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THE MECHANISM OF ACTIVATION OF PROTEIN KINASE FA (THE ACTIVATOR OF TYPE-1 PROTEIN PHOSPHATASE) IN BRAIN SYNAPTOSOMES

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The ATP.Mg-dependent type-1 protein phosphatase and its activating factor (protein kinase FA) were identified to exist in brain synaptosome. The inactive protein phosphatase was found to exist in the synaptosomal cytosol whereas its activating factor (protein kinase FA) was present in the synaptosomal membrane, indicating that the inactive protein phosphatase and its activating factor FA are localized in two separate subcellular compartments. The membrane-bound FA was found to exist in two forms; approximately 75% of FA is inactive and trypsin-resistant, whereas 25% of FA is active and trypsin-labile. When membranes were incubated with exogenous phospholipase C, the inactive/trypsin-resistant FA could be activated and sequestered to become the active/trypsin-labile FA in a time-and dose-dependent manner. Taken together, the results provide initial evidence that the activation-sequestration of membrane-bound protein kinase FA may represent one mode of control modulating the activity of protein kinase FA and thereby to activate protein phosphatase in brain synaptosome, representing an efficient regulatory mechanism for regulating neurotransmission in the central nervous system.

An ATP.Mg-dependent multisubstrate/multifunctional type-1 protein phosphatase has been identified in mammalian nervous and non-nervous tissues (1, 2). The phosphatase exists in an inactive form which can be activated in the presence of ATP.Mg and an activating factor termed FA (1-4). FA has further been identified as a cyclic AMP and Ca^{2+}-independent multisubstrate/multifunctional protein kinase (3-15), representing the first example that a protein kinase can activate a protein phosphatase. By its dual role as a multisubstrate/multifunctional protein phosphatase activating factor and as a multisubstrate/multifunctional protein kinase, FA may simultaneously modulate phosphorylation and dephosphorylation states of many key regulatory proteins and enzymes involved in the regulation of diverse cell functions (3-18). Due

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Abbreviations : FA, the activator of type-1 protein phosphatase; MBP, myelin basic protein.

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to its unique features, there is growing interest in protein kinase FA as a regulatory enzyme, however, there is little information as to how this protein kinase is actually regulated in the cell.

In this report, using brain synaptosome as an excellent model, we found that the inactive protein phosphatase and its activator (protein kinase FA) were localized in two separate cell compartments; the inactive protein phosphatase was found to exist in the synaptosomal cytosol whereas its activator (kinase FA) was present in an inactive form in the synaptosomal membrane. The inactive membrane-bound kinase FA could be activated and sequestered by phospholipase C. This is the first report providing initial evidence that an activation-sequestration system may well be involved in the regulation of protein kinase FA activity in the synaptosomal membrane and thereby to mediate the activation of protein phosphatase in the cytosol of brain synaptosome, representing an efficient control mechanism for regulating neurotransmission in the central nervous system.

MATERIAL AND METHODS

Materials --- [γ-^{32}P] ATP was purchased from ICN. ATP, cyclic AMP, trypsin, polylysine, histone, phosvitin, bovine serum albumin, protamine, D-glucose and HEPES were obtained from Sigma. Ascorbic acid, dithiothreitol, 2-mercaptoethanol and trichloroacetic acid were from E. Merck; DEAE-Sephadex A-50, Sepharose 4B, Sephacryl S-200, CNBr-activated Sepharose 4B and Ficoll were from Pharmacia. DEAE-cellulose, CM-cellulose and phosphocellulose P-11 were from Whatman. Phosvitin-Sepharose 4B and histone-Sepharose 4B were prepared essentially as previously described (1).

Protein purification --- phosphorylase b (19) and phosphorylase b kinase (20) were purified to homogeneity from rabbit skeletal muscle. The catalytic subunit of cAMP-dependent protein kinase (21) was isolated from pig heart. The ATP.Mg-dependent inactive type-1 protein phosphatase (2) and its activating factor protein kinase FA (9) and encephalitogenic myelin basic protein (MBP) (9) were purified to near homogeneity from pig brain.

