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PHOSPHORYLATION OF BOVINE CARDIAC CALCIUM-ACTIVATED NEUTRAL PROTEASE BY PROTEIN KINASE-C

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Protein kinase C prepared from rat brain was used to phosphorylate a calcium-activated neutral protease, purified from bovine cardiac muscle. Attempts to phosphorylate the enzyme in the presence of calcium were unsuccessful, unless the protease inhibitor leupeptin was also present. Phosphorylation of the 74K subunit of the protease was completely inhibited in the absence of phosphatidylserine and diolein, indicating that phosphorylation of the enzyme was catalysed by the calcium and phospholipid-dependent protein kinase C.

Calcium-activated neutral protease (CANP) is an intracellular thiol protease which exists ubiquitously in different tissues and organisms. While many characteristics of the enzyme are known (for reviews see references 1 and 2), its physiological role is still uncertain, probably due to the fact that the intracellular control mechanism which regulates the activity of the enzyme is unknown. Activation of the enzyme may involve lifting of the tonic inhibition exercised by its co-existing, specific inhibitor. Although calcium is obligatory for enzyme function, inhibition is not abrogated by the sequestration of calcium (2). Investigations of the possibility of enzyme activation by phosphorylation have

The abbreviations used are: EGTA, ethylene glycol bis (β-aminoethyl ether) -N,N,N^1,N^1-tetraacetic acid; Hepes, N-2-hydroxethyl piperazine-N^1-2-ethanesulfonic acid; TCA, trichloroacetic acid; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; CANP, calcium-activated neutral protease; PK-C, protein kinase C; STI, soybean trypsin inhibitor.

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yielded equivocal results thus far. Waxman (3) has found that neither phosphorylase b kinase nor the catalytic subunit of cAMP-dependent protein kinase were able to activate the bovine cardiac enzyme by phosphorylation, while Zimmerman and Schlaepfer (4) reported co-purification of the rat skeletal muscle enzyme and the cAMP-dependent protein kinase. In the latter case, the phosphorylated enzyme (mCANP) showed a diminished level of proteolytic activity.

In the present communication we wish to report the phosphorylation of the 74K subunit of bovine cardiac CANP by the cyclic nucleotide-independent protein kinase C.

MATERIALS AND METHODS

Materials: [γ-^{32}P]ATP (25 Ci/mMole) was obtained from ICN Biomedicals. Histone III-S, phosphatidylserine, 1,2-diolein and leupeptin were from Sigma. All other chemicals were of the highest available purity.

Preparation of protein kinase C: a modification of a published procedure (5) was used. The brains of 2 to 4 male Wistar rats (250-300g) were homogenized in 8 volumes of buffer A (0.3 M sucrose, 20 mM Hepes pH 7.4, 10 mM EGTA, 2 mM EDTA, 1 mM DTT, 10 ug/ml leupeptin and 10 ug/ml STI). The homogenate was clarified by centrifugation and the supernatant was applied to a DEAE-Sephacel (Pharmacia) column (1.5 x 10 cm) which was equilibrated with buffer B (20 mM Hepes pH 7.4, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM DTT). A gradient of 0-0.3 M NaCl (50 ml per side) in buffer B was applied. PK-C activity eluting in a broad peak below 0.1 M NaCl was pooled and leupeptin (20 ug/ml) and STI (0.2 mg/ml) added. PK-C was concentrated by ammonium sulfate precipitation (80%). After centrifugation the pellet was dissolved in 1.5 ml of buffer C (0.2 M NaCl, 20 mM Hepes pH 7.4, 2 mM EDTA, 0.5 mM EGTA, 0.5 mM DTT) and applied to a column of Fractogel TSK HW-55(S) (EM Reagents) (1.6 x 90 cm). The active peak was pooled and leupeptin (20 ug/ml) and STI (0.2 mg/ml) added. This solution was dialysed overnight against 500 ml buffer B and then applied to a column of DEAE-Trisacryl M (LKB) (1 x 5 cm). The column was washed in the following sequence: 25 ml of buffer B, 15 ml of buffer B containing 0.1% Triton X-100 and 50 ml buffer B. A 0-0.4 M NaCl gradient in buffer B (30 ml per side) eluted a peak of PK-C activity at 0.1 M NaCl which was completely dependent upon phosphatidylserine/diolein.

PK-C activity was pooled and dialysed against 30% (w/v) glycerol, 20 mM Hepes pH 7.4, 0.5 mM EGTA, 2 mM DTT for 2 days and then stored at 4°C for several months with little loss in activity. The specific activity of this final product ranged between 64 and 135 nmol P_i incorporated/min/mg, using histone III-S as substrate. Analysis by SDS-PAGE indicated that this preparation contained 10-12 protein bands, only one of which became phosphorylated by [γ-^{32}P]ATP in the presence of calcium

and phosphatidylserine/diolein. This band of 82,000 daltons corresponds to the autophosphorylated protein kinase C. This material was utilized for phosphorylation studies because of its ease of preparation (1 week), good stability in storage, and the absence of any endogenous brain proteins which were phosphorylated by protein kinase C.

