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Inhibition by calmodulin of calcium/phospholipid-dependent protein phosphorylation

(phosphoproteins/protein kinase/phosphatidylserine/nervous system)

KATHERINE A. ALBERT, WILSON C.-S. WU*, ANGUS C. NAIRN, AND PAUL GREENGARD

Laboratory of Molecular and Cellular Neuroscience, Rockefeller University, 1230 York Avenue, New York, NY 10021

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ABSTRACT **Calmodulin was previously found to inhibit the Ca^{2+}/phospholipid-dependent phosphorylation of an endogenous substrate, called the 87-kilodalton protein, in a crude extract prepared from rat brain synaptosomal cytosol. We investigated the mechanism of this inhibition, using Ca^{2+}/phospholipid-dependent protein kinase and the 87-kilodalton protein, both of which had been purified to homogeneity from bovine brain. Rabbit brain calmodulin and some other Ca^{2+}-binding proteins inhibited the phosphorylation of the 87-kilodalton protein by this kinase in the purified system. Calmodulin also inhibited the Ca^{2+}/phospholipid-dependent phosphorylation of H1 histone, synapsin I, and the δ subunit of the acetylcholine receptor, with use of purified components. These results suggest that calmodulin may be a physiological regulator of Ca^{2+}/phospholipid-dependent protein kinase.**

Intracellular calcium is recognized to play a key role in the regulation of numerous physiological processes (e.g., exocytosis and muscle contraction). Many of the intracellular actions of calcium appear to be mediated by calcium-dependent protein phosphorylation (1, 2). Two classes of calcium-dependent protein kinases have been identifed—namely, Ca^{2+}/calmodulin-dependent protein kinases (2) and Ca^{2+}/phospholipid-dependent protein kinase (also known as protein kinase C) (3). Ca^{2+}/phospholipid-dependent protein kinase has been purified to homogeneity from rat brain (4) and pig spleen (5) and has been characterized in detail. This kinase has been suggested to play a physiological role in the regulation of hormone and neurotransmitter release. For example, serotonin release from platelets may be mediated through the Ca^{2+}/phospholipid-dependent phosphorylation of an endogenous 40-kilodalton (kDa) substrate (6, 7).

A physiological role for Ca^{2+}/phospholipid-dependent protein kinase in neurons has been suggested by evidence that depolarization-induced calcium influx into intact synaptosomes increased the phosphorylation of an 87-kDa protein (87kDa) through activation of this kinase (8). The Ca^{2+}/phospholipid-dependent stimulation of 87kDa phosphorylation, observed in a calmodulin-depleted preparation of synaptosomal cytosol, was inhibited by the addition of exogenous calmodulin (8). We report here the demonstration of this calmodulin inhibition, using Ca^{2+}/phospholipid-dependent protein kinase and 87kDa that had been purified to homogeneity.

MATERIALS AND METHODS

Materials. Fresh bovine brains were transported on ice to the laboratory from a local slaughterhouse. Rat brain cytosol was depleted of calmodulin as described for rat brain synaptosomal cytosol (8). Calmodulin was purified from rabbit brain by the procedure of Grand *et al.* (9). Human erythrocyte calmodulin, bovine brain S-100 protein, and rat muscle parvalbumin were purchased from Calbiochem–Behring. Rabbit skeletal muscle troponin C was a gift from P. Leavis (Boston Biomedical Research Institute). S-100 protein, parvalbumin, and troponin C were found to be free of calmodulin contamination by their inability to activate Ca^{2+}/calmodulin-dependent protein kinase I (1). Synapsin I was purified from bovine brain by a modification (unpublished results) of the original procedure (10). Purified nicotinic acetylcholine receptor was a gift from R. Huganir. H1 histone (III-S), bovine serum albumin, bovine brain L-α-phosphatidyl-1-serine, dithioerythritol, EGTA, Tris, and ATP were purchased from Sigma.

Other reagents and enzymes were obtained from the following commercial sources: [γ-^{32}P]ATP (New England Nuclear); 2-mercaptoethanol (Baker); low molecular weight protein standards kit for electrophoresis (Bio-Rad); leupeptin (Calmed); DE-52 (Whatman); DEAE-Sephacel (Pharmacia); hydroxylapatite (Bio-Gel HTP) (Bio-Rad); and thermolysin (Worthington). All other chemicals used were reagent grade.

