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A transitory activation of protein kinase-A induces a sustained tau hyperphosphorylation at multiple sites in N2a cells-imply a new mechanism in Alzheimer pathology

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Summary. Overactivation of protein kinase in the end stage of Alzheimer's disease brain has not been established. The purpose of the present study was to explore the possible mechanism for protein kinases in leading to Alzheimer-like tau hyperphosphorylation. We found that incubation of N2a/tau441 with forskolin, a specific activator of cAMP-dependent protein kinase (PKA), induced an increased phosphorylation level of tau at both PKA and non-PKA sites in a dose- and time-dependent manner, and the hyperphosphorylation of tau was positively correlated with the elevation of PKA activity. When the cells were transitorily incubated with forskolin, a temporary activation of PKA with a sustained and almost equally graded tau hyperphosphorylation at some non-PKA sites was observed. In either case, the activity of glycogen synthase kinase-3 (GSK-3) was not changed. It is suggested that only transitory activation of PKA in early stage of Alzheimer disease may result in a sustained tau hyperphosphorylation at multiple sites, implying a new mechanism to Alzheimer-like tau hyperphosphorylation.

Keywords: Alzheimer's disease, tau, cAMP-dependent protein kinase, glycogen synthase kinase-3, abnormal hyperphosphorylation.

Introduction

The normal function of microtubule associated protein tau, a phosphoprotein abundantly expressed in the brain, is to promote microtubule assembly and maintain the stability of microtubules (Weingarten et al., 1975). In Alzheimer's disease (AD) brain, phosphorylation of tau is 3- to 4-fold higher than normal level (Grundke-Iqbal et al., 1986; Ksiezak-Reding et al., 1990; Kopke et al., 1993), and the hyperphosphorylated tau is incompetent in its biological activity and aggregates to form paired helical filaments (PHFs)/neurofibrillary tangles (Iqbal et al., 1986; Lee et al., 1991). Hyperphosphorylation of tau is believed to be an early event in the formation of neurofibrillary tangles in AD patients and other tauopathies (Braak et al., 1994; Kosik, 1992;

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Tolnay and Probst, 1999). Hence, elucidating the regulation of tau phosphorylation is a critical step for understanding the molecular mechanism of neurodegenerative diseases.

Until now, at least 37 abnormal phosphorylation sites have been described in tau proteins associated with AD (for review, see Gong et al., 2005) and more than a dozen of protein kinases are shown to phosphorylate tau (for review, see Iqbal et al., 2002; Lau et al., 2002; Avila et al., 2004). Most of them are on Ser/Thr-Pro motifs which can be phosphorylated by proline-dependent protein kinases (PDPKs) and others are on Ser/Thr-X motifs that are phosphorylated by non-PDPKs (Morishima-Kawashima et al., 1995; Wang et al., 1998). Glycogen synthase kinase-3 (GSK-3) and cAMP-dependent protein kinase (PKA) are among the most important kinases to phosphorylate tau both *in vitro* and *in vivo* and fall respectively into PDPK and non-PDPK (Wang et al., 1998; Liu et al., 2003, 2004; Sun et al., 2005). Several sites-specific antibodies have been developed for study of tau phosphorylation, such as PS214 (recognizes Ser214 phosphorylated tau) (Singh et al., 1996; Sun et al., 2005), PHF-1 (recognizes Ser396/404 phosphorylated tau) (Greenwood et al., 1994) and Tau-1 (recognizes Ser199/202 non-phosphorylated tau) (Binder et al., 1988; Biernat et al., 1992). Also, phosphorylation-independent antibody R134d could be used to quantify total tau and used for inner control of phosphorylated tau.

Recent studies have suggested that several kinases may work coordinately with GSK-3 to phosphorylate tau protein. For example, prephosphorylation of tau by PKA, protein kinase C (PKC), calcium/calmodulin-dependent kinase II (CaMKII), casein kinase-1 (CK-1) and CK-2 could significantly promote subsequent tau phosphorylation by GSK-3 at multiple phosphorylation sites *in vitro*, and the most potent inhibition of microtubule assembly by tau hyperphosphorylation was seen in the combined phosphorylation of PKA and GSK-3 (Singh et al., 1996; Wang et al., 1998). We have recently reported in rat brain that prephosphorylation of tau by PKA primes it for phosphorylation by GSK-3 at Ser199/202 and Ser396/404 (Liu et al., 2004). However, no increased biochemical activity of PKA was detected in the end stage of AD brain until now. Therefore, the significance of PKA in AD-like tau hyperphosphorylation is not understood.

