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# Protein Kinase A Functions as a Negative Regulator of c-Jun N-terminal Kinase but not of p38 Mitogen-activated Protein Kinase in PC12 Cells

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**Abstract:** Cyclic-AMP-dependent protein kinase (PKA) seems to function as a negative regulator of the c-Jun $NH_2$-terminal kinase (JNK) signaling pathway. We demonstrate here that the activity of the PKA catalytic subunit (PKAc) is reduced in apoptotic PC12 pheochromocytoma cells. Apoptotic progress was inhibited by dibutyryl cyclic AMP (dbcAMP), an analog of cAMP. The rescue by dbcAMP was attributable to inhibition of the JNK but not of the p38 signaling pathway, due to the induction of PKA activity. JNK was present in immunocomplexes of PKAc, and PKAc phosphorylated JNK *in vitro*. Presence of p38 kinase, however, was not prominent in immunocomplexes of PKAc. Our data suggest that JNK is a target point of negative regulation by PKAc in the JNK signaling pathway.

**Key words:** apoptosis, cAMP, cAMP-dependent protein kinase A (PKA), JNK, p38 kinase

The discovery of PKA and studies on its regulatory role provided a general scheme for transmission of cAMP-mediated signals as a cascade of protein phosphorylation (Krebs, 1989; Taylor, 1989). There has been evidence that compounds increasing intracellular cAMP rescue PC12 cells from cell death induced by omitting serum from their culture media and stimulate neurite outgrowth. Expression of the catalytic subunits of PKA in rat sympathetic neurons extended cell survival (Rydel and Greene, 1988; Rukenstein et al., 1991; Buckmaster and Tolkovsky, 1994). Cross-talk, however, between cAMP-mediated and MAP kinase pathways has only been revealed rather recently. Compounds activating PKA were found to inhibit PDGF-induced ERK signaling in smooth muscle cells and EGF-induced signal transduction in Rat1 cells (fibroblasts) (Graves et al., 1993; Wu et al., 1993). In other cell types including PC12 cells, cAMP does not inhibit growth factor induced activation, but rather stimulates the ERK pathway and shows synergistic activation with nerve growth factor (NGF) (Frodin et al, 1994; Vossler et al., 1997). It has been proposed that the differential effects of PKA on cellular responses following extracellular ligand stimulation may be achieved via the regulatory status of Raf isoforms (Cook and McCormick, 1993; Miller et al., 1998; MacNicol and MacNicol, 1999). Most of the effects by cAMP on physiological responses have been attributed to the binding of cAMP to PKA, producing active kinase. However, newly identified Epac (exchange protein directly activated by cAMP) directly binds cAMP then activates Rap1, a small Ras-like GTPase, in a PKA-independent manner (Kawasaki et al., 1998; de Rooij et al., 1998). Additional evidence of cross-talk between cAMP-mediated and MAP kinase pathways comes from regulation by HePTP (haematopoietic protein tyrosine phosphatase). HePTP binds and dephosphorylates MAP kinase, ERK and p38 but not JNK, then inhibits kinase activity. Phosphorylation of HePTP by PKA releases MAP kinase from HePTP (Saxena et al., 1999). Considering the effect of PKA on JNK or p38 pathway, there have been controversial lines of evidence depending on cell lines. While PKA stimulates both p38 and JNK in neuroblastoma cells (Xia et al., 1995; Zhen et al., 1998) and activates JNK in macrophages (Delgado and Ganea, 2000), PKA shows no effect on the stress-dependent JNK pathway in rat liver epithelial cells (Li et al., 1997)

Intracellular organization of protein components into higher order complexes seems to provide a means of specifically regulating when and where a particular signaling pathway is active. Such protein complexes include three-member protein kinases (MAPK, MAPKK and MAPKKK) which are cascades of catalytic activators