Purification of Ca^{2+}/Phospholipid-Dependent Protein Kinase and 87kDa. The enzyme was purified from bovine cerebral cortex by modification of the procedure of Kikkawa *et al.* (4). The final peak of kinase activity ran as a single protein band (M_r, 87,000) on $NaDodSO_4$/8% polyacrylamide gel electrophoresis (Fig. 1). The soluble 87kDa was purified to homogeneity from bovine cerebral cortex by DEAE-cellulose chromatography, acid precipitation, hydroxylapatite chromatography, gel filtration, and DEAE-cellulose chromatography (unpublished data). The final peak corresponded to a single protein band on $NaDodSO_4$/8% polyacrylamide gel electrophoresis (Fig. 1).

Measurement of Ca^{2+}/Phospholipid-Dependent Protein Phosphorylation. The standard reaction mixture (final volume, 100 μl) contained 20 mM Tris·HCl (pH 7.4), 10 mM Mg acetate, 2 mM dithioerythritol, 0.01% leupeptin, Ca^{2+}/phospholipid-dependent protein kinase (0.04–0.08 μg of protein; specific activity, ≈500 nmol of P per min/mg), 0.13 μg of 87kDa, 1 mM EGTA, and, where indicated, 5 μg of rabbit brain calmodulin, 1.5 mM $CaCl_2$, and 5 μg of phosphatidylserine. The mixture was preincubated for 1 min at 30°C. The reaction was initiated by the addition of [γ-^{32}P]ATP (final concentration, 2 μM; specific activity, 50–100 × 10^3 cpm/pmol), carried out for 1 min, and terminated by the addition of 20 μl of $NaDodSO_4$ stop solution [20% glycerol/10% (wt/vol) $NaDodSO_4$/10% (wt/vol) 2-mercaptoethanol/0.25 M Tris·HCl, pH 6.7, containing a trace of bromophenol blue] and boiling for 5 min.

The boiled samples were electrophoresed on $NaDodSO_4$/8% polyacrylamide gels by the method of Laemmli (11). The gels were stained, destained, dried, and subjected to autoradiography as described (10). Silver staining of the enzyme

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Abbreviations: kDa, kilodalton; 87kDa, 87-kDa protein.

*Present address: Harvard Medical School, Boston, MA 02115.

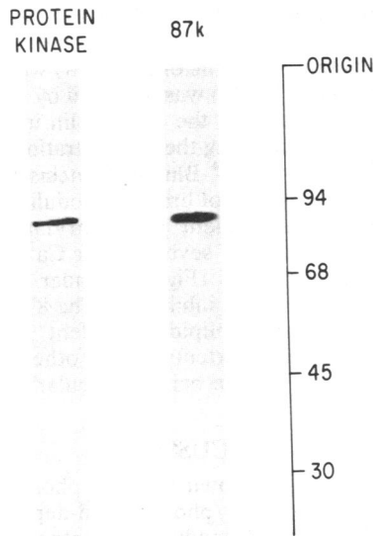

FIG. 1. Protein stain of the final purified preparations of protein kinase and 87kDa. The proteins were subjected to one-dimensional $NaDodSO_4$/8% polyacrylamide gel electrophoresis and silver-stained. Protein kinase, Ca^{2+}/phospholipid-dependent protein kinase; 87k, 87kDa. Size markers are shown in kDa.

(12) and of 87kDa (13) was performed as described. The phosphorylated substrate was located by the autoradiogram and excised, and the radioactivity was quantitated by liquid scintillation counting. All assay conditions were such that phosphorylation was linear with time, substrate concentration, and enzyme concentration. In all cases, a low enough ratio of enzyme to 87kDa was used so that enzyme autophosphorylation did not contribute to phosphorylation measured in the M_r 87,000 region of the gels. Two-dimensional peptide mapping was performed as described (14), except that thermolysin was used instead of trypsin/chymotrypsin to digest the protein. Protein determination was performed by the procedure of Peterson (15) with bovine serum albumin as standard.

