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Role of protein phosphatase-2A and -1 in the regulation of GSK-3, cdk5 and cdc2 and the phosphorylation of tau in rat forebrain

Malika Bennecib, Cheng-Xin Gong, Inge Grundke-Iqbal, Khalid Iqbal*

Department of Neurochemistry, New York State Institute for Basic Research in Developmental Disabilities, 1050 Forest Hill Road, Staten Island, NY 10314-6399, USA

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Abstract In Alzheimer disease brain the activities of protein phosphatase (PP)-2A and PP-1 are decreased and the microtubule-associated protein tau is abnormally hyperphosphorylated at several sites at serine/threonine. Employing rat forebrain slices kept metabolically active in oxygenated artificial CSF as a model system, we investigated the role of PP-2A/PP-1 in the regulation of some of the major abnormally hyperphosphorylated sites of tau and the protein kinases involved. Treatment of the brain slices with 1.0 μM okadaic acid inhibited ∼65% of PP-2A and produced hyperphosphorylation of tau at Ser 198/199/202, Ser 396/404 and Ser 422. No significant changes in the activities of glycogen synthase kinase-3 (GSK-3) and cyclin dependent protein kinases cdk5 and cdc2 were observed. Calyculin A (0.1 μM) inhibited ∼50% PP-1, ∼20% PP-2A, 50% GSK-3 and ∼30% cdk5 but neither inhibited the activity of cyclin AMP dependent protein kinase A (PKA) nor resulted in the hyperphosphorylation of tau at any of the above sites. Treatment of brain slices with 1 μM okadaic acid plus 0.1 μM calyculin A inhibited ∼100% of both PP-2A and PP-1, ∼80% of GSK-3, ∼50% of cdk5 and ∼30% of cdc2 but neither inhibited PKA nor resulted in the hyperphosphorylation of tau at any of the above sites. These studies suggest (i) that PP-1 upregulates the phosphorylation of tau at Ser 198/199/202 and Ser 396/404 indirectly by regulating the activities of GSK-3, cdk5 and cdc2 whereas PP-2A regulates the phosphorylation of tau directly by dephosphorylation at the above sites, and (ii) that a decrease in the PP-2A activity leads to abnormal hyperphosphorylation of tau at Ser 198/199/202, Ser 396/404 and Ser 422. **© 2000 Federation of European Biochemical Societies. Published by Elsevier Science B.V. All rights reserved.**

Key words: Alzheimer disease; Abnormally hyperphosphorylated tau; Protein phosphatase-2A; Protein phosphatase-1; Glycogen synthase kinase-3; Cyclin dependent protein kinase

1. Introduction

Alzheimer disease (AD) is a neurodegenerative disorder characterized clinically by a progressive and irreversible deterioration of cognitive functions and neuropathologically by the presence of two major histopathological lesions, neuritic senile plaques (SP) and intraneuronal neurofibrillary tangles (NFTs). NFTs consist of paired helical filaments (PHFs) the major protein subunit of which is the hyperphosphorylated microtubule-associated protein tau [1–4]. Unlike normal tau which promotes microtubule assembly and stabilizes the structure of microtubules [5–7] the abnormally hyperphosphorylated tau from AD brain sequesters normal microtubule-associated proteins and causes inhibition and disruption of microtubules in vitro [8–10].

Tau is abnormally hyperphosphorylated at least at 21 sites in AD and about half of tau sites are serine/threonine followed by proline [11,12]. These Ser/Thr–Pro sites are among the major abnormally phosphorylated sites in AD tau. Thus, only proline-directed protein kinase (PDKs) will be expected to phosphorylate tau at these sites. In vitro and cell culture studies have shown that GSK-3 and cdk5/cdc2 are likely to be the major PDPKs that phosphorylate tau [13–16].

All sites of tau phosphorylation including the abnormally hyperphosphorylated sites seen in the AD brain are at Ser/Thr residues [11,12]. Hence the candidate phosphatases are restricted to phosphoseryl/phosphothreonyl protein phosphatases (PP) such as PP-1, PP-2A, PP2B and PP2C. In vitro studies have shown that abnormally phosphorylated tau isolated from AD brain can be dephosphorylated by PP-2A, PP2B and to a less extent by PP-1 but not by PP2C [17–20]. Moreover, a decrease of ∼20% in the activities of PP-2A and PP-1 have been found in AD brain as compared to age-matched controls [21,22]. However, whether this decrease in PP-2A/PP-1 activities contributes to the abnormal hyperphosphorylation of tau by merely a reduction in dephosphorylation of this protein or also in addition indirectly by stimulating the activities of one or more tau kinases is not understood.

