Inhibition of PP-2A upregulates CaMKII in rat forebrain and induces hyperphosphorylation of tau at Ser 262/356

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Abstract The regulation of the activity of CaMKII by PP-1 and PP-2A, as well as the role of this protein kinase in the phosphorylation of tau protein in forebrain were investigated. The treatment of metabolically active rat brain slices with 1.0 μM okadaic acid (OA) inhibited ∼65% of PP-2A and had no significant effect on PP-1 in the 16 000×*g* tissue extract. Calyculin A (CL-A), 0.1 μM under the same conditions, inhibited ∼50% of PP-1 and ∼20% of PP-2A activities. In contrast, a mixture of OA and CL-A practically completely inhibited both PP-2A and PP-1 activities. The inhibition of the two phosphatase activities or PP-2A alone resulted in an ∼2-fold increase in CaMKII activity and an ∼8-fold increase in the phosphorylation of tau at Ser 262/356 in 60 min. Treatment of the brain slices with KN-62, an inhibitor of the autophosphorylation of CaMKII at Thr 286/287, produced ∼60% inhibition in CaMKII activity and no significant effect on tau phosphorylation at Ser 262/356. The KN-62-treated brain slices when further treated with OA and CL-A did not show any change in CaMKII activity. In vitro, both PP-2A and PP-1 dephosphorylated tau at Ser 262/356 that was phosphorylated with purified CaMKII. These studies suggest (i) that in mammalian forebrain the cytosolic CaMKII activity is regulated mainly by PP-2A, (ii) that CaMKII is the major tau Ser 262/356 kinase in brain, and (iii) that a decrease in PP-2A/PP-1 activities in the brain leads to hyperphosphorylation of tau not only by inhibition of its dephosphorylation but also by promoting the CaMKII activity.

Key words: Alzheimer disease; Abnormally hyperphosphorylated tau; Protein phosphatase-2A; Protein phosphatase-1; Calcium calmodulin protein kinase II; Metabolically active brain slice

1. Introduction

Type II Ca^{2+}/calmodulin-dependent protein kinase (CaMKII) is a key regulatory enzyme and a number of studies have been carried out to understand its functions in the brain. It is the most abundant of the known brain Ca^{2+}-regulated protein kinases [2], constituting about 1% of total brain protein [3]. It is expressed primarily in neurons and had been found in several subcellular pools including cytosolic, membrane, cytoskeletal and nuclear fractions [4]. Rat brain CaMKII is an oligomeric enzyme with a native molecular weight of about 540 000 and five subunits (α, β/β′, γ, δ) have now been identified using molecular techniques. The α and β subunits are predominantly expressed in the nervous system. The α subunit predominates in the forebrain [3,4], while the β and the β′ subunits predominate in the cerebellum.

Several studies have focused on the characterization of the structural and regulatory properties of the CaMKII. In the absence of Ca^{2+}/calmodulin (CaM), CaMKII is essentially inactive toward exogenous substrates due to interaction of an autoinhibitory domain (residues 281–302) with the catalytic domain (residues 1–260) [5,6]. Ca^{2+}/CaM binding to residues 296–309 [6] disrupts this inhibitory interaction. Therefore, in the presence of Ca^{2+}/CaM the kinase can phosphorylate exogenous substrates. CaMKII is known to be regulated by autophosphorylation at multiple sites with profound effects on its regulatory properties. In the presence of Ca^{2+}/CaM, Thr 286/287 (α/β subunits, respectively) are the major sites of autophosphorylation (e.g. [7]). This autophosphorylation results in the conversion of the kinase to a Ca^{2+}-independent form (e.g. [7,8]), full activation of the total activity [9–11], and trapping of Ca^{2+}/CaM [12]. Removal of Ca^{2+}/CaM from the autophosphorylated kinase stimulates Ca^{2+}-independent autophosphorylation at Thr 305 and/or Thr 306, as well as Ser 314 [13,14]. This Ca^{2+}-independent autophosphorylation inhibits consequent Ca^{2+}/CaM binding to CaMKII and results in a decrease in the activity of the kinase [13–15]. Therefore, the identification of protein phosphatases that dephosphorylate CaMKII is of great interest to understand the regulation of its activity. It has so far been reported that protein phosphatase-1 (PP-1) [16], protein phosphatase-2A (PP-2A) [7,17,18], and PP-2C [19] dephosphorylate Thr 286 in vitro and that the dephosphorylation is catalyzed by distinct phosphatases in distinct subcellular compartments [20]. Thr 306 was shown to be dephosphorylated by PP-2A [15]. However, which protein phosphatases regulate CaMKII in the brain was not reported.

CaMKII phosphorylates a broad range of substrate proteins including the microtubule-associated protein tau [21,22]. In Alzheimer disease (AD), one of the hallmark neuropathological lesions is the accumulation of paired helical filaments (PHFs) of which the major protein subunit is the abnormally hyperphosphorylated tau [23,24]. Among 21 sites identified in PHF tau, one critical site is Ser 262, the only site located within the microtubule binding domain of tau. Phosphorylation of tau at this site reduces the ability of tau to bind to microtubules and to promote their assembly [25,26].

Tau can be phosphorylated at Ser 262/356 in vitro by CaMKII [26], protein kinase A (PKA) [27,28], $Mark^{110}$ kinase [29],

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glycogen synthase kinase-3 (GSK-3) [30], casein kinase-1 [26], protein kinase C [26] and phosphorylase kinase [31]. In a previous study, we have shown that the phosphorylation of tau at Ser 262/356 in metabolically active rat brain slices is positively and negatively modulated in the presence of CaMKII activator, bradykinin, and inhibitor, KN-62, respectively [32]. In the present study we describe the role of PP-2A and PP-1 in the regulation of the activity of CaMKII and the phosphorylation of tau at Ser 262/356 by this protein kinase in rat forebrain cytosol.

2. Materials and methods

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

Female Wistar rats, 3 months old, were killed by a lethal dose of Nembutal (intraperitoneal injection of 75 mg Nembutal per kg body weight) 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 was sliced vertically and cross-sectionally at every 500 μm using a Mcllwain Tissue Chopper (Brinkman Instruments). The slices were washed two times with aCSF and then incubated at 35°C in oxygenated aCSF either alone or in the presence of phosphatase and or kinase inhibitors as described in Section 3. The incubation medium was continuously oxygenated. After different periods of time the brain slices were removed, washed two times and homogenized at 4°C using a Teflon-glass homogenizer in 50 mM Tris–HCl, pH 7.0, containing 10 mM β-mercaptoethanol (β-ME) and a cocktail of protease inhibitors (1 mM EDTA, 0.1 mM phenylmethyl sulfonyl fluoride (PMSF), 2.0 mM benzamidine and 2.0 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, 2.0 mM Na_3VO_4 and 100 mM NaF, pH 7.0) and either used for Western blots, or centrifuged at $16\,000\times g$ for 20 min and the resulting supernatant used to determine the activities of CaMKII and cyclic AMP-dependent protein kinase (PKA).