RESULTS

Inhibition by Calmodulin of Phosphorylation of 87kDa. Calmodulin inhibited the Ca^{2+}/phospholipid-dependent phosphorylation of 87kDa in a calmodulin-depleted preparation of rat brain cytosol (Fig. 2). The results are similar to those observed with a calmodulin-depleted preparation of rat brain synaptosomal cytosol (8). This effect of calmodulin also could be demonstrated with purified preparations of the kinase and 87kDa (Fig. 3). With calmodulin at 5 μg/100 μl, an inhibition of ≈80% was observed (Fig. 3). Two-dimensional peptide mapping of 87kDa after thermolysin digestion indicated the presence of two major phosphorylation sites (Fig. 4*A*). The phosphorylation of these two sites was inhibited to about the same extent by the addition of calmodulin (Fig. 4*B*). The amino acid phosphorylated at each site was serine (data not shown).

Inhibition by Calmodulin of Phosphorylation of Various Substrates. The effect of calmodulin on the phosphorylation of 87kDa was compared with its effect on the phosphorylation of other substrates for Ca^{2+}/phospholipid-dependent protein kinase. For this series of experiments, the standard reaction mixture was used, except that 87kDa was replaced by other substrates. These substrates and their final concentrations included: H1 histone (0.2 μg), an effective substrate for Ca^{2+}/phospholipid-dependent protein kinase (16); synapsin I (0.1 μg), a synaptic vesicle-associated protein that can be phosphorylated at site II (1) by Ca^{2+}/phospholipid-

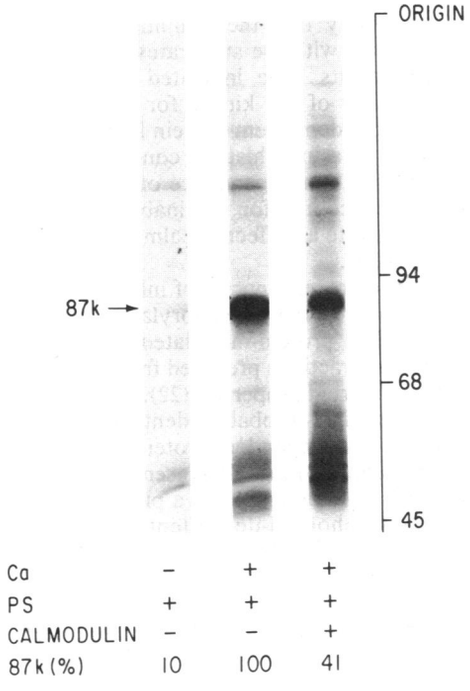

FIG. 2. Autoradiogram showing Ca^{2+}/phospholipid-dependent phosphorylation of 87 kDa and its inhibition by calmodulin in a calmodulin-depleted preparation of rat brain cytosol. The indicated additions were present in the following final concentrations: free Ca^{2+}, 0.5 mM; PS (phosphatidylserine), 5 μg/100 μl; rabbit brain calmodulin, 1 μg/100 μl. After termination of the phosphorylation reaction, samples were subjected to one-dimensional $NaDodSO_4$/6% polyacrylamide gel electrophoresis, followed by autoradiography. 87k, 87kDa; 87k (%), relative amounts of ^{32}P found in the 87kDa band for the various conditions used. Size markers are shown in kDa.

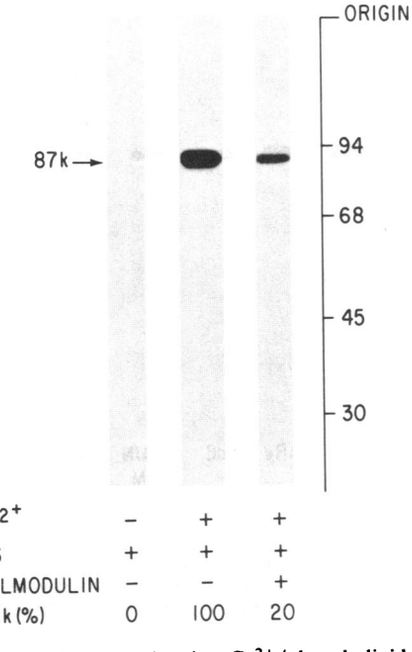

FIG. 3. Autoradiogram showing Ca^{2+}/phospholipid-dependent phosphorylation of 87kDa and its inhibition by calmodulin, with use of purified enzyme and substrate. The indicated additions were present in the following final concentrations: free Ca^{2+}, 0.5 mM; PS (phosphatidylserine), 5 μg/100 μl; rabbit brain calmodulin, 5 μg/100 μl. After termination of the phosphorylation reaction, samples were subjected to one-dimensional $NaDodSO_4$/8% polyacrylamide gel electrophoresis, followed by autoradiography. 87k, 87kDa; 87k (%), relative amounts of ^{32}P found in the 87kDa band for the various conditions used. Size markers are in kDa.