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and scaffolding proteins that interact with a specific set of MAP kinases to enhance the efficiency of signal transduction. This positive effect of activating kinases may be counteracted by many isoforms of MAPK phosphatases (Garrington and Johnson, 1999; Keyse, 2000). The JNK group of MAP kinases, one of three groups extensively characterized, is required for a number of cellular processes in early embryonic development, apoptosis, oncogenic transformation, and immune response. JNK is activated by MAPKKs, MKK4 and MKK7, which are stimulated by members of the MEKK and MLK groups of MAPKKKs (Ip and Davis, 1998). Two scaffolding mechanisms for regulating JNK activity have been reported. JIP-1 binds HPK1 (MKKKK), MLK3 or DLK (MKKKs), MKK7 and JNK, leading to enhanced JNK activation (Whitmarsh et al., 1998). The other scaffolding complex includes MEKK1, MKK4 and JNK. MEKK1, in addition to acting as an upstream activator of MKK4, is able to bind JNK and functions as a scaffolding protein (Xu and Cobb, 1997). The activation of JNK by the positive activators is possibly counteracted by isoforms of MAPK phosphatases, MKP1 and MKP2 (Hirsch and Stork, 1997). A large number of protein components regulating signal transduction pathways have now been identified and the figure of three-dimensional organization of proteins inside of the cell is beginning to emerge.

Here we present data that activity of PKA is reduced in apoptotic cells compared to normally grown PC12 cells and also that PKA, not cAMP, inhibits the activation of JNK, thereby protecting PC12 cells from apoptosis. The presence of a complex containing PKAc and JNK, but not p38, has been confirmed. Phosphorylation of JNK by PKAc, acting as a negative regulator, seems to provide a means of differential regulation between JNK and p38 MAPK pathways.

## MATERIALS AND METHODS

### Materials

Theophylline and dibutyryl cyclic AMP (dbcAMD) were purchased from Sigma Chemical Co. H-89 was obtained from Calbiochem. Histone H2B was provided by Boehringer Mannheim. Catalytic subunit of PKA (PKAc) was purchased from Upstate Biotechnology, Inc.. Protein A Sepharose was obtained from Amersham Pharmacia Biotech. BCA Protein Assay Reagents were provided by Pierce. Enhanced chemiluminescence (ECL) Western blotting detection reagents and [$\gamma$-$^{32}$P]ATP were purchased from NEN Life Science Products, Inc.. Polyhistidine tagged JNK1 (H-JNK1) and antibodies against MKK4 and MKK7 were obtained from Santa Cruz Biotechnology. Factor Xa and antibodies against phospho-Bad, Akt and p38 were provided by New England BioLabs, Inc.. Antibody against PKAc was purchased from Transduction Laboratories. Antibody against JNK was obtained from PharMingen. Culture medium and serum were provided by GIBCO BRL. Purified proteins of GST-c-Jun, GST-ATF2, GST-JNK K55R and GST-p38 were kind gifts from Dr. Eui-Ju Choi (Graduate School of Biotechnology, Korea University, Seoul, Korea). Cell Culture and Preparation of Cell Lysates: PC12 cells were maintained in RPMI medium 1640 with 5% FBS and 10% heat-inactivated horse serum, in a humidified atmosphere containing 10% $CO_2$ at 37°C (Greene et al., 1987). For serum deprivation, cells were washed 3 times with serum-free RPMI 1640 prior to plating. To test the effect of cAMP, cells were washed 3 times with serum-free RPMI 1640 and then plated in serum-free RPMI 1640 with dbcAMP (500 μM) and theophylline (10 μM). Samples were collected 18 hrs after plating, when over the half population of cells underwent apoptosis in serum-free media, and lysed in 10 mM of Tris (pH 7.5), 1% of Triton X-100, 0.5% of Nonidet P-40 (NP-40), 150 mM of NaCl and 1 mM of EDTA (pH 8.0) with the cocktail of phosphatase inhibitors and protease inhibitors. After 5 min spinning in microfuge to remove cell debris, proteins in the supernatant were quantitated with BCA Protein Assay Reagents. Immunoprecipitation and Immunoblotting: Cell lysates containing 1 mg of proteins in 1 ml of volume were incubated with primary antibodies (1 μg/ml) overnight at 4°C with gentle rocking. The mixtures were further incubated with 50 μl of 10% Protein A Sepharose for 1 h at 4°C. Thereafter, beads were washed 3 times with lysis buffer to remove unbound proteins. Immunocomplexes were boiled for 5 min in reducing sample buffer, and then subjected to SDS-PAGE and immunoblotting for the epitopes of interest. Antibody binding was detected using the ECL system.