2.2. Western blots

[^{125}I] Western blots of brain homogenate were developed as described previously [33]. To determine the phosphorylation state of tau protein, the blots were probed with the following tau antibodies: monoclonal antibodies (mAbs) PHF1 (1:200) to P-Ser 396/404 [34], 12E8 (1:500) to P-Ser 262/356 [35], M4 (1:2000) to P-Thr 231/Ser 235 [36], Tau-1 (1:25 000) to Ser 195/198/199/202 [23,37], or polyclonal antibodies (pAbs) R145 (1:3000) to P-Ser 422 [38] and 92e (1:5000) to total tau [39].

2.3. Protein phosphatase assays

Activities of PP-2A and PP-1 were assayed towards [^{32}P]phosphorylase a as a substrate as we described previously [33,40]. The phosphatase activity was assayed in 50 mM Tris, pH 7.0, 10 mM β-ME, 0.1 mM EGTA, 7.5 mM caffeine, 2 mg/ml [^{32}P]phosphorylase and 0.1 mg/ml brain extract. A PP-1 specific inhibitor, phosphorylated inhibitor-1 [41], was included in the assays for PP-2A activity. PP-1 activity was calculated by subtracting the PP-2A activity from the total phosphatase activity (PP-1+PP-2A) assayed in the absence of inhibitor-1.

2.4. Protein kinase assay

CaMKII activity was measured in 25 μl of buffer containing 60 mM HEPES, pH 7.5, 10 mM $MgCl_2$, 2.0 mM $CaCl_2$, 10 mM β-ME, 10 mM EGTA, 10 μg/ml calmodulin (Sigma, St. Louis, MO, USA), 20 μM Syntide 2 (Sigma, St. Louis, MO, USA), 0.2 mg/ml brain extract and 200 μM [γ-^{32}P]ATP. The reaction was initiated by adding [γ-^{32}P]ATP. After incubation for 10 min at 30°C, 25 μl of 300 mM phosphoric acid was added to terminate the reaction and 10 μl of sample was spotted on to phosphocellulose membrane. The membrane was then washed three times in 75 mM phosphoric acid to remove non-protein incorporated ^{32}P, dried and counted by Cerenkov radiation. The activity of PKA was determined as above except the reaction mixture contained 0.05 mg/ml brain extract, 70 mM $NaHPO_4$, pH 6.8, 14 mM $MgCl_2$, 1.4 mM EGTA, 5.0 μM cyclic AMP (Sigma, St. Louis, MO, USA), 30 μM malantide (Sigma, St. Louis, MO, USA) and 200 μM [γ-^{32}P]ATP.

2.5. In vitro phosphorylation of tau_{410} by CaMKII followed by dephosphorylation by PP-2A or PP-1

The human tau clone 39 which encodes tau isoform 3L (tau_{410}) was subcloned in *Escherichia coli* and the recombinant protein was purified from cell extracts as described previously [42]. Tau_{410} was phosphorylated at 30°C in a reaction mixture containing 1.6 μg/ml purified CaMKII (Calbiochem, San Diego, CA, USA), 0.4 mg/ml tau_{410}, 5.0 mM $MgCl_2$, 2.0 mM $CaCl_2$, 10 mM β-ME, 60 mM HEPES, pH 7.5, 1.0 mM PMSF, 0.1 mg/ml bovine serum albumin, 10 μg/ml of each leupeptin, pepstatin, aprotinin, 10 μg/ml calmodulin and 0.1 mM ATP. The reaction was initiated by addition of ATP. After 60 min of incubation, the reaction mixture was heated for 5 min at 95°C and centrifuged at $16\,000\times g$ for 5 min. Tau, which is heat stable under these conditions, was then removed as supernatant and aliquoted. The dephosphorylation of tau_{410} was conducted by adding to 30 μl of supernatant either the catalytic subunit of PP-2A (final concentration 0.9 μg/ml) or PP-1 (final concentration 0.45 μg/ml), both purified from bovine brain according to the method of Cohen et al. [43]. After 2 h of incubation at 30°C the reaction was stopped by addition of SDS–PAGE sample buffer and 10 ng of tau_{410} per lane was then loaded for Western blots.

3. Results

3.1. Okadaic acid (OA) and calyculin A (CL-A) inhibit PP-2A and PP-1 in rat brain slices

Unlike in vitro and in cultured cells where OA can completely inhibit both PP-2A and PP-1 activities [38,44], in brain slices only up to ∼70% of PP-2A and insignificant PP-1 activities are inhibited [40]. Since OA and CL-A are chemically quite different and probably act on different sites of the phosphatases, we investigated the inhibition of the activities of PP-2A and PP-1 by OA and CL-A separately and in combination. OA (1.0 μM) inhibited ∼65% of PP-2A activity while PP-1 activity remained practically unchanged (Table 1). During 1 h of treatment of the brain slices with 0.1 μM CL-A, a potent inhibitor of both PP-1 and PP-2A, ∼50% of PP-1 and ∼20% of PP-2A activities were inhibited. Interestingly, when rat brain slices were treated with both CL-A (0.1 μM) and OA (1.0 μM), neither PP-2A nor PP-1 activity could be detected. Thus, a combination of protein phosphatase inhibitors proved

Table 1
Inhibition of PP-2A and PP-1 by OA and CL-A in rat brain slices

Treatment	Protein phosphatase activity (%)			
	PP-2A		PP-1	
	30 min	60 min	30 min	60 min
aCSF	100	100	100	100
OA (1.0 μM)	34 ± 2	37 ± 2	91 ± 2	89 ± 2
CL-A (0.1 μM)	88 ± 3	78 ± 3	44 ± 2	52 ± 3
OA and CL-A	ND	ND	ND	ND

Rat brain slices were treated with 1.0 μM OA, 0.1 μM CL-A or both for 30 or 60 min. The tissue was then homogenized and $16\,000\times g$ extract used for assaying the activities of PP-2A and PP-1. Protein phosphatase activity was determined using [^{32}P]phosphorylase a as a substrate. Data are expressed as a percentage of the phosphatase activity detected in aCSF-treated rat brain slices. Means ± S.D. of three experiments are presented. ND, not detectable.

to be a very useful approach to achieve a complete inhibition of both PP-2A and PP-1 in brain.

