

The subcellular localization of CaM kinase activity was studied in adrenal medullary cells. About 75% of CaM kinase activity was associated with the supernatant fraction of the cells (data not shown).

Immunoprecipitation of CaM kinase with the anti-CaM kinase II antibody. Because the protein kinases in peaks I and III had broad substrate specificities (Table 1), we examined whether the adrenal medullary CaM kinases were related to CaM kinase II. Peak I and peak III were incubated at 0° for 90 min with or without the anti-rat brain CaM kinase II antibody. The immunoprecipitates were incubated with [γ-^{32}P]ATP and myosin light chain in the presence of Ca^{2+} and CaM. As shown in Fig. 2, the immunoprecipitates of peak I and rat brain CaM kinase II phosphorylated myosin light chain, whereas the immunoprecipitates derived from peak III did not. In addition to the phosphorylation of myosin light chain, peak I showed a faint band at approximately 50 kDa, which migrated to a position similar to that of the autophosphorylated α subunit of CaM kinase II in SDS-PAGE. These results suggest that peak I is an isozyme of CaM kinase II in adrenal medullary cells.

After incubation of peak I with [γ-^{32}P]ATP in the presence of Ca^{2+} and CaM, but in the absence of exogenous substrates, the autophosphorylated 50-kDa subunit was separated by SDS-PAGE and hydrolyzed with 6 M HCl at 110° for 1 hr. High-voltage paper electrophoresis of the acid hydrolysate showed that serine and threonine residues were mainly phosphorylated (^{32}P-serine, 141 cpm; ^{32}P-threonine, 53 cpm) and that the ratio of phosphate incorporated into threonine to that incorporated into serine was approximately 0.38:1.

Phosphorylation of the protein in peak II by peak I kinase. To investigate whether the protein in peak II is phosphorylated by the CaM kinase activities in peaks I and III, we incubated peak II with or without peak I and peak III under standard phosphorylation conditions (Fig. 3). The phosphorylation reaction required CaM (Fig. 3, *lanes 1-5*). In the presence of CaM, peak II was slightly phosphorylated by a faint kinase associated with the peak II fraction (probably contaminants from peak I or peak III) (Fig. 3, *lane 6*). Peak I kinase strongly enhanced the phosphorylation of the peak II protein (Fig. 3, *lane 10*), whereas peak III kinase did not produce significant phosphorylation of the peak II protein (Fig. 3, *lane 9*). This result indicates that the peak II protein is an endogenous substrate for the peak I kinase but not for the peak III kinase.

Analysis of the protein in peak II by immunoblotting with the anticaldesmon antibody. Because the purification procedure and the molecular mass of the protein in peak II are similar to those of caldesmon, a calmodulin-binding protein (26), we examined the possibility that the peak II protein is caldesmon. As shown in Fig. 4, samples were taken after each purification step, separated by SDS-PAGE, and analysed by immunoblotting. The anticaldesmon antibody recognized a protein of 77 kDa in the crude supernatant (Fig. 4, *lane 6*), the effluent fraction that did not bind to the DEAE-cellulose column (Fig. 4, *lane 7*), and the cell lysate (Fig. 4, *lane 10*). The antibody did not recognize any proteins (Fig. 4, *lanes 3* and *4*) in peak II (Fig. 4, *lanes 8* and *9*). The results indicate that peak II does not contain caldesmon.

Phosphorylation of purified tyrosine hydroxylase and several proteins from chromaffin granule membranes by peak I kinase. Several investigators (11, 27, 28) have reported that CaM kinase II from rat brain or pheochromocytoma tumor cells phosphorylates tyrosine hydroxylase *in vitro*. Fig. 5 shows that tyrosine hydroxylase was phosphorylated by the CaM kinase present in peak I, as well as by the catalytic subunit of cAMP-dependent protein kinase (Fig. 5, *lanes 5* and *7*). The phosphorylation of tyrosine hydroxylase by the peak I kinase was dependent on the presence of CaM (data not shown). Furthermore, the autophosphorylation of the 50-kDa subunit

