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STIMULATION OF SYNAPTIC MEMBRANE PHOSPHORYLATION BY A CALCIUM AND CALMODULIN INDEPENDENT HEAT STABLE CYTOSOL FACTOR

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SUMMARY: Synaptosomal cytosol contains a heat-stable factor that stimulates the endogenous phosphorylation of synaptic plasma membranes in a calcium-independent fashion. This factor can be distinguished from the calcium binding protein, calmodulin, and appears to affect the incorporation of phosphate into a specific synaptic membrane protein.

The specific phosphorylated proteins found in neuronal membranes have been implicated as physiological effectors for the diverse actions of the second messenger candidates, cyclic AMP and calcium (1,2,3). In the presence of cAMP, the phosphorylation of several phosphate accepting proteins in synaptic plasma membranes is stimulated due to the activation of a membrane-bound cAMP-dependent protein kinase (4). These phosphorylated substrates include the regulatory subunit of cAMP-dependent protein kinase (4,5) and two neuron-specific proteins (6) with similar molecular properties, designated as proteins Ia and Ib (7). Calcium ion, perhaps through an activation of a specific calcium-dependent protein kinase (8), has also been found to regulate the phosphorylation of specific proteins found in preparations of synaptic membranes (9,10), synaptic vesicles (11), cortical slices (12) and synaptosomes (13). As with cAMP-dependent phosphorylation, proteins Ia and Ib are major substrates for calcium regulated phosphorylation (13). Thus, some of the effects of both cAMP and calcium on neuronal function may be mediated through the phosphorylation of these two neuron-specific proteins (3).

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Recently it was shown that calcium-dependent phosphorylation of neuronal membranes required the heat stable calcium-binding protein, calmodulin (14). Since the calmodulin-regulated protein phosphorylation of synaptic vesicle proteins have been linked to the release of neurotransmitter from isolated vesicles (16), this phosphorylation system may play an important role in synaptic function.

In addition to those described, other factors may be present in the cytosol compartment that are important in determining the steady or dynamic phosphorylation states of specific synaptic membrane proteins. In this report we present evidence for the existence of a heat-stable factor found in synaptosomal cytosol that stimulates the phosphorylation of a specific synaptic membrane protein in a calcium and calmodulin-independent fashion.

MATERIALS AND METHODS

Materials. The following chemicals obtained from commercial sources were used in this study: cAMP and DTT (Sigma), HEPES (Calbiochem), Tris (Schwarz/Mann). Sepharose was purchased from Pharmacia. Fluphenazine HCl was generously provided by Dr. S.J. Lucania, Squibb Institute of Medical Research, Princeton, N.J. The materials used in the preparation of the polyacrylamide gels and associated buffers were of the highest purity commercially available. [^{32}P] ATP (10-40 Ci/mmole) was purchased from New England Nuclear.

Tissue Preparation. The subjects were male Wistar rats (Taconic Farms, Germantown, N.Y.) weighing 150-200 grams. Synaptosomes were isolated from a crude P_2 fraction of whole brain (minus cerebellum) by flotation on a discontinuous ficoll gradient as described by Gurd, et al. (16). A synaptosomal lysate was prepared by exposure of the synaptosome fraction to hypotonic conditions at basic pH (17). The synaptosomal membranes were obtained from the lysate by centrifugation and were used to prepare a synaptic plasma membrane fraction by discontinuous sucrose gradient centrifugation as described (18). The synaptosomal cytosol was heated to 90-100°C for 10 minutes and then centrifuged at 100,000 x g for 30 minutes. The pellet was discarded and the supernatant, hereafter referred to as heated cytosol, was used as a source of calmodulin or was used directly in the phosphorylation assay without further purification. Proteins were assayed according to the method of Bradford (19) using bovine plasma gamma globulin as a standard.