Protein kinase C activity was assayed at 30°C in a solution of 20 mM Hepes pH 7.4, 5 mM DTT, 10 mM $MgCl_2$, 500 uM $CaCl_2$, 1 mg/ml histone III-S in the presence and absence of 50 ug/ml phosphatidylserine, 2.5 ug/ml 1,2 diolein. Under these conditions stimulation of phosphorylation by phospholipids was considered to be the measure of PK-C activity. Assays were carried out in a final volume of 60 ul and initiated by adding [γ-^{32}P]ATP (100-200 cpm/pmol) to a concentration of 50 uM. At timed intervals, 20 ul of assay solution was pipetted onto a square (1.5 x 1.5 cm^2) of Whatman 3MM paper which was then washed in an ice cold bath of 10% TCA, 2% sodium pyrophosphate. The dried squares were counted in 10 ml of scintillant (6 g PPO, 75 mg POPOP per liter of toluene).

Preparation of Ca^{+2}-activated neutral protease:

The purification of bovine cardiac CANP has been previously described in detail (6). The purity of the preparation was monitored by SDS-PAGE, where the enzyme consistently resolved into 3 protein bands, corresponding to 73,6000, 30,500 and 28,600 daltons, respectively (see Figure 2B).

Phosphorylation of CANP by PK-C: the phosphorylation of CANP by PK-C was tested in a solution containing 20 mM Hepes pH 7.4, 5 mM DTT, 10 mM $MgCl_2$, 40 uM $CaCl_2$, 50 ug/ml phosphatidylserine and 2.5 ug/ml 1,2-diolein. Each solution contained 0.5 ug PK-C (specific activity of 116 nmol/min/mg towards histone III-S) and 25 ug CANP. The reaction was carried out at 30°C, started by the addition of [γ-^{32}P]ATP (500-800 cpm/pmol) to a final concentration of 50 uM. In some instances, leupeptin (20 ug/ml final concentration) was included. In these cases, the leupeptin and CANP were pre-incubated at 30°C for 5 min prior to the addition of PK-C. In control reactions CANP was replaced by the buffer (20 mM Tris-acetate pH 7.5, 1 mM EDTA, 5 mM 2-mercaptoethanol). The reaction was performed in a volume of 180 ul. At timed intervals, 50 ul aliquots were removed and rapidly mixed with 50 ul of SDS-stop solution (125 mM Tris-HCl pH 6.8, 4% SDS, 25% (w/v) glycerol). After heating at 100°C for 4 min, 10 ul of 0.5 M DTT and a drop of 0.05% bromphenol blue were added in preparation for electrophoresis.

Electrophoresis: SDS-PAGE was performed on 5-15% gradient gels using the method of Laemmli (7). After drying the gels were submitted to autoradiography for 7-10 days with Kodak XRP-1 film and a Cronex lightning-plus intensifying screen at -80°C. The following molecular weight markers were used: myosin (200,000), β-galactosidase (116,250), phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500).

RESULTS AND DISCUSSION

Initial experiments designed to detect phosphorylation of CANP by PK-C were unsuccessful, apparently because relatively

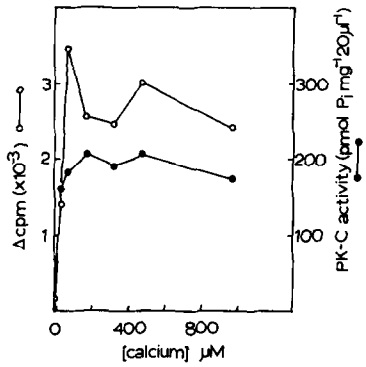

Figure 1. Calcium dependence of the phosphorylation of CANP and histone III-S by PK-C. [^{32}P]-phosphate incorporation into CANP was measured following TCA precipitation onto 3MM paper squares (methods). Autophosphorylation of PK-C was measured separately and subtracted from the phosphorylation obtained with PK-C and CANP (ΔCPM). The reaction was terminated after 60 min (O——O). Protein kinase C phosphorylation of histone III-S was measured for 3 and 6 min periods (methods). These phosphorylation rates (●——●) were averaged for each calcium concentration. 'Zero' calcium was obtained with 1 mM EGTA.

