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Activation of Signal Transduction Pathways Changes Protein Phosphorylation Patterns in the Rat Hypothalamus

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Although alteration in protein phosphorylation by specific protein kinases is of importance in transducing cellular signals in a variety of neural/endocrine systems, little is known about protein phosphorylation in the hypothalamus. The present study aims to explore whether activation of the second messenger-dependent protein kinases affects phosphorylation of specific proteins using a cell free phosphorylation system followed by SDS-polyacrylamide gel electrophoresis. Cytoplasmic fractions derived from hypothalami of immature rats were used as substrates and several activators and/or inhibitors of cAMP-, phosphatidylinositol- and Ca^{2+}-calmodulin-dependent protein kinases were assessed. Many endogenous proteins were extensively phosphorylated and depending on the signal transduction pathways, phosphorylation profiles were markedly different. The present data indicate that extracellular signals may affect cellular events through protein phosphorylation by second messengers-protein kinases in the rat hypothalamus.

KEY WORDS: Protein phosphorylation, Signal transduction, Hypothalamus, cAMP, Ca^{2+}, Calmodulin, Phosphatidylinositol

Increasing evidence indicates that protein phosphorylation is of fundamental importance in the signal transduction pathways in a variety of neuronal/endocrine systems (Nestler and Greengard, 1983; Nestler *et al.*, 1984; Narin *et al.*, 1985b). The effect of numerous extracellular signals have been known to be mediated through activation of specific second messengers. The action of second messengers is achieved through activation of protein kinases (PKs) which in turn regulate the state of phosphorylation of specific proteins. Indeed, it is evident that the brain contains many PKs, such as cAMP-dependent PK (PKA), phosphatidylinositol (PI)-dependent PK (PKC) and Ca^{2+}-calmodulin (CaM)-dependent PKs (Greengard, 1979; Berridge and Irvine, 1984; Nishizuka, 1984; Narin *et al.*, 1985a). As far as the hypothalamus is concerned, we are not aware of evidence indicating changes in protein phosphorylation patterns in response to activation of specific signal transduction pathways.

It has been known that several intracellular messengers, Ca^{2+}, cAMP and PI cascades are involved in the regulation of neurohormone release from the hypothalamus, for instance, gonadotropin releasing hormone (GnRH) release (Drova *et al.*, 1981, 1984; Ramirez *et al.*, 1985; Kim *et al.*, 1986; Negro-Vilar *et al.*, 1986; Ojeda *et al.*, 1986). Recently, we found that activation of two intracellular pathways such as cAMP-dependent PKA and PI turnover-diacylglycerol-dependent PKC increases GnRH gene expression (Lee *et al.*, 1990). It appears then that protein phosphorylation is a critical step between activation of second messengers-PK systems and cellular events, such as secretion and gene

expression. Therefore, the present study aims to clarify whether activation of specific cellular pathways may trigger specific changes in protein phosphorylation in the rat hypothalamus.

Materials and Methods

Animals and tissue preparation

Immature female Sprague-Dawley rats (weighing 80-90 g at 28 days of age, Seoul National University Animal Breeding Center) were kept in temperature-controlled environment of a 14-h light and 10-h dark photocycle (light on at 06.00 h) with food and water supplied *ad libitum*. After decapitation, the brains were rapidly removed, and the entire hypothalamic tissues including the mediobasal hypothalamic-anterior hypothalamic-preoptic area (MBH-AHA-POA) were immediately dissected. The boundaries of the MBH-AHA-POA were posteriorly the anterior border of the mammillary bodies, rostrally 2 mm anterior to the optic chiasm, laterally the lateral hypothalamic sulci, and transversely at a depth of 2-3 mm as previously defined (Kim and Ramirez, 1986; Kim *et al.*, 1989). The hypothalamic tissues (more than 5 for each experimental group) were immediately used to obtain cytoplasmic fractions.

Preparation of cytoplasmic fractions

The hypothalamic tissues were scrutinizingly washed for removing blood cell contaminants, then homogenized in 10 volume of homogenization buffer containing 0.3 M sucrose, 0.1% soybean trypsin inhibitor (Sigma) and 1 mM phenylmethylsulfonylfluoride (PMSF, Sigma) with glass homogenizer (Kinder *et al.*, 1987). Homogenates were centrifuged at 1,000 x g for 10 min to remove cellular debris. The supernatant was ultracentrifuged at 100,000 x g at 4°C for 60 min in Beckman SW 50.1 rotor to obtain the cytoplasmic soluble fractions. These cytoplasmic fractions were aliquoted to 500 μl in microcentrifuge tube and stored in −70°C until used. Protein concentration was measured by Bradford method using BSA (Sigma) for standard reference (Bradford, 1976).