3.2. OA and CL-A stimulate CaMKII activity

In vitro studies have suggested that PP-2A can have both inhibitory and stimulatory effects on CaMKII because it can dephosphorylate the kinase at Thr 286/287 [45] and at Thr 305/306 [15] which inhibits and stimulates its activity, respectively. CaMKII in post synaptic densities can be dephosphorylated by PP-1 at Thr 236/287 [45]. Thus, from in vitro studies it is difficult to predict the effect of the activities of PP-2A and PP-1 on CaMKII in vivo. In the present study we investigated the regulation of the cytosolic CaMKII activity by PP-1 and PP-2A in rat brain.

Rat brain slices were treated with a mixture of 1.0 μM OA and 0.1 μM CL-A in aCSF. At different incubation times the slices were removed, homogenized and 16 000 × *g* extract prepared from them. The activities of CaMKII and PKA were determined in the extract. The activity of cytosolic CaMKII was markedly and specifically increased after treatment of the brain slices with OA plus CL-A (Fig. 1A), whereas no significant change in PKA activity was observed (Fig. 1B). These studies suggested that in rat forebrain PP-2A or PP-1 or both might regulate CaMKII activity by dephosphorylation of the kinase at Thr 286/287. In rat brain slices where both PP-2A and PP-1 activities were totally inhibited by OA plus CL-A, CaMKII activity increased up to 220% after 10 min of treatment and slightly decreased to ~170% by 60 min (Fig. 1A). This decrease in CaMKII activity might be the result of an unchecked autophosphorylation of the kinase at Thr 305/306 due to complete inhibition of PP-2A [15], or a slow dephosphorylation of CaMKII at Thr 286/287 by other protein phosphatases such as PP-2C or CaMKII phosphatase, an OA and CL-A insensitive phosphatase [19,46].

3.3. OA and CL-A increase tau phosphorylation at Ser 262/356

The state of tau phosphorylation was investigated using a battery of phosphorylation-dependent antibodies as described in Section 2. The treatment of brain slices with OA plus CL-A resulted in hyperphosphorylation of tau at Ser 262/356 as determined with the mAb 12E8 (Fig. 1C,D). The level of total tau as determined by [^{125}I] Western blots developed with pAb

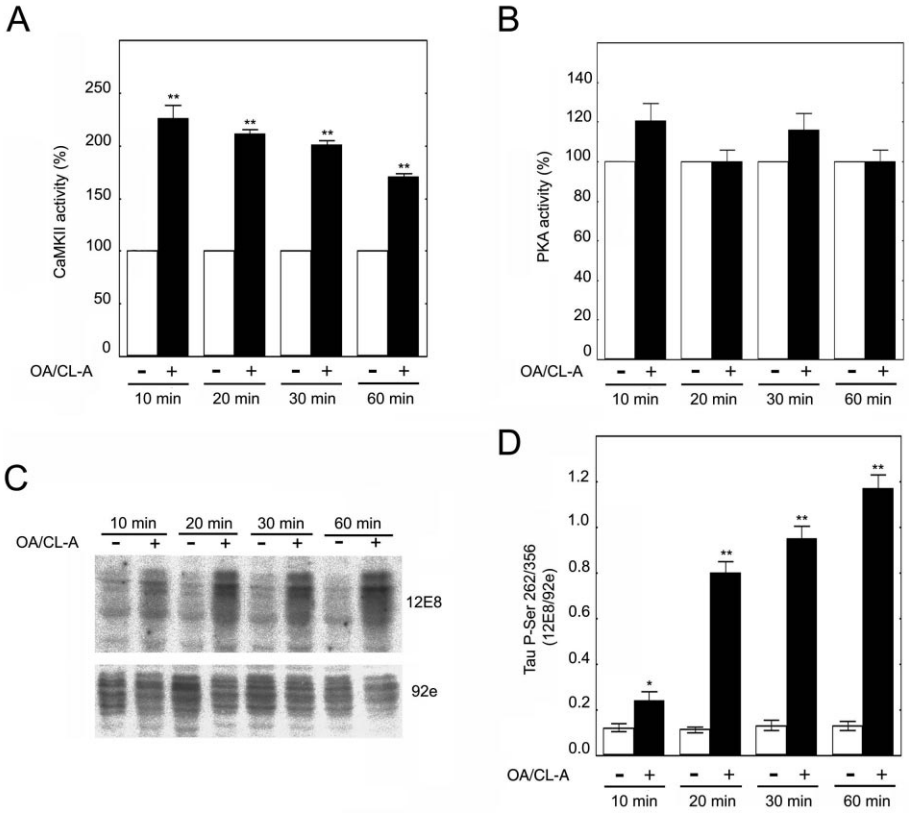

Fig. 1. OA plus CL-A stimulate CaMKII activity and increase phosphorylation of tau at Ser 262/356 in rat forebrain. Rat brain slices were treated with aCSF alone or in the presence of 1.0 μM OA and 0.1 μM CL-A for up to 60 min, and then homogenized. The tissue homogenate was either used for Western blots or centrifuged at 16 000 × *g* for 20 min and the extract used for the kinase assays as described in Section 2. The activities of (A) CaMKII and (B) PKA of inhibitors-treated samples (black bars) were expressed as the percentage of the activities of the control samples (open bars) treated with aCSF alone for the same period of incubation. OA/CL-A stimulated the activity of CaMKII by ~2-fold in rat brain slices whereas the PKA activity was not significantly affected. (C) Western blots were developed with the mAb 12E8 (1:500) which recognizes tau phosphorylated at Ser 262/356 (upper panel; 10 μg protein per lane), and the pAb 92e (1:5000) directed against total tau (lower panel; 5 μg protein per lane). Treatment of rat brain slices with OA/CL-A induced phosphorylation of tau at Ser 262/356. Hyperphosphorylation of tau at Ser 262/356 was observed at 10 min and increased until 60 min of incubation with OA/CL-A. (D) Western blots such as in C were scanned and the immunoreactivity at Ser 262/356 obtained with the mAb 12E8 was normalized against the level of total tau detected with the pAb 92e. Data are expressed as 12E8/92e immunoreactivity. The level of phosphorylation at Ser 262/356 in OA/CL-A-treated samples (black bars) was ~2-fold increased compared to the control (open bars) after 10 min of incubation. Hyperphosphorylation was ~8-fold higher in rat brain slices treated for 60 min with OA/CL-A than in control. Data in A, B, and D are presented as mean ± S.D. obtained from at least three experiments. $^{*}P < 0.05$; $^{**}P < 0.01$.

tau 92e was not affected by OA plus CL-A treatment. The increase of tau phosphorylation at Ser 262/356 was detectable as early as at 10 min of treatment and by 60 min was ~8-fold higher than in the aCSF-treated control brain slices (Fig. 1D). A lack of quantitative correlation between the increase in the CaMKII activity and increase in the phosphorylation of tau suggested (i) that the dephosphorylation of tau at Ser 262/356 might be regulated by PP-2A/PP-1 and (ii) that the maximal inhibition of these phosphatases in the brain slices probably occurred between 10 and 20 min after the OA/CL-A treatment (compare Fig. 1A with Fig. 1D). No significant change in the phosphorylation of tau at Tau-1 (Ser 195/198/199/202), M4 (Thr 231/Ser 235), PHF1 (Ser 396/404) or R145 (Ser 422) sites was observed (data not shown).