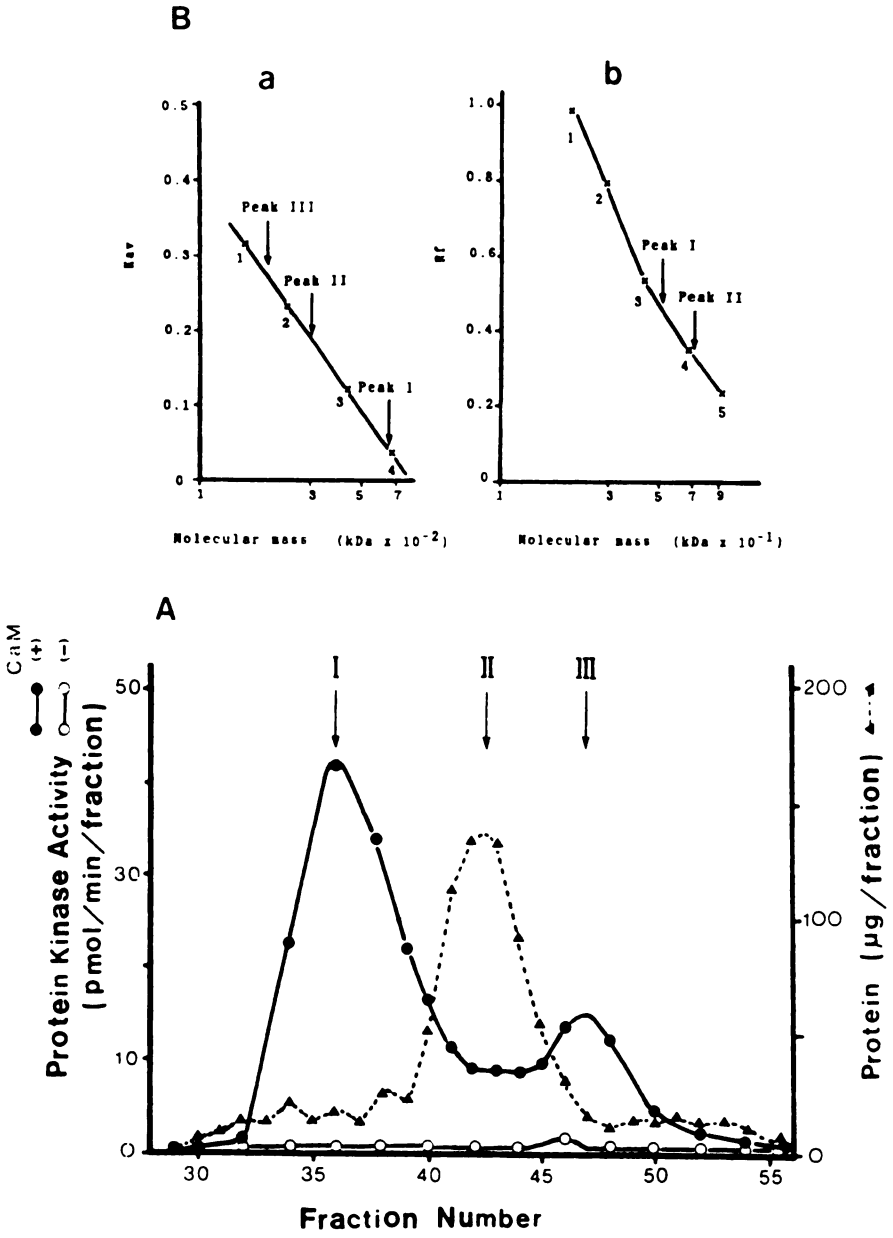


Fig. 1. Separation of CaM kinases and CaM-binding protein by Sephacryl S-300 column chromatography. A, The supernatant fraction from cultured bovine adrenal medullary cells (1.5×10^9) was subjected to three steps of chromatography using DEAE-cellulose, calmodulin affinity, and Sephacryl S-300 columns, in that order, with a 50% ammonium sulfate precipitation of the active fraction of the DEAE-cellulose column. Fractions 34–38, 42–43, and 47–48 from the Sephacryl S-300 column were collected and designated peaks I, II, and III, respectively. Each fraction was concentrated by dialysis with a buffer containing 50% glycerol, 0.2 M NaCl, 10 mM 2-mercaptoethanol, and 10 mM Tris·HCl, pH 7.5, and was stored at −80° until use. *I*, peak I; *II*, peak II; *III*, peak III. B, The molecular masses of peaks I, II, and III were estimated by Sephacryl S-300 column chromatography (a) and by SDS-PAGE (b). Protein standards were as follows: a, *1*, aldolase (158 kDa); *2*, catalase (232 kDa); *3*, ferritin (440 kDa); *4*, thyroglobulin (669 kDa); b, *1*, soybean trypsin inhibitor (20 kDa); *2*, carbonic anhydrase (30 kDa); *3*, ovalbumin (43 kDa); *4*, bovine serum albumin (67 kDa); *5*, phosphorylase *b* (94 kDa). *Arrows*, chromatographic and electrophoretic positions of peaks I, II, and III.

protein in peak I was enhanced in the presence of tyrosine hydroxylase (Fig. 5, *lane 5*).

To seek endogenous substrates of the peak I kinase in the secretory granule membranes, we incubated chromaffin granule membranes with peak I under phosphorylation conditions. As shown in Fig. 6, CaM kinase in peak I phosphorylated several proteins in the chromaffin granule membranes in the presence of Ca^{2+} and CaM (Fig. 6, *lane 5*). These proteins were estimated to be 121 ± 3, 70 ± 2, 58 ± 2, 49 ± 2, and 26 ± 1 kDa. As a positive control, Fig. 6, *lane 6*, shows that purified tyrosine hydroxylase subunits (58 kDa) were phosphorylated by the catalytic subunit of cAMP-dependent protein kinase.

Effect of 56 mM K^+ on the autonomous activity of CaM kinase II in cultured adrenal medullary cells. The activation of CaM kinase II in intact cells has recently been reported as an increase in Ca^{2+}-independent activity (autonomous activity) using syntide 2, a synthetic peptide substrate for CaM kinase II (23, 25, 29). In the present study, we investigated whether the activity of CaM kinase II is increased after high-K^+-evoked depolarization of cultured adrenal medullary cells. The basal Ca^{2+}-independent activity of CaM kinase II in the nonstimulated cells was not significantly changed during incubation for 5 min (Fig. 7A). Stimulation of the cells with 56 mM K^+ caused a rapid increase in Ca^{2+}-independent activity (autonomous activity) in a time-dependent manner. This increase was evident at 0.5 min and reached a plateau at 3 min. When the cells were incubated with 20 mM $MgSO_4$, an inhibitor of voltage-dependent Ca^{2+} channels, the high-K^+-stimulated autonomous activity was significantly inhibited. During incubation for the indicated periods of time (0–5 min), the Ca^{2+}/CaM-dependent activity of CaM kinase II (total CaM kinase II activity) remained the same under all conditions (basal, 56 mM K^+, and 56 mM K^+ plus 20 mM $MgSO_4$). Treatment of the cells with 56 mM K^+ also produced an increase in $^{45}Ca^{2+}$ influx in a time-dependent manner (Fig. 7B), similar to the time course for increases in the autonomous activity of