Preparation of ^{32}P-labeled protein substrates --- ^{32}P-phosphorylase a was prepared from phosphorylase b, phosphorylase b kinase and [γ-^{32}P] ATP basically as described in Ref. 22. ^{32}P-MBP was prepared from purified MBP, the catalytic subunit of cAMP-dependent protein kinase and [γ-^{32}P] ATP.

Preparation of brain synaptosomes --- 10 grams of brain cortex were homogenized in 60 ml of ice cold 0.32 M sucrose, 5 mM Tris/HCl at pH 7.4 (buffer A) using Teflon pestle and glass homogenizer at 900 rpm. The homogenate was centrifuged at 1,000 x g for 10 min and the resulting supernatant was then centrifuged at 12,000 x g for 20 min. The pellet was washed two times using buffer A and then dissolved in a final Ficoll concentration of about 14% in buffer A. A discontinuous density gradient was formed by overlaying 15 ml of this suspension, first with 10 ml of 7.5% Ficoll in buffer A and then with 5 ml of buffer A alone. This gradient was then centrifuged at 90,000 x g for 2 h. in a Beckman SW 25.1 swinging bucket rotor. The material at the 7.5% to 14% Ficoll interface was harvested and further purified with the second discontinuous Ficoll-sucrose density gradient as described above. The purified synaptosomes which were located at 7.5% to 14% Ficoll interface were collected and used as the source for brain synaptosomes in the present study. The procedure for preparation of brain synaptomes was basically as described in a previous report (23). The synaptosome preparation was finally suspended at a concentration of 1-2 mg/ml in buffer containing 10 mM HEPES at pH 7.4, 140 mM

NaCl, 2 mM KCl, 1 mM $MgSO_4$, 5 mM D-glucose and 0.5 mM ascorbic acid. The synaptosomal membrane and cytosol were separated by using osmotic shock of synaptosomes in the presence of 20 volumes of 5 mM Tris buffer at pH 7.0 basically as described in ref. 24.

Enzyme assays --- The activities of ATP.Mg-dependent protein phosphatase and its activator kinase FA were determined by methods described in previous reports (1, 2, 9). Briefly, the activity of ATP.Mg-dependent protein phosphatase was typically measured after a 10-min preincubation with 0.1 mM ATP, 0.5 mM Mg^{2+} ions and saturated amount of FA required for full activation of the inactive phosphatase. 1 mg/ml ^{32}P -phosphorylase a or ^{32}P-MBP was used as the substrate. The activity of kinase FA in brain synaptosomes was measured by the formation of the activated ATP.Mg-dependent protein phosphatase. The assay mixture contained appropriate dilutions of kinase FA, 0.1 mM ATP, 0.5 mM Mg^{2+} ions and excess amount of inactive phosphatase to ensure linear activation of the phosphatase activity. A unit of protein phosphatase activity is that amount of enzyme that catalyzes the release of 1 nmol of phosphate per min. A unit of FA is that amount of enzyme that produces one unit of activated ATP.Mg-dependent protein phosphatase in 1-min preincubation. For determination of the activity of the inactive/trypsin-resistant kinase FA in the synaptosomal membrane, the membrane was first pretreated with 0.02 mg/ml trypsin at 30°C for 3 min, followed by the addition of 0.2 mg/ml trypsin inhibitor to terminate the action of trypsin. The trypsinized synaptosome membrane was next solubilized in 1% Triton X-100 prior to the assay for FA activity.

Analytic Method --- Protein concentration was determined by the method of Lowry et al. (25) using bovine serum albumin as the standard.