The present study was to explore the nature of PKA in priming tau and the possible mechanism involved in AD-like tau pathology. It was found that a transitory activation of PKA could induce a sustained tau hyperphosphorylation at both PKA and some non-PKA sites in same degree as a durative activation did. Thus, we propose that a transitory stimulation of PKA in early stage of AD may play a role in initiating AD-like tau pathology.

Materials and methods

Chemicals and antibodies

Rabbit polyclonal antibody (pAb) R134d against total tau was a gift from Drs. K. Iqbal and I. Grundke-Iqbal (NYS Institute for Basic Research, Staten Island, NY, USA), pAb Ser214 against tau phosphorylated at Ser214 was purchased from Biosource Inc. (Carmarillo, CA, USA). Monoclonal antibody (mAb) PHF-1 against tau phosphorylated at Ser396/404 was a gift from Dr. P. Davies (Albert Einstein College of Medicine, Bronx, NY, USA), and mAb Tau-1 against tau non-phosphorylated at Ser199/202 was from Chemicon International Inc. (Temecula CA, USA). Phospho-GS peptide, a specific GSK-3 substrate, was purchased from Upstate Biotechnology Inc. (Lake Placid, NY, USA), and Kemptide Peptide Substrate, a specific PKA substrate, was from Promega Corporation (Madison, WI, USA). Goat anti-rabbit or goat anti-mouse peroxidase-conjugated secondary antibody, chemiluminescent substrate kit and phosphocellulose units were from Pierce Chemical Company (Rockford, IL, USA). [γ-^{32}P]ATP was from Beijing Yahui Biologic and Medicinal Engineering Co. (Beijing, P.R. China). Forskolin, a specific PKA activator and Rp-Adenosine 3′,5′-cyclic monophosphorothioate triethyl ammonium salt (Rp-cAMPs), a specific inhibitor of PKA, were purchased from Sigma Chemical Co. (St Louis, MO, USA).

Cell culture, treatment and lysate preparation

N2a/tau441 cells (Neuroblastoma N2a/wt cells stably transfected with the longest human tau cDNA) were maintained in 1:1 DMEM: OPTI-MEM in the presence of 200 μg/ml G418 (Gibco BRL, Gathersburg, MD, USA) with 5% fetal bovine serum (FBS) (vol:vol) (Gibico BRL, Gathersburg, MD, USA). Cells were used for experiment when it was in 70–80% confluence. Forskolin was freshly resolved in medium before use in different concentrations according to the experimental design. The cells were firstly treated with different doses of forskolin (i.e., 0, 1, 2, 4, 8, 16 or 32 μM forskolin) for 24 h or one fixed concentration of forskolin for different time periods (i.e., 4 μM forskolin for 0, 1, 3, 6, 12, 24, 48 or 72 h) to investigate tau phosphorylation at Ser214, Ser199/202 and Ser396/404 induced by forskolin. Then, the cells were divided into three groups: normal control group received no treatment; transitory forskolin-treated group was firstly cultured in medium containing 4 μM forskolin for 3 h, then washed for 3 times and continually cultured with medium deprived of forskolin; durative forskolin-treated group was cultured in medium containing 4 μM forskolin till harvest time. Cells were harvested at different time points (i.e., 3, 6, 12, 24, 48, 72 h) counted from the beginning of treatment.

Cells were rinsed twice in ice-cold phosphate-buffered saline (PBS, pH 7.5). For Western blot analysis, cells were lysed with buffer containing 50 mM Tris-Cl, pH 8.0, 150 mM sodium chloride, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.02% sodium azide, 100 μg/mL phenylmethysulfonyl fluoride and 10 μg/mL protease inhibitors (leupeptin, aprotinin and pepstatin) followed by sonication for 5 s on ice. After centrifugation at 12,000 g for 5 min at 4°C, supernatants were fetched out and added with equal volume of 2 × Laemmli sample buffer (125 mM Tris-HCl, pH 6.8, 8% SDS, 17% glycerol, 10% β-mercaptoethanol and 0.05% bromophenol blue). Samples were boiled for 10 min before electrophoresis. For kinase activity assay, cells were lysed with buffer containing 50 mM Tris-Cl, pH 7.4, 150 mM sodium chloride, 1 mmol/L Na_3VO_4, 10 mmol/L NaF, 10 mM β-ME, 5 mM EDTA, 1 mM PMSF, 2 mM benzamidine, 1 μg/mL of each leupeptin, pepstain A and aprotinin each at 4°C, then centrifuged at 16,000 g for 15 min. The supernatants were stored at −80°C until used for kinase activity. Protein concentration was estimated by bicinchoninic acid kit (BCA, Chemical Company Rockford, IL, USA).