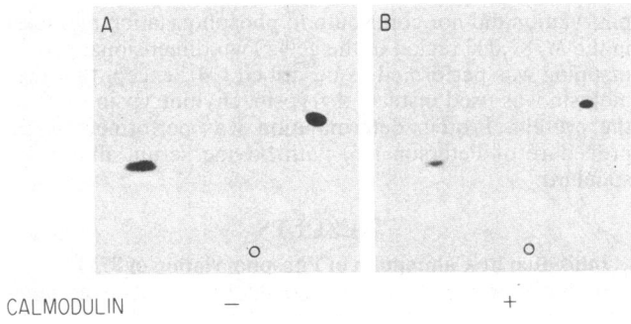

FIG. 4. Autoradiogram showing Ca^{2+}/phospholipid-dependent phosphorylation of the two major sites of 87kDa and inhibition of their phosphorylation by calmodulin, with use of purified enzyme and substrate. Phosphorylation was carried out in the presence of Ca^{2+} (0.5 mM free) and phosphatidylserine (5 μg/100 μl) and in the absence (*A*) or presence (*B*) of calmodulin (5 μg/100 μl). After termination of the phosphorylation reaction, samples were subjected to one-dimensional $NaDodSO_4$/8% polyacrylamide gel electrophoresis, followed by autoradiography. The bands of 87kDa, located by the autoradiogram, were excised and subjected to thermolytic digestion followed by separation in two dimensions on cellulose plates, first by electrophoresis in the horizontal direction (negative pole, left; positive pole, right) and then by ascending chromatography in the vertical dimension. O, origin.

dependent protein kinase (unpublished results and personal communication from Y. Takai, R. Minakuchi, and Y. Nishizuka); and the purified nicotinic acetylcholine receptor (4 μg), the δ subunit of which has recently been found to be phosphorylated by Ca^{2+}/phospholipid-dependent protein kinase (17). Calmodulin was found to inhibit the phosphorylation by Ca^{2+}/phospholipid-dependent protein kinase of all of these substrates, although the extent of inhibition varied depending on the concentration of calmodulin and the type of substrate used. As examples, with calmodulin at 5 μg/100 μl, phosphorylation of H1 histone (93 nM) was inhibited by ≈85% and of 87kDa (14 nM) was inhibited by ≈80%. Preliminary results indicated that the calmodulin inhibition could be surmounted by increasing the concentration of substrate.

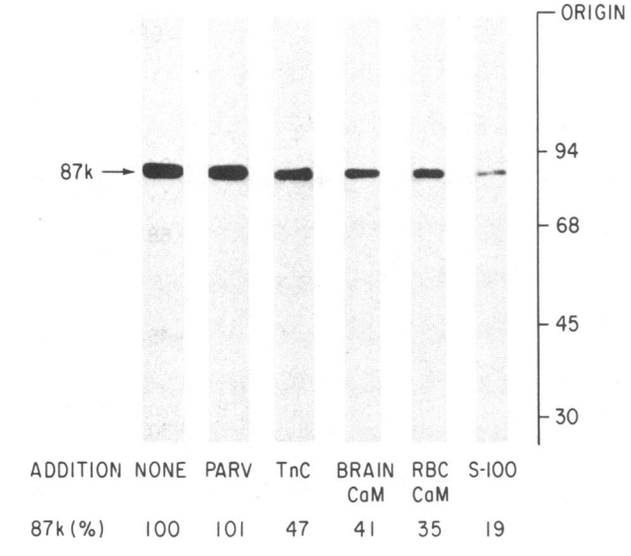

FIG. 5. Autoradiogram showing Ca^{2+}/phospholipid-dependent phosphorylation of 87kDa and its inhibition by several Ca^{2+}-binding proteins, with use of purified enzyme and substrate. Phosphorylation was carried out in the presence of Ca^{2+} (0.5 mM free), phosphatidylserine (5 μg/100 μl), and the indicated Ca^{2+}-binding protein (5 μg/100 μl). After termination of the phosphorylation reaction, samples were subjected to one-dimensional $NaDodSO_4$/8% polyacrylamide gel electrophoresis, followed by autoradiography. 87k, 87kDa; PARV, parvalbumin; TnC, troponin C; BRAIN CaM, rabbit brain calmodulin; RBC CaM, human erythrocyte calmodulin; S-100, S-100 protein; 87k (%), relative amounts of ^{32}P found in the 87kDa band for the various conditions used. No measurable phosphorylation was observed in the absence of Ca^{2+} plus phosphatidylserine. Size markers are in kDa.