TABLE 1

Characterization of CaM kinases (peaks I and III) and a CaM-binding protein (peak II)

Molecular masses of peaks I, II, and III and substrate specificities of peaks I and III were determined. The enzymes in peak I (0.21 μg) and peak III (0.23 μg) were each incubated with 40 μg of myosin light chain and 31 μg of other substrates as indicated, in the presence or absence of 2 μg of CaM, under standard conditions. The value determined with CaM for each substrate was subtracted from that determined without CaM, and this subtracted value for myosin light chain was taken as 100%. The specific activities of CaM kinase in peaks I and III were 9.0 and 1.5 nmol/min/mg of protein, respectively, when myosin light chain was used as a substrate. The recovery and fold purification of CaM kinase activity in peak I were 1.5% and 160-fold, respectively, compared with that found in crude supernatants from adrenal medullary cells.

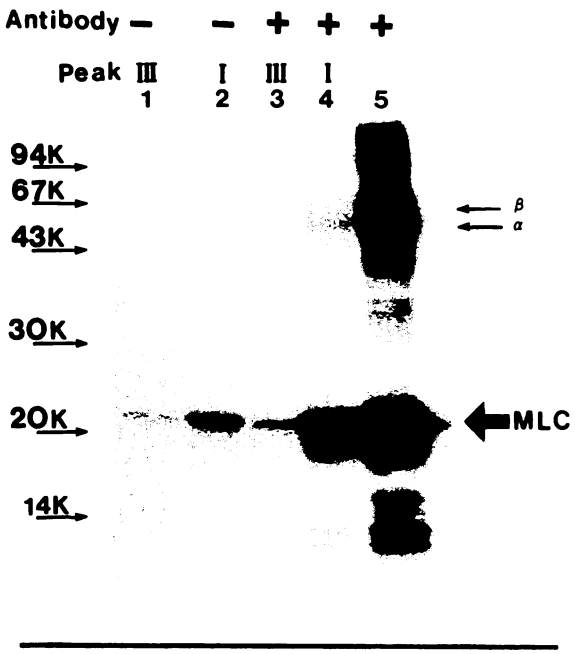
	CaM kinases		CaM-binding protein, peak II
	Peak I	Peak III	
Molecular mass (kDa)			
Sephacryl S-300	650	200	300
SDS-PAGE	50		70
Substrate specificity (%)			
MLC[a]	100	100	
Casein	44	51	
MBP	35	35	
Lys histone	7.0	13	
Whole histone	5.4	1.7	
Arg histone	0.9	7.2	
Phosphorylase *b*	0	0	

[a] MLC, myosin light chain; MBP, myelin basic protein; Lys histone, lysine-rich histone; Arg histone, arginine-rich histone.

CaM kinase II. The high-K^+-evoked $^{45}Ca^{2+}$ influx was also inhibited by 20 mM $MgSO_4$. The activation of CaM kinase II was closely associated with the increase in $^{45}Ca^{2+}$ influx ($y = 5.37 + 4.13x$; $r = 0.97$, $p < 0.001$).

Discussion

Evidence that peak I contains a CaM kinase that is related to CaM kinase II. We have partially purified two CaM kinases (peaks I and III) and one major CaM-binding protein (peak II) from cultured bovine adrenal medullary cells. The molecular mass of the native CaM kinase in peak I is estimated to be approximately 650 kDa, using Sephacryl S-300 gel filtration chromatography, and the subunit molecular mass is 50 kDa, using SDS-PAGE (Fig. 1). Peak I CaM kinase has broad substrate specificities but does not use phosphorylase *b* as a substrate (Table 1). Furthermore, peak I kinase is immunoprecipitated with the antibody against rat brain CaM kinase II (Fig. 2). Hence, peak I kinase has the general properties of CaM kinase II, which are similar to those of brain CaM kinase II. CaM kinase II isolated from rat brain is a heteropolymer composed of at least two kinds of subunits, i.e., the α subunit (49 kDa) and the β subunit (60 kDa). Unlike the brain enzyme, adrenal medullary CaM kinase is apparently composed of a single subunit of about 50 kDa, suggesting that the holoenzyme (650 kDa) comprises 13 subunits (50 kDa), on the basis of the findings for brain CaM kinase II. The adrenal medullary enzyme may be comparable to that found in several non-muscle tissues such as lung, liver, and testis, which express an isozyme of CaM kinase II composed of a single subunit of 51 or 52 kDa (19). Brain CaM kinase II is known to undergo autophosphorylation in the presence of Ca^{2+} and CaM (15, 30). In the present study, the 50-kDa protein in peak I is observed to be autophosphorylated (Figs. 2 and 6). The amino acids autophosphorylated on the enzyme are serine and threonine. From these results, we



	Antibody	M L C Phosphorylation (cpm)
Peak III	–	900
Peak I	–	2500
Peak III	+	1200
Peak I	+	14900
Brain CaM K II	+	48400