Protein phosphorylation by a cell free system

The phosphorylation reaction mixture was 40 μl buffer containing 20 mM Tris-maleate (pH 7.0), 10 mM $MgCl_2$, 100 μM dithiothreitol (DTT), and 3.0 mM EGTA as previously described (Kinder *et al.*, 1987). For the activation of specific PKs, cAMP (Sigma), Ca^{2+} (Sigma), phosphatidyl serine (PS, Sigma) and calmodulin (CaM, Boerhinger Manheim Biochem.) were added to the reaction mixture. Several PK inhibitors, such as cAMP-dependent PK inhibitor (PKAI, Sigma) (Cheng *et al.*, 1986), galactosyl-sphingosine (psychosine, Sigma) (Hannun and bell, 1987) and trifluoperazine (TFP, Sigma) (Weiss and Wallace, 1980) were used to inhibit cAMP-, PI- and Ca^{2+}-CaM-dependent PKs, respectively. Before adding specific inhibitors, the phosphorylation reaction mixture was preincubated with cAMP (5 μM), Ca^{2+} (200 μM), PS (20 μl) and CaM (100 ng). Hypothalamic cytoplasmic fractions (20 μg) were added to reaction buffer at 4°C, and preincubated with kinase activators and/or inhibitors at 37°C for 30 sec. The reaction was initiated by adding 5 μl ATP (gamma-^{32}P-ATP; 10 Ci/mmole, 160 μM; New England Nuclear) to give final concentration of 20 μM. Reaction mixtures were agitated at 37 °C for 60 sec, and the reactions were terminated by addition of 10 mM EDTA, 5% sodium dodecyl sulfate (SDS, Sigma), and 10% glycerol and boiling. Phosphoproteins were reduced by addition of 100 mM DTT and analyzed by gel electrophoresis.

SDS-polyacrylamide gel electrophoresis

Phosphoproteins were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) by the method of Laemmli (Laemmli, 1978) using a 0.1% SDS and 10% polyacrylamide gel. The electrophoretic mobility of molecular weight (Mr) markers was used to construct the standard curve and the apparent molecular weights were estimated from the electrophoretic mobility. After electrophoresis, gels were fixed, stained and destained. The gels were then dried in vacuum at 80°C and exposed to X-ray film under intensifying screen at −70°C for 4-6 days.

Results

In a cell-free hypothalamic preparation, many endogenous phospho-proteins were markedly phosphorylated, depending on the activation of intracellular signal pathways. As shown in Fig. 1, several proteins with Mr of 105, 93, 86, 68, 43, 39, 35 and 29 kDa were highly phosphorylated in a cAMP-dependent manner. Aside from these proteins, the intensity of many other phosphoproteins was also changed by preincubation with cAMP, but the degree of phosphorylation was not markedly affected by different concentrations of cAMP. Fig. 2 shows that increasing doses of PKA inhibitor (PKAI) substantially inhibited phosphate transfer from ATP to several substrates, indicating that these endogenous phosphoproteins appear to be specific substrates for the cAMP-dependent PK pathways.

The preincubation of cytoplasmic fractions with phosphatidyl serine (20 μg) in the presence of Ca^{2+} (200 μM) increased the intensity of several phosphoproteins with Mr of 130, 93, 60, 46, 37, 35 and 29 kDa (Fig. 3, left pannel). From the estimated Mr in the phosphorylation patterns, phosphoproteins of Mr 93, 39 and 35 kDa appear to be the same as those found in response to cAMP-dependent PKA pathways (compare Figs. 1 and 3). The increasing doses of phosphatidyl serine in the combination with Ca^{2+} (200 μM) were ineffective in increasing phosphorylation intensities (Fig. 3, right pannel). Inhibition of PI-dependent PK with psychosine, inhibitor of PI-PKC pathways, markedly decreased the phosphorylation intensity (Fig. 4).