Affinity Chromatography. The calcium-dependent binding protein, calmodulin, was purified from the heated synaptosomal cytosol by affinity chromatography using fluphenazine as the attached ligand. The adsorption of calmodulin to fluphenazine linked to sepharose by means of bifunctional oxiranes has been described (20). Calmodulin obtained in this manner appeared as a single band in SDS polyacrylamide gels and comigrated with calmodulin prepared according to the method of Klee (21). Fractions eluted from the fluphenazine-linked sepharose column were assayed for the presence of calmodulin according to the method of Schulman and Greengard (14).

Phosphorylation of Synaptic Plasma Membranes. The phosphorylation of the synaptic plasma membrane fraction (100 µg protein) was assayed at 30°C in a mixture containing (200 µl final volume) 50 mM HEPES, pH 7.0, 10 mM $MgCl_2$, 1.0 mM DTT, 5 µM [γ ^{32}P] ATP (1×10^7 dpm/nmole) in the absence or

presence of 300 μM $CaCl_2$. Where indicated, other drugs or tissue components were included in the assay as follows: cAMP, 10 μM; fluphenazine HCl, 50 μM; heated synaptosomal cytosol, 30 μg; void volume proteins, 30 μg; calmodulin, 0.50 μg. Following preincubation, the phosphorylation reaction was initiated with the addition of membrane. After 15 seconds the reaction was terminated and membrane proteins were solubilized by the addition of 100 μl of an electrophoresis sample buffer (22).

Gel Electrophoresis and Autoradiography. Synaptic membrane proteins were resolved on SDS polyacrylamide slab gels (6% and 10% in the stacking and resolving gels, respectively) according to the method of O'Farrell(22). For autoradiography the dried gels were placed in close contact with Kodak RP X-ray film for a period of 1-2 days.

RESULTS

During the course of experiments designed to assess the effects of various psychoactive drugs on the calcium-calmodulin-dependent phosphorylation system in brain membranes, it appeared to us that all of the effects of heated synaptosomal cytosol on membrane phosphorylation were not mediated by calmodulin. This observation was prompted by the use of fluphenazine to antagonize the actions of calmodulin on the phosphorylation of neuronal membranes and led us to perform the present experiment.

Figure 1 shows the autoradiograph of synaptic plasma membranes that were resolved on SDS polyacrylamide slab gels following phosphorylation under various conditions. These data were replicated seven times. When synaptic plasma membranes were incubated in the presence of γ^{32}P ATP, several proteins were phosphorylated (Fig. 1, lane 1). As reported previously (14), the addition of calcium in the absence of calmodulin does not alter the extent or pattern of protein phosphorylation (Fig. 1, lane 2). Cyclic AMP in the absence or presence of calcium stimulated the phosphorylation of two proteins that corresponded to the molecular weights of the previously described (7) neuron-specific proteins, Ia and Ib (Fig. 1, lanes 3 and 4). The addition of heated synaptosomal cytosol, which exhibits no endogenous phosphorylation (data not shown), to the assay mixture resulted in a markedly enhanced phosphorylation of several proteins, including proteins Ia and Ib (Fig. 1, lanes 5 and 6). Calcium was required for the effects of heated cytosol on the phosphorylation of all proteins with the exception of a protein with an electro