RESULTS AND DISCUSSION

As shown in Fig. 1, the cytosolic fractions isolated from brain synaptosome contained some basal protein phosphatase activity. Addition of 0.1 mM ATP, 0.5 mM Mg^{2+} ions and exogenous inactive ATP.Mg-dependent protein phosphatase could not slightly stimulate the protein phosphatase activity, indicating that protein kinase FA, the activator of ATP.Mg-dependent protein phosphatase (1-4) does not exist in the cytosol of brain synaptosome. In sharp contrast, addition of ATP.Mg and exogenous protein kinase FA could dramatically stimulate the protein phosphatase activity (see Fig. 1A), indicating that a substantial amount of inactive ATP.Mg-dependent protein phosphatase exists in the cytosol of brain synaptosome (1-4). In similarity with the synaptosomal cytosol, the synaptosomal membrane also contained some basal protein phosphatase activity. However, in sharp contrast to the synaptosomal cytosol, addition of ATP.Mg and exogenous protein kinase FA could not slightly stimulate the protein phosphatase activity in the synaptosomal membrane, indicating that the inactive ATP.Mg-dependent protein phosphatase does not exist in the membrane fractions of brain synaptosome (see Fig. 1B). By contrast, addition of ATP.Mg and inactive protein phosphatase could dramatically stimulate the protein phosphatase activity in the membrane (see Fig. 1B), demonstrating that a substantial amount of protein kinase FA exists in the membrane fractions of brain synaptosome (1-4). The results point out that the inactive

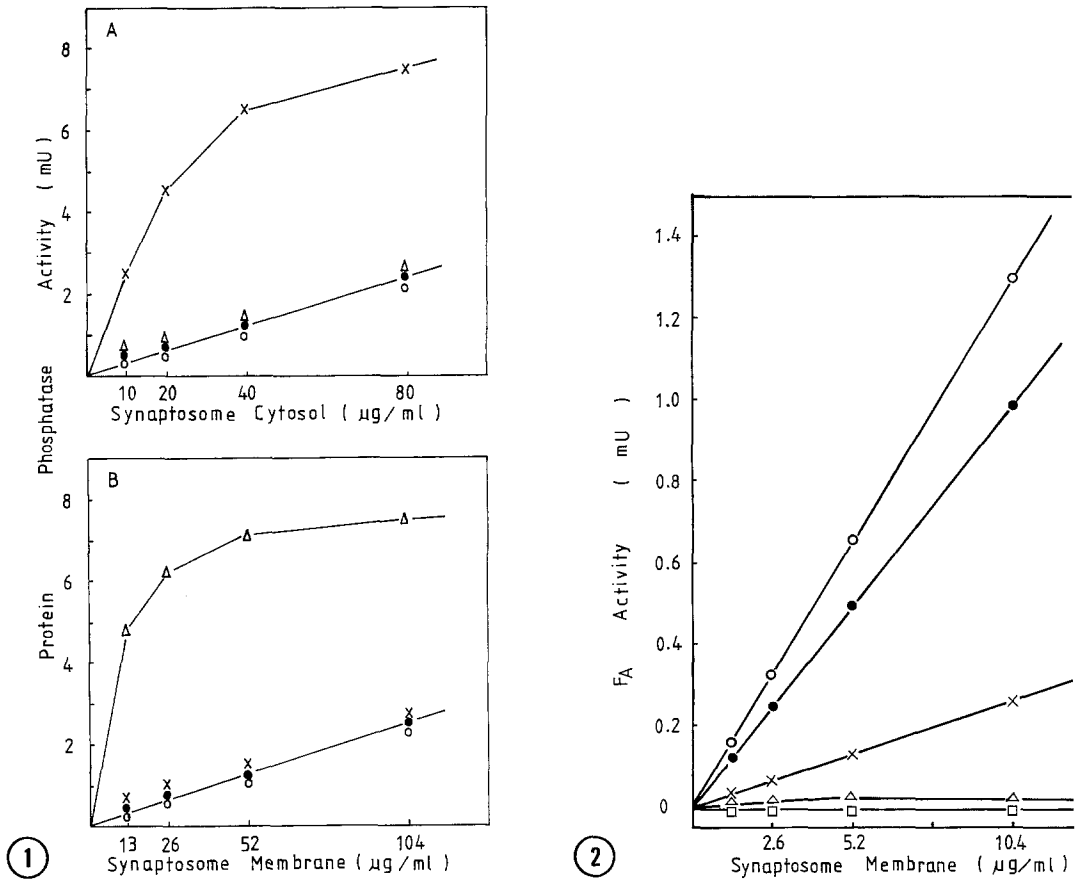

Fig. 1. Identification of ATP.Mg-dependent protein phosphatase and its activator (kinase FA) in brain synaptosome.