Western blotting

Western blot was performed as described previously (Bennecib et al., 2000). In brief, samples of 20 μg protein were firstly separated by 10% SDS-polyacrylamide gel electrophoresis (PAGE) and then electrically transferred onto nitrocellulose membrane. Tau was probed with antibodies PS214 (1:1,000), Tau-1 (1:3,000), PHF-1 (1:500), and R134d (1:1,000), visualized by enhanced chemiluminescent substrate kit (Pierce, Rockford, IL, USA) and exposed to X-ray film (Pierce, Rockford, IL, USA). All tau bands, which may result from differential posttranslational modifications and degradation that is commonly seen in tau proteins, have been quantitatively analyzed as a one band per lane by Kodak Digital Science 1D software (Eastman Kodak Company, New Haven, CT, USA), and were expressed as sum optical densities.

GSK-3 activity assay

The activity of GSK-3 in cell extracts was measured using phospho-GS peptide 2 as described previously (Pei et al., 1997; Tanaka et al., 1998; Tsujio et al., 2000). In brief, 7.5 μg protein was reacted for 30 min at 30°C with 20 μM peptide substrate and 200 μM [γ-^{32}P]ATP (1,500 cpm/pmol ATP) in 30 mM Tris, pH 7.4, 10 mM $MgCl_2$, 10 mM NaF, 1 mM Na_3VO_4, 2 mM EGTA, and 10 mM β-mercaptoethanol in a total volume of 25 μl. The reaction was stopped with 25 μl of 300 mM o-phosphoric acid. The reaction mixture was applied in duplicates to phosphocellulose units. The filters were washed 3 times with 75 mM o-phosphoric acid, dried and counted by liquid scintillation counter. Relative activity of GSK-3 activity was expressed.

PKA activity assay

The PKA activity in cell extracts was measured using kemptide as described previously (Kemp et al., 1977; Casnellie, 1991). In brief, 7.5 μg protein was reacted for 10 min at 30°C with 100 μM peptide substrate, 5 μM cAMP and 100 μM [γ-^{32}P]ATP (2,000 cpm/pmol ATP) in 40 mM Tris-HCl (pH 7.4), 20 mM $MgCl_2$ and 0.1 mg/ml BSA. The reaction was stopped and counted as what we do in GSK-3 activity assay.

Statistical analysis

Data were analyzed using SPSS 10.0 statistical software. The One-Way ANOVA procedure followed by LSD's post hoc tests was used to determine the statistical significance of differences of the means.

Results

Forskolin induces tau hyperphosphorylation at Ser214, Ser199/202 and Ser396/404 in a dose- and time-dependent manner in N2a/tau441 cells

To find a proper concentration of forskolin on tau phosphorylation in the cell line, we first treated the cells with different doses of forskolin (i.e., 0, 1, 2, 4, 8, 16 or 32 μM) for 24 h and then measured the phosphorylation level of tau. It was shown that phosphorylation of tau at Ser214 (PS214) and Ser396/404 (PHF-1) increased in a dose-dependent manner starting at 2 μM of forskolin, and phosphorylation of tau at Ser199/202 (Tau-1) was decreased. No obvious change in phosphorylation of tau was observed at 1 μM of forskolin (Fig. 1A–C). Thereby, cells were further treated with 4 μM forskolin for 0, 1, 3, 6, 12, 24, 48 or 72 h. The immunoactivity of tau at PS214 and PHF-1 epitopes were elevated in a time-dependent manner starting at 3 h except PS214 at 72 h (Fig. 2A, B), along with a time-dependent decrease in immunoactivity of tau at Tau-1 epitope starting at 6 h (Fig. 2C). No significant difference in the