3.4. CaMKII phosphorylates tau at Ser 262/356 in brain

To elucidate whether activation of CaMKII activity was responsible for the elevated level of phosphorylation at Ser 262/356, rat brain slices were incubated with KN-62, a selective inhibitor of CaMKII. CaMKII activity was inhibited by ~60% in rat brain slices treated with KN-62 for 60 min as compared to aCSF-treated tissue (Fig. 2A, lanes 1 and 2). The addition of OA and CL-A for 30 more min into the incubation medium did not stimulate CaMKII activity (lane 5); the level of activity remained lower than the control (compare lane 5 with lanes 1 and 4). Whereas aCSF pretreated rat brain slices showed an increase in CaMKII activity when OA and CL-A were added to the medium (compare lane 6 with lanes 4 and 5). This increase in CaMKII activity was, however, smaller than that observed when the brain slices were not pretreated with aCSF for 1 h prior to the OA/CL-A treatment (compare Fig. 2A, lane 6 with lane 3, and with Fig. 1A, 30 min). A likely cause of this difference is that during 60 min incubation of the brain slices in aCSF, there was ~60% decrease in CaMKII activity (data not shown). This decrease in the kinase activity in the slices might be due to a gradual loss of the intracellular calcium homeostasis, resulting in a shift from the kinase switch on/autophosphorylation at Thr 286/287 to switch off/autophosphorylation at Thr 305/306 position.

Corresponding to the CaMKII activity, the phosphorylation of tau at Ser 262/356 was increased in the brain slices treated with aCSF for 60 min and then with OA plus CL-A for 30 min, but not in the tissue which was treated first with KN-62 and then with the phosphatase inhibitors (Fig. 2B, compare lanes 4, 5 and 6). Taken altogether, these results suggest that the stimulation of CaMKII is responsible for the hyperphosphorylation of tau observed at Ser 262/356 in rat forebrain. The phosphorylation of tau at Ser 262/356 by CaMKII was also confirmed in vitro. Recombinant human tau_{410} was phosphorylated at Ser 262/356 by purified CaMKII as determined by Western blots developed with tau antibody 12E8; the phosphorylation of tau by CaMKII also induced a marked mobility shift on SDS–PAGE (Fig. 2C). Tau_{410} phosphorylated at Ser 262/356 by CaMKII could be dephosphorylated in vitro both by PP-2A and PP-1. This dephosphorylation of tau reversed its mobility shift which was induced by phosphorylation with CaMKII (Fig. 2C). The mobility shift might not be solely due to phosphorylation of Ser 262/356.

3.5. CaMKII is regulated by phosphorylation

Rat brain slices were incubated with OA and CL-A with or without KN-62. CaMKII activity was monitored at different time points (Fig. 3A). The activity of CaMKII was stimulated after treatment with both OA and CL-A. The maximum activity which was observed at 10 min slowly and gradually decreased with time up to 60 min studied. The CaMKII activity also decreased in the brain slices incubated in aCSF alone; at 20, 30, and 60 min, the kinase activity was about 80, 60 and 40%, respectively, of that at 10 min incubation

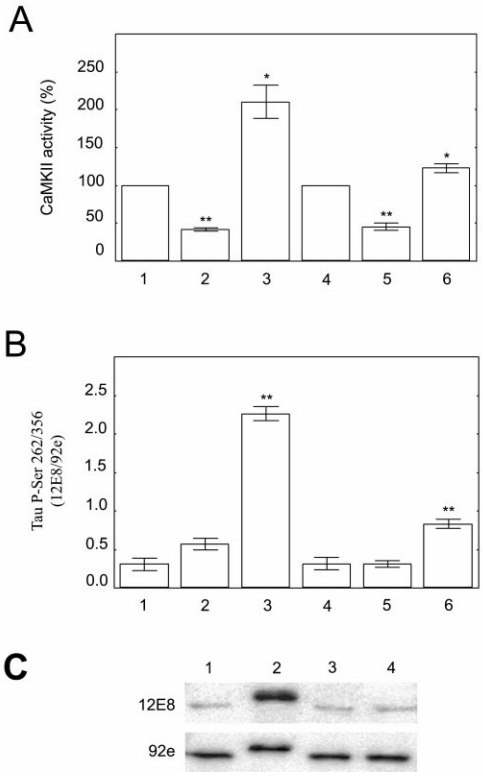

Fig. 2. KN-62 inhibits CaMKII activity and phosphorylation of tau at Ser 262/356 in rat forebrain. (A) CaMKII activity and (B) phosphorylation of tau at Ser 262/356 determined as in Fig. 1. The brain slices were treated as follows: 1, aCSF for 60 min; 2, KN-62 (10 μM) for 60 min; 3, 1.0 μM OA plus 0.1 μM CL-A for 60 min; 4, aCSF for 90 min; 5, KN-62 (10 μM) for 60 min and followed by 1.0 μM OA plus 0.1 μM CL-A for 30 min; and 6, aCSF for 60 min, followed by 1.0 μM OA and 0.1 μM CL-A for 30 min. Not shown in this figure there was a ~60% decrease in CaMKII activity during 60 min incubation in aCSF-treated brain slices. Treatment of rat brain slices with OA plus CL-A for 60 min (bar #3) increased CaMKII activity by about 2-fold. KN-62 inhibited ~60% of CaMKII (bar #2) and the inhibition was maintained even after treatment with OA plus calyculin for 30 more min (bar #5). The brain slices incubated in aCSF for 60 min and then further treated with OA plus CL-A for 30 min, however, resulted in an increase in CaMKII activity (bar #6). Significant phosphorylation of tau at Ser 262/356 was detected only in brain slices treated with OA and CL-A (bars #3 and #6). Data are presented as mean ± S.D. of three experiments. $*P < 0.05$, $**P < 0.01$ as compared with bars #1 and #4. (C) Tau_{410} was phosphorylated with CaMKII followed by dephosphorylation with PP-2A or PP-1. Phosphorylation of recombinant human tau_{410} was determined by Western blots developed with the mAb 12E8 (upper panel; 10 ng tau_{410} per lane) and the pAb 92e (lower panel; 10 ng tau_{410} per lane); recombinant human tau_{410} (lane 1), phosphorylated by purified CaMKII (lane 2), phosphorylated by CaMKII and then dephosphorylated by PP-2A (lane 3), and phosphorylated by CaMKII and then dephosphorylated by PP-1 (lane 4). Phosphorylation of recombinant tau_{410} induced a mobility shift (lane 2). CaMKII-phosphorylated human tau_{410} was dephosphorylated by both PP-2A and PP-1. Note that after dephosphorylation the mobility shift was abolished.

