Calcium Calmodulin Dependent Phosphorylation of Proteins: Fetal Cortical Neurons and Adult Cortex

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In the present investigation, changes in the calcium calmodulin–dependent phosphorylation of proteins have been examined in murine fetal cortical neurons and adult cortex. An ~80-kD protein in the fetal neurons was not phosphorylated/dephosphorylated in a calmodulin-dependent manner. However, this protein was phosphorylated by PMA both in the presence and absence of calcium. These data suggest that calmodulin inhibits the phosphorylation of a ~80-kD protein by inhibiting PKC in murine fetal cortical neurons but not in the adult cortex. More importantly, we demonstrate that the calmodulin-mediated inhibition of phosphorylation was restored by preincubating the cortical neurons with KN-62, a CaM kinase inhibitor.

KEY WORDS: CaM kinase II; development; autophosphorylation.

INTRODUCTION

Protein phosphorylation is a major mechanism by which extracellular signals are translated into intracellular reactions (1). The extent of phosphorylation depends on the availability of second messengers such as cAMP, cGMP, and calcium. Calcium calmodulin–dependent protein kinase II (CaM kinase II) is one of the most abundant protein kinases in the central nervous system (2). It is a multifunctional enzyme implicated in a variety of cellular responses that utilize calcium as a second messenger. The holoenzyme contains a 50-kD α subunit and a 58–60 kD β subunit, both of which can bind calcium and calmodulin and show autophosphorylation (3). The composition of the subunits varies with the region of brain and the stage of development (4). The α and β subunits in the adult rat brain are in the ratio of 3:1 and 1:4 in forebrain and cerebellum, respectively (5,6). In the current study, we examined the calcium calmodulin–mediated \textit{in-vitro} phosphorylation of proteins in the homogenates of murine fetal cortical neurons (prenatal) and compared it with adult cortex (postnatal) under identical conditions.

EXPERIMENTAL PROCEDURES

Cell Culture. The time pregnant mice (strain C57BL/6) purchased from Harlan (Indianapolis, IN, USA) were used in accordance with institutional guidelines, and procedures were approved by the animal welfare committee. Murine cortical neurons were isolated and cultured as described previously (7). Briefly, cerebral hemispheres were dissected from 14 to 15-day-old fetuses, and cells were dissociated by trituration. Approximately 24 × 10^6 cells were used to plate each 75 cm² poly-L-lysine-coated plastic flask. Cells were grown in minimum essential medium (MEM) containing 33.3 mM glucose, 26.2 mM NaHCO₃, 100 μM glutamine, 10% fetal bovine serum, and 10% heat-inactivated horse serum (MEM 10/10) in an incubator containing 5% CO₂ at 37°C. On the second day, a mixture of 5-fluoro-2'-deoxyuridine and uridine at a final concentration of 10 μg/ml was added to the medium to inhibit cell proliferation. The cultures contained more than 92% neurons.

The cultured neurons were washed with PBS and homogenized in 10 mM Tris-HCl, pH 7.5, containing 1 mM EDTA and 1 mM DTT using Dounce homogenizer. Homogenates were centrifuged at 100 × g for 4 min to remove debris and the supernatant was used for further experiments. Similarly, the adult mouse cortex was homogenized in the
same buffer and prepared as described above. Protein content was estimated as reported earlier (8).

**Endogenous Phosphorylation.** In vitro phosphorylation was performed in a buffer containing 50 mM HEPES, pH 7.4, 10 mM MgCl₂, 1 mM DTT, 2 μM calmodulin, 1 mM EGTA or 1 mM CaCl₂, and 20 μg homogenate. Reaction was initiated by the addition of 2 μCi γ³²P ATP (3000 Ci/mmol) containing 10 μM ATP, and phosphorylation was carried out for 1 min at 30°C. The reaction was terminated by the addition of 6 vol of cold acetone and kept for 2 h at −80°C. After centrifuging, the samples, the phosphorylated proteins were solubilized in 30 μl of Laemmli buffer (0.0625 M Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 5% 2-mercaptoethanol), resolved by SDS-polyacrylamide gel electrophoresis and detected by autoradiography. In vitro phosphorylation reaction was also performed under appropriate conditions to study the effect of CaM Kinase II inhibitor KN-62 (10 μM), PKC activator PMA (100 nM), and protein phosphatase 2B inhibitor Fenvelare (30 μM).

**CaM Kinase Assay.** CaM kinase II activity was assayed by the phosphorylation of syntide 2, a synthetic peptide specific for CaM kinase II, as described (9). Reaction mixture contained 50 mM HEPES, pH 7.5, 10 mM MgCl₂, 40 μM syntide-2, 2 μM calmodulin, 1 mM CaCl₂, 0.1 mM EGTA, 100 μM ATP containing 1 μCi of ³²P ATP, and 10 μg of homogenate protein in a total volume of 50 μl. Control reaction was performed in the presence of 1 mM EGTA. After incubation at 30°C for 1 min, the reaction was terminated by adding 10 μl of 0.4 M EDTA and spotting it on phosphocellulose paper (P-81). The filter papers were washed with 75 mM phosphoric acid (3 × 10 min), rinsed with ethanol and dried. The radioactivity associated with the filters was counted and expressed as cpm/10 μg protein.

**Electrophoresis and Immunoblotting.** Protein (30 μg) was separated by SDS-PAGE (10). Two gels were run in parallel; one was stained with Coomassie blue and the other was used for immunoblotting. Proteins were transferred (11) onto a polyvinylidene fluoride membrane (BioRad, CA, USA). The membrane was washed with TBS-T (20 mM Tris base, 150 mM sodium chloride, and 0.1% Tween-20) and then blocked with 5% milk powder made in TBS-T. Following washing, membrane was incubated with anti-CaM kinase II antibody (Monoclonal antibody, Boeringer Mannheim, Germany; 1:5000 in blocking solution) for 1 h. Specific band was visualized on a film (Kodak X-Omat, LS, Kodak, Rochester, NY, USA) using super signal detection kit (Pierce, Rockford, IL, USA). The relative changes in the CaM kinase II levels were evaluated by measuring the intensity of the immunoreactive bands using the NIH IMAGE system as described earlier (12).

**RESULTS**

Figure 1 shows the incorporation of ³²P into syntide, demonstrating a very high activity in the adult tissue relative to prenatal tissue. Consistent with the enzyme activity, CaM kinase II content was also higher in adult tissue. Because the activity of CaM kinase II was found to be increased in the adult cortex, we performed in vitro phosphorylation to detect the substrates of CaM kinase II in fetal and adult tissue. Endogenous phosphorylation of proteins performed in the presence of EGTA was weak (Fig. 2, lane 1), while addition of calcium induced the phosphorylation of a ~80-kD protein in the fetal neurons (Fig. 2, lane 2) and 80-, 60-, and 50-kD proteins in the adult cortex (Fig. 2, lane 2). However, the phosphorylation of 50-kD protein was more than 60, kD protein. When calmodulin was added to the reaction mixture, the phos-