Phosphorylation of Chromogranin A and Catecholamine Secretion Stimulated by Elevation of Intracellular Ca\textsuperscript{2+} in Cultured Bovine Adrenal Medullary Cells*  

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We have recently isolated a new endogenous substrate of 70 kDa for Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaM kinase II) from bovine adrenal medullary cells (Yanagihara, N., Toyohira, Y., Yamamoto, H., Ohta, Y., Tsutsui, M., Miyamoto, E., and Izumi, F. (1994) Mol. Pharmacol. 46, 423–430). Here we report the sequence analysis of the 70-kDa protein and examine its phosphorylation by various protein kinases in vitro and by depolarization of the cultured cells. Protein sequencing and immunoblotting revealed that the 70-kDa protein is chromogranin A (CgA) or a closely related protein. Partially purified CgA was phosphorylated by cyclic AMP-dependent protein kinase and protein kinase C as well as CaM kinase II. Tryptic phosphopeptide mapping patterns of CgA differed among these protein kinases. In \textsuperscript{32}P-labeled bovine adrenal medullary cells, 56 mM K\textsuperscript{+} increased the phosphorylation of CgA and catecholamine secretion in similar time- and concentration-dependent manners, both of which were inhibited by 20 mM MgSO\textsubscript{4}, an inhibitor of voltage-dependent Ca\textsuperscript{2+} channels. These findings suggest that CgA serves as a substrate for several multifunctional protein kinases and that the elevation of the intracellular Ca\textsuperscript{2+} stimulates the phosphorylation of CgA associated with catecholamine secretion in cultured adrenal medullary cells.  

**Experimental Procedures**  

Materials—The following chemicals and reagents were obtained from the indicated sources as follows: Eagle’s minimum essential medium, Nissui Seiyaku; collagenase, Nitta Zerachin; calmodulin (bovine brain), Calbiochem; DEAE-cellulose, Whatman; CaM-agarose, Sigma; and Sephacryl S-300, Pharmacia Biotech Inc.; \textsuperscript{32}P]ATP (3000 Ci/mmol), Amersham Int.; \textsuperscript{32}P, (500 mCi/ml) ICN Biochemicals. Other chemicals used were of analytical grade from Nakalai Tesque. PKC (19), the catalytic subunit of type II of PKA (20), and CaM kinase II (21) were purified from rat brain.  

Preparation of Cultured Adrenal Medullary Cells and Purification of the 70-kDa Protein from the Cells—Bovine adrenal medullary cells were isolated by collagenase digestion, as described previously (22). The isolated cells were purified by a selective plating method (23) and maintained in a CO\textsubscript{2} incubator under 5% CO\textsubscript{2}/95% air (17). The 70-kDa protein was purified from the cultured bovine adrenal medullary cells on DEAE-cellulose, CaM affinity, and Sephacryl S-300 columns (17).  

Sequence Analysis of the 70-kDa Protein—The N-terminal sequence of the 70-kDa protein was determined as follows. The 70-kDa protein was separated by SDS-PAGE, and the protein was electroblotted onto...
polyvinylidene difluoride membrane (Immobilon, Millipore) using a semidy blotting apparatus (Biometra-Fast-Blot, Biometra/Biomediznische Analytik). After staining with Coomassie Brilliant Blue, the band of 70-kDa protein was cut off and directly analyzed by using an automated gas-phase sequencer (Shimadzu, PPSQ-10) (24). The partial amino acid sequences were determined with four peptides obtained by subfragment 1 digestion of the 70-kDa protein. The N-terminal sequence of the major digestions of the C-terminal half of the 70-kDa protein was obtained by hypothesis (25), using an automated gas-phase sequencer (Applied Biosystems, model 4700A). The determined amino acid sequences were analyzed, using the National Biomedical Research Foundation data base.

Partial Purification of CgA from Bovine Adrenal Chromaffin Granules—Intact chromaffin granules were isolated from fresh bovine adre nal medulla (26). After destruction of granules by hypotonic shock, the soluble fraction of chromaffin granules was subjected to a series of column chromatography of DEAE-cellulose and Sephacryl S-300, with a 50% ammonium sulfate fractionation of the DEAE-cellulose column eluate. The CgA fraction was collected and checked by SDS-PAGE (27).