### *In vitro* kinase assay

*In vitro* kinase assays were performed on renatured, blotted proteins, using a modified protocol of the Celenza and Carlson assay (Ferrell and Martin, 1989; 1991). Cell lysates containing 500 μg of proteins were subjected to 10% SDS-PAGE and blotted onto Immobilon-PVDF membrane. Blots were incubated for 1 h at room temperature with gentle rocking in denaturation buffer [7 M guanidine HCl, 50 mM Tris (pH 8.3), 50 mM DTT, 2 mM EDTA (pH 8.0)]. Proteins on guanidine HCl-treated blots were allowed to renature in 140 mM of NaCl, 10 mM of Tris (pH 7.4), 2 mM of DTT, 2 mM of EDTA (pH 8.0) and 0.1% (w/v) of NP-40 with gentle rocking overnight at 4°C. After washing the blot with 30 mM of Tris (pH 7.5), membranes were incubated in 40 mM of HEPES (pH 7.3), 10 mM of $MgCl_2$, 2 mM of $MnCl_2$ and 10 μCi/ml [$\gamma$-$^{32}$P]ATP for 30 min at room temperature. After thorough washing with 30 mM of Tris (pH 7.5), radiolabeled blots were incubated in 1 M of

KOH for 10 min at room temperature to hydrolyze unreacted ATP. The alkali-treated blot was rinsed with $H_2O$ and 10% (v/v) of acetic acid. The air-dried blot was subjected to autoradiography. For further analyses of blotted proteins such as western analysis, the dried membranes were rewetted with MeOH before processing.

### Kinase assay

For kinase assays, immunocomplexes were further washed 3 times with kinase buffer [10 mM Tris (pH 7.4), 150 mM NaCl, 10 mM $MgCl_2$ and 0.5 mM DTT]. Bead pellets were suspended in 40 μl of kinase assay buffer [20 mM HEPES (pH 7.5), 10 mM $MgCl_2$, 10 mM $MnCl_2$ and 1 mM DTT]. 10 μCi of [$\gamma$-$^{32}$P]ATP and a proper kinase substrate were then added. After incubating for 30 min at 30°C, the reactions were terminated by adding SDS sample buffer, followed by boiling for 5 min. Samples were subjected to 12% SDS-PAGE.

Polyacrylamide gels were dried and autoradiography was performed. For assaying phosphorylation of JNK by PKAc, 1 μg of H-JNK and 1 μg of PKAc were mixed in 40 μl of kinase assay buffer. Samples were incubated for 20 min at 30°C with 100 μM of ATP and 10 μCi of [$\gamma$-$^{32}$P]ATP. The reactions were stopped by adding SDS sample buffer and boiled for 5 min. SDS-PAGE and autoradiography were performed. As a control, Factor Xa treated GST-p38 was used. Purified p38 (1 μg) underwent the same reactions described above.

**Abbreviations:** dbcAMP, dibutyryl cyclic AMP; EGF, epidermal growth factor; ERK, extracellular signal regulated kinase; JNK, c-Jun NH2-terminal kinase; MAPK, mitogen-activated protein kinase; MAPKK, MAPK kinase; MAPKKK, MAPKK kinase; MKK, MAPK kinase; MKKK, MKK kinase; MKKKK, MKKK kinase; NGF, nerve growth factor; PDGF, platelet derived growth factor; PKA, cyclic-AMP-dependent protein kinase; PKAc, PKA catalytic subunit

## RESULTS

### Search for kinases related to apoptosis

Serine/Threonine kinases regain their catalytic activities if they are slowly refolded following the unfolding reaction on a blotted membrane (Ferrell and Martin, 1989; 1991). Taking advantage of *in vitro* kinase assays, profiles of autophosphorylating proteins in samples prepared from the cells grown in different conditions were compared to each other. Deprivation of serum from the growth media resulted in reduction of the enzyme activity of at least four different kinases. One kinase resided at the same electrophoretic location as PKAc, which was used as a control enzyme for the *in vitro* treatment (Fig. 1A).