Fig. 2. *Upper*, immunoprecipitation of CaM kinases with the anti-CaM kinase II antibody. The enzyme (2.6 μg) in peak I (*lanes 2* and *4*) or peak III (*lanes 1* and *3*) or rat brain CaM kinase II (0.5 μg) (*lane 5*) was incubated with (*lanes 3*, *4*, and *5*) or without (*lanes 1* and *2*) the anti-CaM kinase II antibody. The antibody was immobilized with Protein A-Sepharose gel and incubated with 20 μM [γ-^{32}P] (2–4 × 10^6 cpm), under standard phosphorylation conditions. Phosphorylated proteins were analyzed by SDS-PAGE in 13.5% acrylamide gels. Protein standards were as follows: *94K*, phosphorylase *b*; *67K*, bovine serum albumin; *43K*, ovalbumin; *30K*, carbonic anhydrase; *20K*, soybean trypsin inhibitor; *14K*, α-lactalbumin. *Arrows* α and β, α and β subunits of brain CaM kinase II. *MLC*, myosin light chain. *Lower*, phosphorylation of myosin light chain by immunoprecipitated CaM kinases (peak I, peak III, and brain CaM kinase II). The radioactivity of phosphorylated myosin light chain in the gel was determined by liquid scintillation counting. Data are the average of two separate experiments.

conclude that the CaM kinase in peak I is closely related to brain CaM kinase II. Yamauchi and Fujisawa (31) have reported that adrenal CaM kinase II activity is not present or is expressed at an extremely low level. The explanation for the discrepancy between their results and ours is not known, but the difference may be due to the fact that they assayed CaM kinase activity by measuring the activation of tryptophan 5-monooxygenase and did not purify the enzyme. The present study directly demonstrates that an isozyme of CaM kinase II exists in adrenal medullary cells.

Peak III contains a CaM kinase activity of approximately 200 kDa on Sephacryl S-300 gel filtration columns. This activity possesses a substrate specificity similar to that found in peak I (Fig. 1; Table 1). However, peak III CaM kinase is not immunoprecipitated with the antibody against rat brain CaM

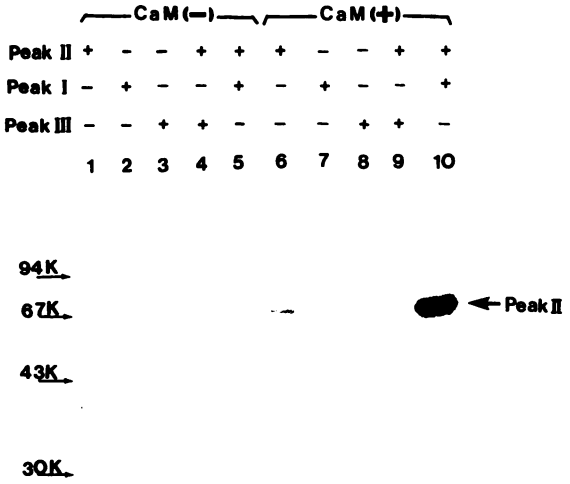


Fig. 3. Autoradiogram of peak II phosphorylated by peak I (CaM kinase II). Peak II (1 μg) was incubated at 30° for 10 min with 20 μM [γ-^{32}P]ATP (4–8 × 10^6 cpm) and 0.2 mM $CaCl_2$, in the presence or absence of 2 μg of CaM and 0.16 μg of peak I or III. SDS-PAGE was carried out in 10% acrylamide gels.

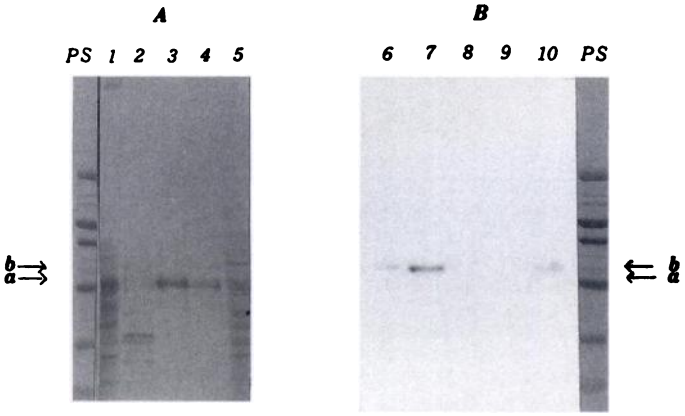


Fig. 4. Immunoblot of the protein in peak II with the anticaldesmon antibody. Samples of the crude supernatant (11.7 μg) (*lanes 1* and *6*), the effluent of the DEAE-cellulose column (2.8 μg) (*lanes 2* and *7*), peak II (*lanes 3* and *8*, 1.3 μg; *lanes 4* and *9*, 0.8 μg), and the lysate of cultured adrenal medullary cells (12 μg) (*lanes 5* and *10*) were separated by SDS-PAGE (9% acrylamide gels) and analyzed by immunoblotting with the anticaldesmon antibody and ^{125}I-Protein A. A, Amido black stain (*lanes 1-5*); B, autoradiogram (*lanes 6-10*). Protein standards (*PS*) were myosin heavy chain (200 kDa), β-galactosidase (115 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), and ovalbumin (43 kDa). *Arrows*, peak II (*a*) and caldesmon (*b*).

kinase II (Fig. 2) and does not phosphorylate the protein in peak II (Fig. 3), suggesting that the peak III kinase is distinct from CaM kinase II. Further investigation is needed to identify the peak III kinase.