Preparation of Antibodies—Antisera to CgA was prepared in female New Zealand White rabbits. One mg of CgA separated from SDS-PAGE gels was emulsified with complete Freund’s adjuvant and was injected into multiple intradermal sites, on four occasions at 3-week intervals.

After separation of the sera by centrifugation, the IgG fraction was precipitated with 40% ammonium sulfate and separated by application to a DEAE-cellulose column (1.1 × 8 cm). The unretained fraction was collected and used as the anti-CgA antibody.

Immunoblotting of the 70-kDa Protein with the Anti-CgA Antibody—The 70-kDa protein (1.9 μg) and partially purified CgA (2.6 μg) were separated by SDS-PAGE in 10% acrylamide. After the electrophoresis, proteins were transferred to a polyvinylidene difluoride membrane, as described above. The membrane was incubated at 4°C overnight with the anti-CgA antibody. The bound antibody was treated with goat anti-rabbit IgG conjugated to horseradish peroxidase (Mextral & Biological Laboratories). The enzyme color was developed with 4-phenylendiamine as a chromogen.

Assay of CgA in the Cell Supernatant—The amount of CgA in the supernatant and the particulate fractions of cell homogenates was analyzed by using the enzyme-linked immunosorbent assay (28). In brief, cultured adrenal medullary cells (4 × 10⁶ cells/dish) were harvested and homogenized with 200 μl of an isotonic 0.27 M sucrose buffer, containing 50 mM Tris-HCl, 7.5, 10 mM EDTA, 25 mM NaF, 4 mM EGTA, 0.43 mM phenylmethylsulfonyl fluoride, 0.05 mM leupeptin, and 50 mM 1,2-diolein tryptic inhibitor. After centrifugation at 15,000 × g for 10 min, the resultant supernatant was reserved at 4°C. Chromaffin granules in the precipitated fraction were disrupted by hypotonic shock and homogenized with 150 mM Tris-HCl buffer, pH 7.5. Polyethylene glycol (PEG) with 96 wells (ImmunoModule, Maxisorp F16, Nunc) were coated with 100 μl of diluted samples (the supernatant or the particulate fraction). The CgA attached in the wells was detected by the enzyme-linked immunosorbent assay (28) using the partially purified anti-CgA antiseraum and a peroxidase-conjugated goat antibody purified to rabbit IgG (PAA Laboratories). The enzyme activity was measured by 4-phenylendiamine method (29). The amount of the supernatant CgA was expressed as percent of the total CgA (the supernatant and the particulate CgA).

Phosphorylation of the 70-kDa Protein or CgA by CaM Kinase II and Other Protein Kinases in Vitro—The standard assay system for protein kinases contained, in 25 μl of a final volume, the following constituents: 50 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 mM EGTA, 0.1 or 0.5 mM (γ-32P)ATP (3000-5000 cpm/pmol), the 70-kDa protein (or CgA), and the indicated amount of the catalytic subunit of PKA. In addition, the assay mixtures contained 1 mM CaCl₂ and 1.5 μM calmodulin for CaM kinase II and 1 mM CaCl₂, 50 μM phosphodiesterase, and 5 μM LiCl, 1.2 mM calmidazolium, and 100 μM FK-506 instead of EGTA. After incubation, 15 μl of each sample was spotted on a phosphocellulose paper (Whatman) and processed, as described (29).

Peptide Mapping—Two-dimensional tryptic peptide mapping was carried out as reported (30). Briefly, the band of CgA was excised from the SDS-PAGE gel and incubated at 37°C overnight with 100 μg of TPCK-trypsin. After centrifugation, the supernatant was incubated with another 100 μg of TPCK-trypsin for 36 h. The supernatant was lyophilized by a "Speed Vac" concentrator and subjected to two-dimensional thin layer chromatography (TLC).