high levels of calcium were utilized (500 uM). Since it seemed possible that activation of CANP under these conditions could lead to the degradation of PK-C (8) or to autodigestion of CANP (9), conditions more optimal for the phosphorylation of CANP were investigated. Decreasing the calcium concentration to 70 uM resulted in optimal phosphate incorporation as determined by TCA precipitation on paper squares (Fig. 1), but autoradiography following SDS-PAGE of these samples revealed a number of phosphorylated bands, including one which coincided with the coomassie-blue stained 74K subunit of CANP. Decreasing the calcium concentration to 40 uM did not modify this observation (data not shown). It seemed possible that activation of CANP and subsequent autodigestion and/or degradation of PK-C occurred in spite of the low calcium levels. Therefore in subsequent phosphorylation reactions leupeptin, which is an inhibitor of CANP, was included. The concentration used (20 ug/ml) completely inhibits CANP activity towards casein

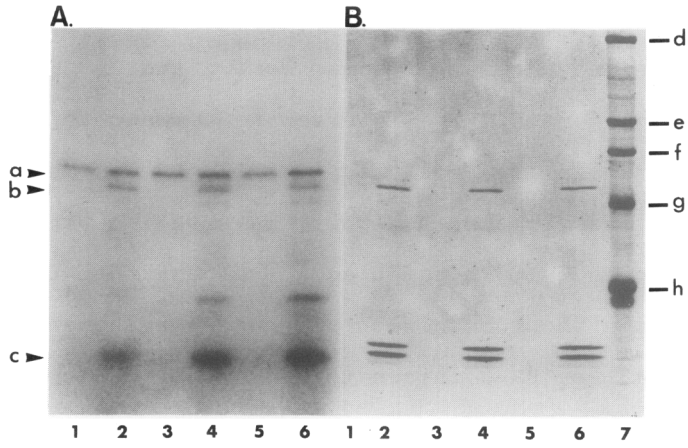

Figure 2. Phosphorylation of CANP by PK-C.
The reactions were performed according to methods, in the presence of 40 uM $CaCl_2$ and 20 ug/ml leupeptin. Lanes 1, 3 and 5 contained only PK-C, while lanes 2, 4 and 6 contained PK-C and CANP. The phosphorylation reactions were stopped at the following times: 1, 2 - 10 min, 3, 4 - 35 min, 5, 6 - 60 min. Lane 7 contained molecular weight markers (methods).
A. Autoradiogram. The indicated bands correspond to the following molecular weights:
a, 82,000; b, 74,000; c, 29,000.
B. Coomassie blue stained gel. Marker molecular weights are the following:
d, 200,00; e, 116,250; f, 92,500; g, 66,200; h, 45,000.

(data not shown). Under these conditions, autophosphorylation of PK-C and definite phosphorylation of the 74K subunit of CANP occurred (Fig. 2a). Phosphorylation was completely inhibited in the absence of phosphatidylserine/diolein or PK-C (data not shown), indicating that the phosphorylation of CANP was catalysed by the calcium- and phospholipid-dependent protein kinase C.

Fig. 2a also demonstrates that a 29K protein is phosphorylated with the same time course as the 74K subunit. This phosphoband corresponds to the lower of the coomassie-blue stained doublet (Fig. 2b). For the reasons outlined below, we suggest that this band represents a fragment of the 74K subunit which contains the site of phosphorylation by PK-C. Recently the amino acid sequence of the 80K subunit of the chicken skeletal muscle CANP has been determined from the

nucleotide sequence (10). From homology considerations, the authors identified a thiol protease domain and an "E-F hand" calcium-binding domain. The intervening sequence of 240 amino acids (domain III, equivalent to 27,400 daltons) contains near its midpoint a highly basic region with 5 arginine residues. It is tempting to suggest that these residues provide the recognition sequence for phosphorylation of threonine 431 by PK-C, since this group of basic residues is unique in the primary structure of the protein. Similar sequences are adjacent to the site of phosphorylation in other proteins which are phosphorylated by PK-C (11,12).

Our proposal that PK-C phosphorylates threonine 431 in domain III lends support to the possibility that this domain represents a regulatory site for the binding of the 30K subunit (10). This 29,000 dalton peptide which is phosphorylated with the same time course as the 74K CANP molecule may represent a co-purifying fragment of the larger molecule. These proposals assume that a large degree of homology exists between bovine cardiac and chicken skeletal CANP molecules.

We can only speculate about the physiological role such phosphorylation could play. Preliminary experiments designed to investigate the effect of phosphorylation upon the calcium-dependence of CANP activation are inconclusive as yet. We note however, that inhibition of the proteolytic activity with leupeptin was necessary in order to prevent degradation of PK-C or autodigestion of CANP even at uM calcium levels. Therefore it is tempting to suggest that phosphorylation by PK-C may provide a mechanism for conversion of mCANP to uCANP and for the activation of proteolytic activity in vivo.

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