Studies of serine–threonine PPs has been facilitated by the availability of potent inhibitors of these enzymes. Among them are okadaic acid and calyculin A which inhibit PP-1 and PP-2A [23,24]. Okadaic acid inhibits PP-2A with an IC_{50} of ∼1 nM; it also inhibits PP-1, but at a 10- to 100-fold higher concentration [24–26]. Calyculin A is structurally different from okadaic acid and inhibits PP-1 and PP-2A with roughly equal potency (IC_{50} of 0.1∼1.0 nM) [24–26].

In this study, employing okadaic acid and calyculin A, we investigated in metabolically active rat brain slices the role of PP-2A and PP-1 in the regulation of the activities of GSK-3, cdk5, cdc2 and PKA and the phosphorylation of tau at several of the sites known to be abnormally hyperphosphorylated in AD. We have found that in rat forebrain (i) the inhibition of PP-2A has no significant effect on the activities of GSK-3, cdk5, cdc2 and PKA and the hyperphosphorylation of tau at Ser 198/199/202, Ser 396/404 and Ser 422 is largely due to reduced dephosphorylation by this phosphatase at these sites; (ii) that PP-1 upregulates the activities of GSK-3, cdk5 and

*Corresponding author. Fax: (1)-718-494 1050.
E-mail: kiqbal@mail.con2.com

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cdc2; and (iii) that GSK-3 and or cdk5/cdc2 phosphorylate tau at Ser 198/199/202, Ser396/404 and Ser 422.

2. Materials and methods

2.1. Preparation and treatment of rat brain slices with phosphatase inhibitors

Female Wistar rats (Charles River Breeding Laboratories), 3 months old, were injected intraperitoneally with 75 mg/kg nembutal, followed by heart puncture. The brain was rapidly removed and cooled in oxygenated (95% O_2/5% CO_2) artificial cerebrospinal fluid (aCSF) containing 150 mM NaCl, 2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 0.5 mM KH_2PO_4, 1.5 mM K_2HPO_4 and 10 mM glucose at pH 7.4. The cerebellum and brain stem were discarded, the forebrain kept and sliced vertically and cross sectionally at every 500 µm using a Mcllwain tissue chopper (Brinkmann Instruments). The slices were washed two times with aCSF and then incubated at 35°C in aCSF either alone or in the presence of 1.0 µM okadaic acid, 0.1 µM calyculin A (both from RBI, Natick, MA, USA) or 1.0 µM okadaic acid along with 0.1 µM calyculin A. The incubation medium was continuously oxygenated. After different periods of time, slices were removed, washed two times and homogenized at 4°C using a Teflon glass homogenizer in 50 mM Tris–HCl, pH 7.0, 10 mM β-mercaptoethanol containing a cocktail of protease inhibitors (1.0 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride, 2 mM benzamidine and 2 µg/ml each of aprotinin, leupeptin and pepstatin). The homogenate was then divided into two parts, one was centrifuged at $16\,000 \times g$ for 20 min and the supernatant was used to assay activities of PP-2A and PP-1. The rest of the homogenate was diluted 1:1 with a phosphatase inhibitor cocktail (20 mM β-glycerophosphate, 2 mM Na_3VO_4 and 100 mM NaF, pH 7.0) and stored at −80°C and used for Western blots and for assaying the activities of GSK-3, cdk5, cdc2 and PKA, as described below.

2.2. Western blots

Samples were subjected to 10% SDS–PAGE as described originally by Laemmli [27]. The protein bands were electrophoretically transferred on Immobilon-P membrane (Millipore, Bedford, MA, USA). The blots were developed with various antibodies to tau (Table 1) followed by ^{125}I-radiolabeled secondary antibodies (Amersham Pharmacia Biotech, Inc) and the bands were visualized and quantitated with a phosphorimager (Fuji film BAS-1500) and TINA 2.0 software (raytest Isotopenmeβgeräte GmbH).

2.3. PP assay

PP activity towards [^{32}P]phosphorylase a was determined by the liberation of ^{32}P as previously described [19]. A PP-1 specific inhibitor, phosphorylated Inhibitor-1 [28], was included in the assay for PP-2A activity. PP-1 activity was calculated by subtracting the PP-2A activity from the total phosphorylase phosphatase activity (PP-1 and PP-2A) assayed in the absence of Inhibitor-1.