Labeling of Cells with [32P]Pi and Immunoprecipitation with the Anti-CgA Antibody—Cultured adrenal medullary cells (4 × 10⁶/dish, Falcon, 35 mm) were labeled with [32P]Pi (0.2 μCi/ml) in phosphate-free Eagle’s MEM medium for 6 h (18). Then, the cells were washed with 1 ml of oxygenated Krebs-Ringer/HEPES buffer, containing 125 mM NaCl, 5.6 mM KCl, 1.1 mM MgSO₄, 2.2 mM CaCl₂, 25 mM HEPES, pH 7.4, and 10 mM glucose. The cells were stimulated with or without high concentrations (25, 56, and 75 mM) of K⁺ at 37°C for the indicated periods. In the high K⁺medium, NaCl was reduced to maintain the isotonicity of the medium. After incubation, the cells were harvested and homogenized in 160 μl of the isotonic 0.27 M sucrose buffer (see above). After centrifugation at 15,000 × g for 10 min, the resultant supernatant was incubated with the anti-CgA antibody (160 μg). The antigen-antibody complex was immobilized on Protein A-Sepharose gel (Pharmacia), and the phosphorylation of CgA was analyzed by SDS-PAGE. In some experiments, the phosphorylation of CgA in the supernatant and the particulate fractions of cell homogenates and the phosphorylation of CgA released from cultured cells was also analyzed by the same method as described above.

Catecholamine Secretion—Cultured cells (4 × 10⁶/dish) were incubated with or without various concentrations (25, 56, and 75 mM) of K⁺ at 37°C for the indicated periods. The catecholamines into the medium were adsorbed to aluminum hydroxide and estimated by the ethylenediamine condensation method (31).

Assay of Dopamine β-Hydroxylase Activity—The activity of dopamine β-hydroxylase in the supernatant and the particulate fractions of cultured cell homogenates was measured with tyramine as the substrate (32). The β-hydroxylated product octopamine was separated by a Dowex 50 × 8 column (0.4 × 4 cm) and determined spectrophotometrically by the periodate method (32).

RESULTS

Sequence Analysis of the 70-kDa Protein—We determined the partial amino acid sequence of the 70-kDa protein. The sequences of N-terminal and another four peptides were analyzed (Fig. 1), and the homology search was performed. These peptides exhibited a complete homology with the published sequences 1-8, 173-179, 195-206, 244-259 and 315-318 of the bovine adrenal CgA (36).

Phosphorylation of the 70-kDa Protein and CgA by CaM Kinase II—Since the 70-kDa protein is phosphorylated by CaM kinase II (17), we examined whether CgA partially purified from chromaffin granules was phosphorylated by CaM kinase II. As shown in Fig. 2, CgA was phosphorylated by CaM kinase II (Fig. 2, lane 5) and conigrated with the 70-kDa protein (Fig. 2, lane 4).

Analysis of the 70-kDa Protein by Immunoblotting with the Anti-CgA Antibody—Further confirmation of the idea that the 70-kDa protein is CgA, we prepared the antibody against bovine adrenal CgA. The samples were separated by SDS-PAGE and analyzed by immunoblotting. The anti-CgA antibody recognized the 70-kDa protein (Fig. 3, lane 3) as well as CgA (Fig. 3, lane 4). These results indicate that the 70-kDa protein is CgA.

Phosphorylation of CgA by Various Protein Kinases—We examined the phosphorylation of CgA by other protein kinases. When the Ca₂⁺ was incubated with CaM kinase II, PKC, and PAK, the phosphorylation of CgA was observed on SDS-polyacrylamide gels (Fig. 4). Based on the calculation of phosphatase by these kinases, the phosphatases incorporated into CgA were 0.9, 1.2, and 0.5 mol/mol of CgA by the incubation for 1 h with CaM kinase II, PKC, and PAK, respectively.

Two-dimensional TLC Separation of Tryptic [32P]Phosphopeptides of CgA Incubated with Protein Kinases—Phosphopeptides of CgA were further examined by two-dimensional TLC after extensive digestion with TPCK-trypsin (Fig. 5).
When CgA was phosphorylated by CaM kinase II, several sites were phosphorylated (Fig. 5A). One phosphorylation site by CaM kinase II (phosphopeptide a) was strongly phosphorylated by PKC (Fig. 5B) and slightly phosphorylated by PKA (Fig. 5C). One minor phosphorylation site by CaM kinase II (phosphopeptide c) was a major phosphorylation site by PKA (Fig. 5C). Another phosphorylation site by CaM kinase II (phosphopeptide b) was not phosphorylated by PKC or PKA. By contrast, one phosphorylation site by PKA (phosphopeptide d) was not phosphorylated by CaM kinase II and PKC. These differences in phosphopeptide mapping patterns by the three protein kinases were clearly shown when two samples were mixed before separation (Fig. 5, D and E).