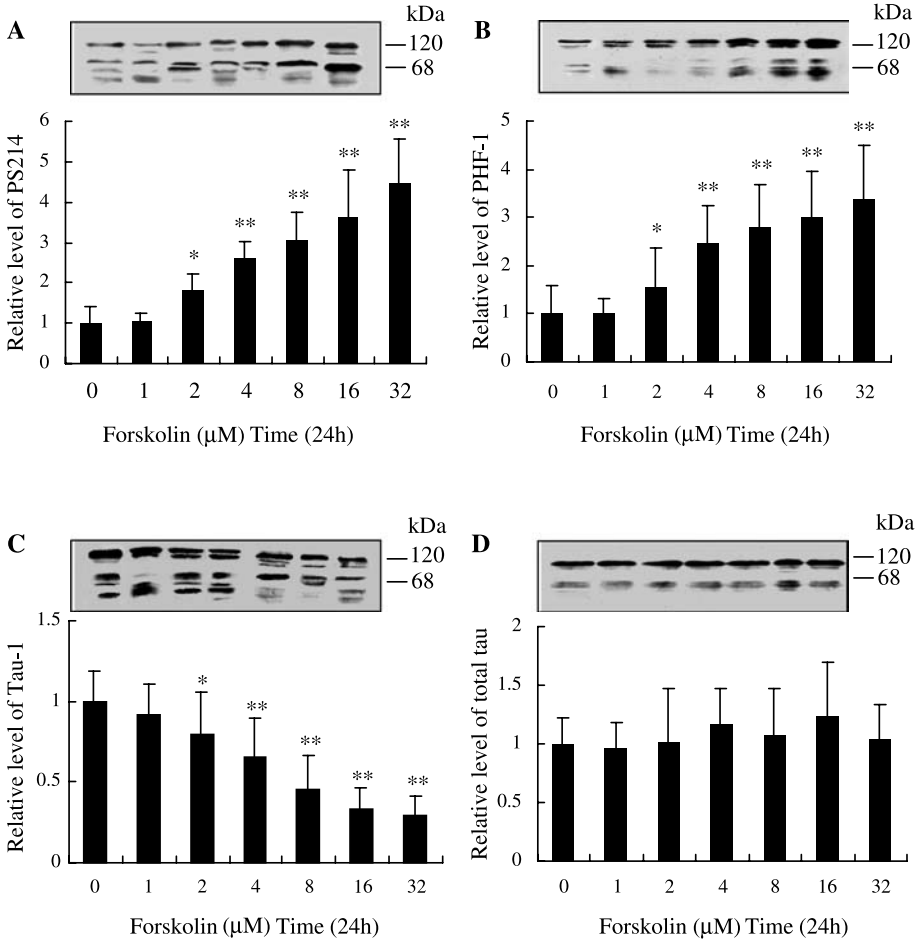

Fig. 1. Concentration-dependent effect of forskolin on tau phosphorylation in N2a/tau441 cells. Forskolin induces tau hyperphosphorylation at PS214 (**A**), PHF-1 (**B**) and Tau-1 (**C**) epitopes in a concentration-dependent manner, it imposed no effect to total tau (**D**). All protein bands were quantitatively analyzed as a one band per lane. The units are arbitrary with control value set at 1.0, and expressed as means ± SD (N = 6). $^{*}P < 0.05$, and $^{**}P < 0.01$ *vs* control