2.4. Protein kinase assay

The activities of cdc2, cdk5 and GSK-3 were assayed after immunoprecipitation of these enzymes from rat brain extract with appropriate specific antibodies. The rat brain extract ($16\,000 \times g$), 50 µg protein was mixed with 2 µg of antibodies directed against cdc2 kinase, cdk5 (both antibodies from Santa Cruz Biotechnology, Heidelberg, Germany) or GSK-3 (polyclonal R133d; [29]) in 50 mM Tris, pH 7.4, 150 mM NaCl, 10 mM NaF, 1 mM Na_3VO_4, 2 mM EGTA, 1.0 mM PMSF, 5 µg/ml leupeptin, 5 µg/ml aprotinin, 2 µg/ml pepstatin and 25 µg/ml phosphoramidon. After incubation at 4°C overnight, 30 µl of immobilized protein G suspension (Pierce, Rockford, IL, USA) was added and the reaction mixture was constantly mixed for 2–4 h. Then the mixture was centrifuged and the precipitates were washed three times with immunoprecipitation buffer. Precipitates were suspended in 25 µl of buffer containing 30 mM Tris, pH 7.4, 10 mM NaF, 10 mM $MgCl_2$, 1 mM Na_3VO_4, 2 mM EGTA, 10 mM β-mercaptoethanol, 200 µM [γ-^{32}P]ATP and 1 mg/ml histone (Sigma, St. Louis, MO, USA) or 0.5 mg/ml myelin basic protein (Sigma, St. Louis, MO, USA). Histone was employed for the cdc2 and cdk5 assays, and myelin basic protein was used as a substrate to assay GSK-3 activity. The reaction was initiated by the addition of [γ-^{32}P]ATP and incubation was carried out at 30°C for 10 min for cdc2/cdk5 assay or for 15 min in the case of GSK-3 assay. The reaction was stopped by addition of 25 µl of 300 mM phosphoric acid and a volume of 10 µl of reaction mixture was then spotted in triplicates on phosphocellulose paper (Pierce, Rockford, IL, USA). The filters were washed three times with 75 mM phosphoric acid, dried and counted by Cerenkov radiation. Phosphorylation in the absence of exogenous substrate was subtracted from each total kinase activity. The activity of PKA was assayed in 25 µl reaction mixture containing brain extract (0.05 mg/ml), 70 mM NaHPO4, pH 6.8, 14 mM $MgCl_2$, 1.4 mM EGTA, 5 µM cyclic-AMP (Sigma, St Louis, MO, USA), 30 µM malantide (Sigma, St Louis, MO, USA) and 200 µM [γ-^{32}P]ATP. The reaction was initiated by adding [γ-^{32}P]ATP, followed by incubation at 30°C for 10 min. The rest of the procedure is the same as described above for cdk5/cdc2 and GSK-3 assays.

3. Results

3.1. Inhibition of PP-2A and PP-1 activities by okadaic acid and calyculin A in rat forebrain

In order to understand the molecular mechanism by which the decrease in PP-2A/PP-1 activities found in AD forebrain might lead to the abnormal hyperphosphorylation of tau, we employed as a model system rat forebrain slices kept metabolically active in oxygenated aCSF. The activities of PP-2A/PP-1 in the brain slices were inhibited by treating them with

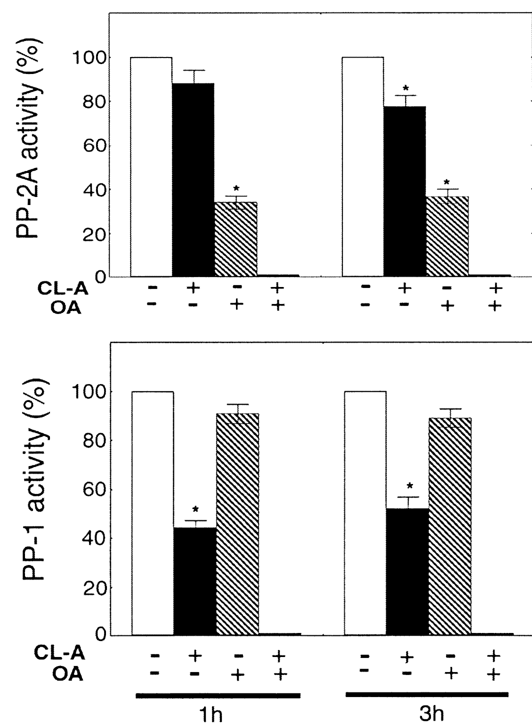

Fig. 1. Inhibition of PP-2A/PP-1 in rat brain slices. Rat brain slices were treated with aCSF as a control, and with either 1.0 µM okadaic acid, 0.1 µM calyculin A or both for 1–3 h. The tissue was then homogenized and the $16\,000 \times g$ extract prepared for assaying the activities of PP-2A and PP-1 as described in Section 2. PP activity was determined using ^{32}P-phosphorylase a as a substrate. Data are expressed as a percentage (mean ± S.D.) of the phosphatase activity detected in aCSF-treated rat brain slices used as a control. Okadaic acid inhibits PP-2A activity by ∼65% ($P < 0.002$) during 1–3 h treatment but had a minimal effect on PP-1 activity. Calyculin A inhibited ∼50% ($P < 0.003$) of PP-1 activity and about 20% ($P < 0.016$) of PP-2A activity. In rat brain slices treated with both okadaic acid and calyculin A, neither PP-2A nor PP-1 activities were detectable.