In the present study, as shown in Experimental Procedures, the purity of adrenal medullary cells is 90%. Therefore, we could not exclude the possibility that the enzyme activities described above are derived from non-chromaffin cells, such as fibroblasts, in the cultured cell preparation. However, we also have partially purified CaM kinases from fresh bovine adrenal medulla that are similar to those from the cultured cells (data not shown). Furthermore, our recent study has demonstrated

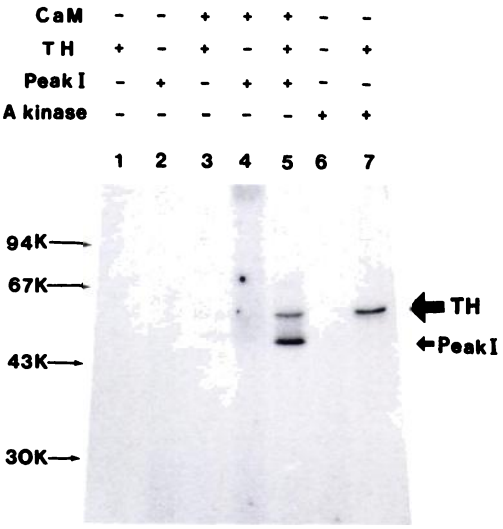


Fig. 5. Phosphorylation of purified tyrosine hydroxylase by peak I kinase. Tyrosine hydroxylase (*TH*) (0.5 μg of protein) (*lanes 1*, *3*, *5*, and *7*) was phosphorylated with 20 μM [γ-^{32}P]ATP (2–4 × 10^6 cpm) by 0.43 μg of peak I (*lanes 2*, *4*, and *5*) or 44 ng of the catalytic subunit of cAMP-dependent protein kinase (*A kinase*) (*lanes 6* and *7*), in the presence (*lanes 3*, *4*, and *5*) or absence (*lanes 1*, *2*, *6*, and *7*) of CaM, under standard phosphorylation conditions. Phosphorylated proteins were analyzed by SDS-PAGE in 10% acrylamide gels.

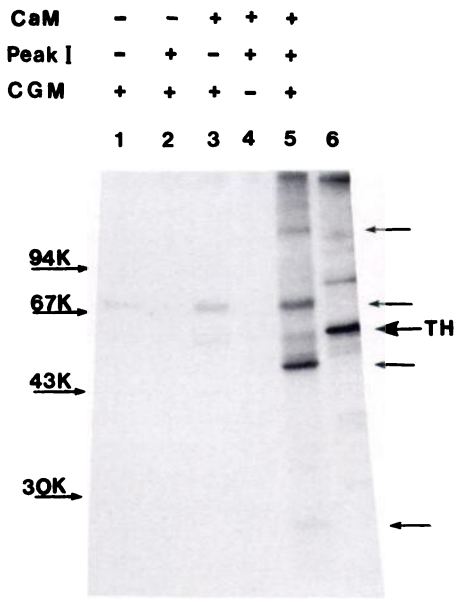


Fig. 6. Phosphorylation of chromaffin granule membranes by peak I kinase. Chromaffin granules were isolated from fresh bovine adrenal medulla, as reported by Pollard *et al.* (40), and lyzed osmotically with 50 mM Tris·HCl buffer, pH 7.5. Chromaffin granule membranes were sedimented by centrifugation and resuspended in 50 mM Tris·HCl buffer, pH 7.5. After heat treatment at 90° for 3 min, chromaffin granule membranes (*CGM*) (13.2 μg) (*lanes 1*, *2*, *3*, and *5*) were incubated at 30° for 10 min with (*lanes 2*, *4*, and *5*) or without (*lanes 1* and *3*) peak I (0.85 μg), in the presence (*lanes 3*, *4*, and *5*) or absence (*lanes 1* and *2*) of CaM, under standard phosphorylation conditions. In *lane 6*, purified tyrosine hydroxylase (*TH*) was phosphorylated with the catalytic subunit of cAMP-dependent protein kinase. Phosphorylated membrane proteins were analyzed by SDS-PAGE in 10% acrylamide gels. *Arrows on the right*, bands (121, 70, 58, 49, and 26 kDa) phosphorylated by peak I kinase.

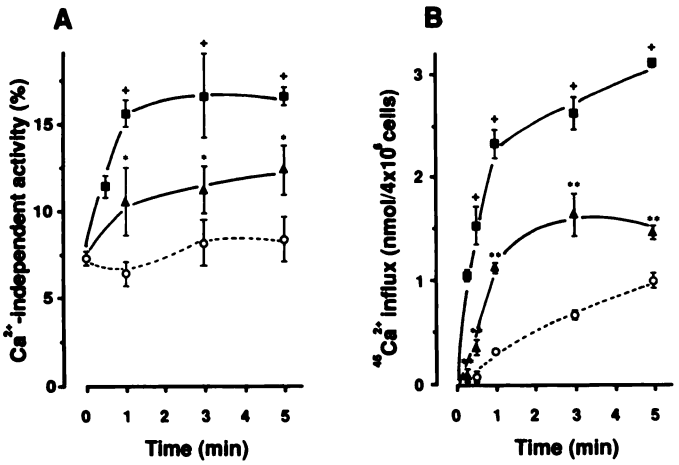


Fig. 7. Effects of 56 mM K^+ on autonomous activity of CaM kinase II and $^{45}Ca^{2+}$ influx in cultured adrenal medullary cells. A, Cultured adrenal medullary cells (4×10^6/dish) were incubated at 37° without (○) or with 56 mM K^+ (■) or 56 mM K^+ plus 20 mM $MgSO_4$ (▲). At the indicated times, cells were frozen and assayed for the percentage of Ca^{2+} independence of CaM kinase II (activity in the presence of EGTA × 100/activity in the presence of Ca^{2+} and CaM). At time 0, the total and Ca^{2+}-independent kinase activities were 675.80 ± 38.67 and 48.94 ± 0.74 pmol/min/mg of protein, respectively. The values are means ± standard deviations of four experiments. B, Cultured cells were incubated with 1.5 μCi of $^{45}CaCl_2$, as described for A. After incubation, the cells were washed four times with 1 ml of ice-cold Krebs-Ringer phosphate buffer and solubilized with 1 ml of 10% Triton X-100. $^{45}Ca^{2+}$ in the cells was counted with a Beckman LS-7000 liquid scintillation counter. The influx of $^{45}Ca^{2+}$ was calculated from the initial specific radioactivity of the ion in the incubation medium. Data are means ± standard deviations of four to seven experiments. *, $p < 0.05$; **, $p < 0.01$, compared with 56 mM K^+ alone at each time, using Scheffe's test. +, $p < 0.01$, compared with normal K^+ at each time, using Dunnett's test.