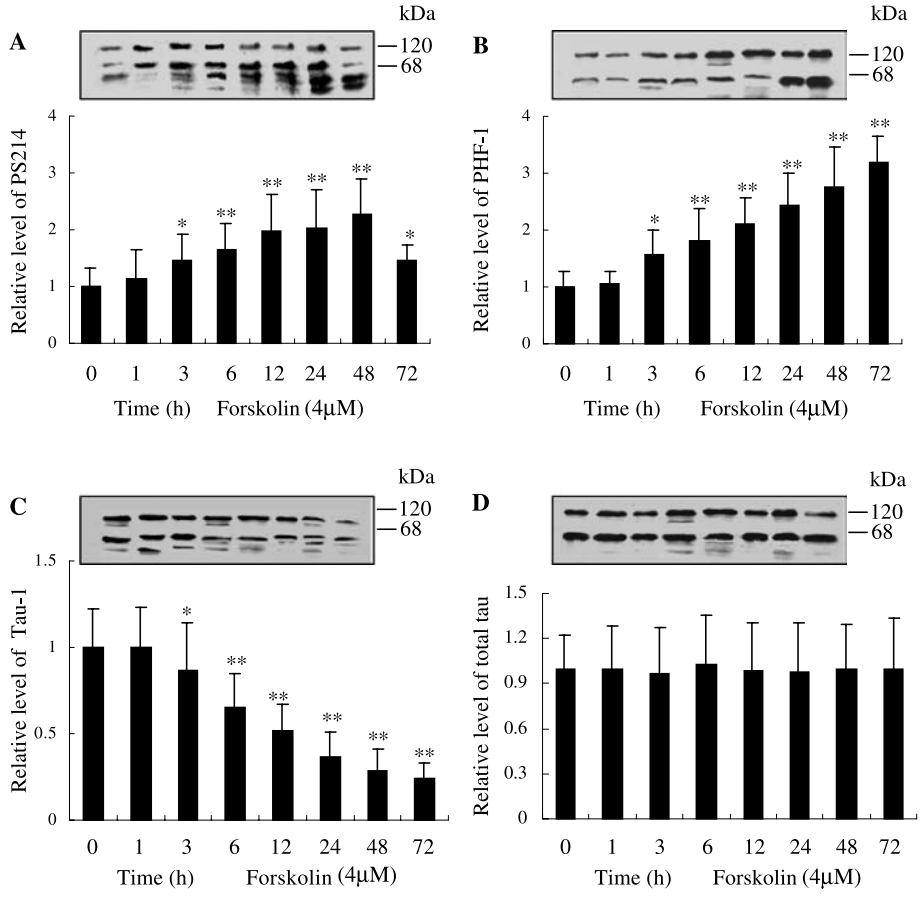

Fig. 2. Time-dependent effect of forskolin on tau phosphorylation in N2a/tau441 cells. Forskolin induces tau hyperphosphorylation at PS214 (**A**), PHF-1 (**B**) and Tau-1 (**C**) epitopes with a time-dependent manner except PS214 at 72 h, it imposed no effect to total tau (**D**). The units are arbitrary with control values set at 1.0, and expressed as means ± SD (N = 6). $^{*}P < 0.05$, and $^{**}P < 0.01$ *vs* control

level of total tau (probed by R134d) was detected among all the above-mentioned detections (Fig. 1D and 2D). These data suggest that forskolin induces tau hyperphosphorylation dose-dependently at both PKA (Ser214) and non-PKA sites (Ser396/404 and Ser199/202) in N2a/tau441 cells.

Activation of PKA is responsible for forskolin-induced tau hyperphosphorylation in N2a/tau441 cells

To confirm the effect of PKA on forskolin-induced tau hyperphosphorylation, we treated the cells simultaneously with 4 μM forskolin and 20 μM Rp-cAMPS, a specific PKA inhibitor, and observed that forskolin-induced hyperphosphorylation of tau at both PKA (Ser214) and non-PKA (Ser396/404 and Ser199/202) sites was reversed to normal level (Fig. 3A–C). The total level of tau probed by R134d was not altered by the treatment of Rp-cAMPS (Fig. 3D). These data suggest strongly that PKA is responsible for the hyperphosphorylation of tau at both PKA and non-PKA sites in this cell line.

Transitory administration of forskolin induces durative tau hyperphosphorylation at non-PKA sites in N2a/tau441 cells