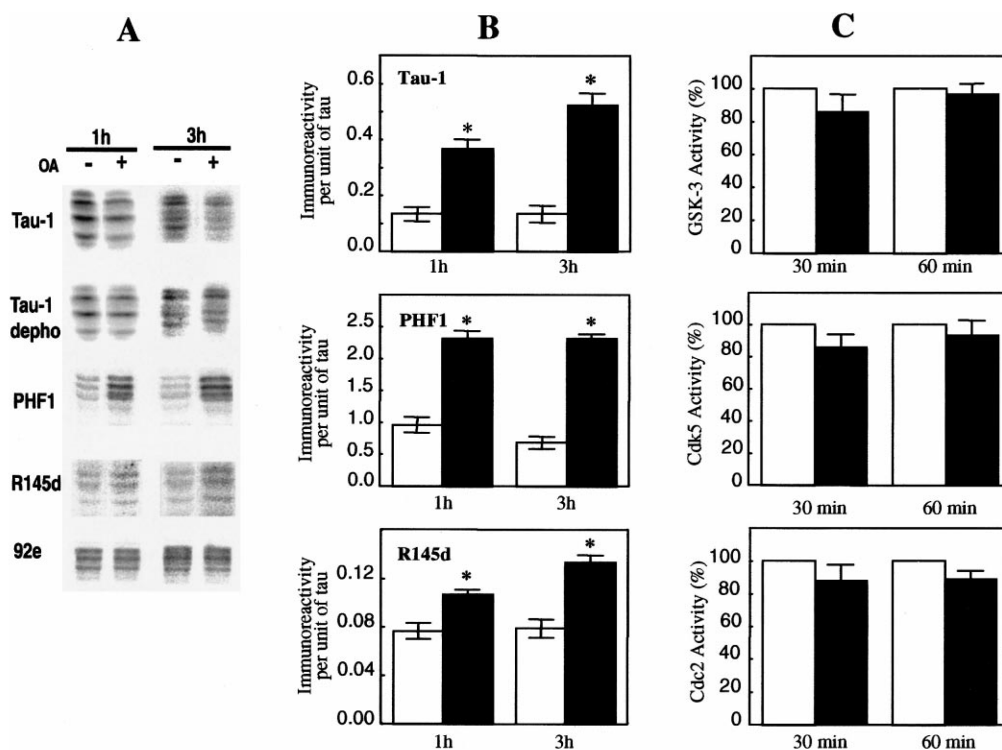

Fig. 2. Effect of inhibition of PP-2A activity on the activities of GSK-3, cdk5, cdc2 and phosphorylation of tau at these specific sites. Rat brain slices were incubated in either oxygenated aCSF as controls or with 1.0 μM okadaic acid, and the changes in phosphorylation status of tau were examined after 1 or 3 h of incubation. A: Homogenates of rat brain slices after treatment for 1 or 3 h were subjected to Western blots developed with phosphorylation dependent tau antibodies (Table 1) as indicated in the right side of each panel. Okadaic acid induced hyperphosphorylation of tau at Ser 198/199/202, Ser 396/404 and Ser 422 as detected by antibodies Tau-1 (before and after treatment of the blot with alkaline phosphatase), PHF1 and R145d respectively. The immunoreactivity at Ser 198/199/202 with Tau-1 antibody decreased in rat brain slices treated with okadaic acid due to phosphorylation at this epitope. The level of total tau as detected with 92e was not affected in rat brain slices treated with okadaic acid. Not shown in this figure, no change was detected at Thr 231/Ser 235 as determined with M4 antibody. B: Immunoblots such as in Fig. 1A were scanned and the immunoreactivity obtained with each antibody was normalized against the total level of tau detected by 92e antibody. Data are expressed as (mean ± S.D.) immunoreactivity per unit of tau. After 1 h of treatment the level of phosphorylation was increased to ∼3.5-fold ($P < 0.005$) at Ser 198/199/202 (Tau-1), >2-fold ($P < 0.014$) at Ser 396/404 (PHF1) and ∼1.5-fold ($P < 0.017$) at Ser 422 (R145d) in okadaic acid-treated rat brain slices (black bars) compared to aCSF samples (open bars). The phosphorylation of tau at Ser 198/199/202 was obtained by subtracting the immunoreactivity detected with Tau-1 antibody from the total immunoreactivity detected with the same antibody in blots pretreated with alkaline phosphatase. The immunoreactivity, which indicates the level of phosphorylation, was then normalized with the total tau immunoreactivity. The immunoreactivity obtained with PHF1 or R145d was normalized with antibody 92 to total tau. Not shown in this figure, no significant change was noticeable with antibody M4 directed against Thr 231/Ser 235. C: The activities of GSK-3, cdc2 and cdk5 were assayed after immunoprecipitation from $16\,000 \times g$ rat brain extract of these enzymes as described in Section 2. Data are expressed as a percentage (mean ± S.D.) of the kinase activity detected in aCSF-treated rat brain slices used as a control. The activities of GSK-3 (toward myelin basic protein), cdc2 and cdk5 (toward histone) were not significantly affected in brain slices after treatment with okadaic acid.