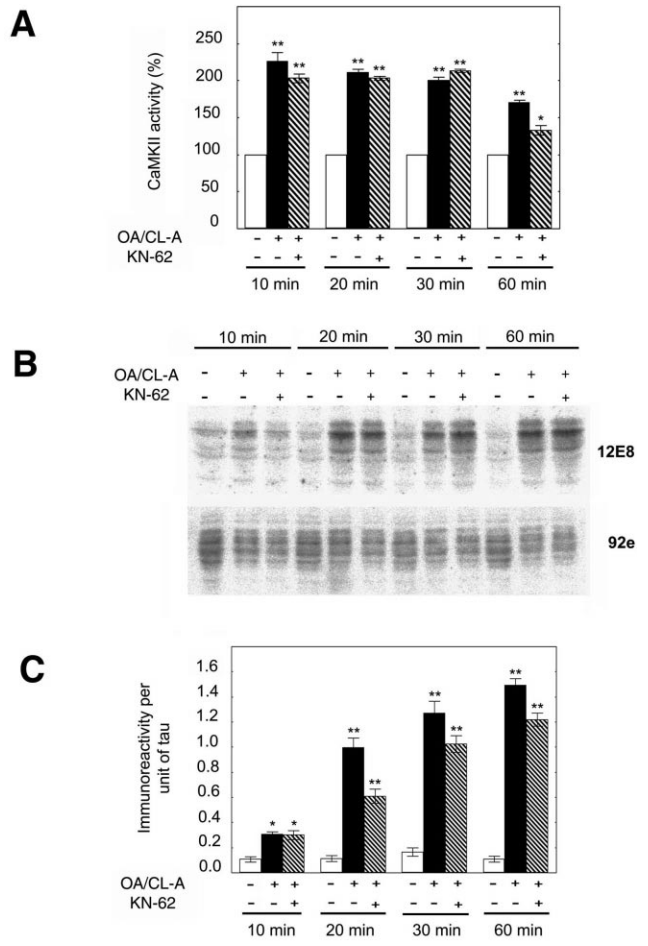

Fig. 3. KN-62 together with OA and CL-A does not inhibit the CaMKII activity stimulated by the phosphatase inhibitors and the phosphorylation of tau at Ser 262/356. Rat brain slices were incubated with aCSF (open bars), 1.0 μM OA and 0.1 μM CL-A (black bars), and with a combination of 10 μM KN-62, 1.0 μM OA and 0.1 μM CL-A (striped bars). After treatment for 10, 20, 30 or 60 min, the brain slices were homogenized and centrifuged at $16\,000\times g$ for 20 min. (A) The CaMKII activity of inhibitors-treated samples was expressed as the percentage of the activity of the control sample treated with aCSF for the same period of incubation. KN-62, a specific inhibitor of CaMKII, when used in combination with OA and CL-A did not inhibit CaMKII activity. (B) Western blots of the brain slices from A were developed with the mAb 12E8 (upper panel; 10 μg per lane) and the pAb 92e (lower panel; 5 μg per lane). KN-62, used in combination with OA and CL-A did not inhibit the phosphorylation of tau at Ser 262/356. (C) Western blots such as in B were scanned and the immunoreactivity obtained with 12E8 was normalized against the level of total tau detected with the pAb 92e. As expected, OA along with CL-A induced an increase of phosphorylation of tau Ser 262/356 (black bars) as opposed to aCSF-treated samples (open bars). The use of KN-62 along with both OA and CL-A resulted in hyperphosphorylation of tau at Ser 262/356 though the increase was lower than that obtained using OA plus CL-A (striped bars). Data in A and C are presented as mean ± S.D. of three experiments. $^{*}P<0.05$, $^{**}P<0.01$ between aCSF- and inhibitor-treated samples.

(data not shown). The presence of KN-62, along with OA and CL-A, did not inhibit CaMKII activity for the first 30 min; at 60 min a small decrease in the KN-62-treated brain slices was, however, observed (Fig. 3A). KN-62 is known to inhibit CaMKII activity by competing with calmodulin [47], which means KN-62 inhibits not only substrate phosphorylation but also autophosphorylation of CaMKII, i.e. the activation of the enzyme. However KN-62 does not inhibit the activity of autophosphorylated CaMKII [47]. Therefore, the lack of inhibition of the CaMKII activity observed in rat brain slices during the first 30 min might be due to the fact that the majority of the enzyme was probably in autophosphorylated form. It has been shown in vitro that autophosphorylation of CaMKII in the presence of Ca^{2+}/CaM is an extremely process [11,17]. To regulate the kinase activity the dephosphorylation of CaMKII might also be rapid in the cell. Thus, KN-62 did not have any effect on CaMKII probably because of its rapid autophosphorylation at Thr 286/287, and because the inhibition of protein phosphatases PP-2A and PP-1 maintained the autophosphorylated status of CaMKII. These data suggest that autophosphorylation of CaMKII occurs and that the regulation of CaMKII in vivo through phosphorylation/dephosphorylation is rapid.

Corresponding to the activation of CaMKII, OA and CL-A increased phosphorylation of tau at Ser 262/356 as early as 10 min and the phosphorylated tau accumulated up to 60 min of treatment studied (Figs. 1C,D and 3B,C). Hyperphosphorylation of tau was also observed in brain slices treated with OA, CL-A and KN-62 (Fig. 3B,C). The total level of tau detected by 92e was not significantly affected by the combination of different drugs used (Fig. 3B). These results confirm that hyperphosphorylation of tau is the result of the activation of CaMKII. The addition of KN-62 in the presence of OA and CL-A only produced a small reduction in the hyperphosphorylation of tau at Ser 262/356; the level of phosphorylation of tau by this treatment, although lower than in OA/CL-A-treated rat brain slices, was significantly higher than control tissue. These findings confirm that the activation of CaMKII in rat brain tissue following treatment with OA and CL-A is due to the inhibition of PP-2A/PP-1. Thus, PP-2A and PP-1 are the main phosphatases to dephosphorylate autophosphorylated CaMKII in brain tissue.