To understand the nature of this PKA-induced tau hyperphosphorylation in AD

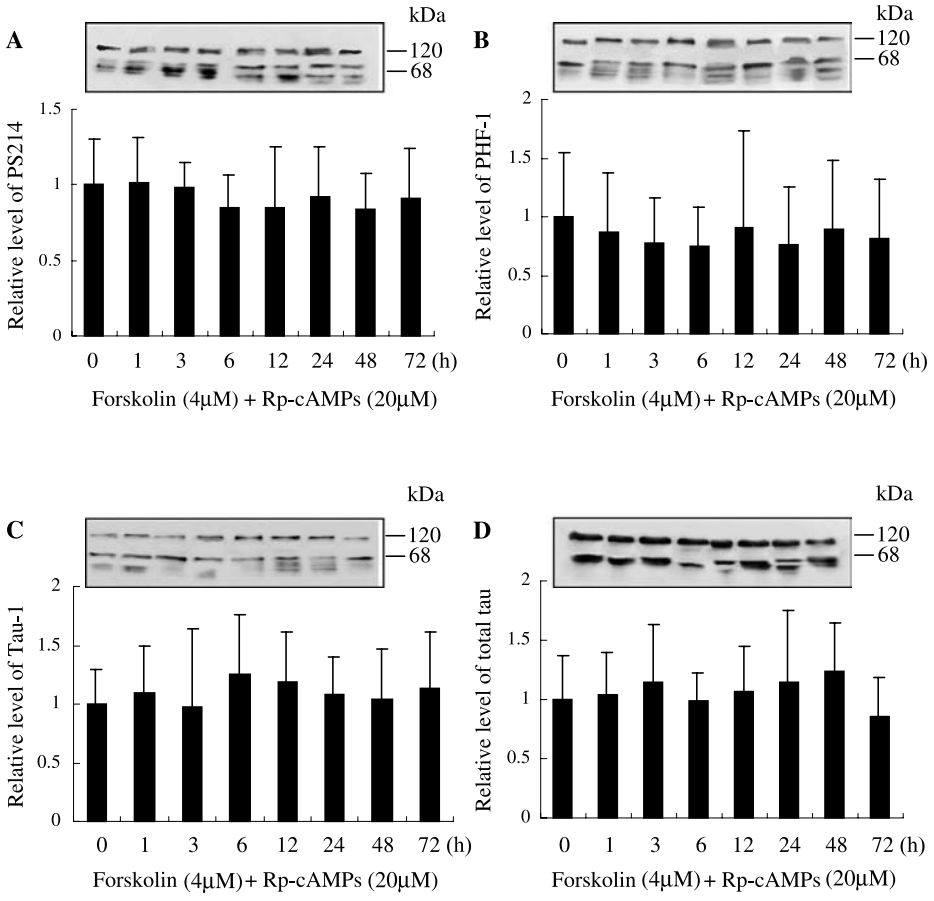

Fig. 3. Simultaneous inhibition of PKA abolishes forskolin-induced tau hyperphosphorylation in N2a/tau441 cells. Forskolin-induced tau hyperphosphorylation at PS214 (**A**), PHF-1 (**B**) and Tau-1 (**C**) epitopes was completely reversed by simultaneous treatment of the cells with 20 μM Rp-cAMPS with no obvious change in total tau (**D**). The units are arbitrary with control values set at 1.0, and expressed as means ± SD (N = 6)

pathology, we compared the difference of tau hyperphosphorylation in cells treated with transitory or durative administration of forskolin (see Materials and methods). The results showed that both transitory and durative forskolin incubation could induce persistent hyperphosphorylation of tau at PS214 (Fig. 4A–C), PHF-1 (Fig. 4D–F) and Tau-1 (Fig. 4G–I) epitopes from 3 h to 72 h. The phosphorylation level of tau at PKA site (Ser214) was lower in transitory group than that of durative forskolin treated group (Fig. 4A–C). On the other hand, the phosphorylation of tau at non-PKA sites (Ser396/404 and Ser199/202) was elevated synchronously without significant difference in both transitory and durative groups, they both reached to the peak immunoactivity at 48 h at PHF-1 sites (Fig. 4D–F) and had a time-dependent decrease in immunoactivity at Tau-1 epitope (Fig. 4G–I), both of them indicate tau hyperphosphorylation. The level of total tau was unchanged for both groups (Fig. 4J–L). These data suggest that a transitory activation of PKA induces a sustained hyperphosphorylation of tau at both PKA and some non-PKA sites, and the effect is more prominent in non-PKA sites.