okadaic acid and calyculin A. The treatment of the brain slices with 1.0 μM okadaic acid inhibited ∼65% of PP-2A in 1h and the level of inhibition remained constant up to 3 h studied (Fig. 1). However, by this treatment no significant inhibition of PP-1 was observed. These findings are in agreement with previous studies where up to ∼70% inhibition of PP-2A with 5 μM okadaic acid was achieved in rat brain slices [30]. Calyculin A which is chemically different from okadaic acid inhibited ∼50% of PP-1 and ∼20% of PP-2A in the brain slices during 3 h. These effects of okadaic acid and calyculin A in rat forebrain are considerably weaker than those reported previously in vitro and in cultured cells where nanomolar concentrations of the drugs were found to completely inhibit these phosphatases [23,31]. As observed previously (Bennecib et al., in preparation) a combination of 1 μM okadaic acid and 0.1 μM calyculin A, however, completely inhibited both PP-2A and PP-1 in the brain slices (Fig. 1).

3.2. Regulation of the activities of tau kinases and the phosphorylation of tau by PP-1 and PP-2A

The activities of PP-2A and PP-1 are compromised in the brain of patients with AD and the abnormally hyperphosphorylated tau from AD brain can be dephosphorylated in vitro by PP-2A at Ser 198/199/202, Ser 396/404 [18] and Ser 422 (unpublished data). Several protein kinases are regulated by reversible phosphorylation and some of these kinases are substrates for PP-2A/PP-1. Thus, a decrease in PP-2A or PP-1 or both could result in the hyperphosphorylation of tau either directly from a decrease in dephosphorylation or from an upregulation of one or more tau kinase activity. In the present study, we investigated in brain the role of these two phosphatases both in the regulation of the phosphorylation of tau at the abnormal sites cited above and in the regulation of the protein kinases involved. The treatment of the rat brain slices with 1.0 μM okadaic acid which inhibited ∼65% of PP-2A

(see Fig. 1) resulted in a significant increase in the phosphorylation of tau at Ser 198/199/202, Ser 396/404 and Ser 422 (Fig. 2A,B). Neither any changes in the level of total tau (Fig. 2A, staining with antibody 92e) nor its phosphorylation at Thr 231/Ser 235 (figure not shown) were observed. Furthermore, assays of GSK-3, cdk5 and cdc2 activities in the immunoprecipitates of these enzymes from the $16\,000\times g$ extract of the okadaic acid treated or control-treated brain slices revealed no significant changes in the activities of these protein kinases (Fig. 2C). These findings suggested that PP-2A regulated the phosphorylation of tau at Ser 198/199/202, Ser 396/404 and Ser 422 in rat brain.