A. The cytosolic fraction of purified brain synaptosome at various concentrations as indicated was assayed for the protein phosphatase activity under the following conditions : (o) with buffer B containing 20 mM Tris-HCl at pH 7.0 and 0.5 mM dithiothreitol, (•) with 0.1 mM ATP and 0.5 mM Mg^{2+} ions in buffer B, (x) with 0.1 mM ATP, 0.5 mM Mg^{2+} ions and 20 mU exogenous kinase FA in buffer B, and (Δ) with 0.1 mM ATP, 0.5 mM Mg^{2+} ions and 20 mU inactive ATP.Mg-dependent protein phosphatase in buffer B. 1 mg/ml ^{32}P-MBP was used as the substrate. Assay conditions were as described in " Methods ".

B. The same as in A except that the membrane fractions of brain synaptosome were used.

Fig. 2. Identification of active/trypsin-labile FA and inactive/trypsin-resistant FA in the brain synaptosomal membrane.

The brain synaptosomal membrane at various concentrations as indicated was assayed for the FA activity under the following conditions : (x) without any treatment, (Δ) with trypsin treatment, (•) first treated with trypsin and then solubilized 1% Triton X-100, (o) solubilized in 1% Triton X-100 only and (□) first solubilized in 1% Triton X-100 and then treated with trypsin. Trypsin treatment was performed using 0.02 mg/ml trypsin at 30°C for 3 min followed by adding 0.2 mg/ml trypsin inhibitor to terminate the action of trypsin. 1 mg/ml ^{32}P-MBP was used as the substrate. FA was assayed as the activating factor of ATP.Mg-dependent protein phosphatase. Assay conditions were as described in " Methods ".

ATP.Mg-dependent protein phosphatase and its activator FA exist in two separate cell compartments of brain synaptosome, suggesting that the membrane-bound FA should be regulated in order to activate protein phosphatase in the cytosol of brain synaptosome.

As shown in Fig. 2, when the synaptosomal membrane was first solubilized in 1% Triton X-100 prior to the assay, the FA activity was further stimulated several-fold, indicating that two FAs exist in the synaptosomal membrane; one form of FA is spontaneously active in situ, whereas the other form of FA is inactive but can be activated by Triton X-100. Quantitative analysis further indicated that approximately 75% of FA is inactive, whereas 25% of FA is active in the synaptosomal membrane (Fig. 2). It is important to note that Triton X-100 has no direct effect on FA activity when the enzyme is in a free solubilized state.Moreover, when the synaptosomal membrane was first treated with 0.02 mg/ml trypsin at 30°C for 3 min prior to the assay, the active FA was destroyed, whereas the inactive FA remained unaffected under the same condition (see Fig. 2). However, when the membrane was first solubilized in 1% Triton X-100 and then treated with trypsin prior to the assay, both active and inactive FA activities in the synaptosomal membrane were completely destroyed by trypsin (Fig. 2). All the results taken together demonstrate that two forms of FA exist in the synaptosomal membrane; one form of FA is active and trypsin-labile whereas the other form of FA is inactive and trypsin-resistant. It is possible that this is due to differences in membrane topography with active/trypsin-labile FA exposed on the outer face of the membrane and inactive/trypsin-resistant FA buried within the membrane. The results further suggest that activation and sequestration of inactive membrane-bound FA may represent one mode of control modulating the activity of protein kinase FA in brain synaptosome. To demonstrate this point, the synaptosomal membrane was next treated with exogenous phospholipase C. As shown in Figs. 3A and 3B, incubation of catalytic amounts of exogenous phospholipase C with synaptosomal membranes resulted in an activation of inactive kinase FA in a time- and dose-dependent manner. Furthermore, when kinase FA was first solubilized from the membrane by 1% Triton X-100, phospholipase C could not bring out any effect on its activity. Moreover, after the treatment with phospholipase C, all the FA activities associated with the synaptosomal membrane were found to become trypsin-labile (data not shown). Taken together, the results support the notion that activation and sequestration of the inactive/trypsin-resistant FA to the active/trypsin-labile FA may represent one mode of control mechanism modulating the activity of kinase FA in brain synaptosome. The results also provide initial evidence that interconversion between inactive FA and active FA in the synaptosomal membrane may represent one of the control mechanisms to regulate the activation of type-1 protein phosphatase. For instance, the inactive membrane-bound FA can be activated and sequestered into the cytosolic face of the synaptosomal