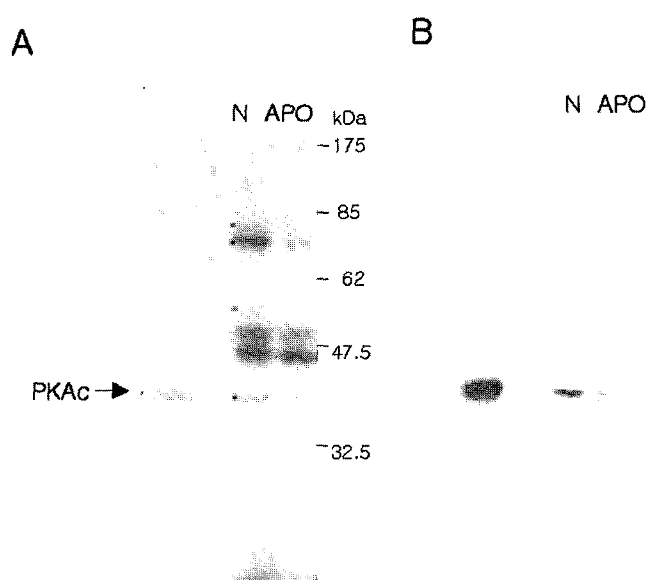


**Fig. 1.** Activity of PKAc is reduced in apoptotic PC12 cells. In order to identify new kinases related to apoptosis, autophosphorylating abilities of kinases were compared among samples prepared from normally grown (N) and apoptotic (APO) cells using an *in vitro* kinase assay. PKAc was used as a control enzyme for treatment (A). To identify the ~40 kDa band, western analysis, using anti-PKAc, was performed against the same radiolabeled PVDF membrane (B).

Since it has been reported that cAMP inhibits apoptosis in cells deprived of survival factors (Rydel and Greene, 1988), it seemed plausible that PKA might have reduced activity from the cells that were harvested and processed at the point of cell death in half of the population. We confirmed the identity of the ~40 kDa protein by western analysis of the radiolabeled membrane using antibody against PKAc. A single western band was superimposed at the location of the ~40 kDa protein shown in Fig. 1A (Fig. 1B).

To test whether PKAc inhibits the apoptotic progress, PC12 cells were plated in serum-free media containing dbcAMP. Up to 90% of cells were alive 18 h after plating with dbcAMP, while more than 50% of cells underwent apoptotic progress without dbcAMP (data not shown).

### Possible mechanisms for PKAc inhibition of apoptosis in PC12 cells

Either induction of anti-apoptotic factors or inhibition of pro-apoptotic factors can protect cells from the death signals. To test the possibility that PKAc induces anti-apoptotic factors, we monitored the changes in Akt signaling pathway, as it has been reported that PKA phosphorylates Ser 112 on Bad (Datta et al., 1997). If Bad molecules are activated by PKA, in addition to being activated by Akt, this synergistic stimulation would facilitate the binding of 14-3-3 molecules to Bad, resulting in the release of anti-apoptotic Bcl-2 and subsequently blocking of cell death (Datta et al., 1997; Zha et al., 1996).

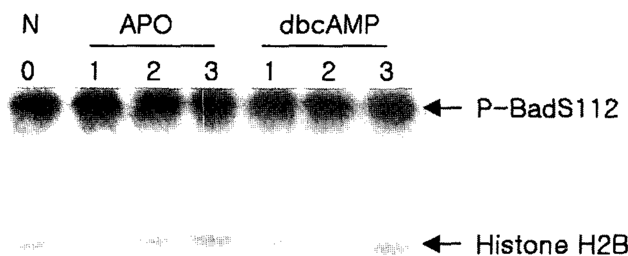


**Fig. 2.** Attenuation of cell death was not achieved by the activation of Akt pathway. The amount of phospho-Bad S112 was monitored by western analysis using antibody against phospho-Bad S112 (top), the activity of PKAc was detected by kinase assay using histone H2B as a substrate (middle), and the activity of Akt was measured by kinase assay using histone H2B as a substrate (bottom). Numbers indicate hours after treatment.

In PC12 cells, neither the activity of Akt nor the amount of phosphorylated Bad at Ser 112 was modified by dbcAMP while the activity of PKA was induced by dbcAMP. The activity of Akt increased over time in the samples from serum deprived media, but that was independent of the presence of dbcAMP in the culture media (Fig. 2).