CgA phosphorylated by three protein kinases was cut out from the SDS-PAGE gel and subjected to partial acid hydrolysis, followed by phosphoamino acid analysis (Fig. 6). PKC and PKA phosphorylated only the serine residue, whereas CaM kinase II phosphorylated both serine and threonine residues.

Effect of High Concentrations of K⁺ on CgA Phosphorylation and Catecholamine Secretion in Cultured Adrenal Medullary Cells—

Fig. 1. Sequence analysis of five peptides cleaved from the 70-kDa protein. Five peptides including the NH₂-terminal region were sequenced. The sequences were analyzed using the National Biomedical Research Foundation database and found to be identical with published bovine adrenal CgA (36). The sequences of the peptides are shown for comparison with those of CgA. Homologous amino acids are boxed. The numbers below the boxes indicate the positions of the residues in CgA. X, not identified.

Fig. 2. An autoradiogram of the 70-kDa protein and CgA phosphorylated by CaM kinase II. The 70-kDa protein (1.0 μg) or partially purified CgA (1.5 μg) were incubated at 30 °C for 10 min with 20 μM [γ-32P]ATP (4 × 10⁶ cpm), 0.2 mM CaCl₂, and 2 μg of calmodulin in the presence or absence of 0.25 μg of rat brain CaM kinase II (CaMKII). SDS-PAGE was carried out in 10% acrylamide. Arrows of α-subunit and β-subunit indicate α- and β-subunits of brain CaM kinase II.

Fig. 3. Western immunoblot analysis of the 70-kDa protein with the anti-CgA antibody. Samples of the 70-kDa protein (1.9 μg) (lanes 1 and 3) and partially purified CgA (2.6 μg) (lanes 2 and 4) were separated by SDS-PAGE in 10% acrylamide, analyzed by immunoblotting with the anti-CgA antibody, and finally developed with horseradish peroxidase-conjugated goat anti-rabbit IgG and O-phenylenediamine. A, Coomassie Blue staining (lanes 1 and 2). B, Immunoperoxidase staining (lanes 3 and 4). Protein standards (PS) were as follows: 94K, phosphorylase b; 67K, bovine serum albumin; 43K, ovalbumin; 30K, carbonic anhydrase; 20K, soybean trypsin inhibitor.

Fig. 4. An autoradiogram of CgA phosphorylated by CaM kinase II, PKC, and PKA. CgA (1.0 μg) was phosphorylated in the absence (–PK) or presence of 100 nm of CaM kinase II (CaMKII), PKC, and PKA at 30 °C for 10 min, under respective standard conditions. The incubation mixtures were subjected to SDS-PAGE in 9% acrylamide, followed by autoradiography. In lane CaMKII (KII), the phosphorylated bands of 50 and 60-kDa are autophosphorylated α-subunit and β-subunit of brain CaM kinase II, respectively.

