Ca\textsuperscript{2+}-Mediated Phosphorylation and Proteolysis Activity Associated with the Cytoskeletal Fraction from Cerebral Cortex of Rats

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We describe a Triton-insoluble cytoskeletal fraction extracted from cerebral cortex of young rats retaining an endogenous Ca\textsuperscript{2+}-mediated mechanism acting in vitro on Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaM-KII) activity and on phosphorylation and proteolysis of the 150 kDa neurofilament subunit (NF-M), α and β tubulin. Exogenous Ca\textsuperscript{2+} induced a 70% decrease in the in vitro phosphorylation of the NF-M and tubulins and a 30–50% decrease in the total amount of these proteins. However, when calpastatin was added basal phosphorylation and NF-M and tubulin content were recovered. Furthermore, exogenous Ca\textsuperscript{2+}/calmodulin induced increased in vitro phosphorylation of the cytoskeletal proteins and CaM-KII activity only in the presence of calpastatin, suggesting the presence of Ca\textsuperscript{2+}-induced calpain-mediated proteolysis. This fraction could be an interesting model to further studies concerning the in vitro effects of Ca\textsuperscript{2+}-mediated protein kinases and proteases associated with the cytoskeletal fraction.

KEY WORDS: Cytoskeletal proteins; phosphorylation; proteolysis; cerebral cortex.

INTRODUCTION

The cytoskeleton is constituted by a cytoplasmic network of protein filaments concerned with cell support and motility. The neuronal cytoskeleton is formed mainly of neurofilaments and microtubules interacting with one another and with a variety of associated proteins. Axons contain many longitudinally oriented neurofilaments and microtubules occupying most of the axoplasm (1).

Neurofilaments are believed to play an important role in the maintenance of neuronal shape and determination of the axonal caliber (2,3). They are composed of three subunits that exhibit apparent molecular masses of 200 kDa (NF-H), 150 kDa (NF-M) and 68 kDa (NF-L) (4). The neurofilament subunits are phosphorylated by different second messenger-dependent and independent protein kinases in their head and tail domains, respectively. Phosphorylation in the head domain is probably related to neurofilament assembly, while phosphorylation in the tail domain is considered to be one means by which neurofilaments cross-link and stabilize the axonal cytoskeleton (5,6). Microtubules are one type of protein filament of the cytoskeleton mainly consisting of α and β tubulin subunits. Tubulins can be phosphorylated on their carboxyl-terminal regions by multiple protein kinases (7). Phosphorylation is known to stabilize neurofilaments as well as microtubules in the axoplasm by distinct mechanisms assuring metabolically stable structures for axonal transport (8).
Calcium is a second messenger in neurons, leading to the activation of various enzymatic processes such as phosphorylation and proteolysis. Calcium-activated kinases and proteases are physiologically associated with the cytoskeleton in axons (8). There are two predominant Ca<sup>2+</sup>-activated protein kinases in brain, Ca<sup>2+</sup>/phospholipid-dependent protein kinase (PKC) and Ca<sup>2+</sup>/calmodulin-dependent protein kinase II (CaM-KII) (9). Calpain is a Ca<sup>2+</sup>-activated neutral protease present in high concentrations in the brain (10). Calpain exists as distinct isoforms that are activated at micromolar or millimolar calcium concentrations (termed calpain I and calpain II, respectively) (11). Calpain specificity appears to be determined by conformational factors as well as primary amino acid sequence and catalysis-limited cleavage of its substrates. Calpain I and calpain II appear to have identical substrates specificities and their endogenous protein substrates are enzymes, myofibrillar proteins, membrane proteins, cytoskeletal proteins and receptor proteins (11). The action of calpain can be inhibited by a number of agents. The most potent of all is the naturally occurring endogenous calpain inhibitor protein, calpastatin (12).

Lasek and Hoffman (13) have hypothesized that cytoskeletal proteins are metabolically stable in the axon but are rapidly degraded by Ca<sup>2+</sup>-dependent proteinases in the axon terminals. Further studies have demonstrated that axoplasm contains little phosphatase activity (14), whereas phosphatase activity is largely located in nerve terminals (15). Furthermore, Pant (8) has suggested that phosphorylation protects neurofilaments against the action of calpain in the axoplasm, while dephosphorylation enhances their susceptibility to degradation by calpain in the nerve terminals.

The aim of this study is to describe a Triton-insoluble cytoskeletal fraction obtained from the cerebral cortex of young rats, retaining an endogenous Ca<sup>2+</sup>-activated mechanism acting in vitro on CaM-KII activity and on phosphorylation and proteolysis of NF-M, and α and β tubulin. These effects are inhibited by calpastatin, suggesting a calpain-mediated activity. This work emphasizes that enzymatic activities regulating the metabolism of neurofilaments and microtubules in vivo would be physically associated with the cytoskeletal proteins, co-purifying in the Triton-insoluble fraction.