We tested the alternative possibility that PKA may function by inhibition of pro-apoptotic factors. Several reports indicate that JNK signaling pathway is required for neuronal apoptosis (Xia et al., 1995; Ip and Davis, 1998). If PKA inhibits and modifies any components in JNK signaling pathway, it would result in cells escaping from apoptosis. As shown in Fig. 3A, JNK activity was induced in the apoptotic cells, and prominent abolishment of enzyme activity was observed in the cells cultured in serum-free media in the presence of dbcAMP. Recently, Delgado and Ganea (2000) also showed that the pituitary adenylate cyclase activating polypeptide (PACAP) inhibited the stress-induced JNK pathway in stimulated macrophages.

The possible prevention of the activity of p38 kinase by dbcAMP was also tested, since both JNK and p38 share a dual specificity Thr/Tyr protein kinase, MKK4, as an upstream signal (Derijard et al., 1995; Lin et al., 1995). In contrast to the pattern of c-Jun phosphorylation by JNK, dbcAMP did not alter the phosphorylating pattern of ATF2 by p38 in PC12 cells (Fig. 3B). This was an unexpected result, since according to Xia et al. (1995), one of anti-apoptotic reagents, cAMP, prevents apoptosis induced by NGF removal from NGF-differentiated PC12 cells and inhibits the activation of both JNK and p38. Moreover, in contrast to Xia et al. (1995) and our data, dbcAMP stimulates both p38 and JNK in SK-N-MC human neuroblastoma cells (Zhen et al., 1998). Taken all together, there seemed be a complex regulation mechanism that may vary depending on cell types or internal and external cellular environment.

Since MKK4 activates both JNK and p38 kinase (Derijard et al., 1995; Lin et al., 1995), changes in MKK4 activities by dbcAMP were examined. MKK4 activities were turned on in the cells plated in serum deprived media, but were not deactivated by dbcAMP in PC12 cells (Fig. 3C). Conversely, in LPS-treated macrophage cells, PACAP significantly inhibited the MKK4-mediated phosphorylation of JNK (Delgado and Ganea, 2000). Activation of MKK7, an upstream activator of JNK (Holland et al., 1997; Moriguchi et al., 1997; Tournier et al., 1997) was not reduced by dbcAMP either (data not shown). Taken together, our results indicated that prevention of apoptotic process by the presence of dbcAMP occurs at the level of MAP kinase and that PKAc may function as a negative regulator which specifically affects JNK, but not p38 kinase.

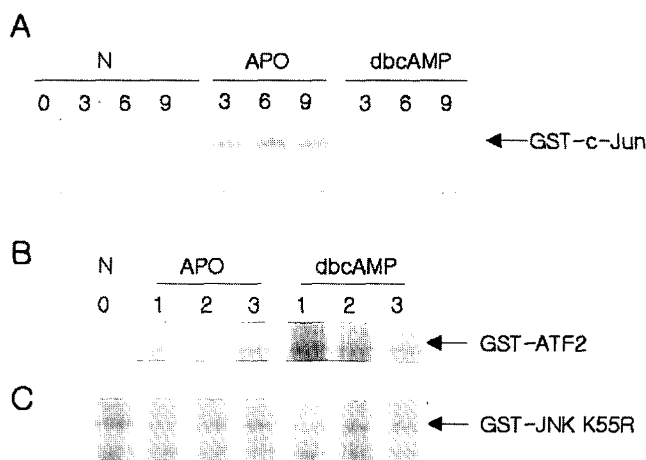


**Fig. 3.** PKAc is a negative regulator of JNK pathway, and JNK is a target of PKAc. A, Phosphorylation of GST-c-Jun was monitored to compare JNK activity. Samples were prepared from normally grown (N), apoptotic (APO) and dbcAMP treated cells. After preparing immunocomplex, kinase assays were performed with the immunoprecipitates and the substrate GST-c-Jun. Radiolabeled immunocomplexes were subjected to SDS-PAGE and autoradiography. Numbers indicate hrs after treatment. B, Phosphorylation of GST-AFT2 was monitored to compare p38 activity. C, Purified protein, GST-JNK K55R, was used as the substrate of kinase, MKK4.