3.6. *The cytosolic CaMKII activity in brain is regulated mainly by PP-2A*

In order to identify which of the two protein phosphatases was responsible for the regulation of CaMKII activity in vivo, we investigated the effect of each protein phosphatase inhibitor separately. Rat brain slices were treated for 30 or 60 min with OA and CL-A individually or in combination. CaMKII was activated with either OA alone (which inhibited ~65% of PP-2A and produced no significant inhibition of PP-1, see Table 1) or in combination with CL-A (Fig. 4). During 60 min, CaMKII activity remained stimulated with OA alone although slightly lower than when both OA and CL-A were used together. When rat brain slices were treated with CL-A alone (which inhibited ~50% of PP-1) for 30 min, CaMKII activity was also upregulated although lower than when both OA and CL-A were used (Fig. 4B). However, after 60 min CaMKII activity in rat brain slices treated with only CL-A was the same as in aCSF-treated control, whereas it remained activated in the OA plus CL-A-treated sample. Since we employed $16\,000\times g$ brain extract for assaying the CaMKII activity, our results suggest that PP-2A upregulates the cytosolic CaMKII activity.

The levels of tau phosphorylation at Ser 262/356 were the same in the brain slices treated with OA or OA plus CL-A at 60 min (Fig. 4E). When CL-A was used alone ~40% increase

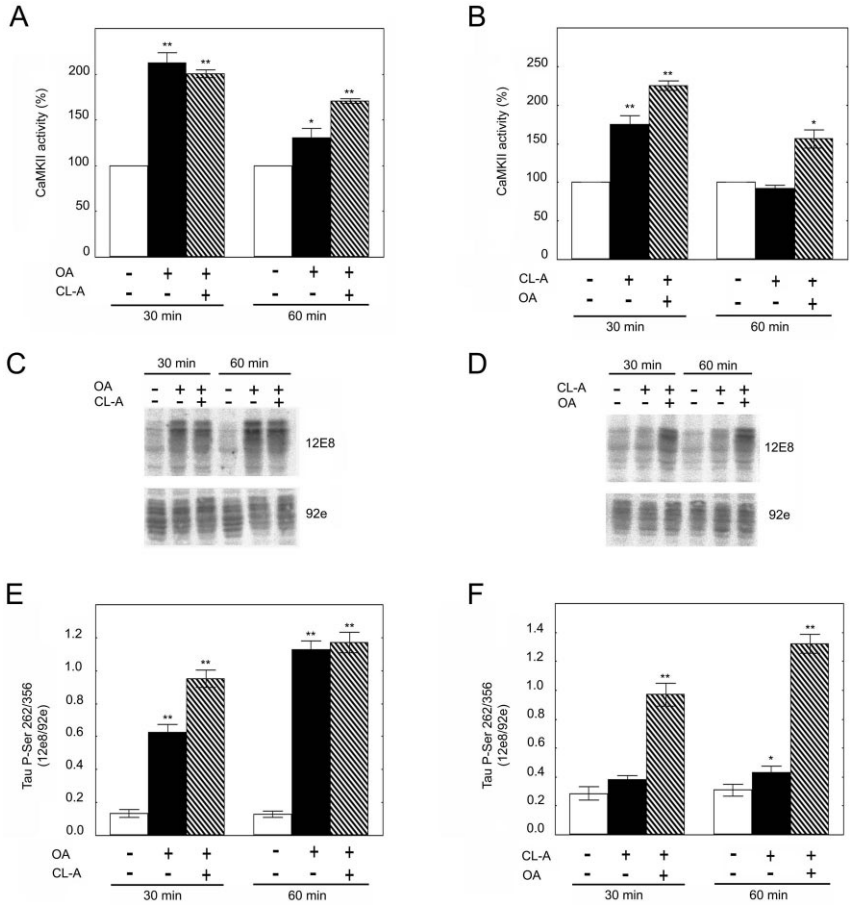

Fig. 4. The activity of cytosolic CaMKII is upregulated by both OA and calyculin. Rat brain slices were incubated for 30 min or 60 min in either aCSF, 0.1 μM CL-A, 1.0 μM OA or both OA (1.0 μM) plus CL-A (0.1 μM) and the CaMKII activity and phosphorylation of tau at Ser 262/356 determined as in Fig. 1. (A) OA alone or used in combination with CL-A increased CaMKII activity up to 200% in rat brain tissue in 30 min. At 60 min CaMKII activity was significantly lower in OA-treated compared to OA/CL-A-treated tissue. Note that CaMKII activity also decreased after 60 min of treatment with OA and CL-A. (B) CL-A alone stimulated CaMKII activity after 30 min of treatment. Although increased, the activity was significantly lower than the CaMKII activity measured in rat brain treated with both OA and CL-A. At 60 min CaMKII activity in rat brain slices treated with CL-A alone was the same as the activity detected in aCSF-treated control tissue. (C–F) Abnormal phosphorylation of tau at Ser 262/356 was detected when rat brain slices were treated with OA alone or in combination with CL-A. In contrast, CL-A induced only a slight increase in the phosphorylation of tau at Ser 262/356. Data in A–F are presented as mean ± S.D. of three experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ as compared with the aCSF-treated samples (open bars).

in phosphorylation of tau at Ser 262/356 was observed whereas as expected OA plus CL-A enhanced several fold the phosphorylation at this site (Fig. 4F). Thus, although CaMKII was activated by 30 min treatment with CL-A (Fig. 4B), no increase in phosphorylation of tau was observed at Ser 262/356. In the OA-treated samples, the upregulation of CaMKII activity (Fig. 4A) was correlated with the increase of tau phosphorylation at Ser 262/356 (Fig. 4C,E). Since CL-A only slightly inhibited PP-2A activity (~20%) and this phosphatase dephosphorylated tau Ser 262/356, no major increase in the phosphorylation was observed by treatment with this drug. These findings suggest that the phosphorylation status of tau protein at Ser 262/356 is probably the result of two major mechanisms: a decrease in protein phosphatase 2A activity and an increase in CaMKII activity. Thus, in vivo tau at Ser 262/356 is a substrate for PP-2A and CaMKII, and CaMKII at Thr 286/287 itself is the substrate for PP-2A.