When CgA was phosphorylated by CaM kinase II, several sites were phosphorylated (Fig. 5A). One phosphorylation site by CaM kinase II (phosphopeptide a) was strongly phosphorylated by PKC (Fig. 5B) and slightly phosphorylated by PKA (Fig. 5C). One minor phosphorylation site by CaM kinase II (phosphopeptide c) was a major phosphorylation site by PKA (Fig. 5C). Another phosphorylation site by CaM kinase II (phosphopeptide b) was not phosphorylated by PKC or PKA. By contrast, one phosphorylation site by PKA (phosphopeptide d) was not phosphorylated by CaM kinase II and PKC. These differences in phosphopeptide mapping patterns by the three protein kinases were clearly shown when two samples were mixed before separation (Fig. 5, D and E).
with or without high K\textsuperscript{+} (25, 56, and 75 mM), and the supernatants were immunoprecipitated with the anti-CgA antibody. The immunoprecipitates were analyzed by SDS-PAGE, followed by autoradiography (Fig. 7). Incubation of cells with the control medium resulted in a small amount of 32P incorporation into CgA (Fig. 7B, lane 1). Stimulation of cells with high K\textsuperscript{+} (25, 56, and 75 mM) increased the phosphorylation of CgA in a concentration-dependent manner (Fig. 7B, lanes 2-4 and Fig. 7C). The maximal effect (a 2.4-fold increase) was observed with 56 mM K\textsuperscript{+}. This concentration-dependent increase was correlated with that of catecholamine secretion (Fig. 7, D) (y = 4.47x + 43.2; r = 0.998, p < 0.002). Fig. 8 shows the time courses of the increases in CgA phosphorylation (Fig. 8, A and B) and catecholamine secretion (Fig. 8C), respectively, produced by 56 mM K\textsuperscript{+}. The phosphorylation of CgA increased rapidly at 30 s after stimulation and reached a plateau at 1 min. The time dependence of the CgA phosphorylation was also similar to that of catecholamine secretion. When the cells were incubated with 20 mM MgSO\textsubscript{4}, an inhibitor of voltage-dependent Ca\textsuperscript{2+} channels, the 56 mM K\textsuperscript{+}-stimulated phosphorylation of CgA and catecholamine secretion were significantly inhibited (Table I).
S.D. from four experiments. *p < 0.01, **p < 0.001, ***p < 0.0001, compared with control.

**DISCUSSION**

The 70-kDa Protein Is CgA or a Protein Closely Related to CgA—In our previous study (17), the 70-kDa protein was copurified with CaM kinase II from the soluble fraction treated with Triton X-100, followed by DEAE-cellulose, CaM-agarose, and Sephacryl S-300 column chromatography. The purification method of the protein differed from the method reported for CgA. Therefore, we considered that the 70-kDa protein was a new substrate for CaM kinase II. In the present study, we isolated CgA from bovine adrenal chromaffin granules and compared the two proteins. We demonstrated that (i) the amino acid sequences of five peptides deft from the 70-kDa protein reveal a high homology with those of CgA (Fig. 1); (ii) CaM kinase II phosphorylates CgA as well as the 70-kDa protein (Fig. 2); and (iii) the 70-kDa protein is immunoblotted with the anti-CgA antibody (Fig. 3). From these findings, we concluded that the 70-kDa protein is CgA or a closely related protein.

CgA, an acid glycoprotein, first identified in chromaffin granules of the adrenal medulla, is the major member of the secretogranin/chromogranin class of proteins and has a widespread distribution in endocrine tissues and the brain (see reviews Refs. 37, 38). CgA is comprised of 431 amino acid residues, corresponding to an unmodified protein of 48,000 of the molecular mass (36). The deduced molecular weight is considerably less than that we obtained (apparent molecular mass, 70 kDa) and other previous estimates based on SDS-PAGE (apparent molecular mass, 70–80 kDa) (37). The discrepancy is well explained by Benedum et al. (36) that even the in vitro translation product of CgA has a highly abnormal mobility by SDS-PAGE (the major product of CgA has an apparent molecular mass of 72 kDa).

Phosphorylation of CgA by Several Multifunctional Protein Kinases—The DNA encoding CgA (36) shows the presence of several potentially accessible consensus sites (39) for the action of PKA and PKC in addition to CaM kinase II. Indeed, in the present study, we directly demonstrated the phosphorylation of CgA by PKA and PKC (Fig. 4). Therefore, this is the first report to show that CgA is the substrate for these three protein kinases. By the two-dimensional peptide mapping, we identified at least four distinct 32P-phosphopeptides derived from CgA phosphorylated by three protein kinases (Fig. 5). In these peptides, CaM kinase II and PKA phosphorylated three peptides, a, b, and c, respectively, whereas PKC phosphorylated only one peptide (a). The phosphoamino acid analysis demonstrated that the phosphorylated amino acid residue of CgA by three protein kinases is exclusively serine. The threonine residue was slightly phosphorylated by CaM kinase II (Fig. 6). A previous in situ study (40) indicated that CgA was phosphorylated on the serine residue and to a small extent on the threonine residue in nonstimulated adrenal medullary cells. Therefore, CgA seems to be phosphorylated by the endogenous protein kinases(s) in the cells.