### Distinction between PKA and cAMP

While we have considered that changes caused by dbcAMP are due to inducing catalytic activity of PKA, two independent groups have reported that Epac, a Rap1 guanine-nucleotide-exchange factor, is regulated in a cAMP-dependent but PKA-independent manner (Kawasaki et al., 1998; de Rooij et al., 1998). Therefore, we further considered whether the deactivation of JNK by dbcAMP was dependent on the chemical dbcAMP or the protein PKA. H-89, a potent and selective inhibitor of PKA (Chijiwa et al., 1990), was applied with dbcAMP, then the effect of H-89 was monitored by measuring the activity of JNK. The ability of JNK phosphorylating c-Jun was abolished by dbcAMP, but was reactivated in the presence of 10 μM of H-89. Data in

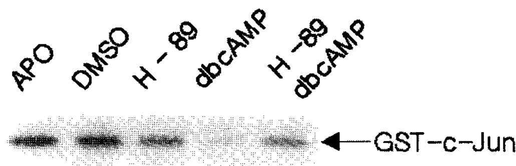


**Fig. 4.** JNK is deactivated not by chemical cAMP, but by protein PKA. To exclude the possibility that dbcAMP directly inhibits JNK, JNK activities were compared among samples prepared from PC12 cells grown serum-free media with dbcAMP and dbcAMP together with H-89, a specific inhibitor of PKAc. Samples of PC12 cells grown in serum depleted media with DMSO were prepared as a control since H-89 stock was solved in DMSO.

Fig. 4 confirm that the negative regulation of JNK activity is achieved by the protein PKA.

## PKA Forms a Complex with JNK in the Cell, and Phosphorylates JNK *in vitro*

If PKA modulation occurs at the level of MAP kinase in the JNK pathway, then we were curious as to how the kinase, whose function is phosphorylating its target protein, inhibits the activity of JNK. To search for a clue on this matter, we explored the possibility that PKA and JNK form a complex. Immunocomplexes were prepared from extracts of PC12 cells using antibody against PKAc, and then the presence of JNK or p38 kinase was detected by western analysis using antibodies against JNK and p38. The presence of JNK in the immunocomplex of PKAc was prominent whereas the western band of p38 was not clearly detectable. Conversely, PKAc was also present in the immunocomplex of JNK (Fig. 5A). Taken together, co-immunoprecipitation data suggested the presence of a complex containing both PKA and JNK, but the conclusion of the direct physical interaction between PKA and JNK could not be drawn from these data. The effect of PKA on JNK may be either direct or indirect via some other proteins in the complex. If the interaction between PKA and JNK is physically direct, one of the possible outcomes may be the phosphorylated JNK. Both JNK and p38 were tested as substrates for PKAc. p38 was chosen as a control because its activity was not altered by dbcAMP, while the activity of JNK was, in PC12 cells. As shown in Fig. 5B, phosphorylation of JNK by PKAc was clearly detected whereas there was no additional phosphorylation of p38 by PKAc as compared to the autophosphorylation of p38 kinase.