4. Discussion

OA and CL-A are the most studied cell permeable potent inhibitors of PP-2A and PP-1. In cultured SY5Y cells, a treatment with 10 nM OA for 24 h completely inhibited PP-2A and ~75% of the PP-1 activity [38]. However, unlike in vitro and in cultured cells, maximally ~70% inhibition of PP-2A activity by 1.0–5.0 μM OA was observed in brain [40]. In the present study, treatment of rat brain slices with 1.0 μM OA resulted in ~65% inhibition of PP-2A and no significant change in PP-1 activity. Furthermore, unlike somewhat known higher to ~equal affinity of CL-A towards PP-2A than PP-1 in vitro, in rat brain slices we observed ~50% inhibition of PP-1 and ~20% inhibition PP-2A by 0.1 μM CL-A. A combination of OA and CL-A practically completely inhibited both PP-2A and PP-1 activities in rat brain slices suggesting a synergistic effect of the two inhibitors. These studies demonstrated that the individual potency of OA and CL-A to inhibit PP-2A/PP-1 activities in brain is not only considerably less than that in vitro but also in the case of CL-A its specificity towards PP-2A and PP-1 is somewhat opposite. A complete inhibition of PP-2A and PP-1 activities in brain observed by employing these two compounds in combination is a promising new approach by which the role of

PP-2A/PP-1 can be investigated by the use of pharmacological compounds. This approach is especially attractive because the selective knock-out of PP-2A in transgenic mice is fatal [48].

In rat brain slices treated with protein phosphatase inhibitors, an increase of CaMKII activity was observed. CaMKII activity was upregulated in rat brain after treatment with OA, CL-A or both in combination. It is well known that CaMKII activity is regulated by autophosphorylation and that autophosphorylation at Thr 286/287 is necessary for full activation of the activity [9–11]. Previous studies have reported that PP-2A [7,17,18] and PP-1 [16] and PP-2C [19] dephosphorylate autophosphorylated CaMKII. However, although the activity of PP-2C has been reported to be present in the brain [1,49], the localization of the mRNA showed that the enzyme was mostly localized in the cerebellum [50]. PP-2B has been shown to lack the capacity to dephosphorylate CaMKII [51]. Most of these studies were conducted in vitro and autophosphorylation of CaMKII in vivo had not been elucidated. The present study shows that cytosolic CaMKII in forebrain is regulated primarily by PP-2A. Inhibition of PP-2A upregulated CaMKII activity and brought its total activity to 2-fold as opposed to aCSF-treated rat brain slices. The inhibition of ~50% PP-1 by CL-A also increased CaMKII activity, however, markedly less than when ~65% of PP-2A was inhibited by OA.

CaMKII activity was associated with the increase in tau phosphorylation at Ser 262/356 in rat brain. The use of the specific CaMKII inhibitor, KN-62, inhibited CaMKII activity and no hyperphosphorylation at Ser 262/356 was then detected. Moreover, the activity of PKA which also can phosphorylate tau at this particular site in vitro [27,28] was unaffected by the use of protein phosphatase inhibitors. Although the role of other protein kinases in the phosphorylation of tau could not be ruled out, taken altogether our results strongly suggest that CaMKII is the major mammalian kinase to phosphorylate tau at Ser 262/356. In a recent study we have shown that the treatment of the brain slices with OA and CL-A not only inhibits PP-2A, PP-1 but also ~80% of GSK-3, ~50% of cdk5 and ~30% of cdc2 [33].

The increase in CaMKII activity was associated with an increase in tau phosphorylation. However, in rat brain tissue treated with CL-A for 30 min the upregulation of CaMKII activity was not associated with any abnormal phosphorylation of tau at Ser 262/356. Since the treatment with CL-A for 30 min did not result in any significant inhibition of PP-2A, tau at Ser 262/356 was probably dephosphorylated by this phosphatase. Consistent with these findings, an ~20% inhibition of PP-2A produced by 60 min of treatment with CL-A resulted in a small increase in the phosphorylation of tau at Ser 262/356.

In conclusion, the present study suggests that in the forebrain PP-2A and to a lesser extent PP-1 downregulate the activity of the cytosolic CaMKII probably by inhibiting its autophosphorylation at Thr 286/287. Furthermore, tau at Ser 262/356 is phosphorylated primarily by CaMKII and dephosphorylated by PP-2A. Thus, PP-2A regulates the activity of CaMKII and both CaMKII and PP-2A regulate the phosphorylation of tau at Ser 262/356 in forebrain. A decrease in PP-2A/PP-1 activities leads to the upregulation of CaMKII activity and the phosphorylation of tau at Ser 262/356. The decrease in PP-2A/PP-1 activities and the abnormal hyperphosphorylation of tau at Ser 262/356 in AD cerebral cortex reported previously [1,52] might involve an increase in the phosphorylation of tau by CaMKII and a decrease in the dephosphorylation by PP-2A as shown in rat forebrain slices. An increase in CaMKII immunocytochemical staining in hippocampal pyramidal neurons in AD has been reported [53].

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References

[1] Gong, C.-X., Singh, T.J., Grundke-Iqbal, I. and Iqbal, K. (1993) J. Neurochem. 61, 921–927.
[2] Hunter, T. (1987) Cell 50, 823–829.
[3] Erondu, N.E. and Kennedy, M.B. (1985) J. Neurosci. 5, 3270–3277.
[4] Bulleit, R.F., Bennett, M.K., Molloy, S.S., Hurley, J.B. and Kennedy, M.B. (1988) Neuron 1, 63–72.
[5] Colbran, R.J., Smith, M.K., Schworer, C.M., Fong, Y.-L. and Soderling, T.R. (1989) J. Biol. Chem. 264, 4800–4804.
[6] Payne, M.E., Fong, Y.-L., Ono, T., Colbran, R.J., Kemp, B.E., Soderling, T.R. and Means, A.R. (1988) J. Biol. Chem. 263, 7190–7195.
[7] Miller, S.G., Patton, B.L. and Kennedy, M.B. (1988) Neuron 1, 593–604.
[8] Ikeda, A., Okuno, S. and Fujisawa, H. (1991) J. Biol. Chem. 266, 11582–11588.
[9] Kwiatkowski, A.P., Shell, D.J. and King, M.M. (1988) J. Biol. Chem. 263, 6484–6486.
[10] Katoh, T. and Fujisawa, H. (1991) J. Biol. Chem. 266, 3039–3044.
[11] Ishida, A., Kitani, T. and Fujisawa, H. (1996) Biochim. Biophys. Acta 1311, 211–217.
[12] Hanson, P.I., Meyer, T., Stryer, L. and Schulman, H. (1994) Neuron 12, 943–956.
[13] Hashimoto, Y., Schworer, C.M., Colbran, R.J. and Soderling, T.R. (1987) J. Biol. Chem. 262, 8051–8055.
[14] Hanson, P.I. and Schulman, H. (1992) J. Biol. Chem. 267, 17216–17224.
[15] Colbran, R.J. (1993) J. Biol. Chem. 268, 7163–7170.
[16] Schworer, C.M., Colbran, R.J. and Soderling, T.R. (1986) J. Biol. Chem. 261, 8581–8584.
[17] Lai, Y., Nairn, A.C. and Greengard, P. (1986) Proc. Natl. Acad. Sci. USA 83, 4253–4257.
[18] Saitoh, Y., Yamamoto, H., Fukunaga, K., Matsukado, Y. and Miyamoto, E. (1987) J. Neurochem. 49, 1286–1292.
[19] Fukunaga, K., Kobayashi, T., Tamura, S. and Miyamoto, E. (1993) J. Biol. Chem. 268, 133–137.
[20] Strack, S., Barban, M.A., Wadzinski, B.E. and Colbran, R.J. (1997) J. Neurochem. 68, 2119–2128.
[21] Lindwall, G. and Cole, R.D. (1984) J. Biol. Chem. 259, 5301–5305.
[22] Baudier, J. and Cole, R.D. (1987) J. Biol. Chem. 262, 17577–17583.
[23] Grundke-Iqbal, I., Iqbal, K., Tung, Y.-C., Quinlan, M., Wisniewski, H.M. and Binder, L.I. (1986) Proc. Natl. Acad. Sci. USA 83, 4913–4917.
[24] Iqbal, K., Grundke-Iqbal, I., Smith, A.T., George, L., Tung, Y.-C. and Zaidi, T. (1989) Proc. Natl. Acad. Sci. USA 72, 1858–1862.
[25] Drewes, G., Trinczek, B., Illenberger, S., Biernat, J., Schmitt-Ulms, G., Meyer, H.E., Mandelkow, E.M. and Mandelkow, E. (1995) J. Biol. Chem. 270, 7679–7688.