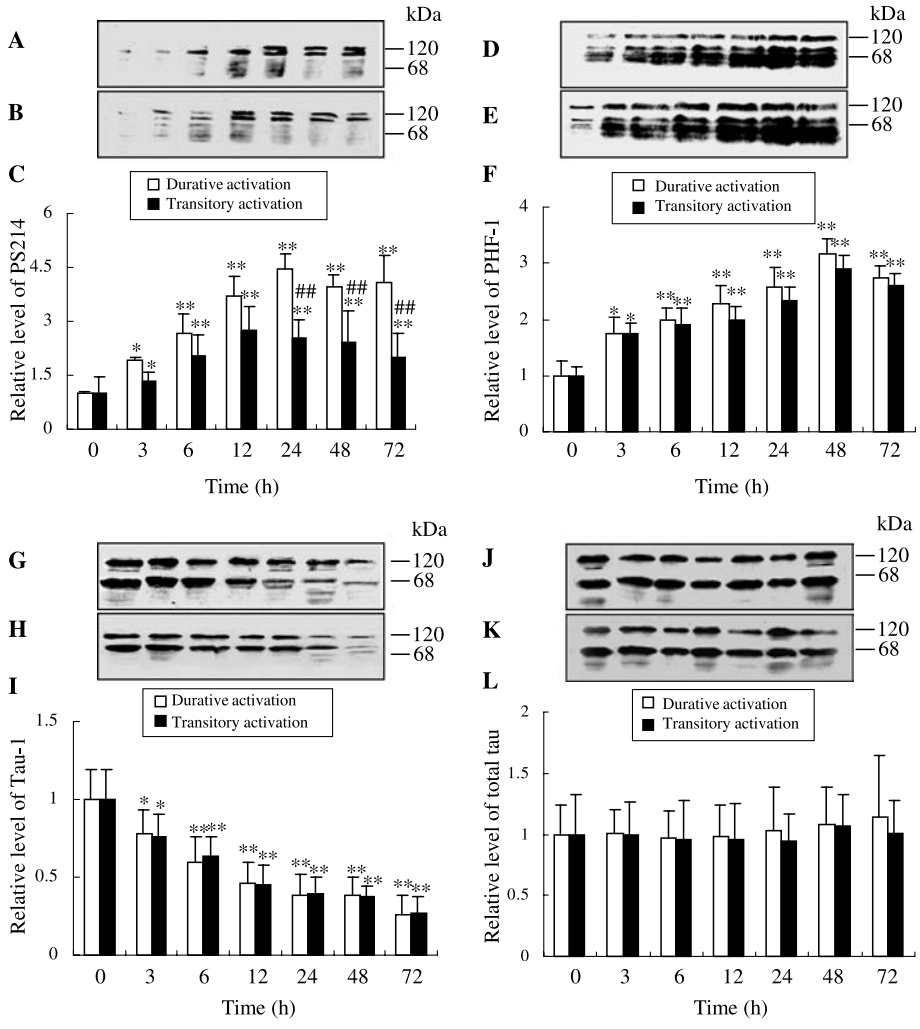

Fig. 4. Effect of durative and transitory forskolin treatment on tau phosphorylation in N2a/tau441 cells. Both durative (**A**, **D**, **G**, **J**) and transitory (**B**, **E**, **H**, **K**) forskolin treatment induce tau hyperphosphorylation at PS214 (**A**, **B**), PHF-1 (**D**, **E**) and Tau-1 (**G**, **H**) epitopes with no change in total tau (**J**, **K**). The units are arbitrary with control values set at 1.0, and expressed as means ± SD (N = 6). $^{*}P < 0.05$, $^{**}P < 0.01$ *vs* untreated control; and $^{\#\#}P < 0.01$ *vs* durative group

Transitory administration of forskolin only induces a transitory activation of PKA with no changes to GSK-3

To further understand the role of PKA in tau hyperphosphorylation in this transitory treated model, we measured the activity of PKA and GSK-3 and compared with durative forskolin group. We found that the activity of PKA was only increased in the first 24 h in transitory forskolin treated group (Fig. 5A), whereas in durative forskolin treated cells, a sustained activation of PKA was observed until 72 h (Fig. 5A). The activity of GSK-3 was not altered under both transitory and durative treated groups (Fig. 5B). This data confirms that only a transitory activation of PKA induces a sustained hyperphosphorylation of tau at some non-PKA sites without changing the activity of GSK-3.

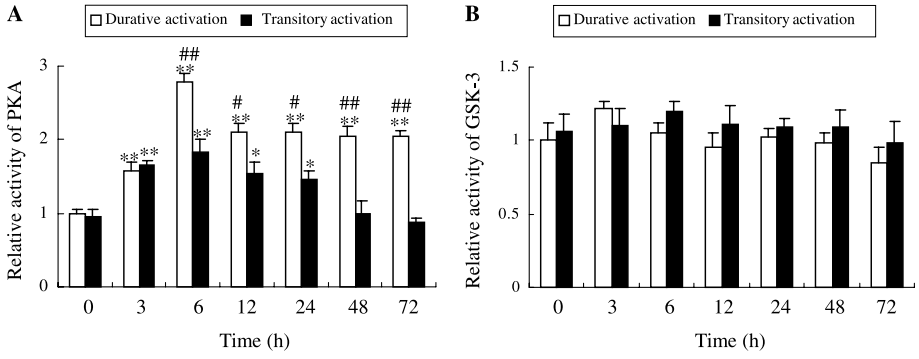

Fig. 5. Effect of durative and transitory forskolin treatment on activities of PKA and GSK-3 in N2a/tau441 cells. Durative treatment induces a sustained PKA activation within 72 h and the highest activation was seen at 6 h after addition of forskolin. Transitory treatment only induces activation of PKA in 24 h with a much lower degree (**A**). No remarkable change of GSK-3 was observed (**B**). The units are arbitrary with control values set at 1.0, and expressed as means ± SD (N = 6). $^{*}P<0.05$, $^{**}P<0.01$ *vs* untreated control; and $^{\#}P<0.05$, $^{\#\#}P<0.01$ *vs* durative group

Discussion

The mechanism of tau hyperphosphorylation has been one of the focal topics in the field of AD research, not only because tau hyperphosphorylation is a pivotal lesion in patients with AD and other tauopathies but also because it is at least one of the primary reasons leading to microtubule destabilization, pretangle stage formation, loss of synapses and eventually cell death (Kosik, 1992; Bhat et al., 2004). The primordial cause for tau hyperphosphorylation is still not understood. Studies have been carried out to search for the candidate kinases and phosphatases as well as related molecular mechanisms involved in the abnormal hyperphosphorylation of tau. In this respect, we have recently reported a novel mechanism by which PKA and GSK-3 abnormally hyperphosphorylate tau where the phosphorylation by the former enzyme PKA primes it for phosphorylation by the latter kinase GSK-3 without the requirement to stimulate its activity to a level above the basal line (Liu et al., 2004).