Inhibition by Various Ca^{2+}-Binding Proteins of Phosphorylation of 87kDa. The ability of brain calmodulin to inhibit the Ca^{2+}/phospholipid-dependent phosphorylation of 87kDa was compared with that of several other Ca^{2+}-binding proteins, each at 5 μg/100 μl (Fig. 5). Under the conditions used, parvalbumin was not inhibitory. The 87kDa phosphorylation by Ca^{2+}/phospholipid-dependent protein kinase was inhibited to varying extents by the other Ca^{2+}-binding proteins, with S-100 protein being particularly potent as an inhibitor.

DISCUSSION

The present study has shown that the phosphorylation of several substrates for Ca^{2+}/phospholipid-dependent protein kinase is inhibited by calmodulin and other Ca^{2+}-binding proteins. Previous studies (8) and the present results (Fig. 2) with a crude cytosol system raised the question of whether the inhibition by calmodulin reflected a direct or indirect action on the kinase or substrate. The ability to demonstrate the effect with purified enzyme and substrates indicates that the action of calmodulin is direct. The fact that the inhibition occurred with all substrates examined suggests that calmodulin acts directly on the kinase rather than the substrates, especially since the substrates differ significantly in their properties; for example, H1 histone and synapsin I (10) are very basic proteins, whereas 87kDa is very acidic (unpublished data). In addition, except for a nonspecific interaction with H1 histone (18, 19), there is no evidence that the substrates interact with calmodulin. However, we cannot exclude the possibility that the inhibition by calmodulin is through interaction with the substrates.

Preliminary results have indicated that calmodulin decreases the affinity of the kinase for its substrates. Since Ca^{2+}/phospholipid-dependent protein kinase activity is generally measured at a high histone concentration (4, 5), the substrate concentration-dependence of the calmodulin inhibition probably accounts for the inability, in previous reports, to demonstrate an effect of calmodulin on the activity of this kinase (4, 5).

There have been recent reports of inhibition by S-100 protein and calmodulin of the phosphorylation of a 73-kDa protein, termed S-100 protein-modulated phosphoprotein, in crude supernatant fractions prepared from brain (20, 21). On the basis of its reported properties (22), S-100 protein-modulated phosphoprotein is probably identical to 87kDa.

Ca^{2+}/phospholipid-dependent protein kinase (4, 8), 87kDa (8), and calmodulin (23) are all present in neurons. Therefore, calmodulin may be acting as a physiological regulator of the Ca^{2+}/phospholipid-dependent phosphorylation of 87kDa in the nervous system. Preliminary results indicate that calmodulin inhibits Ca^{2+}/phospholipid-dependent phosphorylation in extracts of other tissues (data not shown). S-100 protein is found in glial cells (24), and troponin C is found in striated muscle. Whether these Ca^{2+}-binding proteins inhibit the Ca^{2+}/phospholipid-dependent phosphorylation of endogenous substrates in their respective tissues should be investigated.

Several recent reports have described other inhibitory effects of calmodulin. It inhibited the phosphorylation of low molecular weight brain membrane proteins (25), the cAMP-dependent protein kinase-catalyzed activation of phosphorylase kinase (26), and the polyamine-dependent protein kinase phosphorylation of ornithine decarboxylase (27). Calmodulin has also been suggested to play a role in the guanine

nucleotide-mediated inhibition of rat hippocampal adenylate cyclase (28), and to inhibit coupled NaCl transport in membrane vesicles from rabbit ileal brush border (29).

Ca^{2+}/phospholipid-dependent protein kinase has recently been shown to partially inhibit the actin-stimulated Mg-ATPase activity of smooth muscle heavy meromyosin (30), which results from the phosphorylation of heavy meromyosin by myosin light chain kinase (a Ca^{2+}/calmodulin-dependent protein kinase) (30). Those data, together with the results obtained in the present study, suggest that Ca^{2+}/calmodulin-dependent processes and Ca^{2+}/phospholipid-dependent processes may act as physiological modulators of each other.

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