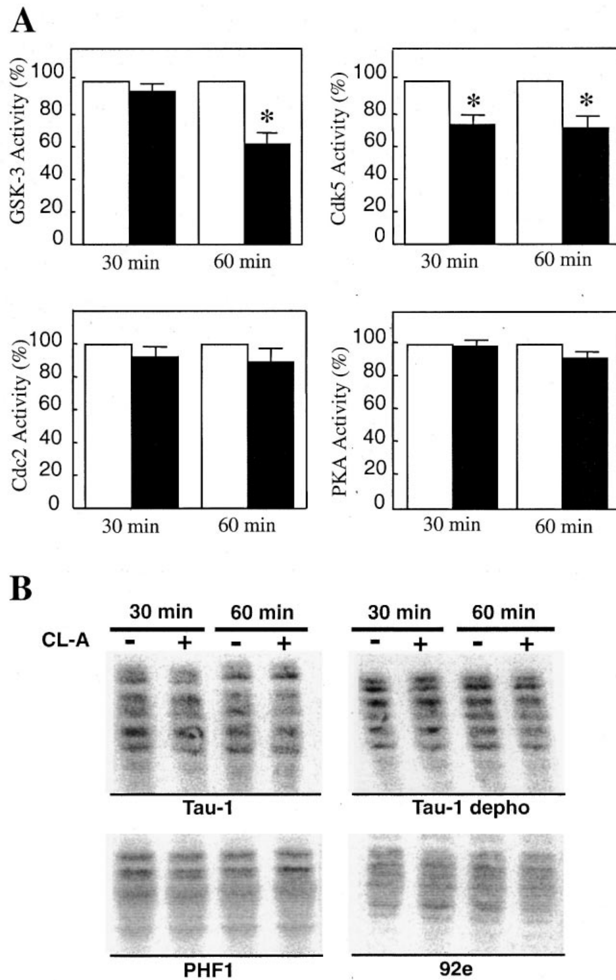

Fig. 3. The effect of inhibition of PP-1 and PP-2 with calyculin A on the activities of GSK-3, cdk5, cdc2 and PKA and the phosphorylation of tau. Rat brain slices were treated in aCSF as controls or with 0.1 μM calyculin A for 30 or 60 min. A: The activities of GSK-3, cdc2 and cdk5 were assayed as in Fig. 2C. The kinase activity of calyculin A-treated samples (black bars) was expressed as the percentage (mean ± S.D.) of the activity of the control sample (open bars). After 60 min of treatment, GSK-3 activity was decreased by ∼40% ($P<0.008$). Cdk5 activity was decreased by ∼30% ($P<0.022$) at 30 min and the inhibition remained constant with time up to the 60 min studied. The activities of cdc2 and PKA were not significantly affected in rat brain-treated with calyculin A. B: Changes in tau phosphorylation were investigated using phosphorylation dependent antibodies as in Fig. 2C. No alteration in tau phosphorylation at any of the sites studied was observed in rat brain slices treated with calyculin A. Not shown in this figure, no significant changes were observed in the phosphorylation of tau at Thr 231/Ser 235 by antibody M4, and at Ser 422 by antibody R145.

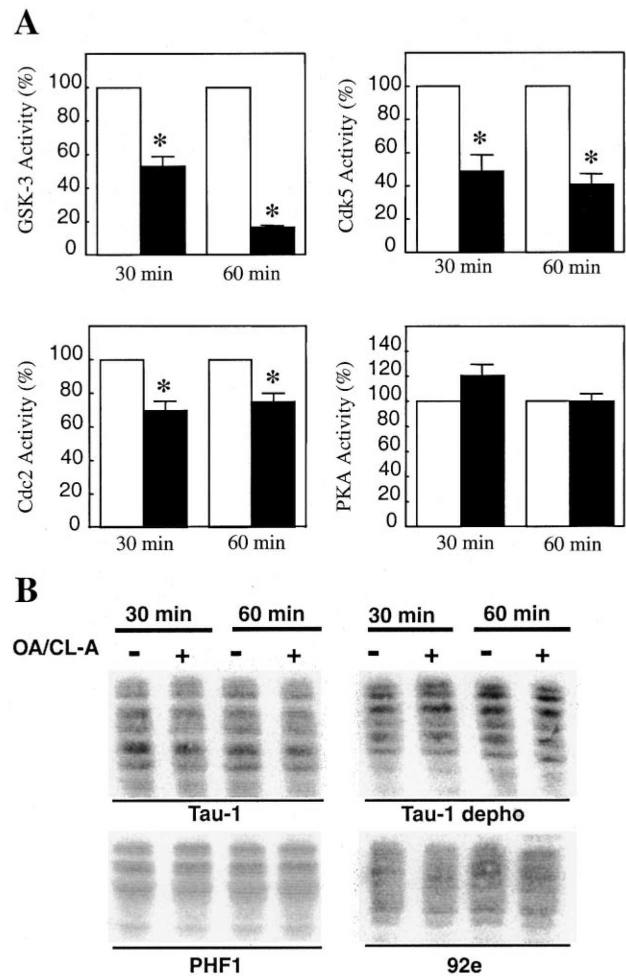

Fig. 4. Effect of inhibition of PP-1 and PP-2A activities with 0.1 μM calyculin A plus 1.0 μM okadaic acid on the activities of GSK-3, cdk5, cdc2 and PKA, and the phosphorylation of tau in rat forebrain slices A: Rat brain slices were treated in aCSF as controls or with 0.1 μM calyculin A plus 1.0 μM okadaic acid for 30 or 60 min. The activities of GSK-3, cdc2 and cdk5 were assayed as in Fig. 2C. The kinase activity of inhibitor-treated brain slices (black bars) was expressed as the percentage (mean ± S.D.) of the activity of the aCSF-treated slices (open bars). The activity of GSK-3 decreased ∼40% ($P<0.004$) in 30 min and ∼80% ($P<0.001$) in 1 h treatment. Cdk5 activity was inhibited by ∼50% ($P<0.012$) at 30 min and the inhibition was maintained up to the 60 min studied. A decrease of ∼30% ($P<0.010$) in cdc2 activity was observed at 30 and 60 min. No significant change in PKA activity was detected. B: Changes in tau phosphorylation were investigated using phosphorylation dependent antibodies as in Fig. 2C. No alteration in tau phosphorylation was observed in rat brain slices treated with calyculin A. Not shown in this figure, no significant changes were observed in the phosphorylation of tau at Thr 231/Ser 235 by antibody M4 and at Ser 422 by antibody R145.