In the present study, we have firstly reproduced in N2a cell line what we have found previously in rat brain. By using a well-known PKA-specific activator, forskolin (Adashi et al., 1986; Laurenza, 1989), we have observed in N2a/tau441 cell line that forskolin induces a dose- and time-dependent enhancement of tau phosphorylation not only at PKA site (Ser214), but also at some non-PKA sites (Ser199/202 and Ser396/404). This tau hyperphosphorylation was blocked by Rp-cAMPs, a specific PKA inhibitor. Though forskolin could activate PKA, it imposed no influence on the activity of GSK-3, the kinase could robustly phosphorylate tau at Ser199/202 and Ser396/404. And furthermore, inhibition of GSK-3 blocked forskolin-induced tau hyperphosphorylation at these sites (Liu et al., 2004). These results not only confirm the role of PKA but also exclude the possibility that the elevated phosphorylation of tau at Ser199/202 and Ser396/404 was directly from an activated GSK-3 in the system. In addition to GSK-3, other proline-directed protein kinases, such as cyclin-dependent kinase-5 (cdk-5), cell division cyclin-2 (cdc-2) kinase and mitogen-activated protein kinase (MAPK) can also phosphorylate tau at Ser199/202 and Ser396/404 sites, we had excluded the involvement of these kinases in forskolin-induced (Liu et al., 2004). These data together further confirms in cell level that the phosphorylation of tau at the non-PKA sites requires and follows the activation of PKA and the enhancement of

tau phosphorylation at these sites is in no sense with elevation of GSK-3 activity in the cell model.

Previous *in vitro* studies also demonstrated that prephosphorylation of tau by PKA, PKC, CaMKII, CK-1 and CK-2 could significantly promote subsequent tau phosphorylation by GSK-3 at multiple sites while the most potent inhibition of microtubule assembly induced by tau hyperphosphorylation was seen in the combined phosphorylation of PKA and GSK-3 (Wang et al., 1998). The phenomenon that the non-PKA sites phosphorylation induced by other PKA activator (such as isoproterenol) has also been observed in *in vivo* studies (Wang et al., 2004; Sun et al., 2005). From the above information, it is demonstrated that the priming effect of PKA on tau works not only in rat but also in cell models. It is also suggested that activation of PKA alone may be sufficient to produce tau pathology through this priming mechanism. Our speculation to this phenomenon is that prephosphorylation of tau by PKA may change its confirmation and thus, render tau a better substrate for other kinases, and therefore, tau can be further hyperphosphorylated by another kinase, such as GSK-3, at basal activity. Further study using specific site mutagenic tau will confirm the speculation. As wild type N2a cells only expresses very low level of endogenous tau proteins, which was hardly detectable by immunocytochemistry and was mainly shown at 120 kDa by Western blot, we stably transfected the longest isoform of tau (tau441) into the cell for the study. We noticed that there were many tau bands with molecular mass from about 60s kDa to 120 kDa even after single tau gene transfection. As tau is phosphoprotein, we believe that these tau bands, variable depending on the antibody employed, must be generated by differential phosphorylation and other post-translational modifications in the cell system.

Although the increased level of activated form of GSK-3 in AD brain was reported (Pei et al., 1997), however, no elevation of PKA activity has been detected in the post-mortem AD brain until now. Therefore, the significance for the priming effect of PKA on tau needs to be illustrated. Our hypothesis is that a transitory stimulation of PKA, which may happen in early stage of AD pathology, may trigger a sustained tau hyperphosphorylation, an early event in formation of neurofibrillary tangles and neurodegeneration. To test this hypothesis, we have investigated in the present study the differences between transitory and durative induction of PKA in tau phosphorylation and the alteration in relevant kinase activity. By transient and durative incubation of the cells with forskolin, we found that there were significant difference between durative and transitory forskolin groups on PKA activity and PKA site phosphorylation level of tau. On the other hand, both groups showed persistent and similar elevation of tau phosphorylation at some non-PKA sites with no change in GSK-3 activity. It is obvious that even a transitory stimulation of PKA can similarly promote tau phosphorylation by other kinases, such as GSK-3, as a durative activation does. Therefore, we believe that PKA may still play a crucial role in AD-like tau pathology although no increased activity has been detected in the end stage of AD brain. GSK-3 has been recognized to be a promising drug target for AD because of its association with PHF tau formation, neuronal death and decline in cognitive performance and as well as the finding of the elevated active form in AD brain (Pei et al., 1997). The present study suggests another promising drug target for arresting AD pathology in early stage.

In conclusion, this work confirms that phosphorylation of tau by PKA primes it for subsequent hyperphosphorylation by GSK-3 in N2a/tau441 cells. Moreover, even a transitory activation of PKA alone is sufficient to produce a sustained hyperphosphorylation of tau at both PKA and some non-PKA sites. Thus, PKA might be an alternative potential

therapeutic target for AD and other tauopathies that are characterized by abnormal hyperphosphorylation of tau.

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