**EXPERIMENTAL PROCEDURE**

*Animals.* Wistar rats from our breeding stock were maintained on a 12-h light/12-h dark cycle in a constant temperature (22°) colony room. On the day of birth the litter size was culled to 8 pups. Litters smaller than 8 pups were not included in the experiments. Free water and a 20% (w/w) protein commercial chow were provided. Rats were sacrificed on the 17th day, the cerebral cortex was immediately removed and the cytoskeletal fraction extracted as described below.

**Preparation of a Triton-Insoluble Cytoskeletal Fraction from Cerebral Cortex.** The fraction was prepared as described by de Mattos et al. (16). Briefly, cerebral cortex (600 mg) was homogenized in 40 ml of ice-cold buffer containing 50 mM Tris-HCl, pH 6.8, 5 mM EGTA, 1% Triton X-100 and the following protease inhibitors: 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM benzamidine, 1 μM leupeptin, 0.7 μM antipain, 0.7 μM pepstatin and 0.7 μM chymostatin (Sigma, St. Louis, MO, USA). The homogenate was centrifuged at 13000 g for 15 min, at 4°C. The pellet was resuspended in 40 ml of the same buffer containing 0.85 M sucrose and centrifuged for 15 min at 13000 g. The pellet was dissolved in 50 mM 2[N-morpholino]ethanesulphonic acid (MES), pH 6.5, and 10 mM MgCl<sub>2</sub> and protein concentration was determined by the method of Bradford (17).

**32P-Incorporation Assay.** Each assay mixture contained 10 μg of protein in 60 μl of a buffer containing 50 mM MES, pH 6.5, and 10 mM MgCl<sub>2</sub>. 32P-incorporation into the cytoskeletal fraction was performed in the presence of 0.01, 0.1, or 1.0 mM CaCl<sub>2</sub> and in the absence or presence of 1.0 μM calpastatin (Sigma, St. Louis, MO, USA). Ca<sup>2+</sup>/calmodulin-dependent protein kinase activity was determined in reaction mixtures containing 1 μM calmodulin (Sigma, St. Louis, MO, USA), 1 mM CaCl<sub>2</sub> and in the absence or presence of 1 μM calpastatin. Control incubations were carried out in the absence of exogenous calcium, calmodulin and calpastatin. The reaction was started by adding 2.0 μ Ci[32P]-ATP (16.6 × 10<sup>6</sup> Bq/nmol) (ICN Biochemicals, Irvine, CA, USA). After incubation for 5 min at 30°C, the reaction was stopped by adding Laemmli sample buffer and the samples were boiled for 3 min. Proteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 10% acrylamide according to the discontinuous system of Laemmli (18). After autoradiograph on Kodak X-Omat K film, phosphoprotein bands were excised from the gel and radioactivity incorporated was measured by liquid scintillation counting (19).

**CaM-KII Assay.** The standard assay system is essentially the same as described above. CaM-KII activity was determined by using the exogenous substrate peptide syntide-2 (Sigma, St. Louis, MO, USA). The assay for CaM-KII contained 1 μM calmodulin (CaM), 1 mM CaCl<sub>2</sub>, 40 μM syntide-2 and in the absence or presence of 1 μM calpastatin. Control incubations were carried out in the presence of 3 mM EGTA and absence of CaM and syntide-2. After 5 min of incubation at 30°C, reactions were stopped by spotting 20 μl of the reaction mixture on P-81 phosphocellulose filters. After, the filters were washed in 75 mM phosphoric acid and the remaining radioactivity was quantified in a scintillation counter. The 32P-incorporation into syntide-2 in the absence or presence of calpastatin (CT) was calculated in cpm by the difference between the assays containing Ca<sup>2+</sup>/CaM + syntide-2 and Ca<sup>2+</sup>/CaM or between the assays containing Ca<sup>2+</sup>/CaM + syntide-2 + Cl and Ca<sup>2+</sup>/CaM + Cl, respectively.

**Ca<sup>2+</sup>-Mediated Proteolysis Assay.** Each assay mixture contained 300 μg protein in 200 μl of a buffer containing 50 mM MES, pH 6.5, and 10 mM MgCl<sub>2</sub> in the presence of 0.01, 0.1, or 1.0 mM CaCl<sub>2</sub> and in the absence or presence of 1 μM calpastatin. Some assay mixtures were pre-incubated in the presence of 0.1 mM ATP for 5 min at 30°C and the procedure for Ca<sup>2+</sup>-mediated proteolysis assay was essentially as described above. Control incubations were carried out in the absence of calcium and calpastatin. The reactions were started by incubating the samples for 5 min at 30°C and were stopped by adding 5% TCA, washed twice with ethanol and acetone, and dissolved in 1% SDS. Proteins were analyzed by SDS-PAGE. Gels were stained with...