Addition of Ca^{2+} (200 μM) intensified the phosphorylation of several proteins. Moreover, treatment with calmodulin (100 ng) in combination with Ca^{2+} was highly effective in increasing phosphorylation of several proteins with Mr of

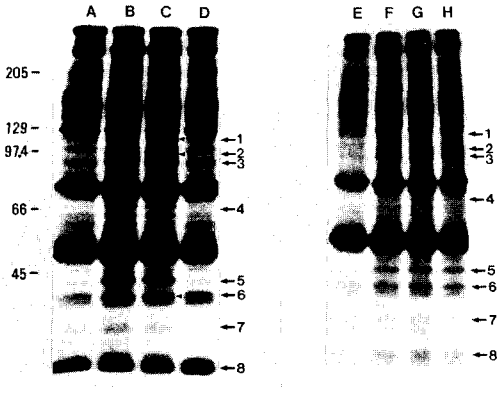

Fig. 1. Autoradiograms showing protein phosphorylation activated by cAMP in a cell free system using hypothalamic soluble fractions. Proteins were separated on a 0.1% SDS and 10% polyacrylamide gel. Left pannel shows: A. control, B. 5 μM cAMP, C. 5 μM cAMP + 0.2 mM IBMX, and D. 0.2 mM IBMX. Right pannel indicates the dose effect of cAMP on the phosphorylation. E. control, F. 0.5 μM cAMP, G. 5 μM cAMP, and H. 50 μM cAMP, respectively. Appropriate MW markers (left) are shown, and arrows and numbers (right) indicate phosphoproteins altered. This denotation is the same as the following figures.

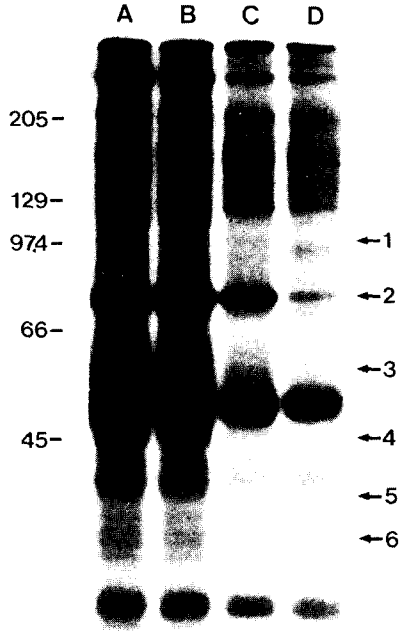

Fig. 2. Inhibition of phosphoproteins by increasing amounts of cAMP-dependent protein kinase inhibitor (PKAI). For the inhibitor treatment experiments, all experimental groups were incubated with cAMP (5 μM), Ca^{2+} (200 μM), CaM (100 ng), and PS (20 μg) in order to activate each phosphorylation reaction. A. control, B. 10 units of PKAI, C. 100 units of PKAI, and D. 1,000 units of PKAI.

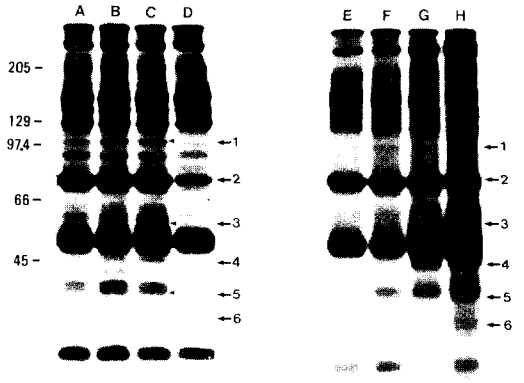

Fig. 3. Activation of protein phosphorylation by phosphatidyl serine (PS) in the presence of Ca^{2+}. Left pannel: A. control, B. 200 μM Ca^{2+}, C. 200 μM Ca^{2+} + 20 μg PS, and D. 200 μM Ca^{2+} + 20 μg PS + 200 μM psychosine. Right pannel shows a dose dependent effect of PS in the presence of 200 M Ca^{2+}. E. control, F. 200 μM Ca^{2+} + 2 μg PS, G. 200 μM Ca^{2+} + 20 μg PS, and H. 200 μM Ca^{2+} + 200 μg PS, respectively.