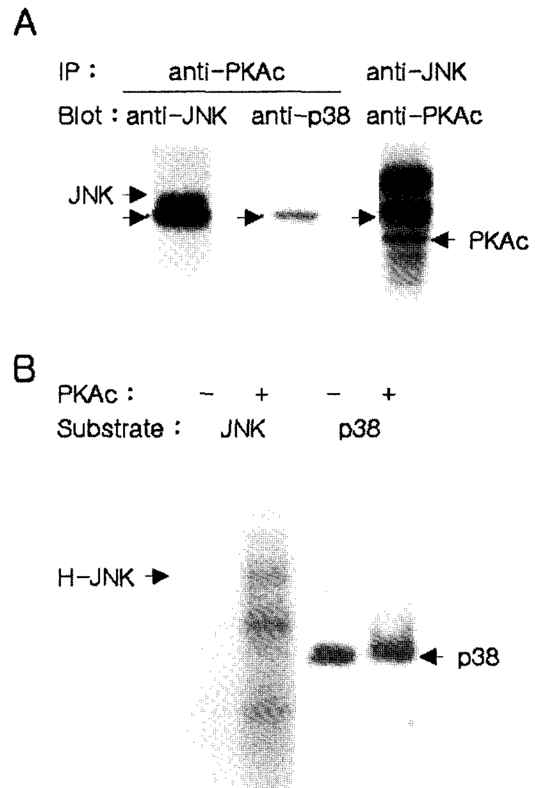


**Fig. 5.** The presence of JNK/PKA complex was detected in PC12 cells, and phosphorylation of JNK by PKAc was examined *in vitro*. A, Immunoprecipitates using antibody against PKAc were prepared using samples of PC12 cells grown in RPMI 1640 with serum, and then subjected to SDS-PAGE and western analysis with antibodies against JNK and p38 kinase. JNK was detected above IgG band while p38 was not detectable. Conversely, PKAc was present in JNK immunocomplex. Unlabeled arrows indicate IgG. B, H-JNK and p38 kinase were underwent kinase assay with PKAc. Phosphorylated JNK was detectable in the reaction with PKAc while none were observed in control reaction. For p38 kinase, the level of phosphorylation by PKAc was not over the level of autophosphorylation.

## DISCUSSION

The functional role of JNK in apoptotic response is rather confusing, since the protein acts both as a pro and anti-apoptotic factor depending on the cell line (Ip and Davis, 1998). Better understanding on how the JNK pathway responds, or is regulated, will help to interpret the variable phenomena related to the status of JNK activity. In neuronal cells, the pro-apoptotic function of active JNK has been clearly demonstrated by Xia et al. (Xia et al., 1995). They showed that both JNK and p38 were activated, while ERKs were inhibited upon withdrawing NGF from NGF-differentiated PC12 cells. They suggested that the intracellular balance between growth factor-activated ERK and stress-activated JNK/p38 pathways might determine the fate of whether cells survive or undergo apoptosis. We considered the possible adaptation of PC12 cells to serum-free media with NGF, so we induced apoptosis directly from the undifferentiated cells by serum deprivation. Omitting serum from the culture media immediately triggered activation of both JNK and p38. Co-activation of these MAP kinase pathways in apoptotic PC12 cells seems to be a general phenomenon, regardless of whether cells are differentiated or not. When considering inhibitory enzymatic activities, however, the JNK pathway seems to differ from the p38 pathway in its

regulatory mechanism. Forskolin, which increases cAMP formation by activating adenylate cyclase, rescued NGF-differentiated PC12 cells from cell death induced by NGF depletion. Activities of both JNK and p38 were repressed (Xia et al., 1995). Apoptotic progress of undifferentiated PC12 cells was also blocked by the addition of dbcAMP to cells in serum-free culture media. However, only JNK, and not p38, was down-regulated by PKA under the experimental conditions we used. Since MKK4, which functions as an upstream activator of both JNK and p38 (Derijard et al., 1995; Lin et al., 1995) is not deactivated by dbcAMP, our data suggest that additional regulation at the level of MAP kinases exists. This negative regulation of JNK signal might provide the critical means for the cells to reverse inappropriate commitment to apoptosis when the activity of p38 still exists.

Based on our results that indicate the JNK formed a complex with PKA and then the PKA phosphorylated JNK. We propose a mechanism regarding the negative regulation of the JNK pathway by PKA. PKAc binds JNK and may simply hold JNK protein, thereby inhibiting further activity. Or possibly, phosphorylation of JNK by PKAc may be unable to transfer the upstream signal to the downstream target due to the loss of kinase activity caused by the structural change of the protein. Alternatively, phosphorylation of JNK may result in the release of JNK from the scaffolding complex, resulting in blocking of signal transduction. Before pursuing experiments on this hypothetical model, we need to demonstrate whether the phosphorylation of JNK by PKAc *in vitro* is relevant *in vivo*. We need to demonstrate whether PKAc modifies JNK proteins regardless of differences in the scaffolding complexes in which JNK molecules are included. A different formulation of components in scaffolding complex seems to be one of factors that affect specific responses to different stimuli (Garrington and Johnson, 1999). It will be important to determine whether the negative regulation of JNK by PKAc is general for any JNK protein or specific for a scaffolding unit.

## ACKNOWLEDGMENT

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