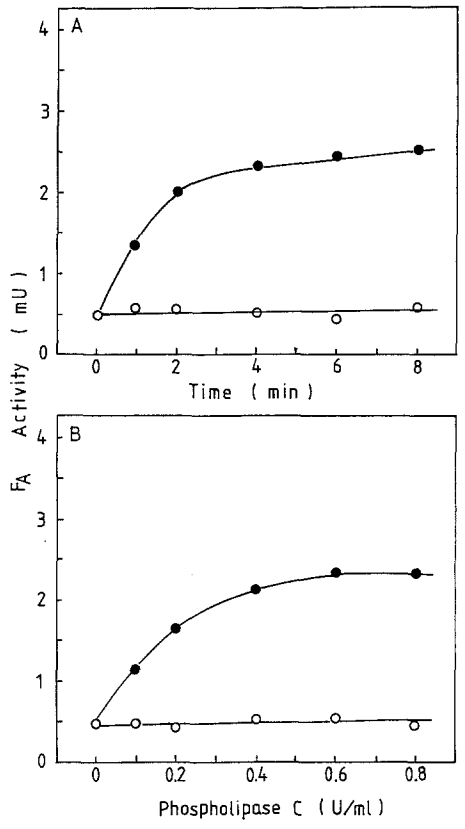

Fig. 3. Time course and concentration dependence of phospholipase C-mediated activation of inactive FA in the brain synaptosomal membrane.

A. The brain synaptosomal membrane was incubated with 0.4 U/ml of phospholipase C at 30°C for various time points as indicated (•). (o) is the control without adding phospholipase C. At each time point, the reaction mixture was diluted 20-fold and the FA activity was determined as described in " Methods ", 1 mg/ml ^{32}P-MBP was used as the substrate and FA was assayed as the activating factor of ATP.Mg-dependent protein phosphatase.

B. The same as in A except that various concentrations of phospholipase C as indicated were used and the reaction time was 3 min.

membrane and thereby promotes the activation of type-1 protein phosphatase in the cytosol of brain synaptosome.

Protein phosphorylation-dephosphorylation is now recognized as an important control mechanism involved in the regulation of diverse cell functions and appears to be a general mechanism by which many hormones, neurotransmitters, and other extracellular signals produce their physiological response in specific target cells (16, 17, 26). It is generally believed that brain synaptosome is far more metabolically active than was previously conceived with a turnover rate of phosphorylation-dephosphorylation in the order of seconds to minutes in vivo (27, 28). The activities of protein phosphatases and kinases endogenous to brain synaptosome are presumably responsible for this rapid turnover. By its dual role as a multisubstrate protein

kinase and as a multisubstrate protein phosphatase activating factor (1-15), FA may simultaneously modulate the phosphorylation and dephosphorylation states of brain synaptosome, which may play an important role in the regulation of neurotransmission (27-30). The activation-sequestration mechanism for modulating the activity of protein kinase FA in brain synaptosome as presented in this study provides an efficient control mechanism highly suited for the regulation of neurotransmission in the central nervous system. On the other hand, although FA has long been recognized as a cytosolic protein kinase (1-10), the results as shown in the present study that FA is an endogenous synaptosome kinase predominantly associated with the synaptosomal membrane provide evidence to support the notion (9, 11-13) that FA is in fact a specific membrane-associated protein kinase, and activation-sequestration of FA in the membrane represents one mode of control mechanism modulating its activity in the cell.

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