that various secretagogues such as acetylcholine and veratridine phosphorylate and activate CaM kinase II in cultured adrenal medullary cells.[1]

Evidence that peak II (70-kDa protein) is a novel substrate for CaM kinase II. Because of the isolation of peak II by CaM affinity column chromatography, peak II apparently contains a CaM-binding protein, with a native molecular mass of approximately 300 kDa and a subunit molecular mass of approximately 70 kDa (Fig. 1). Sobue *et al.* (26) have reported the purification and characterization of the low molecular mass caldesmon from bovine adrenal medulla. They demonstrated that its molecular mass estimated by SDS-PAGE is 77 kDa and a tetramer of polypeptides constitutes the native molecule (300 kDa). The present study (Fig. 4) has shown that the anticaldesmon antibody recognizes a protein (77 kDa) in the cell lysate, the crude supernatant, and the effluent of the DEAE-cellulose column, indicating that the low molecular mass caldesmon indeed exists in our preparation of adrenal medullary cells. However, the antibody does not recognize the protein in peak II. These results suggest that peak II is separated from caldesmon by DEAE-cellulose column chromatography during the purification procedure and, therefore, is distinct from caldesmon.

Previously, the high molecular mass (150-kDa) caldesmon isolated from chicken gizzard has been shown to be phosphorylated by a co-purified CaM kinase (32). The protein kinase associated with caldesmon is reported to be CaM kinase II (33). In the present study, we have observed the phosphorylation of the protein in peak II by CaM kinase II (peak I) (Fig. 3). It is well known that CaM kinase II phosphorylates a number of proteins, such as synapsin 1, tyrosine and tryptophan hydroxylases, glycogen synthase, the microtubule-associated proteins, and myosin light chain (5–7). However, there is no report of a CaM kinase substrate that is a CaM-binding protein and that has a native molecular mass of 300 kDa and a subunit molecular mass of 70 kDa. The present results suggest that the peak II protein is a novel endogenous substrate for CaM kinase II in adrenal medullary cells.

Other endogenous substrates and possible roles of CaM kinase II in adrenal medulla. Protein phosphorylation has been implicated in stimulus-secretion coupling in several tissues such as brain synaptosomes (34) and adrenal medulla (35). In bovine adrenal medullary cells, acetylcholine stimulates the phosphorylation of several proteins; this phosphorylation is inhibited by trifluoperazine, an inhibitor of CaM (36). In the present study, we have demonstrated that adrenal medullary CaM kinase II phosphorylates several proteins in the membranes of chromaffin granules (Fig. 6). Among these phosphorylated proteins, we have observed the phosphorylation of a 70-kDa chromaffin granule membrane protein. However, we do not know whether this 70-kDa protein in the chromaffin granule membranes is the same one that we purified in peak II.

The phosphorylated 58-kDa protein in the chromaffin granule membranes (Fig. 6) is probably tyrosine hydroxylase (58–60 kDa), because this enzyme exists partially in a membrane-bound form attached to bovine adrenal chromaffin granules (37) and is a good substrate for CaM kinase II and cAMP-dependent protein kinase (28). We have also observed that adrenal CaM kinase II (peak I) phosphorylates purified tyrosine hydroxylase (Fig. 5). A previous report (38) has shown that acetylcholine stimulates the phosphorylation of seven tryptic fragments of tyrosine hydroxylase isolated from bovine adrenal medullary cells. Phosphorylation of sites on three of the tryptic peptides correlates with the transient activation of tyrosine hydroxylase (38). These sites also correspond to sites on the enzyme that are phosphorylated *in vitro*, in the presence of Ca^{2+} and CaM, by crude supernatants extracted from the cells (39). These results and the present data suggest that CaM kinase II plays an important role in the activation and phosphorylation of tyrosine hydroxylase in acetylcholine-stimulated adrenal medullary cells. Furthermore, the autophosphorylation of CaM kinase II is enhanced in the presence of tyrosine hydroxylase (Fig. 5, *lane 5*), suggesting that there is an interaction between CaM kinase II and tyrosine hydroxylase.[2]

Activation of CaM kinase II in cultured cells by high-K^+ depolarization. The autophosphorylation on Thr-286 of the α subunit of brain CaM kinase II converts CaM kinase II to an autonomous form that is capable of phosphorylating substrates in the absence of Ca^{2+} and CaM (5, 7). Recent *in situ* studies have demonstrated that cell stimulation by Ca^{2+}-mobilizing agents causes the autophosphorylation of the enzyme, with a concomitant increase in the Ca^{2+}-independent activity (autonomous activity) of CaM kinase II, in cultured

[1] M. Tsutsui, N. Yanagihara, E. Miyamoto, A. Kuroiwa, and F. Izumi. Manuscript in preparation.