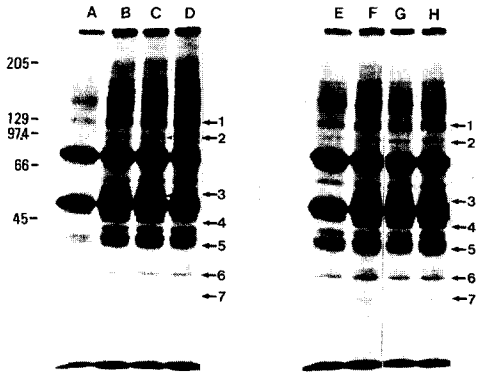

Fig. 5. Activation of protein phosphorylation by Ca^{2+} and/or calmodulin (CaM). Left pannel: A. control, B. 200 μM Ca^{2+}, C. 200 μM Ca^{2+} + 100 ng CaM, and D. 200 μM Ca^{2+} + 100 ng CaM + 20 μM trifluoperazine (TFP). Right pannel shows a dose dependent effect of CaM in the presence of 200 μM Ca^{2+}. E. control, F. 200 μM Ca^{2+} + 10 ng CaM, G. 200 μM Ca^{2+} + 100 ng CaM, and H. 200 μM Ca^{2+} + 1 μg CaM.

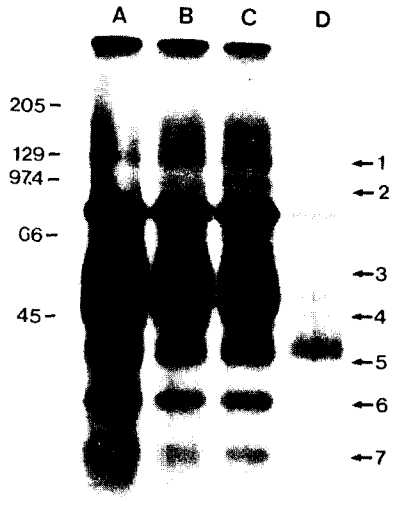

Fig. 4. Inhibition of Ca^{2+}-PS dependent protein phosphorylation by increasing concentrations of psychosine. Samples were preincubated with cAMP (5 μM), Ca^{2+} (200 μM), CaM (100 ng), and PS (20 μg) in order to activate each phosphorylation reaction. A. control, B. 20 μM of psychosine, C. 200 μM of psychosine, and D. 2 mM of psychosine.

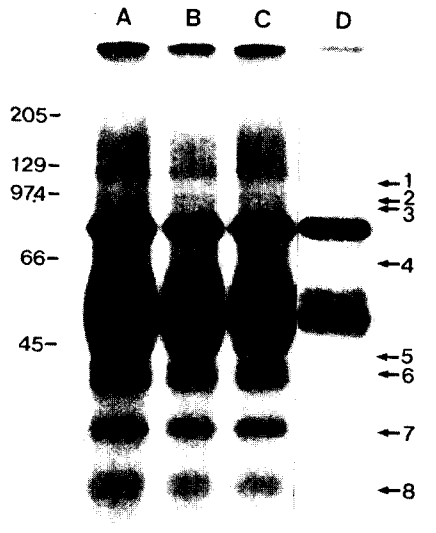

Fig. 6. Inhibition of Ca^{2+}-CaM-dependent protein phosphorylation by increasing doses of TFP. Samples were preincubated with cAMP (5 μM), Ca^{2+} (200 μM), CaM (100 ng), and PS (20 μg) in order to activate each phosphorylation reaction. A. control, B. 2 μM of TFP, C. 20 μM of TFP, and D. 200 μM of TFP.

100, 60, 46, 37 and 35 kDa (Fig. 5, left pannel). Increasing concentrations of calmodulin increased phosphorylation intensities in a dose dependent manner (Fig. 5, right pannel). Increasing doses of TFP, a CaM inhibitor, clearly decreased the intensity of phosphoproteins above mentioned, in particular, protein with Mr 76.6 kDa (Fig. 6).

Discussion

Since protein phosphorylation is one of the major biochemical events in cellular functions (Greengard, 1979; Nestler and Greengard, 1883; Nestler *et al.*, 1984; Nairn *et al.*, 1985b), it is of importance to establish that specific proteins are phosphorylated in response to the second messenger activation. As far as the hypothalamus is concerned, our knowledge in this event is very limited. The present study clearly demonstrates that depending upon activation of specific signal transduction pathways, there are marked changes in phosphorylation patterns of hypothalamic proteins. It seems that some of these PK-dependent phosphorylated proteins are involved in the regulation of neuroendocrine functions, for instance, secretion (Drouva et al., 1981; Drouva *et al.*, 1984; Ramirez *et al.*, 1985; Kim and Ramirez, 1986; Negro-Vilar *et al.*, 1986; Ojeda *et al.*, 1986) and gene expression of GnRH (Lee *et al.*, 1990).