In Situ Phosphorylation of CgA by Cell Depolarization in Cultured Adrenal Medullary Cells—CgA is the major secreted protein that is located in chromaffin granules of adrenal medullary cells (see reviews Refs. 37, 38). Therefore, the question is
whether the phosphorylation of CgA increases by cell stimulation. In the present study, depolarization of cultured adrenal medullary cells with high K⁺ stimulated the phosphorylation of CgA in concentration- and time-dependent manners (Figs. 7 and 8). In the cell homogenizing buffer, we used various inhibitors for protein kinases, protein phosphatases, and proteases such as 10 mM EDTA, 4 mM EGTA, 25 mM NaF, 0.43 mM phenylmethylsulfonyl fluoride, 0.05 mM leupeptin, and 50 mg/liter trypsin inhibitor. In order to attain further complete inhibition of CgA phosphorylation after cell homogenization, staurosporine (100 mM) and β-glycerophosphate (50 mM) were added to the homogenizing buffer as nonselective inhibitors for protein kinases (41, 42) and protein phosphatases, respectively. The stimulation of CgA phosphorylation by 56 mM K⁺ was also observed even when these two inhibitors existed in the homogenizing buffer (data not shown). Therefore, it is unlikely that further phosphorylation of CgA occurs during the subsequent manipulation. Previously, Côté et al. (8) reported that acetylcholine caused an increase (about 30%) in phosphorylation of an 80-kDa protein in bovine adrenal medullary cells. They considered the 80-kDa protein as CgA, because it reacted with the antiserum against CgA, but they did not show the data.

Although the physiological significance of CgA has not been established, there are several proposals for it. They include roles (i) in the involvement in catecholamine or neuropeptides storages, (ii) in the binding of calcium and possible consequences for granule formation, (iii) as a regulatory protein after secretion, and (iv) as a precursor of peptide hormones and neuropeptides (37, 38). Therefore, the phosphorylation of CgA might modify these possible functions. On the other hand, CgA immunoreactivity is most prominent in the cytosol rather than the synaptic vesicles in the brain (43). Somogyi et al. (44) reported that the distribution of CgA immunoreactivity resembled the location of the Golgi apparatus in the brain and that some neurons exhibited a homogeneous staining throughout the cytoplasm, suggesting that CgA in the brain has a cellular function independent of that in the vesicular storage. In the present study, it is important to know whether the phosphorylation of CgA stimulated by high K⁺ occurs in the cytosol or within the chromaffin granules. Stimulation of cells with 56 mM K⁺ did not increase the phosphorylation of CgA in the chromaffin granule fractions (see the "Results"). To check the disruption of chromaffin granules during homogenization, we measured the amount of CgA and the activity of dopamine β-hydroxylase in the supernatant fraction of the cell homogenates. The level of CgA was slightly higher than that of dopamine β-hydroxylase activity in the supernatant fraction of the cell homogenates. The level of CgA was slightly higher than that of dopamine β-hydroxylase activity in the supernatant fraction of the cell homogenates. The level of CgA was slightly higher than that of dopamine β-hydroxylase activity in the supernatant fraction of the cell homogenates. The level of CgA was slightly higher than that of dopamine β-hydroxylase activity in the supernatant fraction of the cell homogenates. The level of CgA was slightly higher than that of dopamine β-hydroxylase activity in the supernatant fraction of the cell homogenates. The level of CgA was slightly higher than that of dopamine β-hydroxylase activity in the supernatant fraction of the cell homogenates. The level of CgA was slightly higher than that of dopamine β-hydroxylase activity in the supernatant fraction of the cell homogenates. The level of CgA was slightly higher than that of dopamine β-hydroxylase activity in the supernatant fraction of the cell homogenates. The level of CgA was slightly higher than that of dopamine β-hydroxylase activity in the supernatant fraction of the cell homogenates. The level of CgA was slightly higher than that of dopamine β-hydroxylase activity in the supernatant fraction of the cell homogenates. The level of CgA was slightly higher than that of dopamine β-hydroxylase activity in the supernatant fraction of the cell homogenates.