[26] Singh, T.J., Wang, J.Z., Novak, M., Kontzekova, E., Grundke-Iqbal, I. and Iqbal, K. (1996) FEBS Lett. 387, 145–148.
[27] Litersky, J.M., Johnson, G.V., Jakes, R., Goedert, M. and Lee, M. (1996) Biochem. J. 316, 660–665.
[28] Schneider, A., Biernat, J., von Bergen, M. and Mandelkow, E. (1999) Biochemistry 38, 3549–3558.
[29] Drewes, G., Ebneth, A., Preuss, U., Mandelkow, E.-M. and Mandelkow, E. (1997) Cell 89, 297–308.
[30] Moreno, F.J., Medina, M., Perez, M., Montejo de Garcini, E. and Avila, J. (1995) FEBS Lett. 372, 65–68.
[31] Paudel, H.K. (1997) J. Biol. Chem. 272, 1777–1785.
[32] Sironi, J., Yen, S.-H., Gondal, J.A., Wu, Q., Grundke-Iqbal, I. and Iqbal, K. (1998) FEBS Lett. 436, 471–475.
[33] Bennecib, M., Gong, C.-X., Grundke-Iqbal, I. and Iqbal, K. (2000) FEBS Lett. 485, 87–93.
[34] Otvos, L., Feiner, L., Lang, E., Szendrei, G., Goedert, M. and Lee, V.M.Y. (1994) J. Neurosci. Res. 39, 669–673.
[35] Seubert, P., Mawal-Dewan, M., Barbour, R., Jakes, R., Goedert, M., Johnson, G.V.W., Litersky, J.M., Schenk, D., Lieberburg, I., Trojanowski, J.Q. and Lee, V.M.Y. (1995) J. Biol. Chem. 270, 18917–18922.
[36] Hasegawa, M., Wabanabe, A., Takio, K., Suzuki, M., Arai, T., Titani, K. and Ihasa, Y. (1995) J. Neurochem. 60, 2068–2077.
[37] Liu, W.-K., Moore, W.T., Williams, R.T., Hall, F.L. and Yen, S.-H. (1993) J. Neurosci. Res. 34, 371–376.
[38] Tanaka, T., Zhong, J., Iqbal, K., Trenkner, E. and Iqbal-Grundke, I. (1998) FEBS Lett. 426, 248–254.
[39] Grundke-Iqbal, I., Vorbrodt, A.W., Iqbal, K., Tung, Y.-C., Wang, G.P. and Wiesniewski, H.M. (1988) Mol. Brain Res. 4, 43–52.
[40] Gong, C.-X., Lidsky, T., Wegiel, J., Zuck, L., Grundke-Iqbal, I. and Iqbal, K. (2000) J. Biol. Chem. 275, 5535–5544.
[41] Aitken, A., Bilham, T. and Cohen, P. (1982) Eur. J. Biochem. 126, 235–246.
[42] Singh, T.J., Haque, N., Grundke-Iqbal, I. and Iqbal, K. (1995) FEBS Lett. 257, 206–211.
[43] Cohen, P., Alemany, S., Hemmings, B.A., Resink, T.T., Strallors, P. and Tung, H.Y.L. (1988) Methods Enzymol. 159, 390–408.
[44] Bialojan, C. and Tabai, A. (1988) Biochem. J. 256, 283–290.
[45] Yoshimura, Y., Sogawa, Y. and Yamauchi, T. (1999) FEBS Lett. 446, 239–242.
[46] Ishida, A., Kameshita, I. and Fujisawa, H. (1998) J. Biol. Chem. 273, 1904–1910.
[47] Tokumitsu, H., Chijiwa, T., Hagiwara, M., Mizutani, A., Terasawa, M. and Hidaka, H. (1990) J. Biol. Chem. 265, 4315–4320.
[48] Gotz, J., Probst, A., Ehler, E., Hemmings, B. and Kues, W. (1998) Proc. Natl. Acad. Sci. USA 21, 12370–12375.
[49] Ingebritsen, T.S., Stewart, A.A. and Cohen, P. (1983) Eur. J. Biochem. 132, 297–307.
[50] Abe, H., Tamura, S. and Kondo, H. (1992) Mol. Brain Res. 13, 283–288.
[51] Goto, S., Yamamoto, H., Fukunaga, K., Iwasa, T., Matsukado, Y. and Miyamoto, E. (1985) J. Neurochem. 45, 276–283.
[52] Morishima-Kawashima, M., Hasegawa, M., Takio, K., Suzuki, M., Yoshida, H., Watanabe, A., Titani, K. and Ihara, Y. (1995) Neurobiol. Aging 16, 375–380.
[53] McKee, A.C., Kosik, K.S., Kennedy, M.B. and Kowall, N.W. (1990) J. Neuropathol. Exp. Neurol. 49, 49–63.