In the present study, cytoplasmic fractions derived from immature rats were subjected to a cell free phosphorylation system and phosphoproteins were separated by SDS-PAGE electrophoresis. Using such cytoplasmic fractions, prominent changes in protein phosphorylation were observed. We did not compare the possible difference in phosphorylation patterns between nuclear and cytoplasmic fractions, neither the recovery nor subcellular compartment of recovered proteins. However, it seems there is no marked differences in protein distribution between tissues and subcellular fractions like other tissues (Steinschneider *et al.*, 1990). It would be, however, of importance to examine changes in protein phosphorylation patterns using nuclear fractions, since many recent studies indicate that specific proteins phosphorylated by activation of specific signal transduction pathways may affect nuclear function including transcription (Comb *et al.*, 1986; Habner, 1990). For instance, transcription of somatostatin, one of the cAMP-inducible gene is regulated by a cAMP-binding phosphoprotein, so called CREB (Montminy *et al.*, 1987; Yamamoto *et al.*, 1988).

Though we are not aware of the molecular characteristics of the phosphoproteins seen in this study, it is possible to speculate about the presumable molecular identity of several proteins. It is of interest that a phosphoprotein with Mr of 86 kDa (Fig. 2) whose phosphoryl intensity was clearly augmented by addition of cAMP and partially increased by Ca^{2+}-CaM addition appears to be similar to synapsin I, as far as Mr is concerned (Camilli *et al.*, 1983; Hunter *et al.*, 1983). Synapsin I, a protein associated with the cytoplasmic side of synaptic vesicle, is a major substrate for both the cAMP- and Ca^{2+}-CaM-dependent PKs in the brain and involved in the regulation of neurotransmitter release (Jullien and Mushynski, 1982). Another cAMP-dependent phosphoprotein with Mr of 68 kDa (Fig. 2) appears to be similar to a small subunit of neurofilament protein (Nestler and Greengard, 1983; Nairn *et al.*, 1985b). It is tempting to speculate that the phosphoprotein with Mr of 46 kDa phosphorylated by Ca^{2+}-PS and Ca^{2+}-CaM might be tyrosine hydroxylase which is widely distributed in catecholaminergic neurons (Nestler and Greengard, 1983). One of the phosphoproteins with Mr of 100 kDa seems to be similar to a major substrate of type III Ca^{2+}-CaM-dependent PK (Nairn *et al.*, 1987). Proteins with Mr of 60 and 37 kDa appear to be autophosphorylated subunits of Ca^{2+}-CaM-dependent PK I and II, respectively, which are known to be widely distributed and common protein kinases existing in the brain including the hypothalamus (Freedman and Jamieson, 1982). It is also possible that protein with Mr of 29 kDa phosphorylated by cAMP- and Ca^{2+}-PS may be a ribosomal protein S6 as previously described (Padel and Soling, 1985). Many other phosphoproteins aside from those mentioned above were also changed by addition of protein

kinase activators and/or inhibitors. Collectively, the present study reveals that in the hypothalamus, there exist a series of specific PKs such as cAMP-, PI- and Ca^{2+}-CaM-dependent PKs that are capable of phosphorylating specific proteins which may be important mediators of intracellular signal transduction in the regulation of neuroendocrine functions. Further study is needed to determine the biochemical identity of these phosphoproteins and their physiological roles in the hypothalamic function.

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흰쥐 시상하부에서 신호전달계의 활성화에 의한 단백질 인산화의 변화

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신경,내분비계의 세포신호전달과정에 특이한 protein kinase들에 의한 단백질인산화의 변화가 중요하다고 알려져 있으나, 시상하부에서의 단백질 인산화에 관해서는 거의 연구된 바 없다. 본 연구에서는 여러 이차 신호전달자에 의존적인 protein kinase들의 활성화에 의한 내인성 단백질 인산화 양상을 전기영동방법으로 조사하였다. 미성숙 흰쥐의 시상하부 조직에서 얻은 세포질 분획을 기질로 사용하여 cAMP, phosphatidylinositol, 혹은 Ca^{2+}-Calmodulin에 의존적인 protein kinase들의 활성제 혹은 억제제를 처리한 결과, 여러 내인성 단백질의 인산화 양상이 바뀌었다. 이는 이차 신호전달자-protein kinase-단백질 인산화를 통한 신호전달계에 의해서 흰쥐 시상하부의 신경내분비 기능이 영향을 받음을 시사한다.