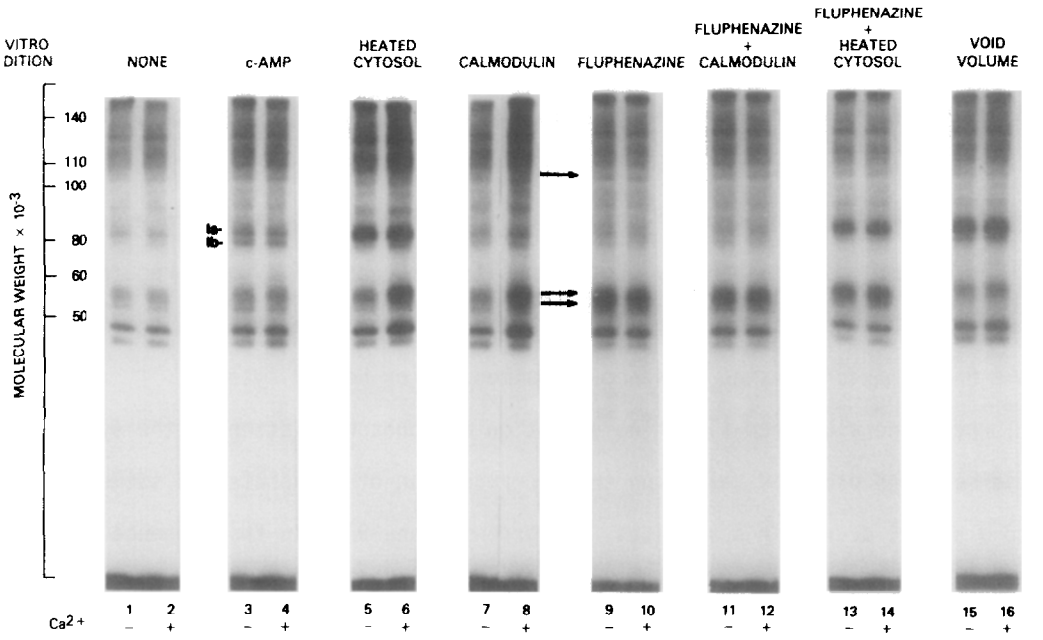

Fig. 1. Autoradiographs showing the phosphorylation of synaptic membrane proteins in the presence and absence of calcium plus the addition of the agents listed above each pair of autoradiographs. The standard incubation conditions and the amount or concentration of the added drug or tissue component are described under "MATERIALS AND METHODS." Void volume refers to the proteins obtained from the effluent of the fluphenazine-sepharose affinity column.

phoretic mobility equal to that of protein 1a (Fig. 1, lanes 5 and 6). This protein has an apparent molecular weight (M_r) of 85,000 and will hereafter be referred to as the 85 K band. That the incorporation of phosphate into the 85 K band was independent of calcium was confirmed by additional experiments where the calcium chelator, EGTA, was included in the assay mixture as described (14). Schulman and Greengard (14) have recently reported results similar to these following the addition of heated cytosol, however, in their studies all protein phosphorylation was calcium dependent (15). Indeed, it has been suggested that all of the effects of heated cytosol on synaptic membrane phosphorylation are mediated by calcium through its specific binding protein, calmodulin (3, 14). When calmodulin was added to the phosphorylation assay it resulted in the expected calcium-dependent phosphorylation of several membrane proteins (Fig. 1, lanes 7 and 8). The pattern of the calcium-cal-

modulin-regulated phosphorylation duplicated the pattern seen in the presence of heated cytosol with the exception that the phosphorylation of the 85 K band was only marginally enhanced (Fig. 1, lanes 7 and 8). Recently, it has been proposed that the biochemical and pharmacological actions of antipsychotics are mediated through an inhibition of calmodulin regulated processes (23). In an attempt to examine only the calmodulin component of cytosol-regulated membrane phosphorylation, the antipsychotic, fluphenazine, was included in the assay mixture in the presence of calmodulin or heated cytosol. Fluphenazine alone had almost no effect on the phosphorylation of the synaptic membrane proteins, although the incorporation of phosphate was stimulated to a slight degree in some cases (see arrows, lane 9). In the presence of fluphenazine, calmodulin did not affect the incorporation of phosphate into any protein with or without the addition of calcium to the assay mixture (Fig 1, lanes 11 and 12). However, in the presence of fluphenazine, the addition of heated cytosol restored, at least partially, the phosphorylation of the 85 K band in a calcium-independent manner, (Fig. 1, lanes 13 and 14). When the void volume proteins obtained following chromatography on fluphenazine-linked sepharose were included in the phosphorylation assay, the phosphorylation of the 85 K band was stimulated in a calcium-independent manner whereas the incorporation of phosphate into most other proteins remained unchanged or elevated to only a slight degree (Fig. 1, lanes 15 and 16). These results indicated that most calmodulin had been removed from the heated cytosol following affinity chromatography whereas the factor affecting the phosphorylation of the 85 K band eluted in the void volume. Coomassie blue staining of the gels also confirmed that calmodulin had been removed following affinity chromatography on fluphenazine-sepharose (data not shown).