[2] N. Yanagihara, H. Yamamoto, E. Miyamoto, E. Tachikawa, M. Tsutsui, and F. Izumi. Manuscript in preparation.

cerebellar granule cells (23), neuroblastoma × glioma hybrid NG108–15 cells (29), and rat hippocampal neurons (25). In the present study, we have shown an increase in the autonomous activity of CaM kinase II and $^{45}Ca^{2+}$ influx in cells stimulated with 56 mM K^+ (Fig. 7). Both responses are inhibited by 20 mM $MgSO_4$, an inhibitor of voltage-dependent Ca^{2+} channels, suggesting that the activation of CaM kinase II is caused by an increase in the intracellular concentration of Ca^{2+}. Furthermore, in other studies we have observed a close relationship among CaM kinase II activation, catecholamine secretion, and tyrosine hydroxylase activation elicited by acetylcholine in cultured adrenal medullary cells.[1]

In conclusion, we have demonstrated that an isozyme of CaM kinase II exists and is activated by cell depolarization in bovine adrenal medullary cells. Furthermore, we have isolated a novel endogenous substrate for CaM kinase II.

Acknowledgments

The authors would like to thank Dr. Eiichi Tachikawa and Dr. Kenji Sobue for their kind gifts of tyrosine hydroxylase purified from rat pheochromocytoma PC-12 cells and anticaldesmon antibody, respectively. We also thank Dr. Akihide Tanimoto for staining adrenal medullary cells and Ms. Mie Sawa for typing this manuscript. We greatly thank Dr. A. William Tank for critical reading of this manuscript.