Treatment of the brain slices with 0.1 μM calyculin A which inhibited ∼50% of PP-1 and ∼20% of PP-2A (see Fig. 1) resulted in 1 h in ∼40% inhibition of GSK-3 and ∼30% inhibition of cdk5 and no significant effect on the activities of cdc2 and PKA (Fig. 3A). However, no significant changes in the level of tau phosphorylation at either Ser 198/199/202, Ser 396/404 (Fig. 3B) or Ser 422 (results not shown) were observed, suggesting that PP-1 was little involved in the dephosphorylation of tau at these sites.

The rat brain slices treated with a combination of 1.0 μM

Table 1
Tau antibodies employed and their site-specific phosphorylation dependence

Antibody	Specificity	Sites	Reference
92e	Un+P		Grundke-Iqbal et al., 1988 [51]
Tau-1	UnP	Ser 198/199/202	Grundke-Iqbal et al., 1986 [2] Liu et al., 1993 [52]
12E8	P	Ser 262/356	Seubert et al., 1995 [53]
M4	P	Thr 231/Ser 356	Hasegawa et al., 1995 [54]
PHF1	P	Ser 396/404	Otvos et al., 1994 [55]
R145	P	Ser 422	Tanaka et al., 1998 [31]

unP, unphosphorylated epitope; P, phosphorylated epitope. All antibodies except 92e were monoclonal.

okadaic acid and 0.1 μM calyculin A, which completely inhibited both PP-2A and PP-1 activities (see Fig. 1) produced ∼50% and ∼80% inhibition of GSK-3 activity by 30 and 60 min, respectively (Fig. 4A). The okadaic acid plus calyculin A treatment also inhibited ∼50% of cdk5 and ∼30% of cdc2 in 30 min which persisted up to 60 min studied. However, no significant change in the activity of PKA between the drug-treated and aCSF-treated control brain slices was observed (Fig. 4A). Quantitative ^{125}I-Western blots of the brain slices developed with tau antibodies did not reveal any significant changes in either the levels or the phosphorylation of tau at Ser 198/199/202, Ser 396/404 (Fig. 4B), Ser 422 and Thr 231/Ser 235 (data not shown). These findings suggested that the activities of GSK-3 and cdk5/cdc2 in brain are regulated by PP-1 and one or more of these kinases are involved in the phosphorylation of tau at Ser 198/199/202 and Ser 396/404 but PP-1 does not appear to dephosphorylate tau at these sites. The phosphorylation of tau at Thr 231/Ser 235 as determined by immunostaining with the phosphorylation dependent antibody M4 was weak in aCSF-treated control brain slices. Neither of the above treatments with PP-2A/PP-1 inhibitors produced any increase in the phosphorylation of tau at Thr 231/Ser 235 (figure not shown) suggesting that tau at this site is not dephosphorylated by either PP-2A or PP-1.

4. Discussion

Identification of the protein kinases and phosphatases involved in the generation of abnormally phosphorylated tau is critical to understanding the pathogenesis of AD. Although the mechanism leading to hyperphosphorylation of tau is still unclear, growing evidence suggests that one or more PP activities might be responsible. The present study suggests (i) that in forebrain PP-2A regulates the phosphorylation of tau at Ser 198/199/202, Ser 396/404 and Ser 422 directly by dephosphorylating these sites; (ii) that tau is phosphorylated at above sites by GSK-3 and or cdk5/cdc2, (iii) that PP-1 upregulates the activities of GSK-3, cdk5 and cdc2 but has no effect on PKA activity; and (iv) that PP-2A has no significant effect on the activities of GSK-3, cdk5, cdc2 or PKA. These findings suggest that the abnormal hyperphosphorylation of tau at Ser 198/199/202, Ser 396/404 and Ser 422 seen in AD are most likely the result of the decreased PP-2A activity.

Treatment of the brain slices with 1.0 μM okadaic acid which inhibited ∼65% of PP-2A activity and showed no inhibition of PP-1 resulted in the hyperphosphorylation of tau at Ser 198/199/202, Ser 396/404 and Ser 422 but not at Thr 231/Ser 235. Furthermore, this treatment of the brain slices did not affect the activity of GSK-3, cdk5 or cdc2. In contrast, the treatment of the brain slices with 0.1 μM calyculin A, which inhibited ∼50% of PP-1 and ∼20% of PP-2A, produced ∼50% inhibition of GSK-3, ∼30% inhibition of cdk5 and ∼20% of cdc2 and did not result in any significant change in the phosphorylation of tau at the above sites. These studies suggest that PP-2A and not PP-1 dephosphorylates tau at Ser 198/199/202, Ser 396/404 and Ser 422. Consistent with our previous in vitro studies which showed dephosphorylation of tau at Thr 231/Ser 235 by PP-2B and not by PP-2A or PP-1 [17–19], the present study shows that this site is not dephosphorylated by either PP-2A or PP-1 in forebrain.