DISCUSSION

The results presented in this report demonstrate the existence of a heat-stable factor in synaptosomal cytosol that stimulates the incorporation of phosphate into a specific synaptic membrane protein. The effects of this

factor on phosphorylation do not require calcium and can be distinguished from the calmodulin-regulated phosphorylation system that has previously been described (14). It appears that the phosphorylation of only one protein band is affected by the unknown cytosol factor. This membrane protein has an apparent molecular weight (M_r) of 85,000 and comigrates in SDS gel with one of the substrates of cAMP-dependent protein kinase, protein Ia (7). Since the phosphorylation of several specific synaptic membrane proteins are regulated in a cAMP- and/or calcium-dependent manner, our results indicate that yet another factor may be responsible for the regulation of the phosphorylation of a specific synaptic membrane protein.

Although we have postulated the existence of a heat stable cytosol factor in addition to calmodulin to explain the observed effects on membrane phosphorylation, other possibilities exist that may account for the data obtained. For example, a substrate present in heated synaptosomal cytosol (protein Ia or a protein with an apparent molecular weight identical to protein Ia) may be phosphorylated by a calcium independent protein kinase present in the membrane fraction. However, this line of reasoning can be discounted based on the following lines of evidence. (1) On the basis of coomassie blue staining no protein in heated synaptosomal cytosol comigrated in SDS gels with the phosphorylated 85 K band. (2) In the presence of magnesium and ATP, we have observed that freshly prepared synaptosomal cytosol will incorporate phosphate into several proteins including a protein corresponding to the 85 K band. However, heat treatment of the phosphorylated synaptosomal cytosol followed by centrifugation results in the removal of all phosphorylated proteins from this preparation. (3) Following the phosphorylation of synaptic membrane proteins in the presence of heated cytosol the phosphorylated membrane proteins can be recovered by centrifugation and the presence of any phosphoprotein remaining in the supernatant can be monitored by electrophoresis and autoradiography. When this experiment was performed the results revealed the presence of a number of phosphoproteins in the supernatant, including one

that comigrated in SDS gels with the 85 K band. However, it is doubtful that these soluble phosphoproteins were derived from heated cytosol since heat treatment followed by centrifugation successfully removed them from solution. More importantly, evidence that the phosphorylated 85 K band was derived from synaptic membranes was obtained by resuspending the phosphorylated membranes in fresh assay buffer containing fresh heated cytosol. The phosphorylation assay was then repeated using the standard assay conditions. The results of this experiment revealed that the incorporation of phosphate into the 85 K band was no longer stimulated in the presence of heated cytosol, although the incorporation of phosphate into proteins that show enhanced phosphorylation in the presence of calmodulin remained unchanged. Thus it appears that the incubation of synaptic membranes under phosphorylating conditions results in the release of proteins (including the phosphorylated 85 K band) into the supernatant.

Cyclic AMP was considered as a possible candidate for the identity of the synaptosomal factor that stimulated the phosphorylation of the 85 K band. However, cyclic AMP is an unlikely candidate since its presence in heated cytosol would be expected to activate the membrane bound cAMP-dependent protein kinase in a manner that would stimulate the phosphorylation not only of the 85 K band (that corresponds to the electrophoretic mobility of protein Ia) but also protein Ib, another specific substrate of this particular membrane kinase (7). The inclusion in the phosphorylation assay of aliquots of the void volume fraction obtained from affinity chromatography of calmodulin enhanced the incorporation of phosphate into the 85 K band. Since the void volume fraction had been extensively dialyzed, this indicates that macromolecular nature of the unknown cytosol factor and provides further evidence against an involvement of cyclic AMP in the enhanced phosphorylation of the 85 K band. The calcium-and calmodulin-independent characteristics of the cytosol-regulated membrane phosphorylation may be due to the artifactual production of a heat-stable factor as a result of the heat step that was

employed in this investigation. To rule out this possibility and to explore the chemical and biochemical characteristics of this unknown modulator of membrane protein kinase activity, we are attempting to purify this factor from both untreated and heat-treated synaptosomal cytosol.

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