References

1. Douglas, W. W., and R. P. Rubin. The role of calcium in the secretory response of the adrenal medulla to acetylcholine. *J. Physiol. (Lond.)* **159**:40–57 (1961).
2. Haycock, J. W., J. A. Meligeni, W. F. Bennett, and J. C. Waymire. Phosphorylation and activation of tyrosine hydroxylase mediate the acetylcholine-induced increase in catecholamine biosynthesis in adrenal chromaffin cells. *J. Biol. Chem.* **257**:12641–12648 (1982).
3. Cheung, W. Y. Cyclic 3′,5′-nucleotide phosphodiesterase: demonstration of an activator. *Biochem. Biophys. Res. Commun.* **38**:533–538 (1970).
4. Kakiuchi, S., R. Yamazaki, and H. Nakajima. Properties of a heat stable phosphodiesterase activity factor isolated from brain extract. *Proc. Jpn. Acad.* **46**:587–592 (1970).
5. Hanson, P. I., and H. Schulman. Neuronal Ca^{2+}/calmodulin-dependent protein kinases. *Annu. Rev. Biochem.* **61**:559–601 (1992).
6. Edelman, A. M., D. K. Blumenthal, and E. G. Krebs. Protein serine/threonine kinases. *Annu. Rev. Biochem.* **56**:567–613 (1987).
7. Colbran, R. J., C. M. Schworer, Y. Hashimoto, Y.-L. Fong, D. P. Rich, M. K. Smith, and T. R. Soderling. Calcium/calmodulin-dependent protein kinase II. *Biochem. J.* **258**:313–325 (1989).
8. Oka, M., M. Isosaki, and N. Yanagihara. Isolated bovine adrenal medullary cells: studies on regulation of catecholamine synthesis and release, in *Catecholamines: Basic and Clinical Frontiers* (E. Usdin, I. J. Kopin, and J. Barchas, eds.). Pergamon Press, Oxford, UK, 70–72 (1979).
9. Weiner, N. Regulation of norepinephrine biosynthesis. *Annu. Rev. Pharmacol.* **10**:273–290 (1970).
10. Yanagihara, N., A. W. Tank, and N. Weiner. Relationship between activation and phosphorylation of tyrosine hydroxylase by 56 mM K^+ in PC12 cells in culture. *Mol. Pharmacol.* **26**:141–147 (1984).
11. Tachikawa, E., A. W. Tank, N. Yanagihara, W. Mosimann, and N. Weiner. Phosphorylation of tyrosine hydroxylase on at least three sites in rat pheochromocytoma PC12 cells treated with 56 mM K^+: determination of the sites on tyrosine hydroxylase phosphorylated by cyclic AMP-dependent and calcium/calmodulin-dependent protein kinases. *Mol. Pharmacol.* **30**:476–485 (1986).
12. Griffith, L. C., and H. Schulman. The multifunctional Ca^{2+}/calmodulin-dependent protein kinase mediates Ca^{2+}-dependent phosphorylation of tyrosine hydroxylase. *J. Biol. Chem.* **263**:9542–9549 (1988).
13. Fukunaga, K., H. Yamamoto, K. Matsui, K. Higashi, and E. Miyamoto. Purification and characterization of a Ca^{2+}- and calmodulin-dependent protein kinase from rat brain. *J. Neurochem.* **39**:1607–1617 (1982).
14. Yanagihara, N., M. Isosaki, T. Ohuchi, and M. Oka. Muscarnic receptor-mediated increase in cyclic GMP level in isolated bovine adrenal medullary cells. *FEBS Lett.* **105**:296–298 (1979).
15. Waymire, J. C., W. F. Bennett, R. Boehme, L. Hankins, K. Gilmer-Waymire, and J. H. Haycock. Bovine adrenal chromaffin cells: high-yield purification and viability in suspension culture. *J. Neurosci. Methods* **7**:329–351 (1983).
16. Miyamoto, E., G. L. Fetzold, J. F. Kuo, and P. Greengard. Dissociation and activation of adenosine 3′,5′-monophosphate-dependent and guanosine 3′,5′-monophosphate-dependent protein kinases by cyclic nucleotides and by substrate proteins. *J. Biol. Chem.* **248**:179–189 (1973).
17. Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**:248–254 (1976).
18. Ohta, Y., T. Ohba, K. Fukunaga, and E. Miyamoto. Serum and growth factors rapidly elicit phosphorylation of the Ca^{2+}/calmodulin-dependent protein kinase II in intact quiescent rat 3Y1 cells. *J. Biol. Chem.* **263**:11540–11547 (1988).
19. Fukunaga, K., S. Goto, and E. Miyamoto. Immunohistochemical localization of Ca^{2+}/calmodulin-dependent protein kinase II in rat brain and various tissues. *J. Neurochem.* **51**:1070–1078 (1988).
20. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–685 (1970).
21. Towbin, H., T. Staehelin, and J. Gordon. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc. Natl. Acad. Sci. USA* **76**:4350–4354 (1979).
22. Miyamoto, E., and S. Kakiuchi. *In vitro* and *in vivo* phosphorylation of myelin basic protein by exogenous and endogenous adenosine 3′:5′-monophosphate-dependent protein kinases in brain. *J. Biol. Chem.* **249**:2769–2777 (1974).
23. Fukunaga, K., D. P. Rich, and T. R. Soderling. Generation of the Ca^{2+}-independent form of Ca^{2+}/calmodulin-dependent protein kinase II in cerebellar granule cells. *J. Biol. Chem.* **264**:21830–21836 (1989).
24. Roskoski, R. Assays of protein kinase. *Methods Enzymol.* **79**:3–6 (1983).
25. Fukunaga, K., T. R. Soderling, and E. Miyamoto. Activation of Ca^{2+}/calmodulin-dependent protein kinase II and protein kinase C by glutamate in cultured rat hippocampal neurons. *J. Biol. Chem.* **267**:22527–22533 (1992).
26. Sobue, K., T. Tanaka, K. Kanda, N. Ashino, and S. Kakiuchi. Purification and characterization of caldesmon$_{77}$: a calmodulin-binding protein that interacts with actin filaments from bovine adrenal medulla. *Proc. Natl. Acad. Sci. USA* **82**:5025–5029 (1985).
27. Yamauchi, T., and H. Fujisawa. Tyrosine 3-monooxygenase is phosphorylated by Ca^{2+}-, calmodulin-dependent protein kinase, followed by activation by activator protein. *Biochem. Biophys. Res. Commun.* **100**:807–813 (1981).
28. Vulliet, P. R., J. R. Woodgett, and P. Cohen. Phosphorylation of tyrosine hydroxylase by calmodulin-dependent multiprotein kinase. *J. Biol. Chem.* **259**:13680–13683 (1984).
29. Yamakawa, T., K. Fukunaga, H. Higashida, and E. Miyamoto. Activation of Ca^{2+}/calmodulin-dependent protein kinase II by stimulation with bradykinin in neuroblastoma × glioma hybrid NG 108–15 cells. *Brain Res.* **597**:220–226 (1992).
30. Bennett, M. K., N. E. Erondu, and M. B. Kennedy. Purification and characterization of a calmodulin-dependent protein kinase that is highly concentrated in brain. *J. Biol. Chem.* **258**:12735–12744 (1983).
31. Yamauchi, T., and H. Fujisawa. A calmodulin-dependent protein kinase that is involved in the activation of tryptophan 5-monooxygenase is specifically distributed in brain tissues. *FEBS Lett.* **129**:117–119 (1981).
32. Ngai, P. K., and M. P. Walsh. Inhibition of smooth muscle actin-activated myosin Mg^{2+}-ATPase activity by caldesmon. *J. Biol. Chem.* **259**:13656–13659 (1984).
33. Abougou, J.-C., M. Hagiwara, T. Hachiya, M. Terasawa, H. Hidaka, and D. J. Hartshorne. Phosphorylation of caldesmon. *FEBS Lett.* **257**:408–410 (1989).
34. Krueger, B. K., J. Forn, and P. Greengard. Depolarization-induced phosphorylation of specific proteins, mediated by calcium ion influx, in rat brain synaptosomes. *J. Biol. Chem.* **252**:2764–2773 (1977).
35. Amy, C. M., and N. Kirshner. Phosphorylation of adrenal medulla cell protein in conjunction with stimulation of catecholamine secretion. *J. Neurochem.* **36**:847–854 (1981).
36. Côté, A., J.-P. Doucet, and J.-M. Trifaró. Phosphorylation and dephosphorylation of chromaffin cell proteins in response to stimulation. *Neuroscience* **19**:629–645 (1986).
37. Kuhn, D. M., R. Arthur, Jr., H. Yoon, and K. Sankaran. Tyrosine hydroxylase in secretory granules from bovine adrenal medulla: evidence for an integral membrane form. *J. Biol. Chem.* **265**:5780–5786 (1990).
38. Waymire, J. C., J. P. Johnston, K. Hummer-Lickteig, A. Lloyd, A. Vigny, and G. L. Craviso. Phosphorylation of bovine adrenal chromaffin cell tyrosine hydroxylase: temporal correlation of acetylcholine's effect on site phosphorylation, enzyme activation, and catecholamine synthesis. *J. Biol. Chem.* **263**:12439–12447 (1988).
39. George, R. J., J. W. Haycock, J. P. Johnston, G. L. Craviso, and J. C. Waymire. *In vitro* phosphorylation of bovine adrenal chromaffin cell tyrosine hydroxylase by endogenous protein kinases. *J. Neurochem.* **52**:274–284 (1989).
40. Pollard, H. B., O. Zinder, P. G. Hoffman, and O. Nikodejevic. Regulation of the transmembrane potential of isolated chromaffin granules by ATP, ATP analogs, and external pH. *J. Biol. Chem.* **251**:4544–4550 (1976).

Send reprint requests to: Nobuyuki Yanagihara, Department of Pharmacology, University of Occupational and Environmental Health, School of Medicine, Kitakyushu 807, Japan.