While inhibition of the PP-2A activity by okadaic acid did not have any effect on the activities of GSK-3, cdk5 or cdc2, inhibition of PP-1 activity by calyculin A and by calyculin A plus okadaic acid resulted in a PP-1 inhibition dependent inhibition of the above kinases. These findings together with the hyperphosphorylation of tau observed by inhibition of PP-2A alone (see above) suggest that PP-1 upregulates the activities of GSK-3, cdk5 and cdc2 and does not dephosphorylate tau at either of the sites studied. The activities of all the three PDPKs investigated in the present study i.e. GSK-3, cdk5 and cdc2 are known to be regulated by phosphorylation. The activity of cdk5 which is enriched in adult brain, especially neurons [32–35] is regulated both by p35/p25 [33,34] and by phosphorylation (reviewed by Morgan [36]). Phosphorylation of cdk5 at Thr 14 leads to its inhibition [37]. In the present study inhibition of PP-1 activity decreased the cdk5 activity to ∼50%. Since the cdk5 activity also depends on the binding of its activator p35/p25, we assayed the level of p35/p25 by quantitative ^{125}I-Western blots and found no significant change (data not shown), suggesting that the decrease in cdk5 activity in the PP-1-inhibited brain slices was not due to any decrease in the level of this activator. Thus, the present study suggests that PP-1 might upregulate the activity of cdk5 by dephosphorylating it at Thr 14.

Cdc2 is subject to both positive and negative regulation by phosphorylation [38]. Cdc2 requires phosphorylation by a second protein kinase, known as CDK-activating kinase (CAK) at Thr 161 (reviewed by Solomon, [39]). CAK is a nuclear protein kinase in the CDK family in which cdk7 is complexed to cyclin H [39]. Interestingly, CAK activity itself requires phosphorylation of cdk7 at Thr 176 by another protein kinase, CAK activating kinase [40,41]. P-Thr 161 can be dephosphorylated by PP-2A in vitro [42,43]. Phosphorylation of cdc2 at Thr 14 or Tyr 15 negatively regulates the enzyme [44]. The major PP that dephosphorylates the cdc2 inhibitory sites is cdc25 [38,45]. Cdc25 is a dual specificity PP that can dephosphorylate both pThr14 and pTyr15. In the present study only ∼30% inhibition of cdc2 was observed when the activities of PP-1 and PP-2A were ∼100% inhibited in the brain slices treated with okadaic acid plus calyculin A. Calyculin A alone (∼50% inhibition of PP-1 and ∼20% inhibition of PP-2A) or okadaic acid alone (∼65% inhibition of

PP-2A) did not induce any significant decrease in cdc2 activity. These results suggest that either the treatment of brain slices with okadaic acid plus calyculin A results in some inhibition of cdc25 or PP-1 also dephosphorylates cdc2 at Thr 14 but poorly. Alternatively, PP-1 might be involved in the regulation upstream of cdc2.

Phosphorylation of GSK-3α at Ser 21 and GSK-3β at Ser 9 results in inhibition of the kinase [46–50]. GSK-3 is also constitutively phosphorylated at Tyr 279 in GSK-3α and at Tyr 216 in GSK-3β and dephosphorylation of these residues results in inactivation of the enzyme. In the present study ∼65% inhibition of PP-2A activity by 1.0 μM okadaic acid in rat brain slices produced no significant changes in GSK-3 activity. These findings are in agreement with our previous study where treatment of SY5Y neuroblastoma-cultured cells with okadaic acid that completely inhibited PP-2A activity did not result in any significant change in GSK-3 activity [31]. However, unexpectedly in the present study the inhibition of PP-1 activity by calyculin A alone or calyculin A plus okadaic acid inhibited up to ∼80% of GSK-3 activity. This unexpected effect of PP-1 on GSK-3 probably involves some upstream regulation the nature of which remains to be determined.

In conclusion the present study in forebrain suggests that tau is phosphorylated at Ser 198/199/202, Ser 396/404 and Ser 422 by GSK-3 and or cdk5/cdc2 and is dephosphorylated by PP-2A. PP-1 upregulates the activities of GSK-3, cdk5 and cdc2. In AD brain the hyperphosphorylation of tau produced by the decrease in PP-2A activity probably more than off sets the decrease in the phosphorylation of tau at the above sites produced by a decrease in PP-1 activity through downregulation of the activities of GSK-3, cdk5 and cdc2. Phosphorylation of tau at Thr 231/Ser 235 does not appear to be regulated by PP-2A. These findings taken together with our recent study in which we found that PP-2A upregulates the cytosolic CaMKII activity and the phosphorylation of tau at Ser 262/356 in forebrain (Bennecib et al., in preparation) suggests that PP-2A probably plays a major role in the abnormal hyperphosphorylation of tau. Thus, PP-2A is a promising therapeutic target for AD and related taupathies that are characterized by the neurofibrillary degeneration associated with the abnormally hyperphosphorylated tau.

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